CHARACTERISATION AND FUNCTIONAL ANALYSIS OF THE CHICKEN MICROPHTHALMIA GENE

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[1] That was a water one.

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Genetic Nomenclature Statement

Wherever possible and/or practical, the genetic nomenclature used for microphthalmia throughout this thesis is adapted from the Trends in Genetics *Genetic Nomenclature Guide* (Wood, Assistant Editor, 1998).

Chicken

Genes (Loci)
Full gene names are not italicised. Gene symbols consist of italic uppercase letters. An adaptation of this nomenclature has been applied throughout this thesis by preceding the *MI* with a "c" for "chicken". Thus the nomenclature used for the gene name and symbol are: microphthalmia; *cMI*.
An exception to this rule is made for the species-specific cmi9 isoform; in this case the nomenclature used is: *cmi9*.

Proteins
Proteins are designated by the same uppercase characters as the gene symbols, but non-italic. An adaptation of this nomenclature has been applied throughout this thesis by preceding the MI with a "c" for "chicken". Thus the nomenclature used for the protein is: cMI. An exception to this rule is made for the species-specific cmi9 isoform; in this case the nomenclature used is: cmi9.

Human

Genes (Loci)
Full gene names are not italicised. Gene symbols consist of italic uppercase. Thus the nomenclature used for the gene name and symbol are: Microphthalmia-Associated Transcription Factor; *MITF*.

Proteins
Proteins are designated by the same uppercase characters as the gene symbols, but non-italic. Thus the nomenclature used for the protein is: MITF.

Mouse

Genes (Loci)
Full gene names are not italicised. Gene symbols consist of italic letters with the initial letter uppercase. Thus the nomenclature used for the gene name and symbol are: microphthalmia, *Mitf*. An exception to this rule is made when discussing mutations; in such cases the gene symbol used is, for example, mi^{vit}.

Proteins
Proteins are designated by the same symbol as the gene, but non-italic. Thus the nomenclature used for the protein is: Mitf.

General

When talking generally about the microphthalmia gene without reference to a specific species, the following nomenclature is used:

Genes (Loci)
Full gene name: microphthalmia
Gene symbol: *mi*

Proteins
Microphthalmia

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Abstract | Characterisation and functional analysis of the chicken microphthalmia gene

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December 2002

Analyses of mammalian melanogenic genes have established that the microphthalmia gene is a putative "master regulator" of melanin biosynthesis; its protein transactivates the tyrosinase gene promoter and the initiation of *tyrosinase* gene transcription is the rate-limiting step of melanogenesis. Despite the fact that the chicken embryo is a model system for developmental biologists, comparatively little is known about the regulation of avian melanogenesis. There has only been one report of the characterisation of the chicken tyrosinase gene promoter, and only one full-length chicken microphthalmia cDNA (*cmi9*) has been identified to date.

A putative chicken microphthalmia clone (*cMI-m)* was previously obtained by screening of a chicken neural crest-derived melanocyte cDNA library (April, 1998). In the present study, restriction mapping, Southern blot hybridisation and sequence analyses revealed that *cMI-m* contains a 2.6 kb insert that encodes a chicken homologue of the mammalian melanocyte-type isoform of microphthalmia: Alignment of *cMI-m* with previously reported mammalian microphthalmia cDNA sequences demonstrates that *cMI-m* shares the conserved bHLH-Zip domains, is missing exon 3 and 6a, and encodes the unique first exon 1M specific to the melanocyte-type isoforms. Thus chicken microphthalmia transcript diversity is generated through the use of alternative first promoters/exons and alternative splicing of the coding region. Preliminary Southern blot hybridisation analysis suggests that the chicken microphthalmia gene is large, spanning at least 39 kb of the genome.

When assessed by RT-PCR, chicken microphthalmia transcripts are widely expressed in the developing chicken embryo, both in pigmented and non-pigmented cells. However, two chicken isoforms have restricted patterns of expression in pigmented cells: *cMI-m* is exclusively expressed in neural crest-derived melanocytes, and *cmi9* (an additional chicken microphthalmia isoform, isolated by others, with an amino terminus differing from that of *cMI-m*) is exclusively expressed in RPE cells. *cMI-m* is the most likely source of illegitimate transcription in non-pigmented cells (chicken embryonic fibroblasts), suggesting that the melanocyte-type promoter is "leaky". These transcripts are not, however, translated into proteins, as assessed by western blotting.

To begin to assess the role(s) of chicken cMI proteins in avian melanogenesis, western blotting of non-pigmented (HeLa and chicken embryonic fibroblasts) cells transfected with *cMI-m* and *cmi9* were carried out. Transfections with the cMI-m construct resulted in a cMI protein doublet of 53-63 kDa, while transfection of the *cmi9* construct resulted in a cMI protein doublet of 65-69 kDa. These differences in size are expected because the encoding sequence of *cmi9* is 55 aas longer than that of *cMI-m.* To study the trans-activational activity of these two proteins, co-transfections of cMI-m or cmi9 with chicken tyrosinase gene promoter reporter constructs in HeLa and B16 cells were carried out. The activity of the tyrosinase gene promoter was shown to be pigment cell-specific in B16 cells. In all co-transfections, cMI-m and cmi9 increase the activity of tyrosinase gene promoter-reporter constructs to equal levels, suggesting that the different amino termini of the proteins do not transactivate the tyrosinase gene promoter differently, at least *in vitro.*

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Chapter 1 | Introduction and Aims

The mechanisms underlying the production of pigmentation first captured researchers' attention over a century ago when, in 1895, Bourquelot and Bertrand reported that a substance present in a mushroom (*Russula nigricans*) was turned black by an enzyme also present in the mushroom. One year later, Bertrand identified the substance as the amino acid tyrosine, and Bourquelot named its catalysing enzyme "tyrosinase". From those early beginnings, studies have elucidated many complexities involved in melanin production, ranging from the activity and regulation of melanogenic-specific enzymes such as tyrosinase, through to "master genes" that may determine the embryological development or mature functions of melanogenic cells.

The product of the microphthalmia locus is one such putative master regulator and evidence gathered over the past decade shows that it has a dual function in melanogenic cells. First, microphthalmia is one of the earliest detectable molecular markers of presumptive melanogenic cells, suggesting that it determines, at least in part, the development of these cells from embryological precursors. Secondly, the Microphthalmia protein transactivates the tyrosinase gene promoter and up-regulates the expression of other melanogenic genes, thus representing an important factor in the continued production of melanin in, and the maintenance of the differentiated state of, mature pigment-producing cells.

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While these two roles of microphthalmia are broadly understood and substantiated by much genetic evidence, a full understanding of the function of the microphthalmia gene remains elusive. In particular, the role of microphthalmia in the regulation of melanin production in differentiated cells in lower vertebrates has not been well studied. Therefore the broad aims of this thesis are:

(i) To contribute to the understanding of the role of microphthalmia in mature pigment-producing cells and;
(ii) To do so in an avian model, thereby furthering the relatively sparse knowledge surrounding pigmentation in lower vertebrates.

This chapter provides a review of the current understanding of the microphthalmia gene and protein. Broadly speaking, the literature may be divided into two areas of interest: (i) those factors upstream of microphthalmia which regulate its function, and (ii) the downstream targets

of the Microphthalmia protein. As the focus of the current study is the latter, information regarding the upstream regulation of microphthalmia by such factors as Sox10, Kit, and the Wnt/β-catenin signalling pathway is beyond the scope of this thesis. The reader is referred to Goding (2000) for a thorough review of such information.

Section 1.1 reviews the tyrosinase gene family and its role in the melanogenic pathway. Section 1.2 focuses on the embryological development of two pigment-producing cell types: neural crest-derived melanocytes and retinal pigment epithelial (RPE) cells. Microphthalmia is introduced in terms of its role in the development of the two cell types. Section 1.3 is dedicated to the molecular biology of microphthalmia and the information about alternative splicing patterns and alternative promoter usage, which result in multiple isoforms of its protein. Section 1.4 focuses on the role of the Microphthalmia protein as a master regulator of the *tyrosinase* gene, while Section 1.5 explores the possibility that the different isoforms of microphthalmia may have functional significance(s) as regulators of target promoter(s). Finally, Section 1.6 discusses the chicken tyrosinase gene promoter and begins to introduce the aims of the present study, which are fully detailed in Section 1.7.

1.1 The tyrosinase gene family and the biochemistry of melanogenesis

Much of our current understanding of the production of melanin (melanogenesis) comes from studies of the biochemical pathway illustrated in Fig. 1.1. The end products (melanins) of this pathway are synthesised in melanosomes, which are specialised cellular organelles containing the enzymes required for the production of melanin. The three melanogenic enzymes, tyrosinase, tyrosinase-related protein-1 (tyrp-1) and dopachrome tautomerase (Dct, formerly called tyrosinase-related protein-2), collectively make up the tyrosinase gene family and are thought to form a multi-enzyme complex at the inner side of the melanosomal membrane (Orlow *et al.*, 1994). The tyrosinase gene family is required for the production of melanin, and mutations at these gene loci reveal the role of each enzyme in the pathway.

Tyrosinase, the essential, rate-limiting enzyme of the pathway, is bi-functional and catalyses the hydroxylation of L-tyrosine to L-dopa and the oxidation of L-dopa to L-dopaquinone (Hearing and Jiminez, 1987; Hearing and Tsukamoto, 1991). L-dopaquinone represents the common substrate for the formation of the two major types of melanin produced in mammals, eumelanin (which is black/brown) and phaeomelanin (which is yellow/red). Thus tyrosinase is absolutely required for both eumelanin and phaeomelanin production. Accordingly, mutations at the

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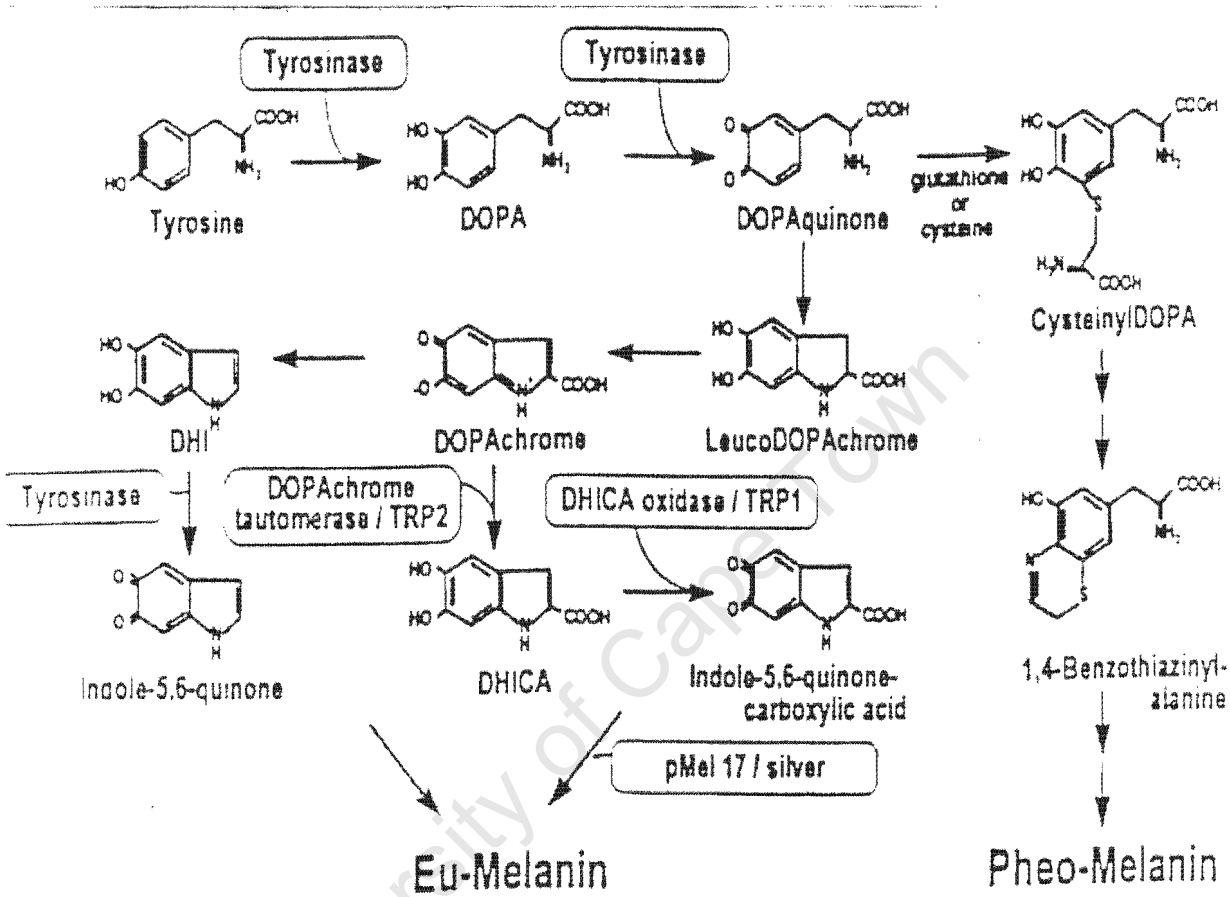

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Fig. 1.1. Schematic diagram of the mammalian melanin biosynthesis pathway (from Furumura *et al.*, 1996).

tyrosinase locus result in hypo-pigmentation in the coat and eyes of mice (Silvers, 1979), and in humans are associated with type I oculocutaneous albinism (OCA) (Tomita *et al.*, 1989; Spritz, 1994).

Tyrp-1 and Dct are involved in the processing of L-dopaquinone to eumelanin polymers. The enzymatic function of TYRP-1 has been the subject of much controversy (Halaban and Moellmann, 1990; Winder *et al.*, 1993, 1994; Zhao *et al.*, 1994). However, it has been shown that mouse tyrp-1 oxidises 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (Kobayashi *et al.*, 1994a; 1994b) to indole-5,6-quinone-2-carboxylic acid. Mutations at the murine locus result in a brown, rather than wild-type black, coat colour (Jackson *et al.*, 1990), indicating that a functional tyrp-1 enzyme is required for the synthesis of black melanin (Zdarsky *et al.*, 1990; Bennett *et al.*, 1990). In humans, TYRP-1 mutations have been associated with type 3 OCA (Spritz, 1994; Boissy *et al.*, 1996; Manga *et al.*, 1997). In addition to its enzymatic role, it has been suggested that tyrp-1 may play a structural role in the melanosome (Rittenhouse, 1968).

Dct isomerises DOPAchrome to DHICA (Jackson *et al.*, 1992; Kameyama *et al.*, 1993; Hearing, 1999). Although *Dct* is involved at a later stage in the melanogenic pathway than *tyrosinase*, it is the first member of the tyrosinase gene family to be expressed during the development of pigmented cells (Steel *et al.*, 1992). Hence it may be used as a molecular marker to detect presumptive melanogenic cells which have not yet begun to pigment. Mutations at the mouse locus result in a grey (slaty) coat colour (Budd and Jackson, 1995); there are no known mutations at the human *DCT* locus.

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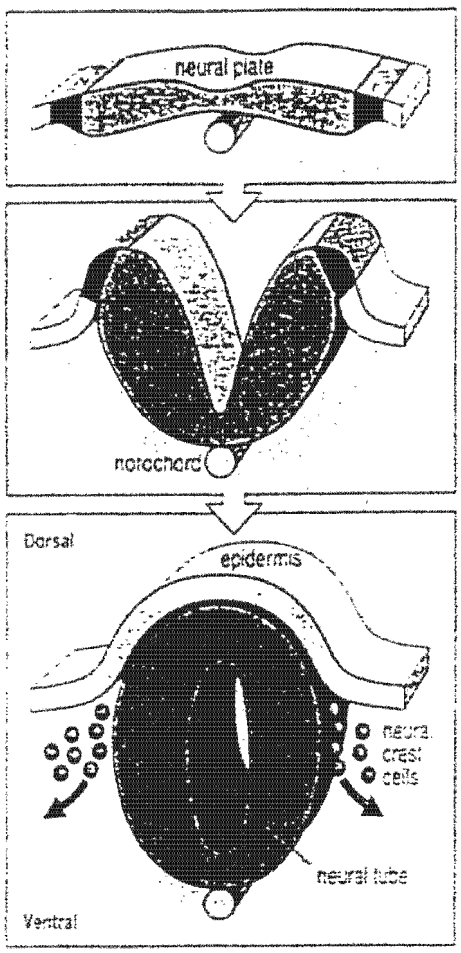
1.2 Melanogenic cells: epidermal melanocytes and RPE cells

The principle locations of pigment-producing cells in vertebrates are the epidermis and the eye. Neural crest-derived melanocytes populate the former, while the retinal pigment epithelial (RPE) cells represent a significant proportion of the pigmented cells of the eye (although neural crest-derived melanocytes also populate the choroid of the eye, and the iris is composed of both RPE-derived pigment cells and neural crest-derived melanocytes). Both cell types utilise the melanogenic pathway but their embryological origins, mature morphology and functions differ.

Epidermal melanocytes develop from neural crest cells. The neural crest (Fig. 1.2.a) is a transient population of embryological progenitor cells with the capacity to differentiate into a number of mature cell types, including, but not limited to: neurons; cartilage; bone; smooth muscle;

(a)



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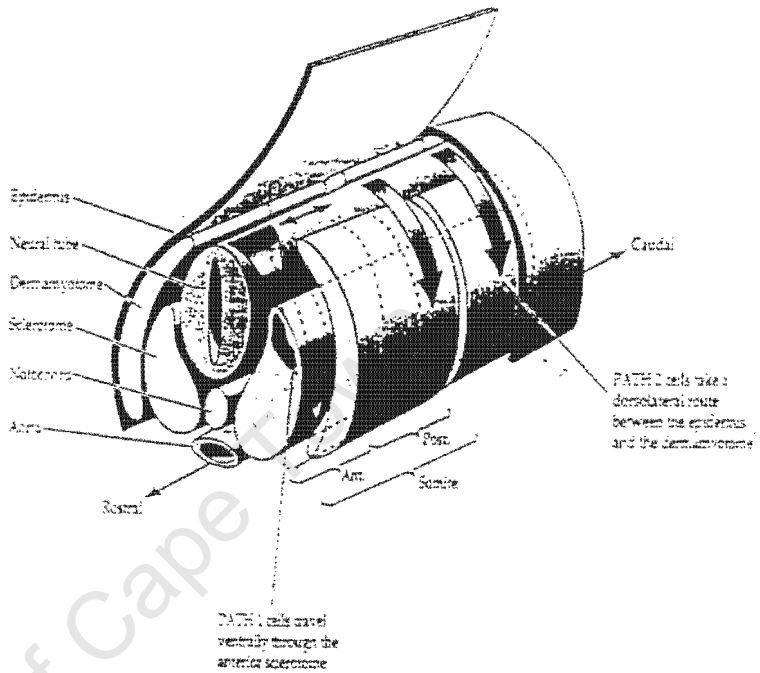

(c)

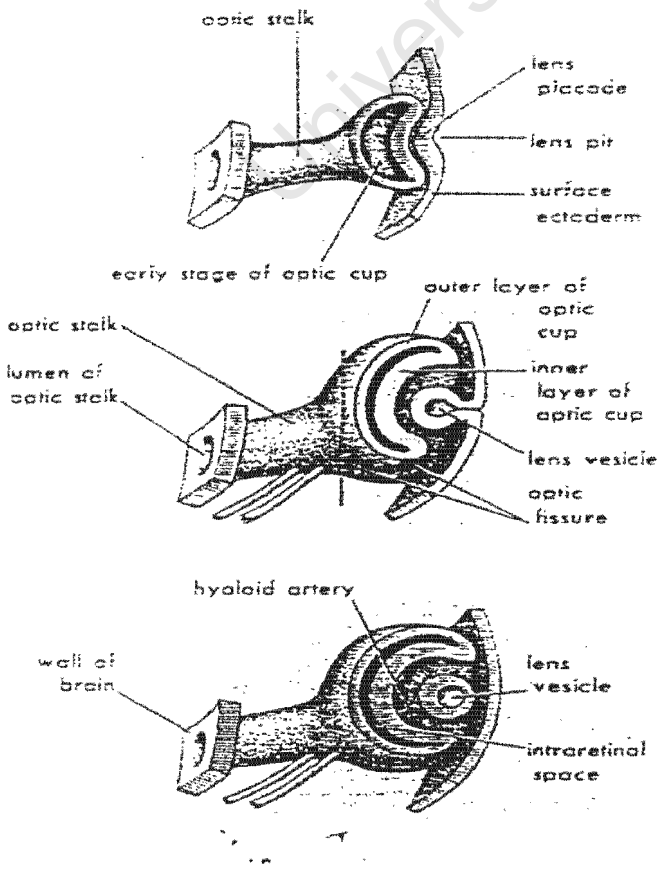

Fig. 1.2. **(a)** Development of the neural crest, the transient population of pluripotent cells from which melanocytes will differentiate. From Wolpert *et al*., 1998.
(b) Migration of neural crest cells in the trunk region of the chicken embryo. Path 1: Cells destined to differentiate into sympathetic ganglia, parasympathetic ganglia, adrenal medullary cells, and dorsal root ganglia migrate through the anterior of the sclerotome along the ventral migration pathway. Path 2: Those cells destined to differentiate into melanocytes follow the dorsolateral migration pathway, beneath the surface ectoderm. From Glibert, 1994.
(c) Development of the eye cup and the RPE. The eye cup, which develops as an evagination of the developing forebrain, invaginates and gives rise to the inner and outer cell layers. The RPE develops from the outer layer of the optic cup. From Moore, 1977.

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connective tissue; and epidermal melanocytes (Hörstadius, 1950; Le Douarin, 1982; reviewed by Bronner-Fraser, 1995). Melanoblasts, the cells destined to become epidermal melanocytes, migrate along a dorsolateral pathway (Fig. 1.2.b) and invade the epidermis, where they become localised in the basal layers of the skin and in hair or feather follicles. Here the cells differentiate into stellate-shaped melanocytes and produce melanin in melanosomes. In epidermal melanocytes, congested melanosomes move from the perinuclear region of the melanocytes to the tips of the dendrites, and are transferred to neighbouring skin keratinocytes. Mature epidermal melanocytes continue to proliferate and to produce and transfer melanin throughout life, and the pigment serves to protect against the harmful effects of ultra-violet radiation. This close interaction between epidermal melanocytes and keratinocytes is important in humans, but is less significant in lower vertebrates. In lower vertebrates such as mice there are few epidermal melanocytes; the melanocytes are concentrated in the hair follicles. In avians, melanin localised in feather follicles also serves for social communication, concealment from predators and in radiative heat exchange (McFarland *et al.*, 1985).

In contrast to the neural crest origin of epidermal melanocytes, RPE cells differentiate from the neuroepithelium of the optic cup. An outpocketing of the lateral wall of the developing forebrain invaginates as it approaches the surface ectoderm, forming a double-walled cup structure (Fig. 1.2.c). The inner surface differentiates into the neural retina while the outer cell layer forms the retinal pigment epithelium. Mature RPE cells exhibit a cobblestone mosaic appearance and, unlike epidermal melanocytes, do not transfer melanin to neighbouring cells. They are reported to exist as post-mitotic cells in the adult and to cease melanogenesis when they become congested with melanosomes (Boissy *et al.*, 1988). Although this point is in some dispute as RPE cells will continue to proliferate *in vitro*, it is clear that the melanin of the RPE serves to absorb scattered light, protect against light damage to the photoreceptors of the eye, and acts as a free radical scavenger.

The differences in development, morphology and mature function of epidermal melanocytes and RPE cells raise the question of how these differences are regulated. Studies over the past decade have shown that microphthalmia is one of the key role players, although the full details of its activity remain to be explained. The remainder of this review is dedicated to the microphthalmia gene.

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1.2.1 The role of microphthalmia in epidermal melanocytes and RPE cells

The role of microphthalmia in the differentiation of mouse epidermal melanocytes has been well studied *in vitro* and may be summarised schematically (Fig. 1.3). The cells progress through a maturation process, beginning with a melanoblast precursor, which is the earliest stage at which presumptive melanocytes are detectable. Microphthalmia expression, which precedes *Dct* expression, defines the melanoblast precursor. Although melanoblast survival is independent of microphthalmia for the first 48 hours of development, if microphthalmia is mutated, the cells do not progress beyond the melanoblast precursor and the development of the melanocyte lineage is aborted and cannot be rescued (Opdecamp *et al.*, 1997). This requirement for microphthalmia has been confirmed *in vivo*. A transgenic mouse study demonstrated that microphthalmia expression is required for the detection of melanoblasts along the dorsolateral pathway of neural crest cell migration, and that mutations blocking the function of microphthalmia compromise melanoblast survival (Hornyak *et al.*, 2001).

Continued expression of microphthalmia, combined with the onset of *Dct* expression and an up-regulation of *Kit* (a signalling molecule essential for neural crest-derived melanocyte development) expression, is required for the development of a late melanoblast. The microphthalmia-expressing melanoblasts invade the epidermis and confer pigmentation to the skin, hair, or feather follicles. In neonates, the expression of microphthalmia becomes restricted to the pigmented cells in these locations (Nakayama *et al.*, 1998). The continued post-natal expression is in contrast to discontinued post-natal expression in other pigmented cells in the body, and suggests a functional role for the continued epidermal expression of microphthalmia. Such a conjecture is consistent with mice homozygous for the microphthalmia *vitiligo* (mi^{vit}) allele. mi^{vit}/mi^{vit} mice show an age-dependent and progressive loss of coat pigmentation (Lerner *et al.*, 1986; Boissy *et al.*, 1987), but apparently no postnatal changes in the RPE (Boissy *et al.*, 1987; Nir *et al.*, 1995), where microphthalmia is not expressed postnatally (see below).

The expression of microphthalmia during mouse eye development precedes its expression in neural crest-derived melanocytes, and thus represents the first known site of microphthalmia expression (Nakayama *et al.*, 1998). Microphthalmia mRNA expression is initiated in the entire optic vesicle and precedes the development of the vesicle into the two mature layers of the retina - the neural retina (NR) and the RPE. Only at a later stage does expression become restricted to the presumptive RPE (Nguyen *et al.*, 1997; Bora *et al.*, 1998; Nakayama *et al.*, 1998). It is thought

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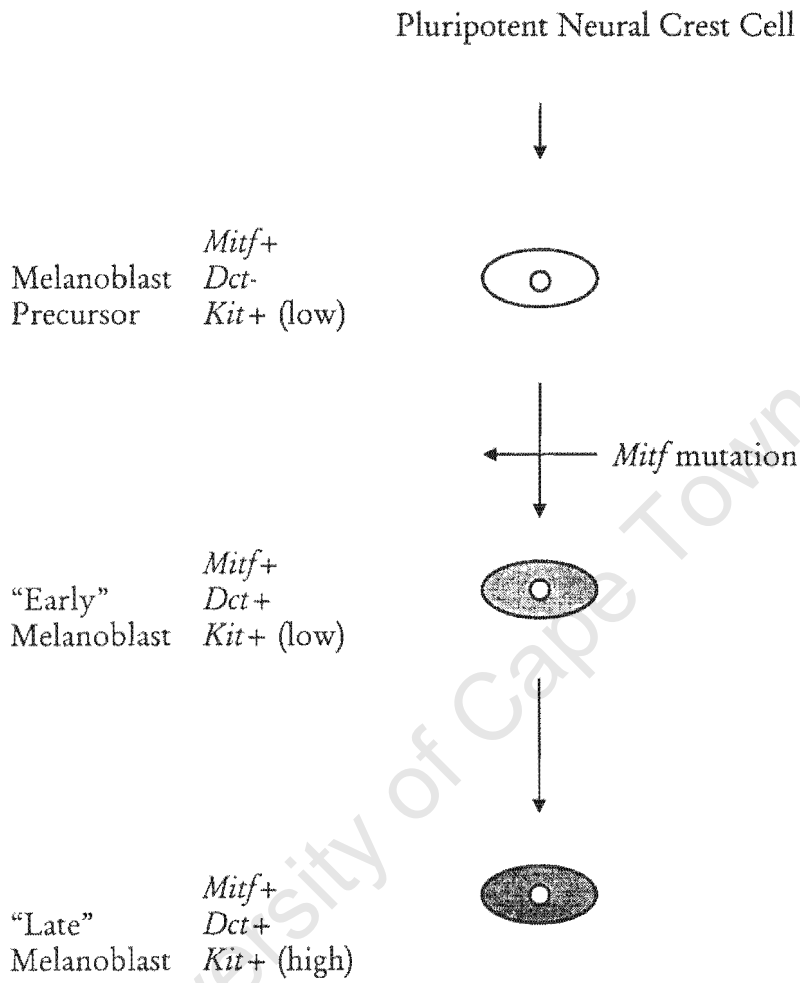

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Figure 1.3. The role of microphthalmia in the development of melanocytes from neural crest cells. The role of microphthalmia at each developmental stage is indicated. In wild type cells a pluripotent neural crest cell gives rise to a melanoblast precursor expressing microphthalmia and low levels of *Kit*. As the cell begins to express *Dct* it develops into an "early" melanoblast, with an upregulation of *Kit* expression progressing the development of the cell through to the "late" melanoblast stage. In microphthalmia mutant cells, *Dct* is never expressed and development is aborted at the melanoblast precursor stage. Adapted from Opdecamp *et al.*, 1997.

that this progressive restriction of microphthalmia expression determines the separation of the optic vesicle into the future NR and RPE. In contrast to the persistent post-natal expression in epidermal melanocytes, microphthalmia expression in the eye becomes less prominent during development and is largely undetectable at birth (Nakayama *et al.*, 1998; Mochii *et al.*, 1998a).

Also, in contrast to neural crest-derived melanocytes, which are few in number and rapidly become undetectable in *Mitf* mutants, the initial steps of RPE development are not affected by *Mitf* mutations and the cells do survive. However, when microphthalmia is rendered completely non-functional, the RPE hyperproliferates, remains unpigmented and displays evidence of the development of a second neural retina (Packer, 1967; Scholtz and Chan, 1987; Mochii *et al.*, 1998b; Nakayama *et al.*, 1998; Bumstead and Barnstable, 2000). This concept that mutations in microphthalmia are able to induce the transdifferentiation of the presumptive RPE into a NR suggests that microphthalmia is absolutely required for differentiation of the RPE. The simplest explanation, proposed by Nguyen and Arnheiter (2000), is that the neuroepithelium initially expresses transcription factors that later become NR or RPE-specific. These factors are activated by surface ectodermal FGFs (or other factors capable of activating signalling pathways), leading to transcriptional repression of microphthalmia in the developing NR. As a result two domains are formed: the NR, which will not express microphthalmia but will still express those transcription factors required for the development of the NR, and the RPE, which continues to express microphthalmia and specifies the presumptive RPE.

In summary, neural crest-derived melanocytes exhibit an absolute requirement for microphthalmia; without a functional microphthalmia gene the cells do not progress beyond the melanoblast stage of development and fail to pigment. In addition, mature neural crest-derived melanocytes exhibit a requirement for microphthalmia as the gene is expressed postnatally, and the cells may be affected by mutations as the organism ages. RPE cells have a different requirement for microphthalmia than do neural crest-derived melanocytes. Without a functional microphthalmia gene the cells do survive, although the RPE exhibits gross changes in morphology. Mature RPE cells do not exhibit a requirement for microphthalmia as the gene is not expressed postnatally; hence those microphthalmia mutations that present themselves at later stages in the life of the organism do not exert their effects on the RPE.

Thus, studies have demonstrated that although these two melanogenic cells of different embryological origins are dependent on the microphthalmia transcription factor for their correct development, the two cell types respond differently to mutations in the gene. Molecular analyses

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of microphthalmia have contributed to an understanding of how these differences may arise, and section 1.3 focuses on the molecular biology of the microphthalmia locus.

1.3 The microphthalmia locus

The microphthalmia phenotype was first described in 1942 (Hertwig, 1942) and was named for the eye defect observed in descendants of an irradiated male mouse. Heterozygotes had white spots on their belly, head and tail and less pigment in the eye than normal, and homozygotes had small (microphthalmic), unpigmented eyes, a white coat, and were deaf (Grüneberg, 1953; Silvers, 1979; reviewed by Green, 1989; reviewed by Jackson and Raymond, 1994).

Half a century later, two independent transgene insertions, one, $mi^{VGA\text{-}9}$, originally generated to study the regulation of the vasopressin promoter (Hara *et al.*, 1990; Tachibana *et al.*, 1992) and the second, mi^{tg}, an insertional mutation of a human globin transgene (Krakowsky *et al.*, 1993), resulted in mice with similar disturbances in pigmentation. This allowed two groups (Hughes *et al.*, 1993; Hodgkinson *et al.*, 1993) to clone the mouse microphthalmia gene. The gene was mapped to chromosome 6 (Moore and Elliott, 1993) and, subsequently, the human homologue (Microphthalmia-Associated Transcription Factor, *MITF*) was cloned and mapped to chromosome 3p14.1-p12.3 (Tachibana *et al.*, 1994). Mutations at both the mouse and human loci result in pigmentation disorders and it was recognised that a key gene involved in melanogenesis had been identified.

Sequence analyses of both the mouse and human cDNAs demonstrated the presence of three conserved domains: a basic domain, a helix-loop-helix domain, and a leucine zipper domain (Fig. 1.4) (Hughes *et al.*, 1993; Hodgkinson *et al.*, 1993; Tachibana *et al.*, 1994). This established that microphthalmia was a novel member of the bHLH-Zip family of transcription factors. This protein family exerts wide-ranging effects in the regulation of gene expression, cell proliferation and differentiation (Jones, 1990) by forming hetero- or homodimers. Specifically, Microphthalmia forms heterodimers with three other bHLH-Zip transcription factors, TFE3, TFEB and TFEC. Thus Microphthalmia belongs to the so-called MiT subfamily of bHLH-Zip proteins (Hemesath *et al.*, 1994, reviewed by Rehli *et al.*, 1999). Biochemical studies indicate that MiT proteins are able to form heterodimers with one another, but not with known bHLH-Zip factors of other subfamilies (Blackwood and Eisenman, 1991; Fisher *et al.*, 1991; Zhao *et al.*, 1993; Hemesath *et al.*, 1994). These early molecular characterisations of the gene paved the way

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   1 TCGGGATGCCTTGTTTATGGTGCCTTCTTTATGCCGGTCGCTCTTCGAATTGGAATTACA
                                     M  L  E  M  L  E  Y  S  H  Y
  61 GAAAGTAGAGGGAGGAGGACTAAGTGGTCTGCGGTGTCTCCTGGGCTTGGGGCTGCCTGA
     Q  V  Q  T  H  L  E  N  P  T  K  Y  H  I  Q  Q  A  Q  R  H
 121 AACCTTGCTATGCTGGAAATGCTAGAATACAGTCACTACCAGGTGCAGACCCACCTGGAA
     Q  V  K  Q  Y  L  S  T  T  L  A  N  K  H  A  S  Q  V  L  S
 181 AACCCCACCAAGTACCACATACAGCAAGCTCAGAGGCACCAGGTAAAGCAGTACCTTTCT
     S  P  C  P  N  Q  P  G  D  H  A  M  P  P  V  P  G  S  S  A
 241 ACCACTTTAGCAAATAAACATGCCAGCCAAGTCCTGAGCTCACCATGTCCAAACCAGCCT
     P  N  S  P  M  A  M  L  T  L  N  S  N  C  E  K  E  A  F  Y
 301 GGCGACCATGCCATGCCACCAGTGCCGGGGAGCAGCGCACCCAACAGCCCTATGGCTATG
     K  F  E  E  Q  S  R  A  E  S  E  C  P  G  M  N  T  H  S  R
 361 CTCACTCTTAACTCCAACTGTGAAAAAGAGGCATTTTATAAGTTTGAGGAGCAGAGCAGG
     A  S  C  M  Q  M  D  D  V  I  D  D  I  I  S  L  E  S  S  Y
 421 GCAGAGAGTGAGTGCCCAGGTATGAACACGCACTCTCGAGCGTCGTGCATGCAGATGGAT
     N  E  E  I  L  G  L  M  D  P  A  L  Q  M  A  N  T  L  P  V
 481 GATGTAATTGATGACATCATCAGCCTGGAATCAAGTTATAATGAAGAAATTTTGGGCTTG
     S  G  N  L  I  D  L  Y  S  N  Q  G  L  P  P  P  G  L  T  I
 541 ATGGATCCGGCCTTGCAAATGGCAAATACGTTACCCGTCTCTGGAAACTTGATCGACCTC
     S  N  S  C  P  A  N  L  P  N  I  K  R  E  L  T  A  C  I  F
 601 TACAGCAACCAGGGCCTGCCACCGCCAGGCCTTACCATCAGCAACTCCTGTCCAGCCAAC
     P  T  E  S  E  A  R  A  L  A  K  E  R  Q  K  K  D  N  H  N
 661 CTTCCCAACATAAAAAGGGAGCTCACAGCGTGTATTTTCCCCACAGAGTCTGAAGCAAGA
     L  I  E  R  R  R  R  R  P  N  I  N  D  R  I  K  E  L  G  T  L
 721 GCATTGGCTAAAGAGAGGCAGAAAAAGGACAATCACAACTTGATTGAACGAAGAAGAAGA
     I  P  K  S  N  D  P  D  M  R  W  N  K  G  T  I  D  K  A  S
 781 TTTAACATAAACGACCGCATTAAGGAGCTAGGTACTCTGATCCCCAAGTCAAATGATCCA
     V  D  Y  I  R  K  L  Q  R  E  Q  Q  R  A  K  D  L  E  N  R
 841 GACATGCGGTGGAACAAGGGAACCATTCTCAAGGCCTCTGTGGACTACATCCGGAAGTTG
     Q  K  K  L  E  H  A  N  R  H  L  L  L  R  V  Q  E  L  E  M
 901 CAACGGGAACAGCAACGAGCTAAGGACCTTGAAAACCGACAGAAGAAGCTGGAGCATGCG
     Q  A  R  A  H  G  L  S  L  I  P  S  T  G  L  C  S  P  D  L
 961 AACCGGCACCTGCTGCTCAGAGTACAGGAGCTGGAGATGCAGGCTAGAGCGCATGGACTT
     V  N  R  I  I  K  Q  E  P  V  L  E  N  C  S  Q  E  L  V  Q
1021 TCCCTTATCCCATCCACCGGTCTCTGCTCGCCTGATCTGGTGAATCGGATCATCAAGCAA
     H  Q  A  D  L  T  C  T  T  T  L  D  L  T  D  G  T  I  T  F
1081 GAACCAGTTCTTGAGAACTGCAGCCAGGAACTTGTACAGCACCAGGCAGACCTGACATGT
     T  N  N  L  G  T  M  P  E  S  S  P  A  Y  S  I  P  R  K  M
1141 ACGACAACTCTGGATCTCACGGACGGTACCATCACCTTTACCAACAACCTCGGCACCATG
     G  S  N  L  E  D  I  L  M  D  D  A  L  S  P  V  G  V  T  D
1201 CCGGAGAGCAGCCCGGCCTACAGCATCCCCAGGAAGATGGGCTCCAACTTGGAAGACATC
     P  L  L  S  S  V  S  P  G  A  S  K  T  S  S  R  R  S  S  M
1261 CTGATGGACGATGCCCTCTCACCTGTTGGAGTCACCGACCCACTGCTGTCATCAGTGTCG
     S  A  E  E  T  E  H  A  C  *
1321 CCAGGAGCTTCAAAAACAAGCAGCCGGAGGAGCAGTATGAGCGCAGAAGAAACGGAGCAT
1381 GCGTGTTAGCGAGCCTGCCTTGCTCTGCCTCTGCACGGACCGCTCCCTCTCTTCTTCAGG
1441 AGACTTTATAATTTACCTGAAGAGGTTTTCTTGATAATTTTCCTTTAATATGAAATTTTC
1501 GTGCTTTATCAGTAGCCCTGCATATATTTTATTTTTAGAATTTTGTGAGCCAGACTTGTA
1561 TATTCTATTTTACAACTACAAAGGCCTCCTAAGTATTGTACCTTCAGCGTGCAGTATCTG
1621 TGAACTGATTTCTCCAAGTGTGAGCTTTCTGAGCAAGGGGATTTTTTTGCTTCAGAGAAG
1681 TAAGTGTCTGTCCGTTTTCATTCAGGGGAAAACTTGGTGTGAGCTTTGTCTGTCTGTGAC
1741 CTCCTTTGAAATTAACATGTAAAGTTTAATTACACGAATGTAAAGCAACCAAAAGAAGAA
1801 AACAAAGAAGATGATGATTATGATAGAAGAAGGAGAAGAGGAGGAAGAGGAAGAGGAGGA
1861 GGAAGAGGAGGAAGAAAGATGGTGGGAAAGACATCCAAGACTTTCTCGCTTTCTAATACG
1921 CG
```

Fig. 1.4. Aligned nucleotide and deduced amino acid sequence of the mouse microphthalmia cDNA (Hodgkinson *et al.*, 1993). The deduced amino acid sequence contains an acidic domain (underlined), a basic domain (boxed and lightly shaded), two helical domains (boxed and darkly shaded) connected by a loop (underlined). The seven leucines, shown in bold, are spaced seven amino acids apart and conform to the prediction of a leucine zipper. The stop codon is marked with an asterisk.

for a more comprehensive understanding of the effect that microphthalmia exerts on vertebrate pigmented tissues.

1.3.1 Alternative splicing of the microphthalmia gene

Perhaps one of the most significant findings about the microphthalmia gene has been that multiple transcripts arise via alternative splicing and the use of alternative promoters. Alternative splicing is an important and widespread mechanism for the generation of protein diversity within many different organisms, from *Drosophila* to human (Breitbart *et al.*, 1987). In contrast to prokaryotes, most protein coding genes of eukaryotes contain the sequences present in the corresponding mature mRNA in discontinuous segments (exons) interspersed among sequences that do not form part of the mature mRNA (introns). Thus, often under developmental and/or tissue-specific regulation, the differential incorporation of exons into mature mRNAs allows a single gene to produce a variety of related but distinct proteins (isoforms) without disturbing the original genomic sequence.

Alternatively spliced genes may employ one or more patterns of differential splice usage (Fig. 1.5). Introns are demarcated by invariant consensus sequences at their 5' (donor splice site) and 3' (acceptor splice site) boundaries (reviewed by Andreadis *et al.*, 1987). The splicing pathway consists of cleavage at the splice donor site, formation of a lariat branch point, and cleavage at the acceptor site with concomitant ligation of the 5' and 3' exons. In principle, a primary gene transcript is said to undergo alternative splicing if at least one pair of donor and acceptor sites that are joined in the formation of one mRNA fail to be joined to each other in the formation of another mRNA. The result is at least two mRNAs with different primary sequences. Should that difference involve translated sequence then the two mRNAs will encode distinct protein isoforms.

The genomic structure, including the intron/exon boundaries and splice donor/acceptor sites, of the human (Tassabehji *et al.*, 1994; Udono *et al.*, 2000) and the mouse (Hallsson *et al.*, 2000) microphthalmia genes have been identified. This has advanced the understanding of the alternatively spliced isoforms generated from this locus. Two alternatively spliced products (involving exon 3 and exon 6a) have been repeatedly reported in a number of species and a recent report has identified at least thirteen alternatively spliced isoforms in mice (Hallsson *et al.*, 2000). Skipping of exon 3 has been identified in a number of species including chicken, human, and zebra fish (Mochii *et al.*, 1998a; Yasumoto *et al.*, 1994; Yasumoto *et al.*, 1997a; Lister *et al.*,

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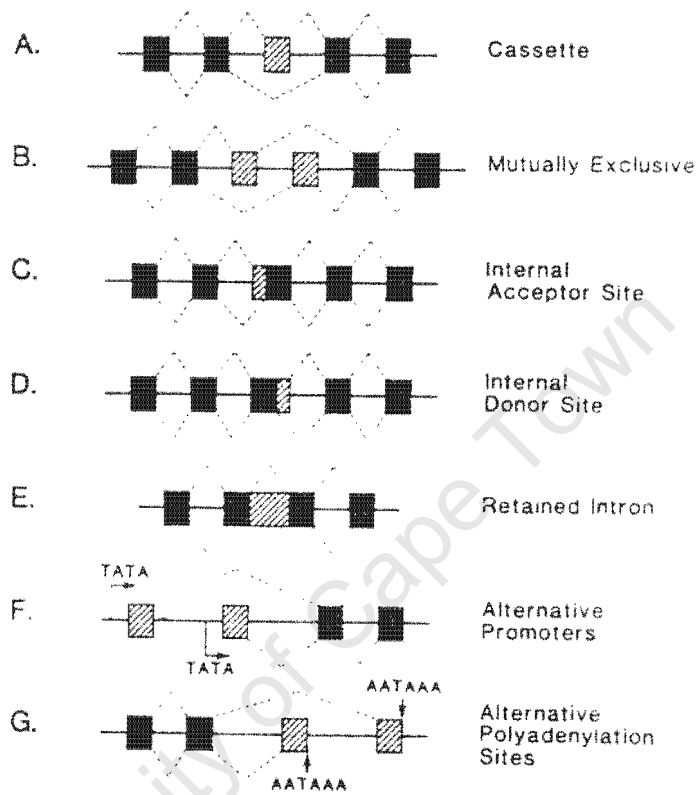

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Fig. 1.5. Patterns of alternative splicing in eukaryotic genes. Constitutive exons (black), alternative sequences (striped) and introns (solid lines) are spliced according to different pathways (dotted lines). Alternative promoters (TATA) and polyadenylation signals (AATAAA) are indicated. (From Breitbart *et al.*, 1987).

1999). Donor/acceptor splice sites are found at the exon 3 boundaries, and the deletion of exon 3 results from alternative splice-acceptor utilisation.

The second deletion, an 18 bp (6 aa) sequence adjacent to the amino-terminal end of the basic region, was reported in the original mouse *Mitf* cDNA (Hodgkinson *et al.*, 1993) and, based on genomic mapping (Tassabehji *et al.*, 1994; Hallsson *et al.*, 2000), is predicted to constitute a deletion of exon 6a. Alternatively spliced products both with and without the insert (commonly referred to as the (+) and (-) forms) are found in many tissues that express microphthalmia (Steingrímsson *et al.*, 1994; Yasumoto *et al.*, 1998). The deletion of exon 6a has been referred to as "one of the most enigmatic features of microphthalmia" (Goding, 2000) and the precise role, if any, of the alternative splice remains unresolved. Lending some insight into the role of the deletion is that it is reminiscent of the mouse mi^{sp} (spotted) mutation. This mutation results in both heterozygotes and homozygotes with no visible phenotype, unless it is combined with another mutation at the locus (reviewed by Hallsson *et al.*, 2000). However, homozygotes present with a reduction in skin tyrosinase activity (Wolfe and Coleman, 1964) and proteins translated from transcripts carrying this deletion are known to bind DNA with a 20% reduced affinity (Hemesath *et al.*, 1994).

1.3.2 Multiple isoforms are generated from the microphthalmia gene: alternative promoters/first exons

In addition to the two "internal" alternative splicing events involving exon 3 and 6a described above, alternative splicing at the 5' terminus of the microphthalmia gene also occurs. Early northern blot analysis suggested that two differently sized transcripts, one exclusively expressed in melanogenic cells (5.5 kb in size) and the other in tissues such as the heart and skeletal muscle (5.7 kb in size), may result from alternative splicing at the 5' end of the mouse microphthalmia gene (Hodgkinson *et al.*, 1993). To explore this further, Steingrímsson *et al.* (1994) used 5'-RACE to analyse the 5' end of the microphthalmia transcripts present in neural crest-derived (melan-c) and non-neural-crest-derived (heart) cells. The melan-c RACE product had a sequence identical to the published mouse cDNA sequence (Hodgkinson *et al.*, 1993), except that it was extended by eight nucleotides at the 5' terminus (Steingrímsson *et al.*, 1994). These additional nucleotides did not, however, change the previously assigned initiation methionine (Met) as an in-frame ATG was not located in the 8 nucleotides. The RACE product from heart RNA was 329 nucleotides longer at the 5' terminus than the RACE product from melan-c RNA. This novel heart sequence was only identical to the melan-c sequence from position 163 onwards of the melanocyte cDNA (Steingrímsson *et al.*, 1994). From the Met that best fits the consensus for

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translation initiation (Cavener and Ray, 1991), this heart-specific 5' end encodes an additional 66 amino acids when compared to the melanocyte-specific 5' terminus. It was at this point that the currently used nomenclature of the microphthalmia gene began to emerge. The melanocyte-type 5' isoform became known as "Mitf-m", and the heart-type 5' isoform as "Mitf-h"; this nomenclature is expanded upon below.

The lack of similarity between the 5' termini of the Mitf-h and Mitf-m isoforms led to the hypothesis that these two proteins are generated from different promoters (Steingrímsson *et al.*, 1994), an idea one step ahead of the research at the time and one which would prove remarkably insightful. Recent studies have confirmed that microphthalmia encodes multiple isoforms by virtue of multiple promoters transcribing more than one first exon. These isoforms are schematically shown in Fig. 1.6, with a schematic representation of the genomic organisation of each detailed in Fig 1.7. For ease of illustration the human isoforms (except for the two mouse mast cell isoforms, MITF-E (Oboki *et* al., 2002) and "Mast cell Mitf (Mitf-mc)" (Takemoto *et* al., 2002) - which have not yet been identified in humans – and cmi9, which was isolated from chicken RPE cells and has also not yet been identified in humans) are shown but the same 5' differences and nomenclature may be applied to the isoforms reported in other species to date. Separate first exons 1mc, 1A, 1C, 1D, 1H, 1E, 1B1a, and 1M which are followed by conserved exons 2-9, encode the different 5' sequences. The 3'-flanking region of each first exon begins with a GT dinucleotide (Udono *et al.*, 2000), which is consistent with a splice donor site and determines that each unique 5' terminus is encoded by a separate first exon. These unique first exons define eight distinct MITF isoforms, commonly referred to as Mitf-mc, MITF-A, MITF-C, MITF-D, MITF-H, MITF-E, MITF-B, and MITF-M. As discussed above, the "-H" and "-M" isoforms are named for the tissues from which they were originally isolated. The same is true for Mitf-mc, isolated from mast cells. The additional nomenclature of "-A, -B, -C and –D, and -E" has presumably evolved following the identification of the "A" isoform by Amae *et al.* (1998). Although the literature at no point explicitly explains this choice, one assumes that the group recognised that multiple isoforms would be isolated in the near future and that not all would be tissue-specific in their expression or function, making it impractical to label isoforms according to the tissue from which the isoform had been isolated.

Thus, the initial simple isolation and cloning of microphthalmia has led to a Pandora's box of complexity. Attempts to understand the function(s) of the different isoforms have necessitated narrow foci by researchers, and the results from individual experiments are only just beginning to emerge as a cohesive picture. Specifically, the complexity of the transcripts generated from the

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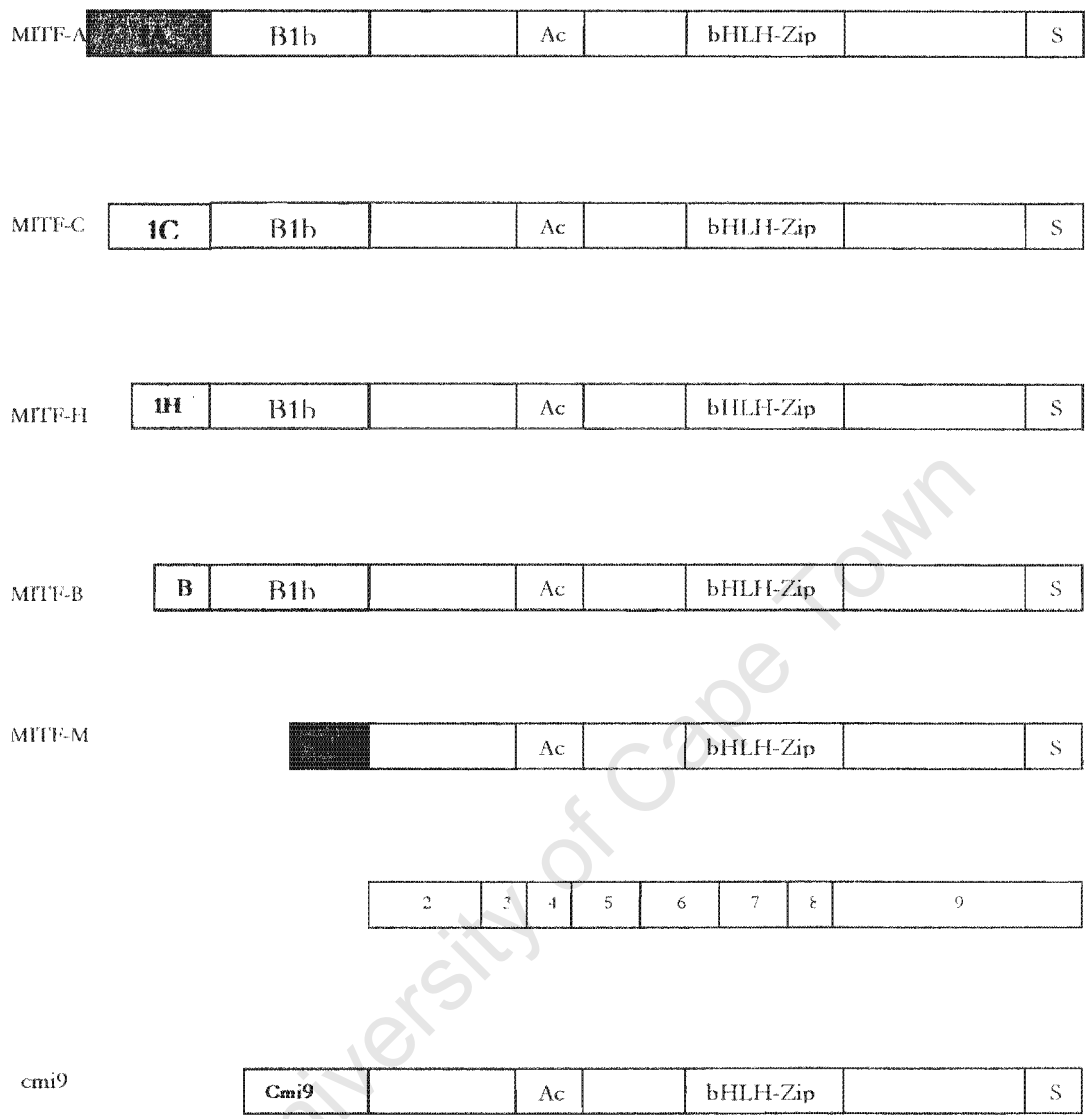

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Fig. 1.6. A schematic diagram of the structures of human MITF isoforms. MITF-A (Amae *et al.*, 1998), MITF-C (Fuse *et al.*, 1999), MITF-H (Amae *et al.*, 1998), MITF-B (Udono *et al.*, 2000), and MITF-M (Tachibana *et al.*, 1994) have different first exons 1A, 1C, 1H, 1B1a, and 1M, respectively. Common exons 2-9 are shown beneath MITF-M. The transcriptional activation domain (Ac) (Sato *et al.*, 1997), the bHLH-Zip domains, and the serine rich region (S) are indicated and are absolutely conserved across all isoforms. cmi9, an avian isoform encoding a unique first exon, has not been identified in mammals.

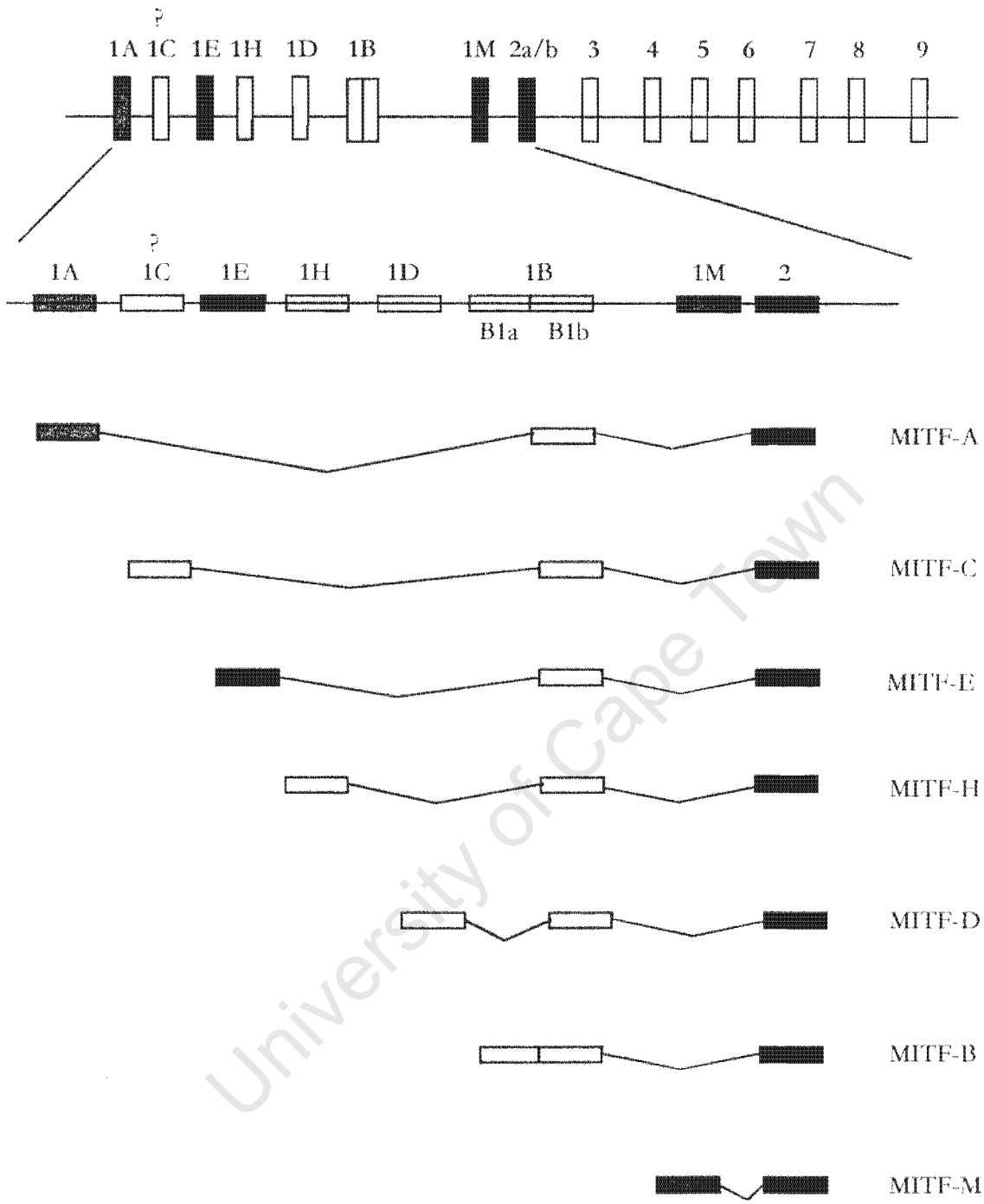

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Fig.1.7. A schematic illustration of the known genomic structure of *MITF/Mitf*. Exons are indicated by boxes and are numbered according to Tassabehji *et al.* (1994), Yasumoto *et al.* (1998), Udono *et al.* (2000), and Hallsson *et al.* (2000). Intron sizes are not drawn to scale but the (mouse) intron sizes are summarised under Table 1.1. The position of exon 1C has not been determined and is therefore indicated with a question mark, but *MITF-C* does contain domain B1b (Fuse *et al.*, 1999). The lower portion of the schematic illustrates alternative use of promoters and splicing patterns of the *MITF* transcripts. The 3' sequence of the 1B exon encoding B1a is consistent with a splice acceptor site and the B1b exon is used as a common second exon when the primary transcripts are initiated from the A, B, C, D, E and H promoters. After Yasumoto *et al.* (1998), Udono *et al.* (2000), and Hallsson *et al.* (2000).

Table 1.1. Exon/Intron boundaries of the mouse microphthalmia gene

Exon	Exon size	Motif	Intron size
1a	≥ 226 bp		unknown
1h	≥ 79 bp		12 kb
1b	250 bp		~20 kb
1m	≥ 171 bp		1.2 kb
2a/b	60/168 bp		1.0 kb
3	84 bp		1.9 kb
4	96 bp	Ac	7.4 kb
5	118 bp		2.1 kb
6a/b	18/57 bp		4.2 kb
7	76 bp	b-HL	3.2 kb
8	148 bp	LH-Zip	3.9 kb
9	396 bp coding	Zip	

Adapted from Hallsson *et al.*, 2000.

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microphthalmia locus leads to two obvious questions for which answers have been sought: (i) do different tissues express different isoforms, and (ii) do the different isoforms have distinct functions?

1.3.2.1 MITF-M is restricted to neural crest-derived melanocytes

MITF-M is perhaps the most well understood isoform. The isoform was isolated from neural crest-derived melanocytes (Hodgkinson *et al.*, 1993; Tachibana *et al.*, 1994) and exists both with and without the alternative 6a exon discussed above. To date, it is the shortest known transcript and is encoded by a unique first melanocyte-type exon, 1M (11 amino acids in length) (Fuse *et al.*, 1996).

Using techniques such as RT-PCR and S1 nuclease mapping analyses, *MITF-M* expression has been reported to be restricted to neural crest-derived melanocytes (Amae *et al.*, 1998; Yasumoto *et al.*, 1998; Fuse *et al.*, 1999). Most significantly, it represents 75% of the total *MITF* mRNAs in normal dermal melanocytes, but it is not expressed in RPE cells (Amae *et al.*, 1998). These results suggest that MITF-M is playing a restricted role in the development and/or function of a specific melanogenic cell type, neural crest-derived melanocytes.

This conjecture has been verified: the insertion of an L1 transposable element in intron 3 of the $Mitf^{mi\text{-}bw}$ murine gene leads to a black-eyed/white coat phenotype. The insertion reduces the amount of *Mitf-a* and *Mitf-h* mRNA by affecting both overall expression levels and pre-mRNA splicing patterns, and completely abolishes the expression of *Mitf-m* mRNA expression (Yajima *et al.*, 1999). The resultant phenotype is normal eye development but a complete loss of neural crest-derived melanocyte populations. That is, the investigators speculate that the normal development of the RPE continues due to the presence of residual full-length Mitf-a protein, but that the lack of Mitf-m results in a loss of the melanocyte population. Taken together, these data provide overwhelming evidence that MITF-M/Mitf-m is absolutely required for the correct development of neural crest-derived melanocytes.

Although there is no question that MITF-M/Mitf-m is essential for the development of neural crest-derived melanocytes, more recent studies have raised some doubt as to whether the expression of the melanocyte-type isoform is absolutely restricted to neural crest-derived melanocyte cell lines. For example, although Takemoto *et al.* (2002) do not find expression of *Mitf-m* in mouse mast cells using RT-PCR and a sense primer specific for the first exon of *Mitf-m*, a second group reports that *Mitf-m* transcripts are detected (by semi-quantitative RT-PCR using a

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sense primer specific for the first exon of *Mitf-m*) in both mouse mast cells and heart tissue (Oboki *et al.*, 2002). In addition, microphthalmia transcripts are detected in young replicating, contact-inhibited, and senescent human fibroblasts using both semi-quantitative RT-PCR and microarray analyses (Semov *et al.*, 2002). Semov *et al.* do not, however, specify which primers were used for PCR amplification of the microphthalmia transcripts and hence it is not known which isoform is being detected.

Thus there are inconsistencies in the literature reported to date for the expression of the melanocyte-type isoform of microphthalmia: some groups report that microphthalmia transcripts (specifically melanocyte-type transcripts) are present in non-neural crest-derived melanocyte cell lines, while other groups do not detect such transcripts in these cells. If melanocyte-type microphthalmia transcripts are indeed present in non-pigmented cells, then at least two key questions arise: Are the transcripts further translated into functional proteins? And if the transcripts are translated into proteins, then what is the function(s) of the so-called melanocyte-type microphthalmia protein in non-pigmented cells? Future work will likely clarify both of these questions.

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1.3.2.2 MITF-A: a role in RPE development?

MITF-A, and its murine counterpart, *Mitf-a*, were isolated from human kidney and mouse kidney cDNA libraries, respectively (Amae *et al.*, 1998). A unique first exon 1A (35 amino acids in length) distinguishes MITF-A from the melanocyte-type isoform (MITF-M). Exon 1A initiates with a Met codon that was presumably identified according to the consensus for a eukaryotic translation initiation site. *MITF-A* lacks the 6 aa insert discussed previously; it is not known whether *Mitf-a* also lacks the insert as only the 5' terminus of *Mitf-a* has been sequenced.

S1 nuclease mapping (Amae *et al.*, 1998) and RT-PCR analyses (Fuse *et al.*, 1999; Udono *et al.*, 2000) have demonstrated that *MITF-A* mRNA is expressed in a large variety of cell types and tissues (including both RPE and normal dermal melanocytes), suggesting that the isoform does not play a tissue-specific role in pigmented cell differentiation or function. However, in human foetal RPE cell lines, *MITF-A* represents 95% of the *MITF* mRNA isoforms tested for using S1 nuclease mapping (Amae *et al.*, 1998). This led the investigators to anticipate a RPE-specific role for the isoform and they explored the functional significance of *Mitf-a* in RPE development using *in situ* hybridisation. The conclusion drawn was that *Mitf-a* expression is limited to the presumptive RPE cell layer and does not extend into the inner layer of the optic cup, which will differentiate into the neural retina (NR). Somewhat confusing, however, is that this *in situ*

expression pattern of *Mitf-a* in murine embryonic eyes was explored using a probe specific for domain B (1b) (Amae *et al.*, 1998; Fig. 1.6). This result, by virtue of the probe used, does not strictly illustrate that *Mitf-a* expression is limited to the RPE, but rather that those microphthalmia isoforms encoding domain B (of which *Mitf-a* is one) are limited to the RPE.

Therefore, the data surrounding the A/a first exon is still far from clear. Although *MITF-A* is the predominant isoform in the RPE, it has not definitively been demonstrated that only the "-A/a" isoform is expressed in the presumptive RPE. All that is known is that the expression of those microphthalmia isoforms encoding domain B are enriched in the presumptive RPE. Despite these shortcomings, it is generally accepted that MITF-A/Mitf-a, now commonly referred to as the RPE-type isoform, is playing a role in the differentiation of the RPE from the optic cup during embryonic development (Amae *et al.*, 1998; reviewed by Goding, 2000). Further investigations will be required to determine an absolute role for the "-A/a" isoform in this function.

1.3.2.3 MITF-E/Mitf-mc: roles in mast cell development?

Very recently, two microphthalmia isoforms have been identified in mouse mast cells. Oboki *et al.* (2002) and Takemoto *et al.* (2002) each identified a novel amino terminus which they have named *MITF-E* and *Mitf-mc*, respectively. *MITF-E* carries 139 bp upstream of exon 1b and is predicted to encode a protein of 474 amino acids. Somewhat confusing, however, is whether the additional 139 bp encode a functional first exon: the authors report that "A single open reading frame of 1422 bp started from the methionine codon at nucleotide 192-194 in exon 1b", but then state that "when transcription was initiated from exon 1e, the exon 1b was used as the second exon." (Oboki *et al.*, 2002). The group does not, however, demonstrate that transcription can be initiated within exon 1e, nor does there appear to be an in-frame initiation Met in the exon. This point aside, semi-quantitative RT-PCR demonstrates that MITF-E transcripts are detected in immature and mature mouse mast cells, but not in B16 melanoma cells or mouse heart tissue, leading the authors to speculate that MITF-E may have a function in the differentiation of mouse mast cells.

Mitf-mc, an additional mouse mast cell microphthalmia isoform (Takemoto *et al.*, 2002), encodes a novel 43 residue amino terminal sequence, contiguous with exon B, that differs from MITF-E. An initiation Met is present in the exon, together with an in-frame upstream stop codon. *Mitf-mc* transcripts are detected using RT-PCR in mast cell lines, but not in NIH 3T3 fibroblasts, mouse heart tissue, B16 melanoma cells, or a macrophage cell line. This tissue-restricted expression,

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together with additional data obtained by the group (see Section 1.5), strongly suggests that *Mitf-mc* is important for the mast cell phenotype.

1.3.2.4 MITF-B, -C, -D and -H are not tissue-specific

The assignment of tissue-specific roles for the -B, -C, -D and -H isoforms of Microphthalmia has not been reported. Whether the N-terminal regions of these isoforms confer distinct properties on each of the proteins, or whether they merely reflect a correct temporal and spatial patterning of microphthalmia through the use of alternative promoters is not known (Goding, 2000). Indeed, it may be both. The different first exons may be translated according to external tissue-specific temporal or spatial cues with the subsequent expression of the different isoforms continuing some aspect of tissue-specificity. However, as this has not been directly tested, these isoforms have not been assigned a "-type" name, and their roles are far from clear. Hence a detailed discussion of these isoforms is beyond the scope of the current discussion. A summary of the key features of the -B, -C, -D, and -H isoforms is presented in Table 1.2 and the reader is referred to references therein for further descriptions of each isoform.

1.3.2.5 cmi9: an avian-specific isoform?

In contrast to the six human and fifteen murine microphthalmia cDNAs, only two chicken microphthalmia cDNA sequences have been reported to date. The first, isolated from chicken embryonic heart (Mochii *et al.*, 1998a), is an assumed chicken homologue of MITF-A/Mitf-a (and is thus here referred to as *cMI-a*/cMI-a). The 5' terminus of the cDNA shows high sequence identity to the mammalian *A/a* cDNA sequences and, most significantly, its predicted protein encodes the same first exon as MITF-A/Mitf-a. However, the expression and function, if any, of this chicken isoform in avian pigmented cells has not been tested.

The second cDNA, *cmi9* (Mochii *et al.*, 1998a), does not have a known mammalian counterpart. Its unique first exon, veritable by the presence of an in-frame stop codon upstream of the ATG initiation codon, has not yet been identified in any other species to date. The *cmi9*-encoded protein is truncated when compared to all but the –M isoform (see Fig. 1.6), and its Met corresponds to codon 53 of MITF-A, codon 52 of MITF-C, and codon 37 of MITF-H.

Mochii *et al.* (1998a) assessed the expression and function of *cmi9* through RT-PCR, *in situ* analysis, and *in vitro* over-expression. RT-PCR demonstrates that the *cmi9*-encoded first exon is exclusively expressed in the RPE of the developing eye. *In situ* experiments confirm this observation. However, as was the case with the mouse *Mitf-a in situ* experiments, the *in situ*

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Table 1.2. Summary of Key Features of the MITF-B, MITF-C, MITF-D, and MITF-H Isoforms

Isoform	Originally isolated from	First exon name/ length	Assigned initiation Met	Protein length	Exon 6a	Expression pattern summary	Reference(s)
MITF-B	human kidney and testis cDNA	B1a 10 aas	Yes	495 aas	✕	In all cell types tested by RT-PCR (Udono *et al.*, 2000)	Udono *et al.* (2000)
MITF-C	human kidney cDNA	1C 34 aas	Yes	519 aas	✕	Not detected by S1 nuclease mapping, but widely detected by RT-PCR; not detected in epidermal melanocytes, but detected in three RPE cell lines (Fuse *et al.*, 1999).	Fuse *et al.* (1999)
MITF-D	human RPE cell lines	1D 25 bp	Yes	468 aas	✕	In RPE and bone marrow-derived cells by RT-PCR (Takeda *et al.*, 2002).	Takeda *et al.* (2002)[1]
MITF-H	mouse heart tissue	1H 19 aas	Yes	504 aas	✕	Problematic and inconsistent; compare S1 nuclease mapping from Amae *et al.* (1998) to that of Udono *et al.* (2000) and RT-PCR results of Fuse *et al.* (1999)	Steingrímsson *et al.* (1994); Amae *et al.* (1998); Yasumoto *et al.* (1998); Fuse *et al.* (1999); Udono *et al.* (2000)

[1] It is noteworthy that, at the amino acid level, MITF-D is equivalent to the chicken cmi9. However, due to differences in the 5'-UTR region – cmi9 is 82 bp, larger than MITF-D (25 bp), and the sequence of the *cmi9* 5'-UTR is divergent from exon 1D and its 5'-flanking region – the authors speculate that it is possible that MITF-D is "not a counterpart of the cmi9-encoded Mitf. Information on the structural organization of the chicken Mitf gene will be required to explore the relationship between the cmi9-encoded Mitf and MITF-D." (Takeda *et al.*, 2002). Due to this uncertainty, and until an absolute homology can be reported, the present study assumes that MITF-D is not the human homologue of cmi9.

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expression patterns reported by Mochii *et al.* (1998a) also make use of a domain B-based probe. Thus this result only confirms the observation discussed above: that those isoforms encoding domain B (or part thereof, as is the case with *cmi9*) may be playing a role in the RPE.

An *in vitro* analysis of cmi9 was also somewhat inconclusive. When RPE cells are infected with RCAS*Mitf* (an avian retroviral vector carrying the *cmi9* coding region; Mochii *et al.*, 1998a), the expression of the pigment cell-specific gene *tyrosinase* is not significantly affected by the over-expression of cmi9, as assessed by northern blotting. However, the over-expression of cmi9 up-regulates the expression of *tyrosinase* in RPE cells treated with bFGF, which markedly reduces the expression of the gene in the control cells (Mochii *et al.*, 1998a). Taken together, these results demonstrate that, in RPE cells cultured under normal conditions, cmi9 does not affect the levels of *tyrosinase* gene expression. However, in the presence of a factor (bFGF) that reduces *tyrosinase* gene expression in RPE cells, cmi9 is able to "rescue" the expression of the gene, suggesting that it has the potential to transactivate the tyrosinase gene promoter *in vitro*.

The role of cmi9 in the regulation of the tyrosinase gene promoter was further (indirectly) investigated using transfection assays. Fuse *et al.* (1999) transfected HeLa cells with an amino-terminally truncated human MITF mutant (MITF-ΔN), which carries an initiation Met at the same location as cmi9. Western blot analysis of the transfected cell cultures show that *MITF-ΔN* is only weakly expressed in the non-pigmented cell line. However, when the HeLa cells are co-transfected with *MITF-ΔN* and the full-length human tyrosinase gene promoter and assessed for promoter reporter function, MITF-ΔN is able to transactivate the promoter as efficiently as any other MITF isoform tested (Fuse *et al.*, 1999). The authors do not, however, make any speculation about what these findings imply for the function of MITF-ΔN or cmi9.

In summary, cmi9 appears to be an avian-specific Microphthalmia isoform. Its amino terminal domain is exclusively expressed in the RPE of the developing chicken eye, but it not clear, as in the developing mouse eye, whether this merely reflects a possible function for the B domain. cmi9 does not appear to up-regulate endogenous *tyrosinase* gene expression in cultured RPE cells, unless the cells are also cultured in the presence of a factor which will inhibit *tyrosinase* expression. That is, cmi9 only appears capable of rescuing the expression of the pigment cell-specific gene in cultured cells. However, transient transfections of a truncated human isoform with homology to cmi9 demonstrate that, although the expression of the protein is low, the isoform is able to transactivate the human tyrosinase gene promoter in non-pigmented cells.

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Thus the precise function, if any, of cmi9 in (avian) melanogenesis is far from clear, and further investigations into the role of this avian-specific isoform are warranted.

1.4 Microphthalmia transactivates the tyrosinase gene promoter *in vitro*: target elements in the promoter

As detailed in section 1.1, tyrosinase catalyses the rate-limiting step in melanin biosynthesis. Given this eminent role, the 5' flanking region of the *tyrosinase* gene has been extensively analysed for regulatory elements (Ferguson and Kidson, 1997); a schematic of the elements conserved between the human, mouse and chicken tyrosinase promoters is presented in Fig. 1.8. The conservation of elements such as the TDE, M-box, Inr, and Sp1 binding site suggests important functional roles for each. Of specific interest to the current discussion is the functional role(s) of the TDE, the M-box, and the Inr.

Although the locations and precise sequences of the tyrosinase distal element (TDE), the M-box, and the Inr differ across species, all three elements contain a core hexamer sequence, CANNTG, termed the E-box. Because the E-box motif is the consensus sequence of the binding site of the bHLH-Zip family of transcription factors (reviewed by López, 1995), and is recognised and bound with high affinity by at least twenty-four members of the family (Anthony-Cahill *et al.*, 1992), it seems likely that the motif will also be recognised by the product of the microphthalmia locus.

The first report of the transactivation of the human tyrosinase gene promoter by the mouse Mitf protein began to confirm this hypothesis. Using transient transfection assays, Bentley *et al.* (1994) demonstrated that the Mitf protein was able to increase tyrosinase transcription by up to 14-fold in pigmented B16 melanoma cells. The group next analysed which promoter element was being targeted by the protein. Linker-scanner mutations of the CATGTG E-box sequence of the M-box in an otherwise intact promoter reduced the promoter activity by approximately three-fold. This suggests that the M-box sequence, although required for full activation, is not crucial. In contrast, *no* activity was observed when the Mitf protein was co-transfected with a promoter mutated at the E-box CATGTG sequence of the Inr motif. This suggests that the E-box sequence at the tyrosinase promoter Inr is essential for transactivation by the Mitf protein.

Similar observations have been made with the mouse tyrosinase gene promoter. Ganss *et al.* (1994a) demonstrate that mutations introduced into the central E-box motif of the M-box

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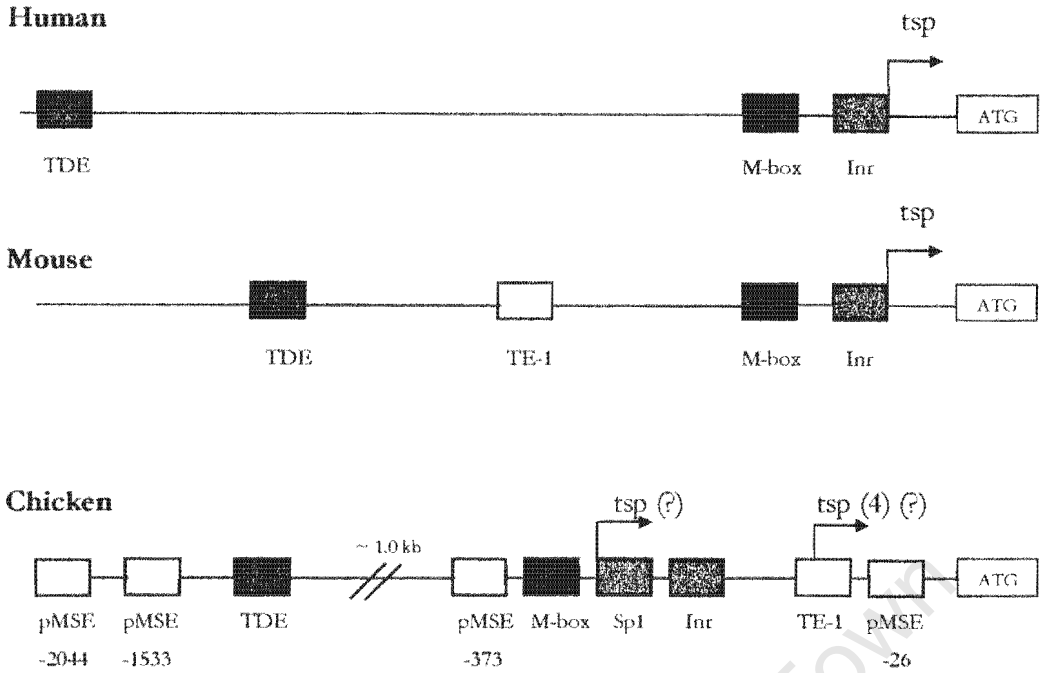

Fig. 1.8. Schematic representation of the conserved elements in the human, mouse, and chicken tyrosinase gene promoters. Adapted from Ferguson and Kidson (1996; 1997).

pMSE: These are M-box like elements, termed pMSE by Yamamoto *et al.* (1992), and have not been identified in mammalian reporters to date.
TDE: The tyrosinase distal element contains an E-box motif at its centre and mutation analysis demonstrated that it possesses protein binding ability and pigment cell-specific activity in the expression of the human *tyrosinase* gene (Shibata *et al.*, 1992).
M box: an 11-bp motif containing an E-box motif at its centre. In the chicken promoter the M-box is situated slightly further upstream than the human or mouse M-box and is not strictly conserved over its 11 bp.
Sp1 binding site: an element important for the initiation of *tyrosinase* transcription by Sp1, a general eukaryotic transcription factor involved in the initiation of transcription by RNA polymerase II.
Inr: This element serves to direct accurate transcription initiation by RNA polymerase II in the absence of a TATA box (reviewed by Smale, 1997).
TE-1: a putative melanocyte-specific enhancer element (Ponnazhagen and Kwon, 1992). TE-1 shares sequence similarity with the human 39 bp TDE, but was unable to compete with the human enhancer for binding of human melanoma nuclear proteins in gel mobility shift assays (Yasumoto *et al.*, 1994).
Transcription start point (tsp): The 5' ends of the mammalian *tyrosinase* mRNAs have been mapped using primer extension and RNAse protection (Ruppert *et* al., 1988; Yamamoto *et* al., 1989; Kikuchi *et* al., 1989; Giebel *et* al., 1991; Ponnazhagan *et* al., 1994). Each independent analysis revealed several (two to four) transcription initiation sites, with a common site positioned at 80 nt upstream from the ATG translation start site. Five potential transcription start sites have been identified in the chicken tyrosinase promoter at nt positions -62, -71, -143, -200, and -248 (Ferguson, PhD Thesis, 1996). Most interestingly, different potential transcription start points were obtained for eye- versus skin-derived *tyrosinase* transcripts. Primer extension products were obtained at the three most poximal sites in skin-derived transcripts, while all five transcription start points were identified in both total eye and RPE-derived transcripts.

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reduce the transactivation potential of Mitf by approximately four-fold. However, in the same year, it was shown that a disruption of the M-box has only a small effect on the activity of the tyrosinase gene promoter in transgenic mice (Ganss *et al.*, 1994b), leading investigators to suggest the importance of the Inr E-box in promoter function. Experiments using different techniques further these observations. In gel mobility shift assays, an oligonucleotide representing a region of the mouse tyrosinase gene promoter in which the E-box motif of the M-box is mutated shows no competition for binding with a GST-MITF fusion protein (Yasumoto *et al.*, 1995). However, the group also reports that the MITF protein transactivates the proximal 82 bp of the mouse tyrosinase promoter, containing only the Inr E-box, as efficiently as a longer promoter construct containing the M-box motif. These transient transfection assays led the researchers to conclude that the M-box motif is not essential for transactivation by MITF, and that the Inr E-box is mainly responsible for transactivation by MITF.

The importance of the E-box motif is further illustrated by investigations into the significance of the human TDE in the transactivation of the tyrosinase gene promoter by MITF. The TDE sequence deviates from the M-box sequence except for the presence of the CATGTG E-box motif. In gel mobility shift assays, a GST-MITF fusion protein forms complexes with a synthetic TDE probe, unless the probe contains an altered E-box sequence (ACTGTG) (Yasumoto *et al.*, 1997a). This indicates that MITF specifically binds the CATGTG motif and that the human TDE element is an additional target for the protein.

Taken together, these results suggest that Microphthalmia, at least *in vitro*, specifically binds the CANNTG E-box. More recently, the precise mechanisms through which Microphthalmia can recognise the E-box element of the tyrosinase gene promoter have come under investigation. The problem that continued to puzzle researchers was how Microphthalmia specifically recognises the tyrosinase promoter. All cells will contain a multitude of transcription factors able to recognise the E-box motif and, equally, multiple promoters within any given cell will contain the sequence in their 5' flanking regions. Since it is unlikely that Microphthalmia will recognise all promoters with E-box motifs, it was postulated that a mechanism must exist which allows Microphthalmia to be specifically recruited to the CATGTG E-box motif of the tyrosinase gene promoter.

An impressively thorough study by Aksan and Goding (1998) has now left little doubt regarding the precise mechanism. Using a combination of band shift assays, mutational analyses, and yeast one-hybrid assays, these authors demonstrate that a single T residue allows the Microphthalmia

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protein to locate its target promoters. The presence of a 5' flanking T residue on either strand at the -4 position of the M-box, the Inr and the TDE (i.e. flanking the CANNTG E-box) is the key determinant in the ability of Microphthalmia to recognise the promoters of melanogenic enzymes. Microphthalmia will bind either TCATGTG, CATGTGA, or preferably TCATGTGA. Replacement of the T residue with either a A, C or G severely inhibits the binding of Microphthalmia to the E box, and this T residue was found to be even more critical *in vivo* than it was *in vitro*. Thus the mechanism by which Microphthalmia is specifically recruited to a subset of E-boxes in target promoters while being prevented from binding to the E-box motifs of other promoters present in the cell was determined. This has enabled accurate predictions to be made as to which E-box motifs are likely to be recognised by Microphthalmia (Aksan and Goding, 1998).

Finally, it should be mentioned that the Microphthalmia protein also transactivates the tyrp-1 promoter (Yasumoto *et al.*, 1995; Bertolotto *et al.*, 1998a) and possibly the Dct promoter (Yokayama *et al.*, 1994; Yasumoto *et al.*, 1997a; Bertolotto *et al.*, 1998a; Udono *et al.*, 2000). However, a detailed discussion of the transactivation of these promoters is beyond the scope of the current review and the reader is referred to references given herein for further information.

1.5 Is there a functional significance (or role) of the different amino termini of microphthalmia?

As discussed to this point of the current review, experimental evidence demonstrates that the mammalian Microphthalmia protein targets and binds the tyrosinase gene promoter through strictly defined elements, at least *in vitro*, thus representing a potentially important regulatory step of melanogenesis *in vivo*. What is less clear is whether the different amino termini of the multiple microphthalmia isoforms play a functional role(s) in this interaction between Microphthalmia and the tyrosinase gene promoter.

At least three microphthlamia isoforms exhibit apparent tissue-restricted expression patterns (as discussed in sections 1.3.2.1 – 1.3.2.3), suggesting that microphthalmia plays a pivotal role in the development and/or maintenance of a diverse number of cell types. Yet does each tissue-specific isoform also exhibit specificity in binding its target promoter(s)? For example, does the melanocyte-type Microphthalmia protein exclusively bind melanocyte-specific gene promoters, such as tyrosinase? Should this be the case, why then does it not transactivate the tyrosinase gene promoter in those unpigmented cells (such as mouse mast cells) that express melanocyte-type

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microphthalmia transcripts? Is it simply that these transcripts are not translated into functional proteins (an intriguing thought in its own right) or is it that the melanocyte-type protein is unable to transactivate the tyrosinase gene promoter in mast cells due to a lack of appropriate partnering proteins, some of which are likely pigment cell-specific?

One approach in beginning to answer these questions, at least *in vitro*, is through co-transfection experiments in which the ability of different Microphthalmia isoforms to transactivate known target promoter(s) is compared. Among the first to compare the transactivation potential of different isoforms using such techniques was Amae *et al.* (1998). The group first performed transient co-transfections of MITF-A and MITF-M with both the human tyrosinase and TRP-1 gene promoters in MeWo melanoma cells. Interestingly, both MITF-A and MITF-M transactivate the tyrosinase gene promoter to the equal levels, but MITF-A increased the activity of the TRP-1 promoter approximately one-fold better than did MITF-M. The authors did not speculate on the significance of this, but it is of interest that MITF-A, an isoform thought to play a key role in the development of the RPE, is able to bind one of its target promoters in MeWo neural crest-derived cells.

Amae *et al.* (1998) next compared the ability of MITF-A, MITF-H, and MITF-M to transactivate the same two promoters (tyrosinase and TRP-1) in an additional melanoma cell line (HMVII). MITF-H does not appear to transactivate either promoter in HMVII cells. However, most intriguing is that although MITF-A and MITF-M both increase the activity of the tyrosinase gene promoter to equal levels, there is also no significant difference (as assessed by standard deviation bars) between the ability of the two microphthalmia constructs to increase the activity of the TRP-1 promoter. Thus MITF-A appears to increase the activity of the TRP-1 promoter to a higher level than MITF-M in MeWo melanoma cells, but not in HMVII melanoma cells. Whether this apparent difference would become less or equalise with a larger number of experiments, or whether it represents a significant functional difference between the two pigmented cell types, is unknown.

One year following the report of Amae *et al.* (1998), Fuse *et al.* (1999) demonstrated, using transient co-transfection assays of MITF-A, -C, -H, and –M with a full length human tyrosinase gene promoter-reporter construct in HeLa cells, that each isoform increases promoter activity equally. The average RLA induced by MITF-M was higher than the average RLA induced by the other isoforms, but the difference does not appear to be very different, as assessed by the mean RLAs ± standard deviation.

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More recently, Takemoto *et al.* (2002) compared the ability of two mouse microphthalmia isoforms (Mitf-mc and Mitf-m) to transactivate a number of promoters: the human tyrosinase gene promoter, the mouse tyrosinase gene promoter, a tandem E-box-containing artificial promoter, and a mast cell gene promoter (MMCP6). In co-transfection assays, Mitf-m is able to drive reporter expression from all four promoters in NIH 3T3 fibroblasts, B16 melanoma cells, and HMC-1 mast cells. In contrast, Mitf-mc is only able to increase the activity of the MMCP6 promoter, although it does so in all three cell types. The increase in MMCP6 activity obtained with co-transfection of Mitf-mc and Mitf-m are approximately equal in the three cell types. Thus Mitf-m appears capable of transactivating both pigment cell-specific and non-pigmented cell-specific target promoters in a number of different cell types. On the other hand, Mitf-mc is only able to increase the activity of a known mast cell promoter, although it is able to do so in both mast cells and non-mast cells.

Amongst the most intriguing of these results is the fact that the Mitf-m isoform is able to transactivate the mast cell MMCP6 promoter. As Takemoto *et al.* note, "The DNA sequences in (these) melanocyte-restricted promoters required for Mitf recognition have recently been more stringently defined, and are composed of the hexameric core, CATGTG, flanked by a highly conserved 5' T and/or 3' A. Mast cell promoter recognition sites, however, differ. The stringently defined melanocyte binding sites are rarely found within Mitf mast cell target gene promoters. The single known exception is the α-melanocyte-stimulating hormone receptor." If MMCP6 therefore does not contain the known required binding sites for Mitf, how are both Mitf-mc and Mitf-m able to transactivate its promoter? Takemoto *et al.* speculate that these results suggest that those promoter elements through which Mitf transactivates its target genes are distinct between cell types. However, it is not clear why an apparently melanocyte-specific Microphthalmia protein is able to transactivate a mast cell promoter, yet the apparently mast cell-specific Microphthalmia protein is unable to transactivate a pigmented cell promoter, despite being able to bind the DNA elements in the tyrosinase gene promoter (as assessed with gel shift analyses; Takemoto *et al.*, 2002).

Thus an answer to the question of whether apparently tissue-specific microphthalmia isoforms exhibit specificity in binding target promoter(s) is still far from resolved. The present study investigates this question further by comparing the ability of two chicken microphthalmia (cMI) isoforms to transactivate the chicken tyrosinase gene promoter (which is further discussed below) in pigmented and non-pigmented cells. Finally, it is indeed possible that the different

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proteins do not have any functional significance(s) but may have arisen as a consequence of the different developmental histories of each tissue-specific isoform.

1.6 The chicken tyrosinase gene promoter: targets for Microphthalmia

The 5' flanking region of the chicken *tyrosinase* gene has been cloned, sequenced and functionally assessed by transient transfections/promoter reporter assays (Ferguson and Kidson, 1996). An alignment of the chicken sequence with those of the human and mouse *tyrosinase* genes reveal evolutionarily conserved regions, and these are schematically shown in Fig. 1.8. As discussed above, it is known that the mammalian Microphthalmia protein targets and binds to the conserved TDE, M-box, and Inr motifs of the mammalian tyrosinase gene promoter. In contrast, there is currently no information regarding which chicken tyrosinase gene promoter elements, if any, are targets for the chicken Microphthalmia (cMI) protein.

In addition to the previously discussed TDE, M-box, and Inr motifs, the chicken tyrosinase gene promoter contains four further motifs that may play a role in the transactivation by the cMI protein. These M-box-like elements were termed p-MSE by Yamamoto *et al.* (1992), who identified two short transcriptional elements homologous to each other in the promoter regions of the mouse *b* (brown) and *c* (albino) loci. One p-MSE element has also been identified in the human tyrosinase gene promoter (Kikuchi *et al.*, 1989). The mouse and human p-MSE elements correspond exactly to what are now known as the M-box element (Lowings *et* al., 1992) in each promoter (Fig. 1.8).

Together with the chicken p-MSE element corresponding to the M-box at position –310, four additional p-MSE elements are located at positions –26, -373, -1533, and –2044 of the chicken tyrosinase gene promoter (Fig. 1.8). Two p-MSE elements were also identified in the quail tyrosinase gene promoter (Akiyama *et al.*, 1994); the chicken p-MSE elements at –26 and –373 are the homologues of these quail p-MSE elements. Transient transfections using promoter deletion constructs demonstrate that at least one of the quail p-MSE elements (corresponding to position –373 in the chicken tyrosinase gene promoter) is important for the pigment cell-specific activity of the quail promoter (Akiyama *et al.*, 1994). However, the functional significance of the avian p-MSE elements for the transactivation by the cMI protein has not been reported.

Thus, using the CANNTG E-box motif as the criteria (as discussed in section 1.4), there are six elements in the chicken tyrosinase gene promoter that are potential targets for the cMI protein. These are as follows (E-box motif underlined in each):

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p-MSE (position –2044): GGCACATGAGG
p-MSE (position –1533): TCTCATGTGTA
TDE (position –1195): GGTCATGTGAT
p-MSE (position –373): AGCCAACTGCT
M-box (position –310): AATCATGTGAT
Inr (position –209): AACATGTGAT

The most proximal p-MSE (AGCATAACACT; position –26) does not contain a central E-box motif and is therefore not predicted to be a target for the cMI protein. Taken one step further, an assessment of the motifs based on the presence of a flanking T residue on either strand at the –4 position (as detailed in section 1.4) predicts the following as potential targets for the cMI protein (relevant flanking residue(s) printed in bold in each sequence):

p-MSE (position –2044): GGCACATGAGG
p-MSE (position –1533): TCTCATGTGTA
TDE (position –1195): GGTCATGTGAT
M-box (position –310): AATCATGTGAT
Inr (position –209): AACATGTGAT

That is, although the p-MSE at position -373 is, based on the quail work discussed above, predicted to be important for the pigment cell-specific activity of the promoter, it does not contain a flanking T residue and is therefore not predicted to be a target for the cMI protein.

In summary, the chicken tyrosinase gene promoter contains five potential targets for the cMI protein (TDE, M-box, Inr, and two of the apparently species-specific p-MSE elements). However, a functional assessment of this prediction has not been reported. In the present study, the possible importance of these elements for the transactivation of the promoter by two cMI isoforms is investigated through transient co-transfection assays and promoter deletion constructs.

1.7 Summary and general & specific aims of study

Since the first identification of tyrosine and tyrosinase in the mushroom, significant progress has been made in understanding the biochemical and molecular regulation of melanogenesis. By

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virtue of its rate-limiting role in the melanogenic pathway, the *tyrosinase* gene represents an important regulatory step in the production of melanin. Equally, the products of the microphthalmia locus, as potential "master regulators" of the *tyrosinase* gene, are providing new insight into the development and mature function of melanogenic cells.

During the course of this review of *microphthalmia*/Microphthlamia and the tyrosinase gene promoter, at least three important questions have surfaced: **Firstly**, are some of the microphthalmia isoforms (such as the M/m-, A/a-, and E/mc-encoded isoforms) truly tissue-specific/restricted in their expression pattern(s) and/or function(s)? The latest experimental evidence surrounding this issue has given investigators pause for thought. For example, the melanocyte-type isoform has been extensively analysed on the premise that it has a restricted functional role in neural crest-derived melanocytes. While there is still no question that the M/m isoform plays a pivotal role in neural crest-derived melanocytes, recent reports indicate that the isoform is also detected in non-pigmented cell types, at least at the transcriptional level. This then raises the question of whether melanocyte-type microphthalmia transcripts are translated into functional proteins in non-pigmented cells and, if so, what function(s) do they have? In addition, the presence of apparently tissue-specific transcripts has only been reported to a significant level in mammals; it is not known if the same will hold true in other vertebrates. For example, is the one previously characterised chicken isoform (cmi9; Mochii *et al.*, 1998) truly restricted - both in expression and function - to the developing RPE? Are there chicken melanocyte-type and/or mast cell-type isoforms of microphthalmia that exhibit restricted expression patterns? Further work is clearly required to answer questions such as these.

A **second** major topic that has surfaced is the role of Microphthalmia as a master regulator of tyrosinase gene expression. *In vitro* studies have been undertaken, exclusively in mammals, to determine the interaction of Microphthalmia with the tyrosinase gene promoter. Those mammalian tyrosinase promoter elements that are targets for the Microphthalmia protein have been stringently defined, and require a 5' T and/or 3' A residue flanking the core sequence CATGTG. Three such elements, the TDE, the M-box and the Inr, are conserved in the chicken tyrosinase gene promoter, together with two further (apparently avian-specific) elements, termed p-MSEs, that are also predicted to be targets for the Microphthalmia protein. These predictions regarding the chicken tyrosinase gene promoter elements have not, however, been experimentally tested. Due to the species-specific target elements, it is possible that the transactivation of the tyrosinase gene promoter by the Microphthalmia protein will be subject to different regulation in mammals and avians.

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A **third** question is whether the different amino termini-spliced microphthalmia isoforms have functional significance(s) in terms of regulating the development and/or maintenance of a number of cell types. For example, do the apparently tissue-specific isoforms also exhibit specificity in binding their target promoter(s)? This question has been addressed to a lesser extent than the previous two questions (although still exclusively in mammals), and the results of experiments performed to date are far from clear. It is still not known whether the different amino termini of the microphthalmia isoforms are important in regulating the ability of the resulting different proteins to transactivate their downstream targets. Most relevant to the present study is the question of how different Microphthalmia proteins interact with the pigment cell-specific tyrosinase gene promoter. Specifically, it is of interest to study the transactivation of the chicken tyrosinase gene promoter by different isoforms of the chicken Microphthalmia protein, because there have been no known reports of this interaction in lower vertebrates and it is again possible that the mechanism(s) will differ between mammals and avians.

This burgeoning, intriguing possibility - that chicken and mammalian Microphthalmia genes/proteins may differ in expression and function - has its foundation in the one report of the characterisation of a full-length chicken microphthalmia isoform (cmi9; Mochii *et al.*, 1998a). As discussed in Section 1.3.2.5, the evidence surrounding the ability (or lack thereof) of the cmi9 protein to transactivate the endogenous tyrosinase gene promoter in cultured cells is open to consideration. If cmi9 is unable to increase the activity of the endogenous tyrosinase gene in cultured cells, as demonstrated by Mochii *et al.* (1998a), then is this indicative of a significant difference between the chicken and mammalian Microphthalmia proteins? Or might this simply reflect a difference in the experimental approaches taken to date? That is, will *in vitro* co-transfection of cmi9 with the chicken tyrosinase gene promoter reveal results more similar to those reported with *in vitro* co-transfection assaays using the mammalian microphthalmia gene and tyrosinase promoter?

In addition, an interesting difference between mammals and chickens is notable in the expression of the microphthalmia gene in the RPE: In the mammalian RPE, the MITF-A/Mitf-a isoform represents 95% of the *MITF* mRNA in the cells (Amae *et al.*, 1998). On the other hand, a different isoform (cmi9) is enriched in the developing chicken RPE (Mochii *et al.*, 1998a). Taken one step further, a chicken cDNA encoding the same first exon as MITT-A/Mitf-a has been identified (Mochii *et al.*, 1998a), yet a mammalian cDNA encoding the same first exon as cmi9 has not. This suggests that mammalian and chicken RPE cells differ in terms of their

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microphthalmia isoform expression profile. This in turn raises the possibility that the same will be true in the second major population of vertebrate pigmented cells: neural crest-derived melanocytes. That is, it is possible that chicken neural crest-derived melanocytes will express a different major microphthalmia isoform(s) than that of mammalian neural crest-derived melancoytes.

Consequently, several questions present themselves: (1) do developing chicken neural crest-derived melanocytes express the melanocyte-type microphthalmia isoform, as do mammalian neural crest-derived melanocytes?; (2) if the M/m isoform is expressed in chicken neural crest-derived melanocytes, does its protein transactivate the tyrosinase gene promoter in the same manner as the mammalian M/m protein?; (3) is cmi9 the predominant/only isoform in the chicken RPE?; (4) can cmi9 upregulate the expression of the chicken tyrosinase gene promoter under "normal" conditions, or does it truly only "rescue" the promoter, as a previous report suggests (Mochii *et al.*, 1998a)?; and (5) are there functional difference(s) between chicken microphthalmia isoforms isolated from pigmented cells, such as their ability to transactivate a pigment-specific gene promoter?

As a start to answering some of these questions, the **broad aims** of the present study are three-fold: (i) to further investigate the regulation of melanogenesis in chickens because, despite the fact that chicken embryos are a model system for developmental biologists, our knowledge of the regulation of avian melanogenesis is sparse; (ii) to clone, sequence, and characterise the expression pattern of a/the chicken microphthalmia isoform present in neural crest-derived melanocytes, and to compare its expression pattern to that of the previously described cmi9 isoform; and (iii) to carry out a comparative characterisation of the chicken neural crest-derived melanocyte cell-type Microphthalmia protein with the previously described chicken Microphthalmia cmi9 protein by investigating the ability of each to transactivate the chicken tyrosinase gene promoter.

The **specific aims** of this study are:

1. To clone, characterise, and sequence a cDNA obtained from a chicken neural crest-derived melanocyte cDNA library;
2. To compare the nucleotide and deduced amino acid sequence of the isolated chicken *cMI* cDNA with other reported microphthalmia cDNAs, thus determining which microphthalmia isoform it encodes;

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3. To ascertain the general genomic structure and embryonic expression patterns of the chicken microphthalmia gene;
4. To compare the expression patterns of two chicken microphthalmia isoforms (*cMI-m* and *cmi9*) in pigmented and non-pigmented tissues; and
5. To compare the activity and function of cMI-m and cmi9 through co-transfections with chicken tyrosinase promoter-reporter constructs in pigmented and non-pigmented cells.

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Chapter 2 | Materials and Methods

Some of the experiments described in this thesis were performed at The University of Cape Town (hereafter referred to as UCT), Cape Town, South Africa, while others were performed at The Johns Hopkins University Medical Institutions (hereafter referred to as JHMI), Baltimore, United States of America. Those protocols or reagents which differ between the two institutions within a given experimental procedure are noted in the materials and methods described herein.

2.1 Chicken Embryos

The three chicken breeds used in the present study have the following pigmentary genotypes:

i) Black Australorp: *E/E i+/i+ C+/C+ pk+/pk+*.
This breed has a pure black plumage and pigmented eyes.

ii) White Plymouth Rock x Pile Game: *E/? I/? C+/c pk+/pk+*.
This cross breed has a pure white plumage but pigmented eyes.

iii) White Leghorn: *E/? I/I C+/c pk+/pk+*
This breed has a pure white plumage but pigmented eyes.

The *E (Extended Black)* locus is believed to be primarily involved in black-red melanin distribution in chicken feathers. The *I (Dominant white)* locus is responsible for the completely white plumage of many commercial breeds of fowl, including White Leghorns and Pile Games (Smyth, 1990). The *c (recessive white)* locus is inherited as a recessive mutation and results in the most common form of white plumage (Brumbaugh *et al.*, 1983; Smyth, 1990). The *pink eye (pk)* locus results in a dilution of black melanin, resulting in grey plumage and pink eyes (Warren, 1940).

Fertilised Black Australorp and White Plymouth Rock x Pile Game chicken eggs were obtained from the Animal Unit, UCT, Cape Town, South Africa. Fertilised White Leghorn chicken eggs were commercially obtained from The Charles River Laboratories, SPAFAS Avian Products and Services, Connecticut, United States of America (standard grade eggs tested for 23 pathogens). All fertilised eggs were incubated at 37°C in a humidified (50-60%) incubator (at UCT) or a 37°C warm room humidified using multiple buckets of water (at JHMI), and staged according to Hamburger and Hamilton (1951) prior to use.

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2.2 Isolation of clone M156 from a chicken melanocyte cDNA library

The starting material for the present study was clone M156, a chicken *cMI* cDNA isolated from a previously described Black Australorp x New Hampshire Red chicken neural crest-derived melanocyte cDNA library (April *et al.*, 1996; April, Ph.D. Thesis, 1998). Briefly, April *et al.* (1996) cultured chicken neural crest cells in Hams F-12 medium supplemented with 20% foetal calf serum (FCS), 32 nM TPA and 10 ng/ml bFGF (the addition of which was found to allow for the continued proliferation and survival of pigmented melanocytes beyond three months). Following three months in culture, RNA was extracted from approximately 10^8 cells, 70% of which were visibly pigmented. A cDNA library was next constructed in Lambda ZAPII from approximately 10 µg chicken melanocyte poly (A+) RNA. The chicken neural crest-derived melanocyte cDNA library was screened with a 1.3 kb mouse *Mitf* cDNA probe, carrying the entire coding region of the mouse *Mitf* gene (Yavuzer *et al.*, 1995). 11 positively hybridising isolates were cored and stored at +4°C until *in vivo* excision. To facilitate manipulation, the cDNA inserts were "subcloned" into pBluescript SK- by *in vivo* excision, using ExAssist helper phage and XL1-Blue cells, according to manufacturer's instructions (Stratagene). Colonies were rescued after selection on bacterial plates containing ampicillin, yielding one final positively hybridising clone (clone M156).

2.3 DNA sequencing and deduced amino acid analysis

Caesium-chloride purified cDNAs were prepared according to standard procedures (Sambrook *et al.*, 1989) and sequenced either manually or using automated sequencing technology. Manual sequencing utilised the dideoxy chain termination method (Sanger *et al.*, 1977) and either a Sequenase Kit (USB) or the cycle sequencing-based SequiTherm EXCEL II Kit (Epicentre Technologies) according to manufacturer's instructions. When utilising the Sequenase Kit, plasmid DNA (5 µg) was extracted using alkaline lysis (Davis *et al.*, 1986), alkali denatured and radiolabelled using [α^{35}S]-dATP. cDNA sequencing reactions using the SequiTherm kit were carried out according to the manufacturer's instructions and radiolabelled using [α^{32}P]-dCTP. Reactions were then cycle sequenced using a Perkin Elmer 2400 Thermocycler with PCR cycles of 94°C for 1 minute followed by 26 cycles of 94°C for 15 seconds, 50°C for 30 seconds, 72°C for 1.5 minutes and a final elongation for 1 minute at 72°C. Each manual sequencing reaction was performed at least twice. Sequenced products were resolved on 6% polyacrylamide/8M urea gels electrophoresed on a Hoeffer Poker Face II sequencing apparatus. Gels were vacuum dried

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at 80°C for 1-1.5 hours and autoradiography was performed using x-ray film at room temperature for 2-3 days.

Additional, automated, sequencing reactions were performed in the Department of Human Genetics, UCT, South Africa, using the ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kit (Perkin Elmer) according to the manufacturer's instructions. Products were separated by automated capillary electrophoresis and analysed by an ABI PRISM Genetic Analyser (Perkin Elmer). The automated sequencing of the 3'-UTR of clone M156 was performed by the Core DNA Sequencing Unit, The University of Stellenbosch, Stellenbosch, South Africa. The automated sequencing of the microphthlamia transcripts detected in chicken embryonic fibroblasts was performed by the DNA Analysis Unit at JHMI, Baltimore, United States of America.

The primers used for sequencing in the present study are described under Table 2.1. Universal vector primers (M13F and M13R) were commercially obtained (Boehringer Mannheim). Custom internal primers were designed using the PRIME function of Genetics Computer Group (GCG), version 9.0 (GCG sequence analysis software package, GCG Inc., Madison, WI, USA) and were synthesised by the Department of Medical Biochemistry, The University of Cape Town, South Africa, or the Department of Molecular and Cellular Biology, The University of Cape Town, South Africa, or commercially generated by GenoSys (Europe) Inc. (through Whitehead Scientific, Cape Town, South Africa).

DNA and deduced amino acid sequence analyses were performed using the Genetics Computer Group (GCG) program, version 9.0 (GCG sequence analysis software package, GCG, Inc., Madison, WI, USA), GenBank and EMBL databases. The Swiss-Prot and Prosite databases were used to identify protein patterns and motifs in the deduced protein sequences.

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2.4 Gene constructs and cloning

In the present study four chicken *cMI* cDNA constructs were generated:

1. clone *cMI*-1.1, containing most of the chicken *cMI* coding region;
2. clone *cMI-m*, containing the full-length chicken melanocyte-type *cMI* coding region;
3. RCAS BP(A)/*cMI-m*, carrying the chicken *cMI-m* coding region in the correct orientation relative to the genome of the retroviral vector;
4. RCAS BP(A)/*cMI-mr*, carrying the chicken *cMI-m* coding region in the reverse orientation relative to the genome of the retroviral vector.

Table 2.1. Primers used in the present study

Primer	mer	Sequence
M13F (UF)	17	5' GTTTTCCCAGTCACGAC 3'
M13R (UR)	19	5' GGAAACAGCTATGACCATG 3'
SF	20	5' CTCGGAAATGTGACTGAACC 3'
SR	22	5' GCAGTTTATGAAAGGCGTGCCA 3'
cMIF2	22	5' ATGTGACTGAACCAACTGGCAC 3'
cMIF3	21	5' GTACCTCTCTACCACTCTAGC 3'
cMIF4	19	5' GCCCCTGGTAGAAGCTGAG 3'
cMIF5	28	5' CGATCGATCGATGCTGGAAATGCTTGAG 3'
cMIF6	23	5' GAAGAGTCTGCTTCTATTTGGGG3'
cMIR2	20	5' CGTCCATCAAGATGTCTTCC 3'
cMIR3	19	5' AGCTCAGGGCTTGGTTGGC 3'
cMIR4	29	5' CGATCGATCGCTAACAAGCATGATCAGTG 3'
TRP-2F4	23	5' GACTACCATCCACCCAGACTATG 3'
TRP-2R3	22	5' GTGATAGGATAAGGGAGAATGC 3'
ctyr-F1	20	5' TGATGAATACATGGGTGGCC 3'
ctyr-R1	20	5' CAGCAGTAATTATGCCTCCG 3'

Commonly used primer pairs

Primer pair	Amplifies
cMIF3/cMIR2	The bHLH-Zip domains common to all microphthalmia isoforms.
cMIF5/cMIR2	The unique first exon of *cMI-m* and portions of coding region.
cMIF4/cMIR3	The unique first exon of *cmi9* and portions of coding region.

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2.4.1 Cloning of *cMI*-1.1

Clone M156 was mapped by digestion with various restriction enzymes (see Results, section 3.1) and the products resolved on a 0.8% agarose gel. The gel was subjected to plasmid Southern blot hybridisation as described in section 2.5 using the mouse *Mitf* cDNA probe. A 1.1 kb *Bam*HI fragment of clone M156 that consistently hybridised to the mouse *Mitf* cDNA probe was isolated by gel purification using Gene Clean (Gene Clean II Kit, Bio 101 Inc.) and subcloned into pBluescript SK- digested with *Bam*HI. The resulting clone was called *cMI*-1.1 for chicken Microphthalmia - 1.1 kb in length. Clone *cMI*-1.1 was sequenced manually using a combination of universal vector and custom-designed primers and was found to carry the 5' portion (nts 1-1042) of the chicken microphthalmia gene.

2.4.2 Cloning of *cMI-m*

To generate a construct containing the full-length coding region of clone M156, the 1.1 kb *Bam*HI fragment from *cMI*-1.1 was ligated to a PCR fragment of the 3' portion of the coding region. Briefly, primers SF and SR (Table 2.1) were used to amplify a 230 bp fragment (nts 992-1222) of the coding region of clone M156. The 230 bp fragment was A-tailed and ligated with pGEM-T Easy according to manufacturer's instructions (Promega Corporation). This construct was digested with *Bam*H1 and ligated with the gel-purified *Bam*H1 fragment of clone *cMI*-1.1. The resulting clone was called *cMI*-m for chicken microphthalmia - melanocyte type. Automated sequencing confirmed the presence of the full-length coding region in clone *cMI-m*.

2.4.3 Cloning of the full-length chicken *cMI-m* coding region into RCAS BP(A)

The RCAS BP(A) vector was a kind gift of Dr. Stephen Hughes (National Cancer Institute, Frederick Cancer Research and Development Centre, Frederick, Maryland, U.S.A.). RCAS BP(A) is an avian retroviral vector of the envelope subgroup (A); the reader is referred to http://home.ncifcrf.gov/hivdrp/RCAS/ for a thorough review of the RCAS vectors.

RCAS BP(A) was digested with *Cla*I and an aliquot analysed by gel electrophoresis to confirm that complete digestion had taken place. The 5' *Cla*I sticky-ends were blunt-ended using Klenow polymerase (Promega) and 5' phosphates removed using Calf Intestinal Phosphatase (Promega). Next, clone *cMI-m* was digested with *Eco*RI (present on both sides of the polycloning site of pGEM-T Easy) to release the full-length coding region, which was purified using electroelution. The resulting *Eco*RI sticky-ends product was blunt-ended using Klenow polymerase (Promega)

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and ligated with the blunt-ended RCAS BP(A). A *Kpn*I restriction enzyme digest confirmed the orientation of the insert relative to the retroviral genome. The resulting clone was named RCAS BP(A)/*cMI-m* for Replication-Competent retroviral vector, ASLV LTRs, Splice acceptor, Bryan *pol* and subgroup (A) envelope gene/chicken microphthalmia - melanocyte-type.

The insert was also blunt-end ligated into RCAS BP(A) in a reverse orientation relative to the retroviral genome. This clone served as a control for both translation of the viral genome, as well as non-sense translation of the *cMI-m* insert, in transfected cells. Strategies used were identical to those described for the generation of clone RCAS BP(A)/*cMI-m*. The resulting clone was called RCAS BP(A)/*cMI-mr* for Replication-Competent retroviral vector, ASLV LTRs, Splice acceptor, Bryan *pol* and subgroup (A) envelope gene /chicken microphthalmia - melanocyte-type, reverse orientation.

2.4.4 RCAS/*cmi9*

A chicken cDNA (*cmi9*) encoding a unique amino terminus, cloned into RCAS BP(A) (Mochii *et al.*, 1998a) was a kind gift of Dr. M. Mochii (Himeji Institute of Technology, Faculty of Science, Hyogo, Japan). This construct is here referred to as RCAS/*cmi9* for RCAS/chicken Microphthalmia - *cmi9*-encoded isoform.

2.4.5 Control and tyrosinase promoter constructs used in *in vitro* transfection assays

The following constructs were used as controls in transfection assays:

1. pGL2-control, a positive control vector that contains the firefly *luciferase*-coding region under the control of the SV40 promoter and enhancer sequences (Promega). In any given experiment, duplicate dishes of cells were transfected with pGL2-control, serving as the standard for transfection efficiency. All other relative *luciferase* activities were expressed as a percentage of that of pGL2-control.
2. pGL2-basic contains the firefly *luciferase*-coding region without any promoter or enhancer sequences (Promega) and serves as a control for the background *luciferase* activity of the pGL2 vector backbone. Although never specifically incorporated into final transfection results, all cell types used in the current study were transfected with this construct to control for the background *luciferase* activity (which in all cases was zero).
3. pRL-CMV is a reporter vector that contains the coding region of the *Renilla* luciferase gene under the control of the CMV promoter and enhancer sequences. pRL-CMV serves as an internal control for transfection efficiency, and all cells in any given experiment were co-

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transfected with pRL-CMV. *Luciferase* activities from other constructs co-transfected with pRL-CMV were normalised with respect to the *Renilla* luciferase activity in the cells.

The following chicken tyrosinase promoter-reporter constructs, cloned by Ferguson (Ferguson and Kidson, 1996; Ferguson, Ph.D. Thesis, 1996), were used in transfection experiments:

1. Tyr2.1-Luc, a vector containing the firefly *luciferase*-coding region under the control of the chicken tyrosinase gene promoter. This construct contains 2.1 kb of the 5' flanking sequence of the chicken *tyrosinase* gene.
2. Deletion constructs Tyr1.1-Luc and Tyr0.5-Luc that contain, respectively, 1.1 kb and 0.5 kb deletion fragments of the 2.1 kb chicken tyrosinase gene promoter.

All three constructs drive expression of the firefly *luciferase* reporter in pGL2-basic. These promoter constructs are schematically shown in Figure 2.1, which also illustrates the promoter elements present in each construct.

2.5 Genomic and plasmid Southern blot hybridisation analysis

Genomic DNA was prepared according to a previously described method (Strauss, 1987) from 10 day-old Black Australorp and White Plymouth Rock x Pile Game chicken embryos. The DNAs were digested with various enzymes as described in Results (section 3.3), separated on 0.7% agarose gels for 12-14 hours, and capillary transferred in 0.4 M NaOH to nylon membranes (Amersham) for 12 hours. Membranes were hybridised to the *Bam*HI 1.1 kb fragment of clone *cMI*-1.1 following [α-^{32}P]dCTP random-prime labelling of the cDNA (Random Primed DNA Labelling Kit (Boehringer Mannheim)).

For Southern blot hybridisations of plasmid cDNAs, samples were digested with various restriction enzymes as described in Results (section 3.1), separated on 0.8% agarose gels, and capillary transferred in 0.4 M NaOH to nylon membranes (Amersham) for 10 hours. Membranes were hybridised to the mouse *Mitf* 1.3 kb cDNA or the *Bam*HI 1.1 kb fragment of clone *cMI*-1.1 following [α-^{32}P]dCTP random-prime labelling of the cDNA (Random Primed DNA Labelling Kit (Boehringer Mannheim)).

In all cases, prehybridisation (10% dextran sulphate; 4 x SSC, pH 7.0; 20 mM Tris, pH 7.4; 5 x Denhardt's reagent; 40% formamide and 100 µg/ml denatured salmon sperm DNA) was performed in a hybridisation oven (Hybaid) at 42°C for 16 hours. Hybridisations (42°C for 24

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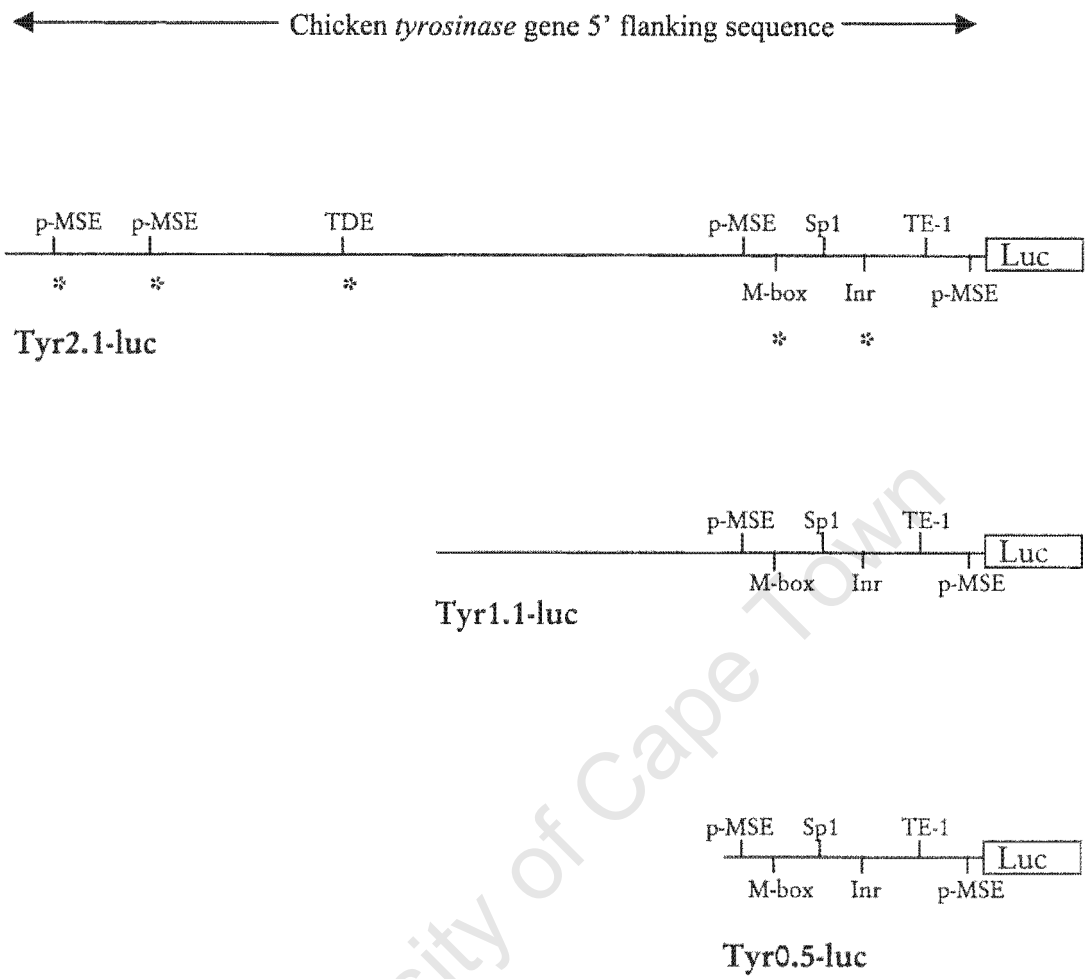

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Fig. 2.1. Maps of tyrosinase promoter-*luciferase* reporter constructs used for transient transfection assays. Tyr2.1-luc contains the entire flanking sequence cloned to date for the chicken *tyrosinase* gene. Tyr1.1-luc excludes the distal TDE element and two p-MSEs. Tyr0.5-luc retains the proximal 504 nt containing the core elements of the M-box, Sp1, Inr and TE-1. Refer to section 1.4 and Fig. 1.8 for a description of each element. Elements indicated with an asterisk (*) in Tyr2.1-luc are potential targets for the cMI protein, as detailed in section 1.5.

hours) were followed by washes under low stringency (2 x SSC; 0.1% SDS; 2 x 20 minutes at 42°C) and then under high stringency conditions (0.1 x SSC; 0.1% SDS; 2 x 20 minutes at 65°C). Autoradiography was performed using x-ray film at -80°C for 10 days to 3 weeks.

2.6 One-tube reverse transcription/polymerase chain reaction

Total RNA was extracted from dissected eyes, skin, brains, hearts and gastrointestinal tracts of chicken embryos according to previously described methods (Chomczynski and Sacchi, 1987; Sambrook *et al.*, 1989). 1 µg of each total RNA was used for each analysis with the Access RT-PCR System (Promega) according to the manufacturer's instructions. RT-PCR was carried out in a Hybaid PCR Sprint machine with reverse transcription at 48°C for 45 minutes, followed by PCR cycles of an initial 2 minute denaturation at 94°C followed by 35 cycles of 1 minute at 94°C, 1.5 minutes at 55°C, and 72°C for 1.5 minutes with a final elongation at 72°C for 4 minutes. The primers used for amplification of transcripts are detailed under Table 2.1. 10 µl of each PCR product was resolved on 1.5-2% agarose gels.

2.7 Two-step reverse transcription/polymerase chain reaction

A two-step RT-PCR approach was adopted in the latter portions of the current study in order to allow for the amplification of multiple gene targets from the same first strand cDNA. Organ and tissue total RNA was extracted as described for one-tube RT-PCR. RNA from cell cultures was extracted as described in section 2.8.6. All reactions were carried out in a Hybaid PCR Sprint machine.

First stand cDNA was synthesised from all RNAs using MuMLV reverse transcriptase (Promega) and an oligo dT_{15} primer incubated at 42°C for 1 hour. A typical RT reaction included: RNA (1 µg); oligo dT_{15} primer (3 µl at 20 pmol/µl); 4 µl of 5 mM dNTPs; 2 µl of 25 mM $MgCl_2$ (Promega); 4 µl of M-MLV RT 5x buffer (Promega); 1 µl of M-MLV RT (Promega); made up to 20 µl with DEPC-dH_20. In each case, as a control for plasmid and/or genomic DNA contamination, RNA was also subjected to RT without the addition of the RT enzyme (MuMLV), thereby generating a "mock" reverse transcribed first strand cDNA for each RNA sample. First strand cDNAs were PCR amplified using a variety of primers and conditions, depending on which gene was being studied, which are described below. A typical PCR reaction included: 1 µl of first strand cDNA; 1 µl of primer A (10 pmol/µl); 1 µl of primer B (10 pmol/µl); 4.85 µl of DEPC- dH_20; 0.4 µl of 5 mM dNTPs; 0.6 µl of 25 mM $MgCl_2$ (Promega);

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1 µl of Mg-Free Thermophilic DNA Pol. 10x buffer (Promega); and 0.15 µl Taq polymerase (Promega). All primer pairs crossed intron/exon boundaries and appropriate positive and negative controls were performed with each experimental reaction. All PCR products were resolved on 1.0-1.5% agarose gels and detected by ethidium bromide staining and UV transillumination.

For all chicken *cMI* primer pairs (Table 2.1) 1 µl of first stand cDNA was amplified using a "touch down" PCR programme. Such a programme begins with a high annealing temperature to generate an initial pool of highly specific gene products. As the cycles progress, the annealing temperature is gradually decreased, thus exponentially increasing the pool of target sequences. Cycling conditions used were: an initial 2 minute denaturation at 94°C followed by: four cycles at 94°C for 15 seconds, 55°C for 30 seconds, 74°C for 30 seconds; sixteen cycles at 94°C for 15 seconds, 55°C for 30 seconds with a decrease of 0.2°C per cycle, 74°C for 30 seconds; eleven cycles at 94°C for 15 seconds, 50°C for 30 seconds, 74°C for 30 seconds with an increase of 5 seconds per cycle; and a final elongation at 74°C for 6 minutes. When performing semi-quantitative PCR, reactions were carried out under the same cycling conditions but reactions were removed after 20, 25, or 30 of the PCR cycles.

Chicken *tyrosinase* amplification from 1 µl of first strand cDNA was carried out using primers designed for the current study. ctyr-F1 and ctyr-R1 (Table 2.1) were designed from the previously reported chicken *tyrosinase* cDNA sequence (April *et al.*, 1996) and synthesised by The Department of Molecular and Cellular Biology, The University of Cape Town, South Africa. PCR cycling conditions used were: an initial denaturation for 2 minutes at 94°C followed by: 35 cycles of 94°C for 15 seconds, 57°C for 30 seconds, and 74°C for 1.5 minutes, and a final 1 minute elongation at 74°C.

Amplification of chicken *Dct* from 1 µl of first strand cDNA was performed using primers TRP-2F4 and TRP-2R3 (Table 2.1; April, Ph.D. Thesis, 1998). PCR cycling conditions used were: an initial denaturation for 2 minutes at 94°C followed by: 35 cycles of 94°C for 15 seconds, 62°C for 30 seconds, and 74°C for 1.5 minutes, and a final 1 minute elongation at 74°C.

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2.8 Isolation, culture and manipulation of cells

2.8.1 Isolation and culture of primary chicken embryonic fibroblasts

Primary chicken fibroblasts were isolated with minor modifications to a previously described method (Morgan and Fekete, 1996). White Plymouth Rock x Pile Game (UCT) or White Leghorn (JHMII) chicken embryos were incubated at 37°C for 8-10 days. All dissections and subsequent manipulations were carried out in a lamina flow hood/tissue culture hood. Eggs were wiped with 70% ethanol to prevent bacterial and fungal contamination from the shells, embryos were removed and transferred to a dry, sterile Petri dish where the heads, limbs and all viscera were removed. The torso was minced using a sterile razor blade and transferred to 10 ml of 0.05% trypsin/0.02% EDTA for every 10 embryos obtained. Cells were dissociated by gentle swirling on a rotor for 12-15 minutes at room temperature. An equal volume of foetal calf serum (FCS; Highveld Biological (Pty) Ltd.) was added to inactivate the trypsin/EDTA and the mixture was allowed to stand at room temperature for 5 minutes to allow clumps to settle. The supernatant was transferred to a sterile 50 ml plastic centrifuge tube and fibroblasts were pelleted by centrifugation at 1000 x g for 5 minutes at room temperature. Cells were re-suspended in 20 ml of Dulbecco's modified Eagle's medium (DMEM) (Highveld Biological (Pty) Ltd, UCT; Sigma, JHMI.) supplemented with 10% FCS, 2mM L-glutamine, and 100 I.U. each of penicillin and streptomycin (UCT) or 0.05 mg/ml gentamicin (JHMI).

In initial experiments performed at UCT, primary cultures of chicken fibroblasts were found to be sensitive to the plasticware used immediately following isolation, with optimal cell health and division observed when using 10 cm Corning tissue culture dishes. Following a first passage, the cells were found to tolerate any other plasticware equally as well. At JHMI, primary cultures of chicken fibroblasts were additionally found to tolerate T75 (Sarstedt Inc.) tissue culture flasks immediately following isolation. In both cases, fibroblasts were maintained in DMEM supplemented with 10% FCS, 2mM L-glutamine, and 100 I.U. each of penicillin/streptomycin (UCT) or 0.05 mg/ml gentamicin (JHMI) in a 37°C/5% CO_2 incubator

For transient transfections of primary chicken embryonic fibroblasts, cells at 80-100% confluence in 10 cm Corning tissue culture dishes were dissociated using 0.05% trypsin/0.02% EDTA, split 1:20, and plated onto 35 mm Nunc tissue culture dishes. This allowed for the use of smaller volumes of FuGENE 6 (Roche) transfection reagent, and resulted in cells being approximately 50-70% confluent on the day of transfections (see section 2.9).

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For western blotting of primary chicken embryonic fibroblast proteins, cells at 80-100% confluence in T75 (Sarstedt Inc.) tissue culture flasks were rinsed once with phosphate buffered saline (PBS), and scraped from the culture flask using a rubber policeman and a second volume of PBS. Cells were centrifuged at 1000 x g for five minutes at 4°C, and cell pellets further processed as described for western blotting (see section 2.11).

2.8.2 Isolation and culture of chicken RPE cells

Primary cultures of chicken retinal pigment epithelial (RPE) cells were prepared according to a previously described method (Eguchi and Okada, 1973) with minor modifications according to Clarke (M.Sc. Thesis, 2000). Briefly, the eyes of 6-8 day-old White Plymouth Rock x Pile Game chicken embryos (UCT) were dissected in Ca^{2+} and Mg^{2+}-free Hanks (CMF Hanks, pH 7.4). The cornea, lens and vitreous were removed and the eye cups incubated for one hour in CMF Hanks containing 0.05% EDTA. Fine forceps were used to gently dislodge the RPE from the sclera and sheets of cells were collected in chilled CMF Hanks. Sheets of cells were gently agitated with a Gilson pipette to obtain small clusters of cells. Freshly dissected clusters of cells were plated in 6 well (35 mm) tissue culture dishes and maintained in Eagles' MEM (Sigma) supplemented with 10% FCS and 100 I.U. each of penicillin/streptomycin (UCT) or 0.05 mg/ml gentamicin (JHMI) in a 37°C/5% CO_2 incubator.

For transient transfection experiments, a "one eye per 35 mm well" rule of thumb was adopted, according to Clarke (M.Sc. Thesis, 2000), and pigmented cultures were obtained which were approximately 50% confluent after four days in culture, at which time they were used for transfection experiments (see section 2.9).

For western blotting, RPE cells were isolated as described above except that 12-14 day old (to obtain larger eyes and thus optimise RPE protein levels) White Leghorn chicken embryos were used (JHMI). Cells at 80-90% confluence in 6-well (Falcon) tissue dishes were rinsed once with phosphate buffered saline (PBS), and scraped from the culture flask using a rubber policeman and a second volume of PBS. Cells were centrifuged at 1000 x g for five minutes at 4°C, and cell pellets further processed as described for western blotting (see section 2.11).

2.8.3 Culture of melan-a cells

Melan-a cells are a spontaneously immortalised mouse melanocyte cell line (Bennett *et al.*, 1987). Cells were grown in RPMI medium (Highveld Biological, South Africa) supplemented with 10% FCS which had not been heat-inactivated, 2mM L-gluatmine, and 100 I.U. each of penicillin and

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streptomycin (Highveld Biological, South Africa) in a 37°C/10% $C0_2$ incubator. TPA (12-O-tetradecanoyl-phorbol-13-acetate) was added to the medium at a final concentration of 200 nM.

For transient transfections, melan-a cells were plated at a density of 2×10^5 cells per 35 mm well 24 hours prior to transfection. This allowed for the use of smaller volumes of FuGENE 6 (Roche) transfection reagent, and resulted in cells being approximately 50-70% confluent on the day of transfections (see section 2.9).

2.8.4 Culture of B16 cells

B16 cells, a mouse melanoma cell line, were obtained from Dr. Cliff Takemoto (Division of Pediatric Hematology, The Johns Hopkins University, Baltimore, United States of America). Cells were grown and maintained in DMEM (Sigma) supplemented with 10% FCS, 2mM L-gluatmine, and 0.05 mg/ml gentamicin in a 37°C/5% CO_2 incubator (JHMI).

For transient transfections of B16 cells, cells at 80-100% confluence in a T75 (Sarstedt Inc.) tissue culture flask were dissociated using 0.05% trypsin/0.02% EDTA, split 1:20, and plated onto 6-well (Falcon) tissue culture dishes. This allowed for the use of smaller volumes of PolyFect (Qiagen) transfection reagent, and resulted in cells being approximately 50-70% confluent on the day of transfections (see section 2.9).

For western blotting of B16 cell proteins, cells at 80-100% confluence in a T75 (Sarstedt Inc.) tissue culture flasks were rinsed once with phosphate-buffered saline (PBS), and scraped from the culture flask using a rubber policeman and a second volume of PBS. Cells were centrifuged at 1000 x g for five minutes at 4°C, and cell pellets further processed as described for western blotting (see section 2.11).

2.8.5 Culture of HeLa cells

HeLa cells, a human cervical cancer cell line, are routinely available in the Biochemical Virology Laboratory at The Johns Hopkins University, Baltimore, United States of America, where all experiments with the cells were performed. HeLa cells were grown and maintained in DMEM supplemented with 10% FCS, 2mM L-gluatamine and 0.05 mg/ml gentamicin in a 37°C/5% CO_2 incubator.

For transient transfections of HeLa cells, cells at 80-100% confluence in a T75 (Sarstedt Inc.) tissue culture flask were dissociated using 0.05% trypsin/0.02% EDTA, split 1:20, and plated onto 6-well (Falcon) tissue culture dishes. This allowed for the use of smaller volumes of

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PolyFect (Qiagen) transfection reagent, and resulted in cells being approximately 50-70% confluent on the day of transfections (see section 2.9).

For western blotting of HeLa cell proteins, cells at 80-100% confluence in a T75 (Sarstedt Inc.) tissue culture flasks were rinsed once with phosphate buffered saline (PBS), and scraped from the culture flask using a rubber policeman and a second volume of PBS. Cells were centrifuged at 1000 x g for five minutes at 4°C, and cell pellets further processed as described for western blotting (see section 2.11).

2.8.6 RNA extraction from cell cultures

RNA was extracted from cell cultures according to the method of Xie and Rothblum (1991) with a few modifications. Briefly, cells were grown to confluence, the medium was removed, and cells were rinsed twice with phosphate-buffered saline (PBS). 1ml of Solution 'A' (0.5 ml water-saturated phenol; 0.5 ml solution 'B' [25 g guanidinium thiocyanate (Merck, 4M); 1.67 ml 0.75M Na Citrate (pH 7.25) made to 50 ml with DEPC-treated dH_20, filter sterilised (0.45 micron filter)]; 0.05 ml 2M NaOAc (pH 4.0); 5 µl 2-mercapto-ethanol (Sigma)] per 5 cm tissue culture dish was added and the cells lysed by repetitive pipetting. 0.2 ml of chloroform/isoamyl alcohol per 1 ml of Solution 'A' was added, the mixture vortexed for 15 seconds, and centrifuged at 12,000 x g at 4°C for 15 minutes.

The upper aqueous phase was transferred to an Eppendorf tube containing 0.4 ml of isopropanol and precipitated at -20°C overnight. The following day RNA was pelleted by centrifugation at 12,000 x g for 15 minutes at 4°C and the pellet rinsed with 1 ml of 75% ethanol. RNA was centrifuged again at 12,000 x g for 5 minutes at 4°C, the pellet vacuum dried, and re-suspended in a suitable volume of DEPC-dH_20.

At a later stage in the present study, RNA was extracted from cell cultures using TriPure according to manufacturers' instructions (Roche).

2.9 Transfection of cell cultures

Primary chicken embryonic fibroblasts, chicken RPE cells, and melan-a cells were transfected using the lipidic transfection reagent FuGENE 6 (Roche), according to the manufacturer's instructions (UCT). B16 and HeLa cells were transfected using the activated dendrimer PolyFect Reagent (Qiagen), according to the manufacturer's instructions (JHMI).

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A DNA:FuGENE 6 complex was prepared by adding FuGENE 6 reagent to plasmid DNA at a ratio of 3µl FuGENE:2 µg plasmid DNA. When using PolyFect, the amount of PolyFect transfection reagent was modified according to the manufacturer's recommendations and the size of the tissue culture dish; the normal ratio used was 10 µl PolyFect: 6 µg plasmid DNA in 6-well (Falcon) tissue culture plates. Both FuGENE and PolyFect transfections were allowed to proceed for 24 or 48 hours without a change of medium, unless indicated otherwise.

Each transfection was performed in duplicate at least thrice. Co-transfection of all dishes with pRL-CMV (section 2.4.5), a reporter vector containing the *Renilla* luciferase gene under the control of the CMV promoter, allowed for the normalisation of transfection efficiency between different dishes of cells in a given experiment. Additional duplicate dishes were co-transfected with pGL2-control (section 2.4.5) and pRL-CMV. pGL2-control is a positive control vector containing the *luciferase* coding region under the control of the strong SV40 promoter and enhancer sequences. pGL2-control serves as the standard for transfection efficiency in any given cell type. The activity of pGL2-control is assigned a value of 100%, with all other luciferase activities being expressed as a percentage of this value.

The amount of each plasmid DNA used in each transfection with FuGENE is: 5 µg of tyrosinase promoter constructs; 5 µg of cMI-coding constructs; 0.05 µg of pRL-CMV; and 5 µg of pGL2-control. The amount of each plasmid DNA used in each transfection with PolyFect is: 5 µg of tyrosinase promoter constructs; 6 µg of cMI-coding constructs; 0.05 µg of pRL-CMV; and 5 µg of pGL2-control. All transfected cells were incubated overnight in a 37°C/5% CO_2 incubator and harvested 24-48 hours post-transfection.

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2.10 Reporter Gene Assays

Cells were lysed *in situ* using 1 x Passive Lysis Buffer (Dual Luc Assay kit; Promega) and removed from tissue culture dishes using a rubber policeman. Lysates were subjected to two freeze-thaw cycles according to the manufacturer's instructions (Dual Luc Assay kit; Promega) and the cell debris pelleted at 12,000 x g for 1 minute at room temperature. The supernatant was transferred to a fresh Eppendorf and was assayed immediately for reporter gene activity and protein concentrations.

Firefly and *luciferase* activity in transfected cells were measured sequentially using the Dual-Luciferase Reporter Assay System (Promega Corporation) according to manufacturer's

instructions and a luminometer (Bio-Orbit 1253 luminometry system, UCT; or Monolight 2010 Analytical Luminescence Laboratory, JHMI). The protein concentration of each cell lysate was assayed using the Bio-Rad Protein Assay according to the manufacturer's instructions. For transfections performed at UCT (chicken embryonic fibroblasts, mouse melan-a cells, and chicken RPE cells), BSA in 1 x Passive Lysis Buffer was used a protein standard and all cell lysate protein concentrations were determined from a standard curve obtained with the protein standard. For transfections performed at JHMI (human HeLa cells and mouse B16 cells), protein concentrations were determined using the Emax Precision Microplate Reader (Molecular Devices) and a blank of 1 x Passive Lysis Buffer.

To allow comparative analyses across all transfections, the activity of each cell lysate was expressed as a relative luciferase activity (RLA). RLA was calculated by division of the light units values obtained for both firefly and *Renilla* luciferase activity by the protein concentration (mg/ml) measured from the same cell lysate. Firefly luciferase activity (light units) was normalised by dividing with the value obtained with the internal *Renilla* construct (light units) divided by the protein concentration (mg protein). The final value was expressed as a percentage of the activity of pGL2-control (which was assigned a value of 100% in each transfection).

2.11 Western blotting

All western blotting was performed in the Biochemical Virology Laboratory at The Johns Hopkins University, Baltimore, United States of America, according to previously described methods (Sambrook *et al.*, 1989) and minor modifications according to methods routinely used in the laboratory.

Briefly, the medium was removed from cells, and cultures were rinsed once with PBS to remove FCS proteins. A second volume of PBS added, and cells were scraped (using a rubber policeman) from the tissue culture dish and transferred to a sterile tube. The cell suspension was centrifuged at 1000 x g at 4°C for five minutes, and the cell pellets were re-suspended in a suitable volume (according to pellet size) of whole cell lysis buffer (20 mM Tris, pH 8.0; 1 mM EDTA; 200 mM NaCl; 0.5% NP-40) containing 1:500 Proteinase Inhibitor Cocktail (Sigma). Cells lysis was achieved by incubating the mixture on ice for twenty minutes with brief vortexing every 3–4 minutes. Lysed cells were centrifuged at 4000 x g for five minutes at 4°C to pellet the cell debris.

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Protein concentrations from the resultant supernatants were assayed using the Bio-Rad Protein Assay according to the manufacturer's instructions. Whole cell lysis buffer was used as a protein standard and all protein concentrations were determined using the Emax Precision Microplate Reader (Molecular Devices). This method of ensuring equal protein levels across samples (as opposed to, for example, using a housekeeping protein antibody with western blotting) is routinely used in the laboratory in which the westerns were performed, and has stood up to rigorous peer-review in numerous published articles in quality journals. If necessary, protein samples were concentrated using the Speed Vac SVC 100 (Savant) to obtain a suitable volume for SDS-polyacrylamide gel electrophoresis. Each protein was mixed with 10 µl of 2 x SDS gel-loading buffer (100 mM Tris-Cl, pH 6.8; 200 mM DTT; 4% SDS (electrophoresis grade); 0.2% bromophenol blue; 20% glycerol), placed in a boiling water bath for 3 minutes, and centrifuged briefly at 5000 x g at room temperature. Equal amounts of protein were loaded and electroporesed on a 10% SDS-polyacrylamide gel at 120 V for one and a half hours. The BenchMark Prestained Protein Ladder (10 µl; Invitrogen) was used to estimate protein size.

Proteins were transferred to a nitrocellulose membrane (BioRad) at 120 V for one and a half hours in western transfer buffer (39 mM glycine; 48 mM Tris base; 0.037% SDS (electrophoresis grade); 20% methanol). Membranes were blocked overnight in 5% Dry Milk/TBST (150 mM NaCl; 10 mM Trizma base; 0.5% Tween 20) with gentle rocking on a platform shaker at 4°C to prevent subsequent non-specific binding of the primary antibody.

The following day, membranes were washed twice with TBST (10 minutes each wash, at room temperature). The primary antibody, a monoclonal mouse anti-Mitf antibody, was a kind gift of Dr. Cliff Takemoto (Division of Pediatric Hematology, The Johns Hopkins University, Baltimore, United States of America). Conditions used were as previously described (Takemoto *et al.*, 2002) with a one hour incubation with gentle rocking on a platform shaker at room temperature. The membrane was washed again with TBST (2 x 10 minutes each at room temperature) and incubated with the secondary antibody (Anti-mouse Ig, Horseradish Peroxidase linked whole antibody (from sheep)) (Amersham Biosciences) at a 1:2000 dilution in 5% Dry Milk/TBST for one hour with gentle rocking on a platform shaker at room temperature.

Detection of proteins was performed using the ECL western blotting detection reagents (Amersham Phamacia Biotech) according to manufacturers' instructions. X-ray films were exposed at room temperature for various times as indicated for each experiment, and were automatically developed and fixed using the Kodak X-OMAT 2000A Processor.

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Chapter 3 | Results

To address the specific aims of the study, this investigation proceeded through three main stages. The first stage was a structural characterisation of the chicken microphthalmia gene, which involved the cloning and sequencing of a chicken microphthalmia cDNA. The second stage was an analysis of the expression patterns of the gene in pigmented and non-pigmented tissues of the developing chicken embryo, as well as a preliminary analysis of the genomic structure of the gene. The final stage was an analysis of two different isoforms of the chicken microphthalmia gene and their ability to transactivate the chicken tyrosinase gene promoter.

3.1 Isolation of a chicken *cMI* cDNA

To isolate a chicken microphthalmia cDNA, a chicken neural crest-derived melanocyte λZAP cDNA library was amplified and screened with a 1.3 kb cDNA probe (April *et al.*, 1996; April, Ph.D. Thesis, 1998), which contains the entire coding region of the mouse microphthalmia gene (Yavuzer *et al.*, 1995). Secondary and tertiary screenings were performed, yielding 11 positive plaques. pBluescript (SK-)-containing inserts were excised *in vivo* by R408 helper phage, yielding one positively hybridising clone (M156). Clone M156 is thus expected to contain an insert, which hybridises to the mouse microphthalmia cDNA probe, cloned into the *Eco*RI/*Xho*I sites of pBluescript (SK-) (April, Ph.D. Thesis, 1998).

At the start of the current project, it was therefore necessary to obtain further information about the insert. Thus, restriction enzyme mapping was carried out. Clone M156 was digested with *Ava*I, *Bam*HI, *Eco*RI, *Kpn*I, *Pst*I, *Sma*I, *Pst*I/*Xho*I, *Sma*I/*Xho*I, and *Eco*RI/*Xho*I and electrophoresed on a 0.8% agarose gel (Fig. 3.1.a). To determine which restriction enzyme fragment(s) correspond to the insert of clone M156, the DNA was subjected to Southern blot hybridisation (Fig. 3.1.b) with the same mouse microphthalmia cDNA probe used to originally screen the library. The *Eco*RI/*Xho*I digest, which should excise the insert of clone M156 from pBluescript SK-, yielded a positively hybridising restriction enzyme fragment of 2.6 kb. A confirmation of this insert size was obtained by polymerase chain reaction (PCR) analysis: using M156 as a template and universal vector primers, one product of 2.6 kb was amplified (Fig. 3.1.c).

The results of restriction enzyme mapping/Southern blot hybridisation of clone M156 revealed some confusing anomalies in the clone, not least of which were extraneous restriction enzyme

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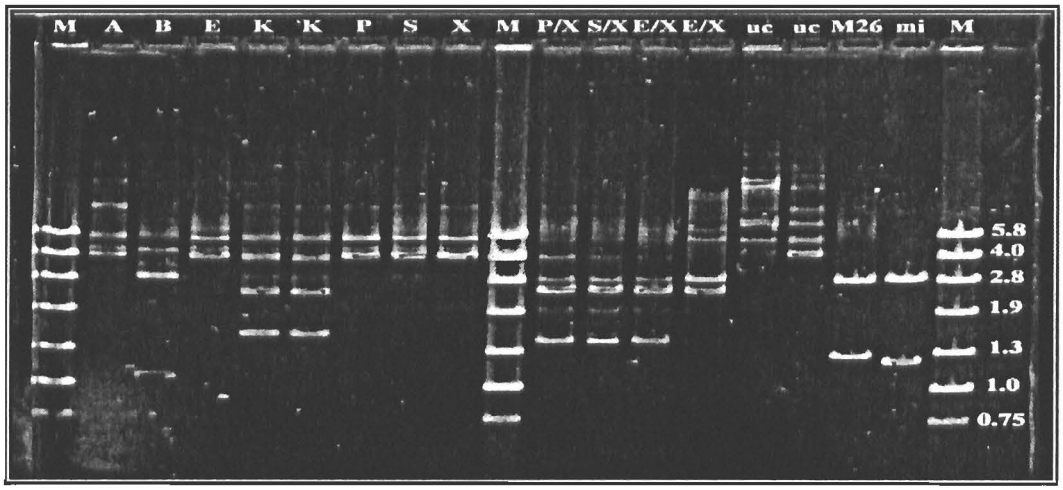
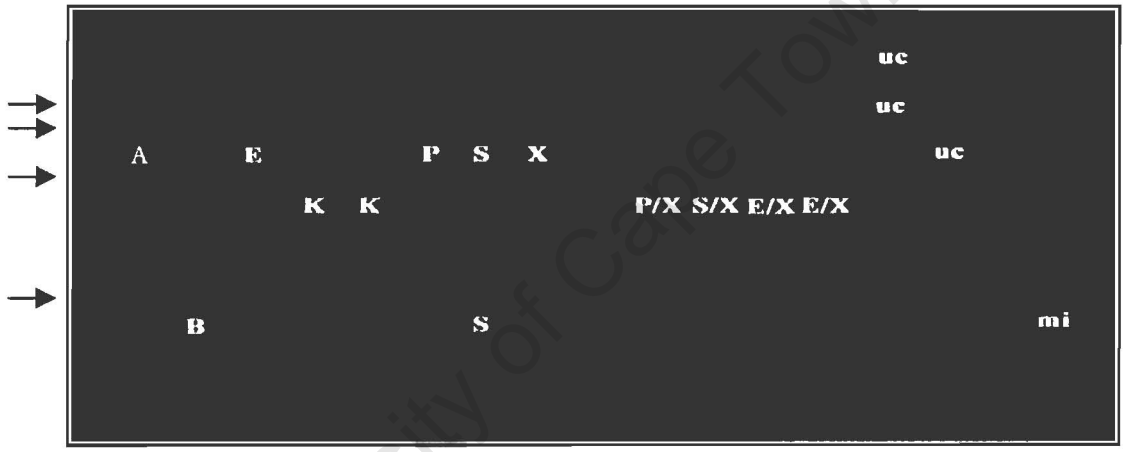
(a)



(b)



(c)

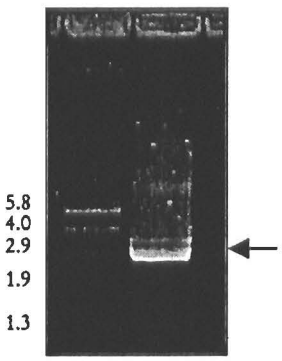


Fig. 3.1. Restriction enzyme mapping of clone M156. (a) DNA gel electrophoresis of clone M156 digested with various restriction enzymes (A: *Ava*I; B: *Bam*HI; E: *Eco*RI; K: *Kpn*I; P: *Pst*I; S: *Sma*I; X: *Xho*I) and electrophoresed on a 0.8% agarose gel. "uc" is uncut cDNA, "M26" was an additional putative chicken microphthalmia cDNA, and "mi" is the mouse *Mitf* cDNA used as a positive control. The molecular weight marker (M) is Pox/*Eco*RI (kb). (b) Autoradiograph of plasmid Southern blot. The Southern blot was probed with a radiolabelled 1.3 kb mouse *Mitf* cDNA fragment. The letters correspond to the restriction enzyme fragments shown in (a). Arrows indicate the positively hybridising fragments obtained with the restriction enzyme digests. (c) PCR of clone M156 using universal vector primers. One product of 2.6 kb is indicated with an arrow. The molecular weight marker (M) is Pox/*Eco*RI (kb).

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products. Despite extensive studies and analysis, the anomalies could not be explained; these are further discussed in Chapter Four. However, subsequent sequencing of clone M156 (see below) definitively demonstrated that the anomalies do not lie in the insert and, as a result, they only gave rise to difficulties while attempting to excise the insert for cloning into RCAS BP(A) for the purposes of *in vitro* studies (see section 3.5.1.1).

3.2 Nucleotide and amino acid sequence analysis of clone M156

Two different methods were employed for the sequencing of clone M156. The coding region was sequenced manually using a combination of universal vector primers and custom designed internal primers, and the 3' untranslated region (UTR) was sequenced with vector and internal primers using automated sequencing technology. The results are described in the following four sections. In all sections the analyses and discussion are restricted to microphthalmia cDNA sequences expressed in pigmented cells reported from two vertebrate classes (Aves and Mammalia) and three species (chicken, human, and mouse).

3.2.1 Clone M156 encodes a chicken microphthalmia cDNA

The nucleotide (nt) and predicted amino acid (aa) sequence of the clone M156 coding region is presented in Figure 3.2, and the salient features of the clone are summarised under Table 3.1. The sequence consists of 34 bp of 5' UTR followed by an open reading frame (ORF) of 1158 nts. This ORF begins with an ATG codon (nts 35-37) and ends with a TAG translation termination codon (nts 1191-1193). FASTA (GCG) homology searches reveal that clone M156 shares extensive nt sequence identities with orthologous microphthalmia cDNAs from other species. Clone M156 shares 80.9%, 83.4%, and 98.6% nt sequence identity with the previously reported full-length mouse (Hodgkinson *et al.*, 1993), human (Tachibana *et al.*, 1994), and chicken (*cmi9*; Mochii *et al.*, 1998a) microphthalmia sequences, respectively (Table 3.2).

The M156 ORF encodes a polypeptide of 385 amino acids with a predicted molecular weight of 44 kDa. The protein encoded by clone M156 shares, respectively, 88.3%, 91.9%, and 96.8% aa sequence identity with the mouse (Hodgkinson *et al.*, 1993), human (Tachibana *et al.*, 1994), and chicken (cmi9; Mochii *et al.*, 1998a) Microphthalmia proteins (Table 3.2). Taken together, the high nt and aa sequence identity observed between clone M156 and previously reported microphthalmia cDNA sequences, demonstrates that a chicken homologue of the microphthalmia gene has been isolated.

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```
1       GTGCAGTCACTTCTCTCACAACCGTATAAGTATTATGCTGGAAATGCTTGAGTATAATCA
                                          M  L  E  M  L  E  Y  N  H
                Exon 1m | Exon 2
61      TTATCAGGTGCAGACTCACCTTGAGAATCCAACCAAATACCATATTCAGCAAGCTCAGCG
         Y  Q  V  Q  T  H  L  E  N  P  T  K  Y  H  I  Q  Q  A  Q  R

121     GCAGCAGGTAAAGCAGTACCTCTCTACCACTCTAGCAAATAAACACGCCAACCAAGCCCT
         Q  Q  V  K  Q  Y  L  S  T  T  L  A  N  K  H  A  N  Q  A  L

181     GAGCTTGCCATGTCCAAACCAGCCCGGTGATCATGTCATGCCGCCTGGAACTGGAAGCAG
         S  L  P  C  P  N  Q  P  G  D  H  V  M  P  P  G  T  G  S  S
                                                        Exon 2 | Exon 4
241     TGCGCCCAACAGTCCGATGGCTATGCTCACCCTCAACTCCAACTGTGAAAAAGAGATGGA
         A  P  N  S  P  M  A  M  L  T  L  N  S  N  C  E  K  E  M  D

301     TGATGTGATTGATGACATAATTAGTCTGGAATCAAGTTACAATGAAGAAATCCTTGGCTT
         D  V  I  D  D  I  I  S  L  E  S  S  Y  N  E  E  I  L  G  L
                                Exon 4 | Exon 5
361     GATGGACCCAGCCTTGCAAATGGCAAATACGTTACCAGTGTCTGGGAATTTGATTGACCT
         M  D  P  A  L  Q  M  A  N  T  L  P  V  S  G  N  L  I  D  L

421     TTATGGCAACCAAAGCATGCCTCCTCCAGGACTTAACATCAGCAACTCCTGTCCAGCTAA
         Y  G  N  Q  S  M  P  P  P  G  L  N  I  S  N  S  C  P  A  N
                             Exon 5 | Exon 6b
481     TCTTCCTAATATAAAAAGGGAGCTCACAGAGTCAGAAGCGAGAGCACTGGCTAAGGAGAG
         L  P  N  I  K  R  E  L  T  E  S  E  A  R  A  L  A  K  E  R
                          Exon 6b | Exon 7
541     GCAAAAGAAAGACAATCACAACTTGATTGAACGAAGAAGAAGATTTAATATTAATGACCG
         Q  K  K  D  N  H  N  L  I  E  R  R  R  R
                                          Exon 7 | Exon 8
601     TATAAAAGAACTGGGCACCTTGATACCCAAATCAAACGACCCGGATATGCGCTGGAATAA
                                  I  P  K  S  N  D  P  D  M  R  W  N  K

661     GGGAACTATTCTAAAAGCATCAGTGGACTACATCCGTAAGCTGCAAAGAGAGCAGCAACG
         G                                                  Q  Q  R

721     CACGAAGGAACTTGAGAACAGACAGAAGAAATTGGAACACGCCAACAGGCACCTGCTGCT
         T  K  E  L  E  N  R  Q  K  K  L  E  H  A  N  R  H  L  L  L
            Exon 8 | Exon 9
781     CAGAATACAGGAACTTGAGATGCAAGCTCGGGCACATGGACTGTCCCTTGTTCCATCCAC
         R  I  Q  E  L  E  M  Q  A  R  A  H  G  L  S  L  V  P  S  T

841     AGGCATTTGCTCCCCTGATATGGTCAACAGGGTCATCAAACAAGAACCTGTGCTGGACAA
         G  I  C  S  P  D  M  V  N  R  V  I  K  Q  E  P  V  L  D  N

901     CTGCAACCAAGACCTCATGCCGCACCACACAGACCTGTCTTGCACTACCACCCTTGATCT
         C  N  Q  D  L  M  P  H  H  T  D  L  S  C  T  T  T  L  D  L

961     CACTGATGGTACCATCACCTTCAGTGACAACCTCGGAAATGTGACTGAACCAACTGGCAC
         T  D  G  T  I  T  F  S  D  N  L  G  N  V  T  E  P  T  G  T

1021    TTACAGTGTTCCTGCAAAAATGGGATCCAAACTGGAAGACATCTTGATGGACGATACCCT
         Y  S  V  P  A  K  M  G  S  K  L  E  D  I  L  M  D  D  T  L

1081    CTCCCCCGTAGGAGTAACTGACCCACTGCTTTCCTCTGTGTCTCCTGGAGCATCGAAAAC
         S  P  V  G  V  T  D  P  L  L  S  S  V  S  P  G  A  S  K  T

1141    GAGTAGCAGGCGGAGCAGCGTGAGCATGGAGGACACTGATCATGCTTGTTAG
         S  S  R  R  S  S  V  S  M  E  D  T  D  H  A  C  *
```

Figure 3.2. Nucleotide and deduced amino acid sequence of the clone M156. The predicted 385-residue protein sequence contains an acidic domain (underlined), a basic domain (lightly shaded), and two helical domains (darkly shaded) connected by a loop (underlined). The bold-faced leucines, spaced seven amino acids apart, conform to the prediction of a leucine zipper domain. The stop codon is marked with an asterisk. Exon/intron boundaries are indicated according to human (Tassabehji *et al.*, 1994; Udono *et al.*, 2000) and mouse (Hallsson *et al.*, 2000) genomic sequences. The nucleotide sequence shown here has been deposited in the GenBank database and assigned the accession number AF145751.

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Table 3.1. Summary of key features of the chicken microphthalmia cDNA

Feature	Size or number	Position
cDNA length (bp)	2533	
5' UTR (bp)	34	nts 1-34
ORF (bp)	1158	nts 35-1193
3' UTR (bp)	1341	nts 1194-2533
poly (A+) signal	1 (18 nts)	nts 2514-2531
mature protein length (aa)	385	
molecular weight (kDa)	44	
acidic domain	1	nts 296-346
basic domain	1	nts 527-583
helix domains	2	nts 585-622;665-712
loop domain	1	nts 623-664
leucine residues	4	nts 731-733; 752-754; 773-775; 794-796
casein kinase II motifs	5	aas 14-17; 82-85;101-104;158-161;374-377
glutamine-rich basic domain	1	aas 20-37
mitogen-activated protein kinase motif	1	aas 71-74
activation domain	1	aas 88-98
serine-rich domain	1	aas 355-382
protein kinase C motif	1	aas 370-372
cAMP- or cGMP-dependent protein kinase motif	1	aas 372-375

Refer to Fig 3.2 and Fig 3.4 for a schematic representation of the locations of the domains in the predicted chicken cMI protein.

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Table 3.2. Nucleotide and deduced amino acid sequence comparisons of avian and mammalian microphthalmia genes.

		Mouse *Mitf*	Chicken *cMI* (*cmi9*)	Chicken *cMI* (M156)
Chicken *cMI* (*cmi9*)	% nucleotide identity % amino acid identity % amino acid similarity	68.532 82.679 85.679		
Chicken *cMI* (M156)	% nucleotide identity % amino acid identity % amino acid similarity	80.915 88.342 91.451	98.611 96.891 96.891	
Human *MITF*	% nucleotide identity % amino acid identity % amino acid similarity	86.637 78.700 80.144	73.375 85.681 88.222	83.420 91.969 94.301

DNA sequences were translated using the "Translate" programme and were then aligned and compared in a pair wise fashion using the "Gap" programme (Genetics Computer Group (GCG) program (GCG sequence analysis software package, version 9.0, GCG, Inc., Madison, WI, U.S.A.). The three values for each comparison are percentage nucleotide sequence identity (top), amino acid sequence identity (middle) and amino acid sequence similarity (bottom), respectively. Mouse (Hogkinson *et al.*, 1993; Accession Number Z23066), human (Tachibana *et al.*, 1994; Accession Number Z29678), chicken (*cmi9*; Mochii *et al.*, 1998a; Accession Number D88363), and chicken (M156; this study; Accession Number AF145751) sequences were used in the analysis.

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3.2.2 Clone M156 encodes a chicken melanocyte-type Microphthalmia isoform

Since it is known that there are multiple isoforms of Microphthalmia, the next step was to determine which isoform clone M156 encodes. To do this, an alignment of the aa 5' termini of clone M156 with the human and mouse melanocyte-type isoforms (MITF-M, Tachibana *et al.*, 1994; Mitf-m, Hodgkinson *et al.*, 1993), the human, partial mouse and partial chicken RPE-type isoforms (MITF-A, Amae *et al.*, 1998; Mitf-a, Amae *et al.*, 1998; here referred to as cMI-a, Mochii *et al.*, 1998a), and the species-specific cmi9 isoform (Mochii *et al.*, 1998a), was generated using the PILEUP GCG command (Fig. 3.3).

The position of the initiation Methionine (Met) of clone M156 corresponds to the position of the initiation Met of the human and mouse melanocyte-type isoforms. The two previously described chicken microphthalmia cDNAs differ from clone M156: the initiation Met of cMI-a corresponds to that of the human and mouse RPE-type isoforms, while the initiation Met of cmi9 is in a location unique to itself. The high nt sequence identity between the RPE-type and melanocyte-type isoforms begins at nts 68-70 of clone M156 which, based on the human genomic mapping of *MITF* (Tassabehji *et al.*, 1994) corresponds to the exon 1/exon 2 boundary or, based on the recent and likely more relevant mouse genomic mapping (Hallsson *et al.*, 2000), corresponds to the exon 1m/exon 2a boundary. Thus clone M156 encodes exon 1m at its 5' terminus while MITF-A, Mitf-a, and cMI-a encode a different first exon (A/a), and cmi9 encodes yet another unique first exon. Due to these defining differences at the 5' termini, it is concluded that clone M156 **(hereafter referred to as *cMI-m*/cMI-m for chicken microphthalmia - melanocyte type)** encodes a chicken homologue of the human and mouse melanocyte-type Microphthalmia isoforms.

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3.2.3 Comparison of predicted amino acid sequence encoded by the chicken and mammalian Microphthalmia gene

To learn more about the level of molecular conservation between avians and mammals, a determination of the conservation of protein domains across species and/or isoforms was undertaken. An aa alignment of the predicted cMI-m protein with the proteins from the species discussed above was generated using the PILEUP GCG command (Fig. 3.4) and domains were identified using the Swiss-Prot and Prosite databases.

```
MITF-M  ---------- ---------- ---------- ---------- ----------
Mitf-m  ---------- ---------- ---------- ---------- ----------
M156    ---------- ---------- ---------- ---------- ----------
MITF-A  ---------- ---------- ---------- ---------- --------MQ
Mitf-a  ---------- ---------- ---------- ---------- --------MQ
cMI-a   ---------- ---------- ---------- ---------- --------MQ

                                          Exon A | Exon B1b
MITF-M  ---------- ---------- ---------- ---------- ----------
Mitf-m  ---------- ---------- ---------- ---------- ----------
M156    ---------- ---------- ---------- ---------- ----------
MITF-A  SESGIVPDFE VGEEFHEEPK TYYELKSQPL KSSSSAEHPG ASKPPISSSS
Mitf-a  SESGIVPDFE VGEEFHEEPK TYYELKSQPL KSSSSAEHPG ASKPPISSSS
cMI-a   SESGIVADFE VGEEFHEEPK TYYELKSQPL KSSSSAEHSG ASKPPLSSSS

MITF-M  ---------- ---------- ---------- ---------- ----------
Mitf-m  ---------- ---------- ---------- ---------- ----------
M156    ---------- ---------- ---------- ---------- ----------
MITF-A  MTSRILLRQQ LMREQMQEQE RREQQQKLQA AQFMQQRVPV SQTPAINVSV
Mitf-a  MTSRILLRQQ LMREQMQEQE RREQQQKLQA AQFMQQRVAV SQTPAINVSV
cMI-a   MTSR
cmi9    MTSRILLRQQ LMREQMQEQE RREQQQKQQA AQFMQQRVPV SQTPAINVSV

                  Exon 1M | Exon 2a
MITF-M  ~~~~-MLEML EYNHYQVQTH LENPTKYHIQ QAQRQQVKQY LSTTLANKHA
Mitf-m  ~~~~-MLEML EYSHYQVQTH LENPTKYHIQ QAQRHQVKQY LSTTLANKHA
M156    ~~~~-MLEML EYNHYQVQTH LENPTKYHIQ QAQRQQVKQY LSTTLANKHA
MITF-A  PTTLPSATQV PMEVLKVQTH LENPTKYHIQ QAQRQQVKQY LSTTLANKHA
Mitf-a  PTTLPSATQV PMEVLKVQ-- ---------- ---------- ----------
cmi9    PASLPPATQV PMEVLKVQTH LENPTKYHIQ QAQRQQVKQY LSTTLANKHA

MITF-M  NQVLSLPCPN QPGDHVMPPV PGSSAPNSPM AMLTLNSNCE KEGFYKFEEQ
Mitf-m  SQVLSSPCPN QPGDHAMPPV PGSSAPNSPM AMLTLNSNCE KEAFYKFEEQ
M156    NQALSLPCPN QPGDHVMPPG TGSSAPNSPM AMLTLNSNCE KE........
MITF-A  NQVLSLPCPN QPGDHVMPPV PGSSAPNSPM AMLTLNSNCE KEGFYKFEEQ
cmi9    NQALSLPCPN QPGDHVMPPG TGSSAPNSPM AMLTLNSNCE KEGFYKFEEQ

MITF-M  NRAESECPGM NTHSRASCMQ MDDVIDDIIS LESSYNEEIL GLMDPALQMA
Mitf-m  SRAESECPGM NTHSRASCMQ MDDVIDDIIS LESSYNEEIL GLMDPALQMA
M156    .......... .......... MDDVIDDIIS LESSYNEEIL GLMDPALQMA
MITF-A  NRAESECPGM NTHSRASCMQ MDDVIDDIIS LESSYNEEIL GLMDPALQMA
cmi9    SRVESECPAL NTHSRASCMQ MDDVIDDIIS LESSYNEEIL GLMDPALQMA

MITF-M  NTLPVSGNLI DLYGNQGLPP PGLTISNSCP ANLPNIKREL TACIFPTESE
Mitf-m  NTLPVSGNLI DLYSNQGLPP PGLTISNSCP ANLPNIKREL TACIFPTESE
M156    NTLPVSGNLI DLYGNQSMPP PGLNISNSCP ANLPNIKREL ......TESE
MITF-A  NTLPVSGNLI DLYGNQGLPP PGLTISNSCP ANLPNIKREL ......TESE
cmi9    NTLPVSGNLI DLYGNQSMPP PGLNISNSCP ANLPNIKREL TACIFPTESE

MITF-M  ARALAKERQK KDNHNLIERR RRFNINDRIK ELGTLIPKSN DPDMRWNKGT
Mitf-m  ARALAKERQK KDNHNLIERR RRFNINDRIK ELGTLIPKSN DPDMRWNKGT
M156    ARALAKERQK KDNHNLIERR RRFNINDRIK ELGTLIPKSN DPDMRWNKGT
MITF-A  ARALAKERQK KDNHNLIERR RRFNINDRIK ELGTLIPKSN DPDMRWNKGT
cmi9    ARALAKERQK KDNHNLIERR RRFNINDRIK ELGTLIPKSN DPDMRWNKGT
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Fig. 3.3. Alignment of deduced amino acid sequences of the 5' termini of the human (MITF-M; Tachibana *et al.*, 1994), mouse (Mitf-m; Hodgkinson *et al.*, 1993) and chicken (**M156**; this study) melanocyte-type Microphthalmia isoforms with the human (MITF-A; Amae *et al.*, 1998), partial mouse (Mitf-a; Amae *et al.*, 1998) and partial chicken (here referred to as cMI-a; Mochii *et al.*, 1998a) RPE-type Microphthalmia isoforms. The boundaries for exons A, B1b, M (boxed) and 2 are indicated according to Hallsson *et al.* (2000). For orientation purposes the basic domain is indicated (shaded).

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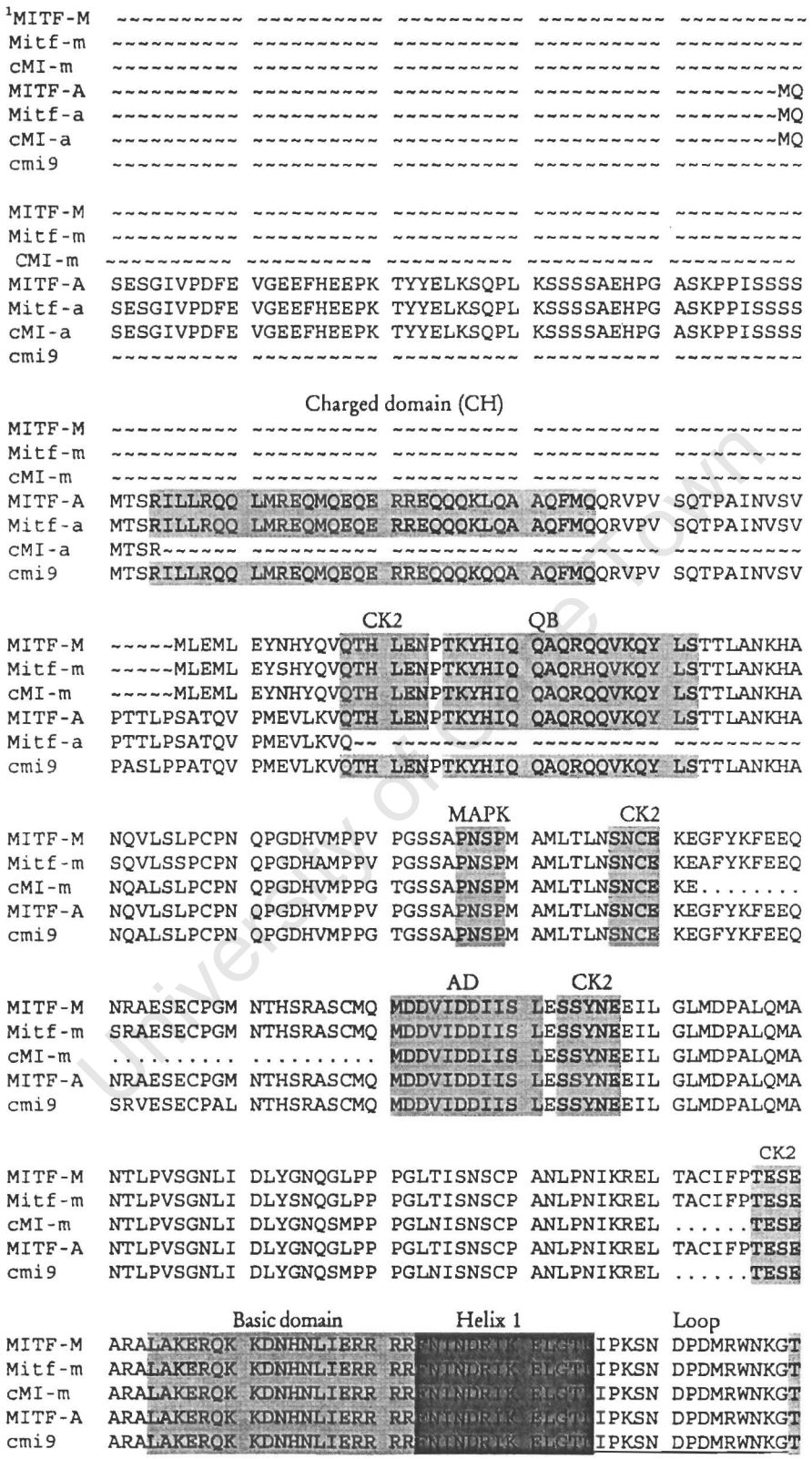

[1] Fig. 3.4. See following page for the remainder of the alignment and figure legend.

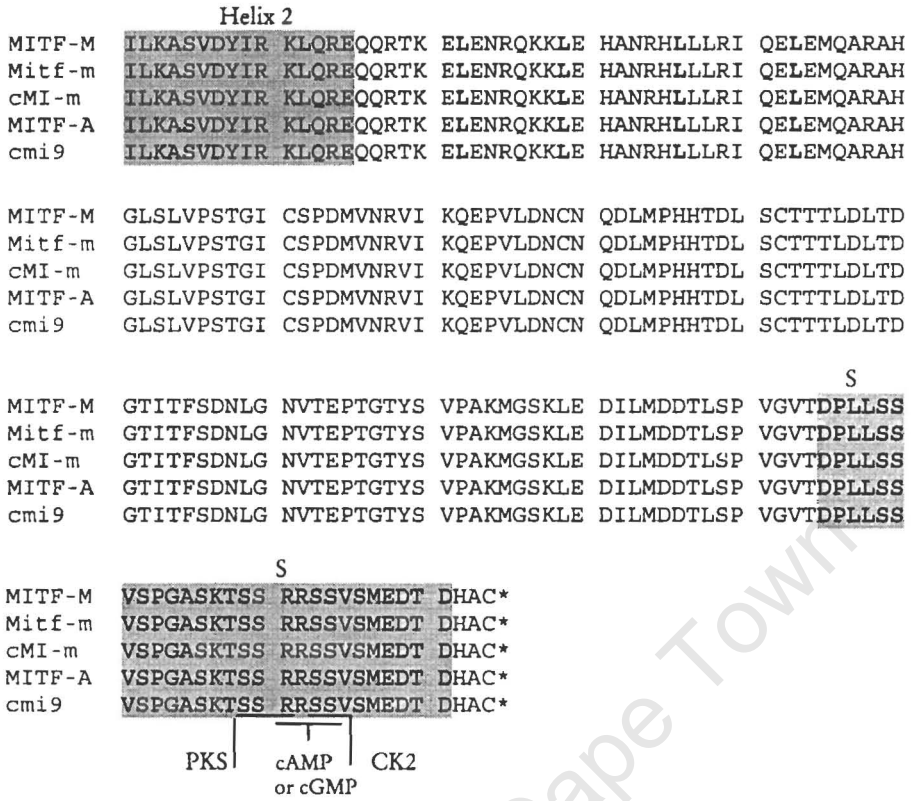

Fig. 3.4. Alignment of deduced amino acid sequences of the human (MITF-M; Tachibana *et al.*, 1994), mouse (Mitf-m; Hodgkinson *et al.*, 1993), and chicken (cMI-m; this study) melanocyte-type microphthalmia isoforms with the human (MITF-A; Amae *et al.*, 1998), partial mouse (Mitf-a; Amae *et al.*, 1998), partial chicken (cMI-a; Mochii *et al.*, 1998a) RPE-type microphthalmia isoforms with the chicken cmi9 (Mochii *et al.*, 1998a) isoform. The characteristic basic, helix, loop, and leucine residues are absolutely conserved, as is the activational acidic domain (AD). In addition there is conservation of casein kinase II (CK2) phosphorylation motifs, a glutamine-rich basic region (QB), a mitogen-activated protein kinase (MAPK) phosphorylation motif, a cyclic nucleotide-dependent protein kinase (cAMP or cGMP) phosphorylation motif, and a serine-rich (S) domain. The charged domain (CH) is only present in the RPE-type and cmi9 isoforms. Termination codons are indicated with asterisks. The deletion of exon 3 and exon 6a is shown in cMI-m.

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An acidic domain, a basic domain, and two helix domains separated by a loop, and followed by four leucine residues, are present in all proteins. The two amphiphilic α-helices form a dimerisation domain which is connected by a loop and followed by the leucine zipper motif (characterised by four leucine residues spaces seven amino acids apart). Immediately NH_2-terminal to the dimerisation domain is a basic region. Together, these three domains form the bHLH-Zip motif (basic region, helix-loop-helix, leucine zipper), which is required for DNA binding and dimerisation (Kadesch, 1993). The HLH region mediates the formation of homo- or heterodimers with related proteins, with a radical change in the protein structure from a random coiled monomer to a α-helical dimer accompanying dimerisation of these domains. This in turn permits the basic region to form a DNA contact surface that determines the DNA binding ability of the protein (Murre *et al.*, 1989; Davis *et al.*, 1990) through the recognition of a canonical CANNTG DNA-binding sequence (the E-box) in its target downstream elements. The basic region then adopts a helical structure when bound to its target DNA sequence (Anthony-Cahill *et al.*, 1992) and the leucine zipper assists the stabilisation of the protein complex. The acidic domain (AD) corresponds to a transcriptional activation domain important for interactions between Microphthalmia and the transcriptional co-activator CBP/p300 (Sato *et al.*, 1997).

In addition, all proteins contain five casein kinase II (CK2) motifs, one glutamine-rich basic domain (QB; thought to be necessary for the strong transactivation properties of MiT subfamily members (Artandi *et al.*, 1995; reviewed by Rehli *et al.*, 1999)), one mitogen-activated protein kinase motif (PXSP; containing a target serine residue for phosphorylation as part of the c-kit pathway (Price *et al.*, 1998)), one serine-rich (S) domain, one protein kinase C (PKS) motif, and one cAMP- or cGMP-dependent protein kinase motif. In contrast to the numerous domains that are conserved across all of the isoforms, cMI-m, MITF-M and Mitf-m do not carry a charged domain (CH). This domain is thought to form a helix that might be an important interface for protein-protein interactions (Rehli *et al.*, 1999).

Further analysis reveals two deletions in *cMI-m*. The first, an 84 bp sequence, has been reported in the cDNA encoding a chicken isoform (Mochii *et al.*, 1998a), the human cDNA encoding the melanocyte-type isoform (Yasumoto *et al.*, 1994; Yasumoto *et al.*, 1997a) and, more recently, in uncharacterised mouse isoforms (Hallsson *et al.*, 2000). The second deletion, an 18 bp sequence adjacent to the amino-terminal end of the basic region, has been extensively reported in the literature (reviewed by Yasumoto *et al.*, 1998). Based on both human (Tassabehji *et al.*, 1994) and mouse (Hallsson *et al.*, 2000) genomic DNA mapping, these missing aas are predicted to constitute complete deletions of exon 3 and 6a, respectively.

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3.2.4 The 3' untranslated region (UTR) of microphthalmia transcripts

Since variations in the 3'-UTR of a transcripts are an additional way in which diversity may be generated, the 3'-UTR of *cMI-m* (clone M156) was sequenced (Fig. 3.5) and comparatively analysed. The 1341 bp-long 3'-UTR begins with the C nucleotide following the TAG termination codon of the coding region and ends with a poly (A+) tail of 18 nts. Five CAYUG recognition elements (Berget, 1984) are present; this element is thought to be involved in the selection of the poly (A+) cleavage site. Three putative polyadenylation signals (Wickens and Stephenson, 1984) are also present; this sequence is absolutely required for the formation of a stable mRNA (Fitzgerald and Shenk, 1981). Two of the polyadenylation signals in *cMI-m* conform to the consensus sequence (AAUAAA), while the third conforms to the most frequent natural variant (AUUAAA, which occurs in 12% of pre-mRNAs), whose activity is comparable to that of the canonical sequence (Zhao *et al.*, 1999).

A comparison of the 3'-UTRs of *cMI-m* and the chicken *cmi9* cDNA revealed 11 nucleotide differences (highlighted in Fig. 3.5). Three are substitutions and eight are additional nucleotides in one of the two sequences. Overall, the *cMI-m* 3'-UTR sequence contains two more nucleotides than the *cmi9* 3'-UTR sequence. None of the differences affect consensus sequences for the selection of the transcription termination point.

There have been three previous reports on the 3'-UTR sequence of the microphthalmia transcripts in normal pigmented cells: the human *MITF-M* cDNA (Tachibana *et al.*, 1994), and the mouse *Mitf-m* cDNA (Hodgkinson *et al.*, 1993; Hallsson *et al.*, 2000). Comparisons of the *cMI-m* 3'-UTR and the mouse and human 3'-UTRs reveal large differences (Fig. 3.6). *cMI-m* carries a 3'-UTR 922 nts longer than *MITF-M*, but 2047 nts shorter than *Mitf-m*. Further comparison indicates diversity at the 3' end of the transcripts. In all cases the size of the major transcript, as assessed through northern blotting, far exceeds the length of the reported cDNA. Using the chicken transcript as an example, the major transcript size is 6.0 kb (Mochii *et al.*, 1998a), yet the two reported cDNA lengths are 2.5 kb (for *cMI-m*) and 2.7 kb (for *cmi9*), leaving at least 3.3 kb of sequence unaccounted for. Because the 5' end of microphthalmia transcripts have been well characterised, it is likely that such differences between the major transcripts and the cDNAs are due to diversity at the 3' end of the transcripts. This is further investigated below.

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```
cMIm Gcaaatactt ggcacgcctt tcataaactg ctttggttct tgatcagtag attaaatatt
cmi9 Gcaaatactt ggcacgcctt tcataaactg ctttggttct tgatcagtag attaaatatt

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cmi9 gtgcgtgtgt gtatatatta tatatatagg attttttta gaatttttgt gaagaaactt

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cMIm cataaaaaaa aaaaaaaaaa
cmi9 cataaaaaaa aaaaaaaaaa
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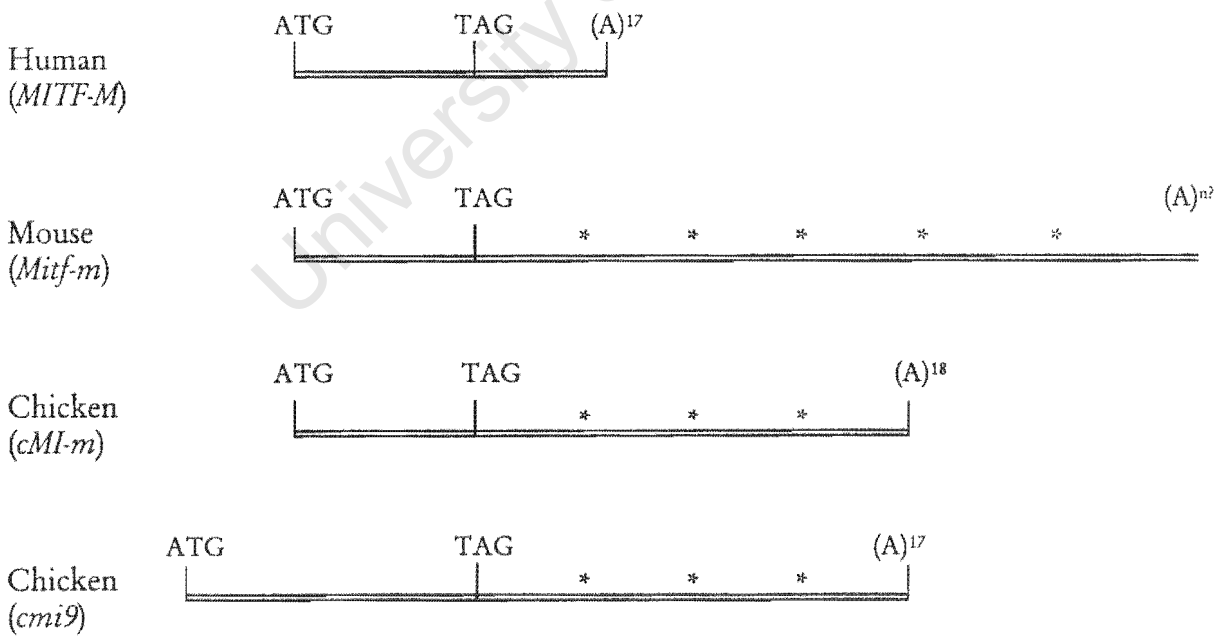
Fig. 3.5. The 3' untranslated region (UTR) nucleotide sequence of clone *cMI-m* (this study) and clone *cmi9* (Mochii *et al.*, 1998a). Sequences begin with the G nucleotide (bold face and underlined) of the TAG termination codon of the coding region of each clone. The 3' UTR of *cMI-m* spans 1341 bp; *cmi9* spans 1339 bp. Both contain three putative polyadenylation signals (AAUAAA, AUUAAA) (bold and underlined), five CAYUG recognition elements (underlined), and one poly (A+) tail. The eleven nucleotide differences are highlighted.

Figure 3.6. A comparison of the mammalian (mouse and human) and avian (chicken) microphthalmia 3' untranslated regions (UTRs). The Table compares the salient features of the cDNA sequences reported to date. The schematic demonstrates the lengths of the cDNAs relative to each other. Asterisks indicate the presence of a polyadenylation signal conforming either to the consensus sequence AAUAAA or the most common variant AUUAAA.

Species	Reported 3'-UTR length	Coding region length	Northern blot major transcript size(s)	Putative polyadenylation sequence (numbers of)	Poly (A+) tail	Reference(s)
Human (*MITF-M*)	419 bp	1257 bp	Unknown	None[1]	17 nts	Tachibana *et al.* (1994)
Mouse (*Mitf-m*)	3388 bp	1257 bp	5.5 kb 5.7 kb	AATAAA (2) ATTAAA (3)	Not reported[2]	Hallsson *et al.* (2000); Hodgkinson *et al.* (1993)
Chicken (*cMI-m*)	1341 bp	1158 bp	6.0 kb	AATAAA (2) ATTAAA (1)	18 nts	This study; Mochii *et al.* (1998a)
Chicken (*cmi9*)	1339 bp	1404 bp	6.0 kb	AATAAA (2) ATTAAA (1)	17 nts	Mochii *et al.* (1998a)



[1] Tachibana *et al.* (1994) did not report a polyadenylation sequence in the human *MITF* cDNA. Examination of the sequence reveals one variation of the consensus sequence, AAUCAA; however, this signal has very low polyadenylation activity (Wickens, 1990).

[2] The reported 3' UTR was from the mouse EST clone AV327306, generated by 3' RACE. Hallsson *et al.* (2000), however, were unable to amplify the 3' sequence of mouse *Mitf* using RACE experiments.

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3.3 The genomic organisation of the chicken *cMI* locus

As a first, preliminary step towards analysing the genomic structure of the chicken *cMI* gene, genomic Southern blot hybridisation was performed on DNA from two breeds of chicken: Black Australorps, which have a black plumage and pigmented eyes, and White Plymouth Rock x Pile Game chickens, which exhibit a white plumage with pigmented eyes. This latter breed is reminiscent of the mi^{bw} mouse, which exhibits a black eyed/white coat phenotype as a result of a genomic rearrangement (Yajima *et al.*, 1999). Therefore, it is possible that the corresponding phenotype of the White Plymouth Rock x Pile Game chicken is also caused by a rearrangement of the chicken *cMI* locus.

Thus, to determine if there are any large genomic differences between the two breeds at the *cMI* locus, genomic DNA from both breeds was digested with *Bam*HI, *Xho*I, *Pvu*II, *Kpn*I, and *Eco*RI and hybridised to the random-prime labelled *Bam*HI *cMI*-1.1 fragment (Fig. 3.7). The average sum of the labelled fragment sizes for the five enzymes used is 26.6 kb, with sizes ranging from 1.4 to 39 kb. Because there are no *Xho*I or *Eco*RI sites, and one *Bam*HI, one *Pvu*II, and one *Kpn*I site in the chicken *cMI-m* cDNA, these results suggest that the chicken *cMI* gene contains several introns. In addition, the fact that the *Xho*I digest generates the largest detected band at ~39 kb, and there are no *Xho*I sites within the chicken *cMI-m* cDNA, suggests that the chicken microphthalmia gene spans at least 39 kb of the genome. Comparisons of the Southern blot hybridisation analysis of Black Australorp and White Plymouth Rock x Pile Game reveals no gross differences (deletions or rearrangements) at the chicken *cMI* locus.

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3.4 Endogenous expression of the *cMI* gene

One of the most interesting aspects of the microphthalmia gene is the difference in the 5' termini between isoforms/species. Although much work has been carried out to elucidate the structure of the differences in the 5' ends of the microphthalmia cDNAs, it is still not clear whether the variations are responsible for functional differences. That is, do the different 5' termini play a role in the function of the proteins or are they merely indicative of different developmental histories in different tissues? In chickens, the isolation of *cMI-m* paves the way for an unravelling of some of the complexity and an expansion of the thus far narrow understanding of the different isoforms generated from the avian gene. A number of questions may now be addressed. Where is *cMI-m* expressed? Does its expression pattern differ from that of the *cmi9*

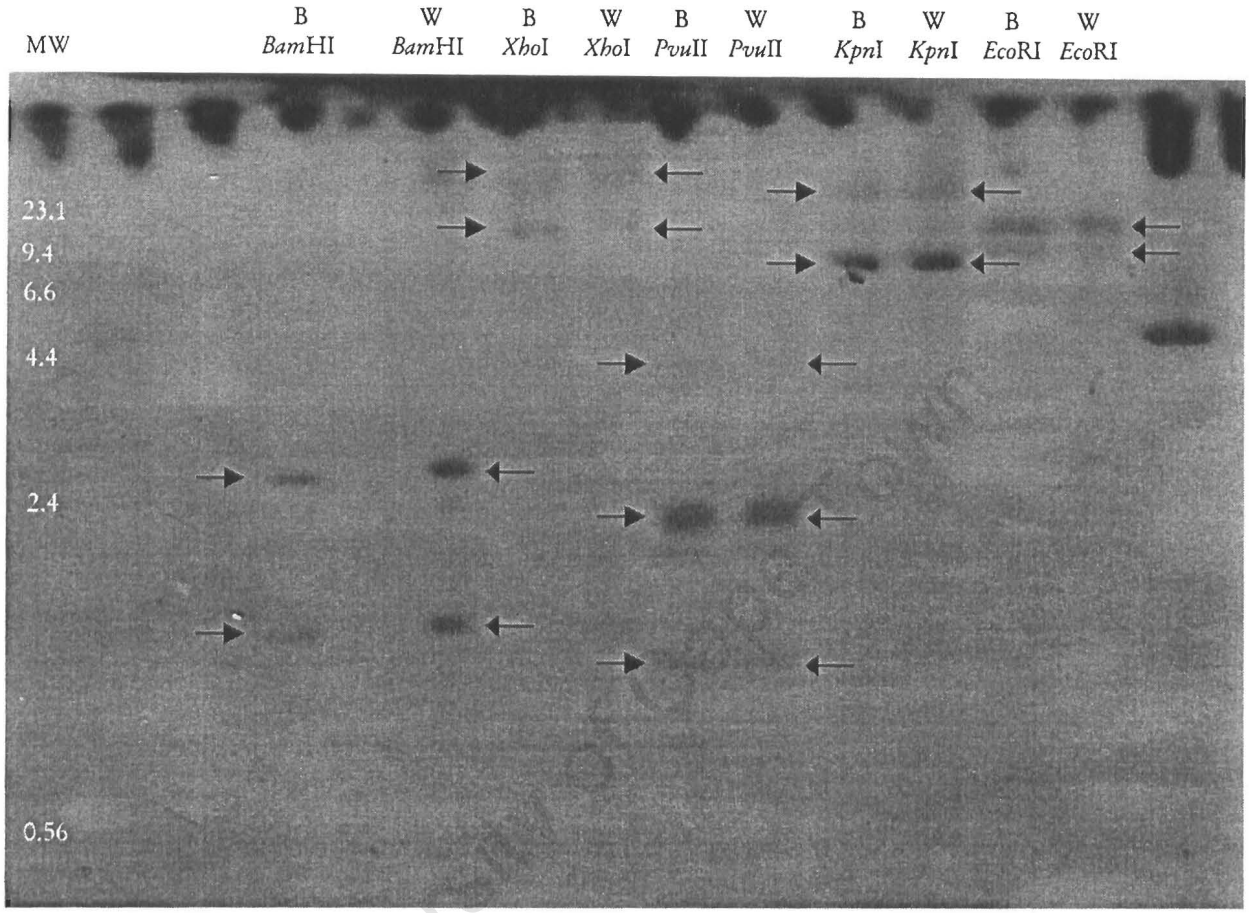

Fig. 3.7. Genomic Southern blot hybridisation analysis of the chicken *cMI* locus in Black Australorp (B) and White Plymouth Rock x Pile Game (W) chicken embryos. Genomic DNA (20 μg) prepared from 10 day-old B and W embryos was digested with *Bam*HI, *Xho*I, *Pvu*II, *Kpn*I, and *Eco*RI and subjected to Southern blot hybridisation using a radiolabeled 1.1 kb *Bam*HI chicken *cMI* cDNA probe. The molecular weight (kb) marker (MW) is λ/*Hind*III. Arrows indicate the positively hybridising bands obtained with each restriction enzyme.

isoform? Will such a comparison allow for predictions of a relationship between structure and function?

To specifically elucidate which tissues express which isoform(s) of microphthalmia, different tissues were analysed by reverse transcription/polymerase chain reactions [RT-PCR] using three sets of specifically designed primers. The first set amplifies the unique amino terminus of *cMI-m*, the second set amplifies the unique first exon of *cmi9*, while the third set of primers amplifies the conserved bHLH-Zip domain (hereafter called the bHLH-Zip primer pair) of all microphthalmia isoforms.

A number of different tissues were assessed for microphthalmia transcripts. Total RNA was extracted from the heart, brain, skin, total eye, and gastrointestinal tracts of 6 day- and 10 day-old Black Australorp and White Plymouth Rock x Pile Game chicken embryos. Using bHLH-Zip primer pair, microphthalmia transcripts were detected in all tissues following 40 PCR cycles (Fig. 3.8). This result is the same as those of Mochii *et. al* who showed that microphthalmia transcripts are present in a variety of developing chicken tissues (brain, neural retina, liver, heart and gizzard) following 35 PCR cycles (Mochii *et al.*, 1998a). These results are somewhat surprising, because *in situ* hybridisation analyses show that chicken microphthalmia transcripts are restricted to tissues known to require microphthalmia for differentiation (i.e. RPE) (Mochii *et al.*, 1998a). The simplest explanation is that the high PCR cycle numbers result in the detection of "illegitimate" transcripts of microphthalmia.

To explore this possibility, semi-quantitative RT-PCR was carried out to determine whether it is possible to distinguish between transcripts in non-pigmented cells and pigmented cells. Four chicken RNAs (skin, eye, brain and cultured fibroblasts) were amplified by RT-PCR and samples analysed at 20, 25 or 30 PCR cycles using the bHLH-Zip primer pair. For the two pigmented tissues (eye and skin), transcripts were detected after 25 PCR cycles (Fig. 3.9.a and 3.9.b) while for non-pigmented tissues (brain and fibroblasts) 30 PCR cycles were necessary before transcripts were detected (Fig. 3.9.c and 3.9.d). These results were obtained at least thrice, are in agreement with a subsequent study performed in this laboratory (J. Key, personal communication), and show that at 25 PCR cycles, it is possible to distinguish between levels of expression of microphthalmia in pigmented tissues versus non-pigmented tissues, and that the levels of expression in pigmented tissues is higher than that in non-pigmented tissues

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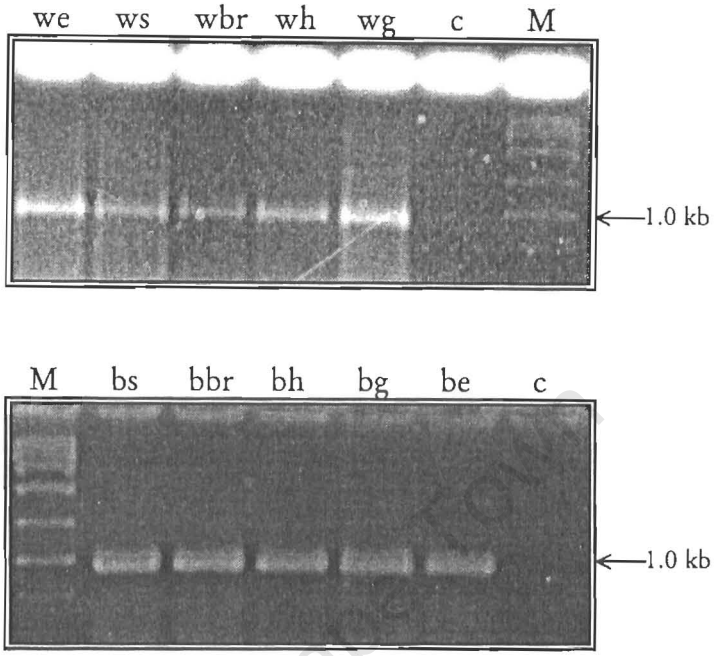
(a) 6-day old embryos



(b) 10-day old embryos

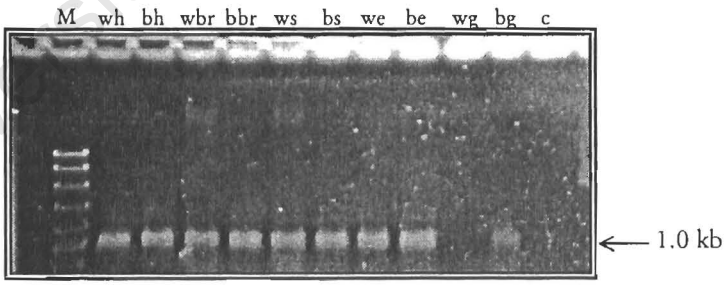


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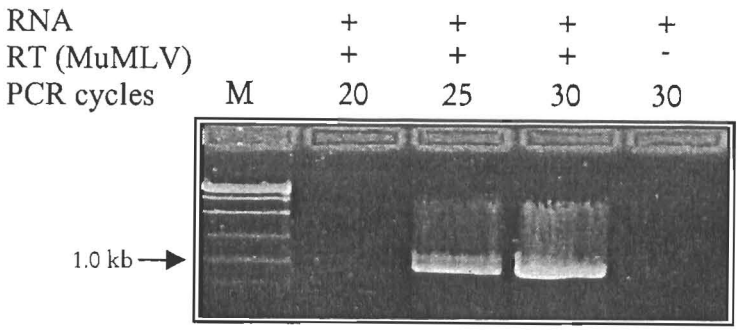
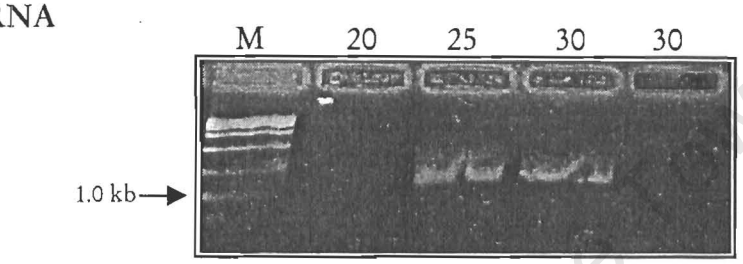
Fig. 3.8. Expression of the chicken *cMI* gene in **(a)** 6-day old chicken embryos and **(b)** 10 day-old chicken embryos. RT-PCR analysis was performed on pigmented and non-pigmented tissues from White Plymouth Rock x Pile Game (w) and Black Australorp (b) chicken embryos. 1 μg of total RNA from heart (h), brain (br), skin (s), eye (e), and gastrointestinal tract (g) was subjected to one-tube RT-PCR using a primer pair (cMIF3/cMIR2) that will amplify the conserved bHLH-Zip domains of all microphthalmia isoforms. Transcripts were detected following 40 PCR cycles by ethidium bromide staining. c: H_20 negative control in which no RNA was added. The lack of a product in the (wg) lane of 10 day-old embryos reflects a failed reaction; the tissue does express *cMI*. The molecular weight marker (M) is Pox/*Eco*RI and the 1.0 kb fragment is indicated in each figure.

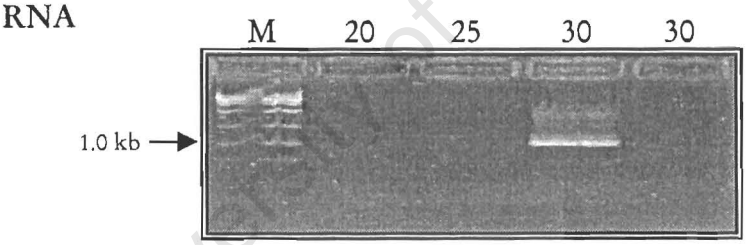
(a) Eye RNA



(b) Skin RNA



(c) Brain RNA



(d) Fibroblast RNA

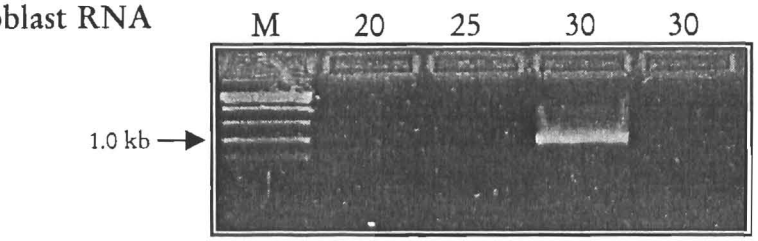


Fig. 3.9. Expression of *cMI* in embryonic chicken pigmented and non-pigmented tissues. Shown are the products of two-step RT-PCR of chicken (a) eye, (b) skin, (c) brain, and (d) fibroblast RNA subjected to reverse transcription and 20, 25, or 30 PCR cycles using the cMIF3/cMIR2 (bHLH-Zip) primer pair. Numbers above each lane indicate the numbers of PCR cycles used. (+) indicates the presence of RNA or RT enzyme in each sample and (-) indicates the absence of RNA or RT enzyme in each sample; this legend is shown only for the eye products but the same applies to each lane of the skin, brain and fibroblast products. The molecular weight marker (M) is Pox/*Eco*RI and the 1.0 kb fragment is indicated in each case.

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3.4.1 Endogenous expression of *cMI-m* and *cmi9* in pigmented tissues

The next question addressed was whether the transcripts detected in the eye and the skin represents the *cMI-m* and/or the *cmi9* isoforms. This is an important question because other investigators have shown that some isoforms are preferentially expressed in some tissues, and it is postulated that these might have functional implications. Experiments were performed as previously described using 20-30 PCR cycles, except that specific primer pairs used were those that amplified either the unique first exon of *cMI-m* or the unique first exon of *cmi9*. In this section of the present study, RPE RNA was used instead of total eye RNA. This allowed for the exclusion of eye choroidal cells, and hence a clean and more meaningful comparison of expression patterns in chicken neuroepithelial-derived pigmented cells (RPE) and neural crest-derived melanocytes (skin) would be obtained. As a control for the tissue-specificity of the *cMI-m* and *cmi9* transcripts, chicken fibroblast RNA was used.

Fig. 3.10.a demonstrates that *cMI-m* expression was detected in the skin RNA after 25 cycles, whereas *cmi9* expression was not detected, even following 30 cycles. Fig. 3.10.b demonstrates that, in contrast, *cmi9* expression was detected in RPE RNA following 25 cycles, but that *cMI-m* expression was not detected in RPE RNA, even following 30 cycles. These results suggest that the expression of *cMI-m* and *cmi9* are restricted to skin melanocytes and RPE, respectively.

A surprising result was obtained with the putative negative control cell line: In Fig 3.10.c it can be seen that *cMI-m* mRNA, but not *cmi9* mRNA, expression was detected in fibroblasts following 30 PCR cycles. This suggests that *cMI-m* may be the source of transcripts in chicken fibroblasts; this result is somewhat surprising, as other researchers have demonstrated that expression of the mammalian melanocyte-type isoform is restricted to pigmented cells. However, more recent reports have also suggested the presence of the melanocyte-type isoform in non-pigmented cells (as detailed in Chapter One). It was therefore important to determine whether the transcripts here amplified from chicken embryonic fibroblasts are indeed microphthalmia transcripts. To do this, the transcripts amplified by RT-PCR were subjected to automated sequencing using a primer (cMIF5) that will anneal to the melanocyte-type first exon. As illustrated in the ABI PRISM automated sequencer output shown on the following page and the BLAST aligment shown in Fig. 3.11, sequencing demonstrates that the transcripts amplified from chicken embryonic fibroblasts are those encoding for the melanocyte-type microphthalmia isoform. The transcripts are missing both exon 3 and 6a.

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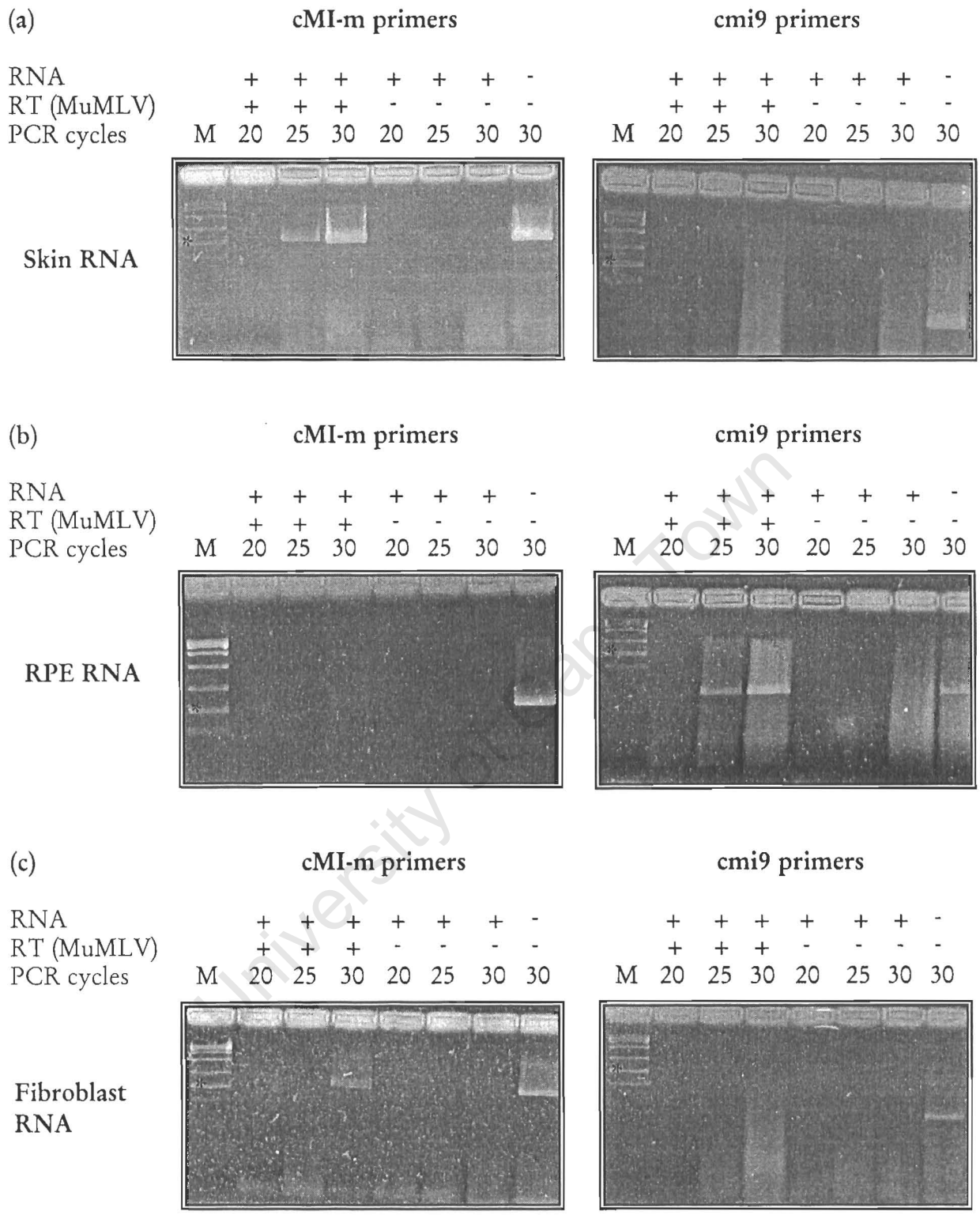

Fig. 3.10. Expression of *cMI-m* and *cmi9* in embryonic chicken pigmented and non-pigmented tissues. Shown are the products of two-step RT-PCR of (a) skin, (b) RPE, or (c) fibroblast RNA subjected to reverse transcription and PCR using 20, 25, or 30 cycles. The cMI-m primers refer to the primer pair that will amplify the unique first exon of *cMI-m*; the cmi9 primers refer to the primer pair that will amplify the unique first exon of *cmi9*. (+) indicates the presence of RNA or RT enzyme in each sample and (-) indicates the absence of RNA or RT enzyme in each sample. Numbers above each lane indicate the numbers of PCR cycles used. The final lane in each figure is a positive plasmid cDNA control for PCR amplification. In the case of *cMI-m* primer amplification, RCAS/*cMI-m* was used; in the case of *cmi9* primer amplification, RCAS/*cmi9* was used. The molecular weight marker (M) is Pox/*Eco*RI and the 1.0 kb fragment is indicated with an asterisk (*) in each case.

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ABI PRISM™

Model 3700
Version 3.4.1
Basecaller-3700POP6.bcfibF5(cMIF5)
BC 1.3.0.0

D021022_G03_020.ab1
Pitha-KP
Lane 20

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Tue, Oct 22, 2002 4:25 PM
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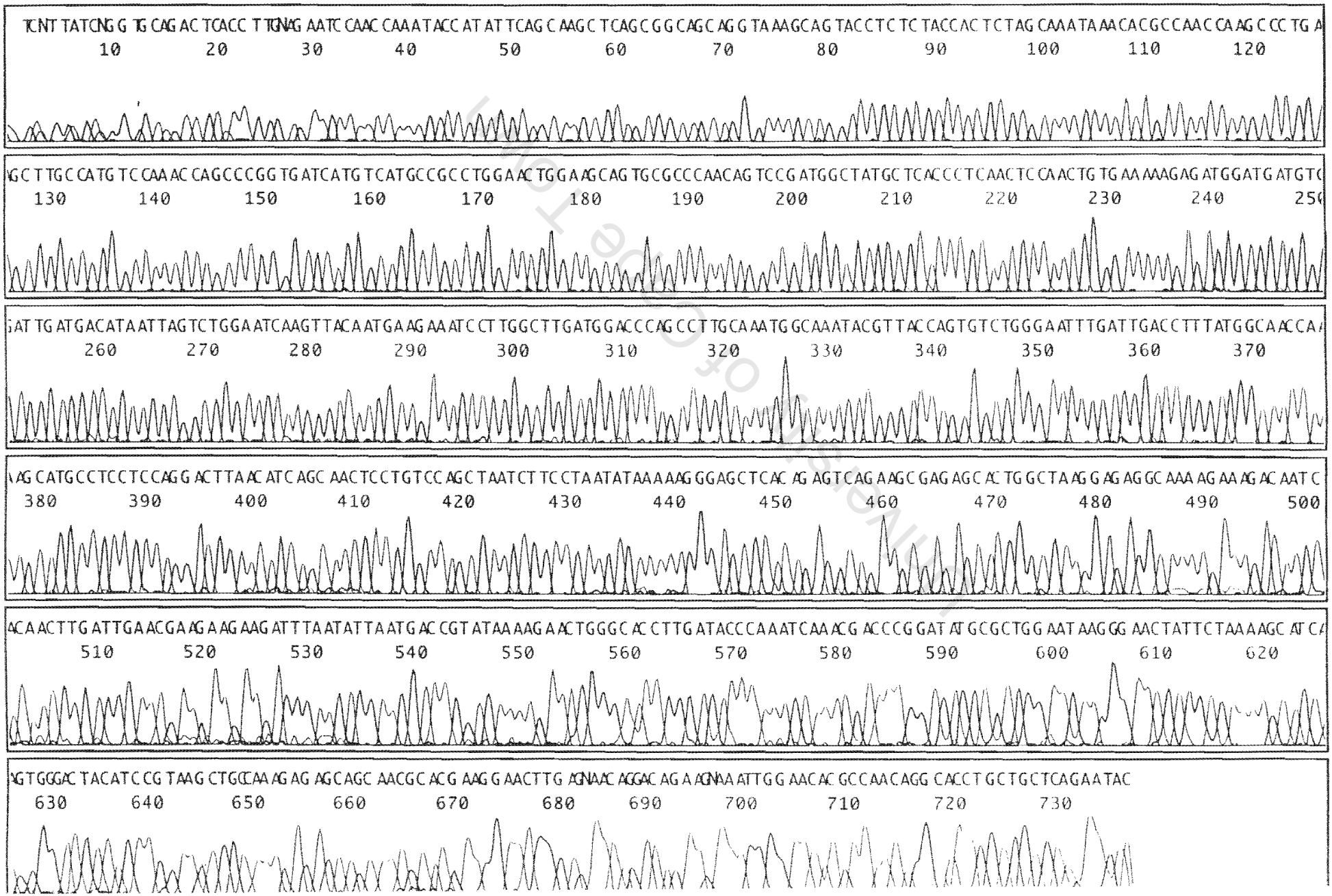

Not to be used for commercial purposes

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Figure 3.11. Results of a BLAST alignment of the sequence obtained from the amplification of chicken embryonic fibroblast RNA using RT-PCR and a primer specific for the first exon of *cMI-m*. The Figure is taken directly from the BLAST output and shows the amplification product from fibroblasts ("query") aligned with the sequence obtained from clone M156 ("sbjct") in the present study (Accession number AF145751). Numbering indicates the nucleotide positions.

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3.4.2 Analyses of Microphthalmia proteins in embryonic tissues and cell lines

In order to determine whether the microphthalmia transcripts detected above are translated into proteins, western blot analyses using a Mitf antibody were carried out. The antibody used was a mouse monoclonal anti-Mitf antibody generated against the domains common to all microphthalmia isoforms (a kind gift of C. Takemoto, Johns Hopkins University; Takemoto *et al.* (2002)). To determine whether this antibody cross-reacts with the chicken Microphthalmia proteins, cell extracts from chicken RPE and fibroblast cultures were analysed by western blotting. Included on the blots were protein extracts from mouse B16 melanoma cells (as a positive control) and HeLa cells (as a negative control). The results are illustrated in Fig. 3.12.a-c.

When the ECL x-ray film was exposed for approximately five seconds, two Mitf proteins of approximately 60 and 65 kDa were detected in B16 cells (Fig. 3.12.a; lanes 1 and 4). This protein doublet represents different phosphorylation forms of the melanocyte-type Microphthalmia protein (Hemesath *et al.*, 1998; King *et al.*, 1999; Takemoto *et al.*, 2002), although the reported sizes of the proteins differ in the various publications (further discussed in Chapter Four). The additional bands located below the two major proteins are likely proteolytic products because they are "cleaned up" by western-immunoprecipitation, while the additional band above the two major proteins is thought to represent the Mitf-a isoform; this isoform is detected using RT-PCR of B16 RNA and a-isoform-specific primers (C. Takemoto, personal communication).

The two non-pigmented cell lines (chicken fibroblasts and HeLa cells) give different results upon a five second exposure of the western blot. A doublet of ~ 65 - 69 kDa is detected in human HeLa cells (Fig. 3.12.a; lane 2), but no protein is detected in chicken embryonic fibroblasts (Fig. 3.12.a; lane 3). However, when the film is further exposed, a faint doublet of ~ 65 - 69 kDa is detected in chicken embryonic fibroblasts after thirty seconds (Fig.3.12.b; lane 3; indicated with an arrow); this band intensifies following an exposure time of two minutes (Fig 3.12.c; lane 3).

Finally, the protein detected in chicken RPE cells (Fig. 3.12.a-c; lane 5) runs as a single band of about 69 kDa. The fact that the RPE protein (i) runs as a single species and (ii) is approximately the same size as the protein detected in both human and chicken non-pigmented cell lines are both interesting points, and are further discussed in Chapter Four.

In summary, the mouse monoclonal Mitf antibody does cross-react with the chicken Microphthalmia protein present in RPE cells and the size of the protein is ~ 69 kDa. The

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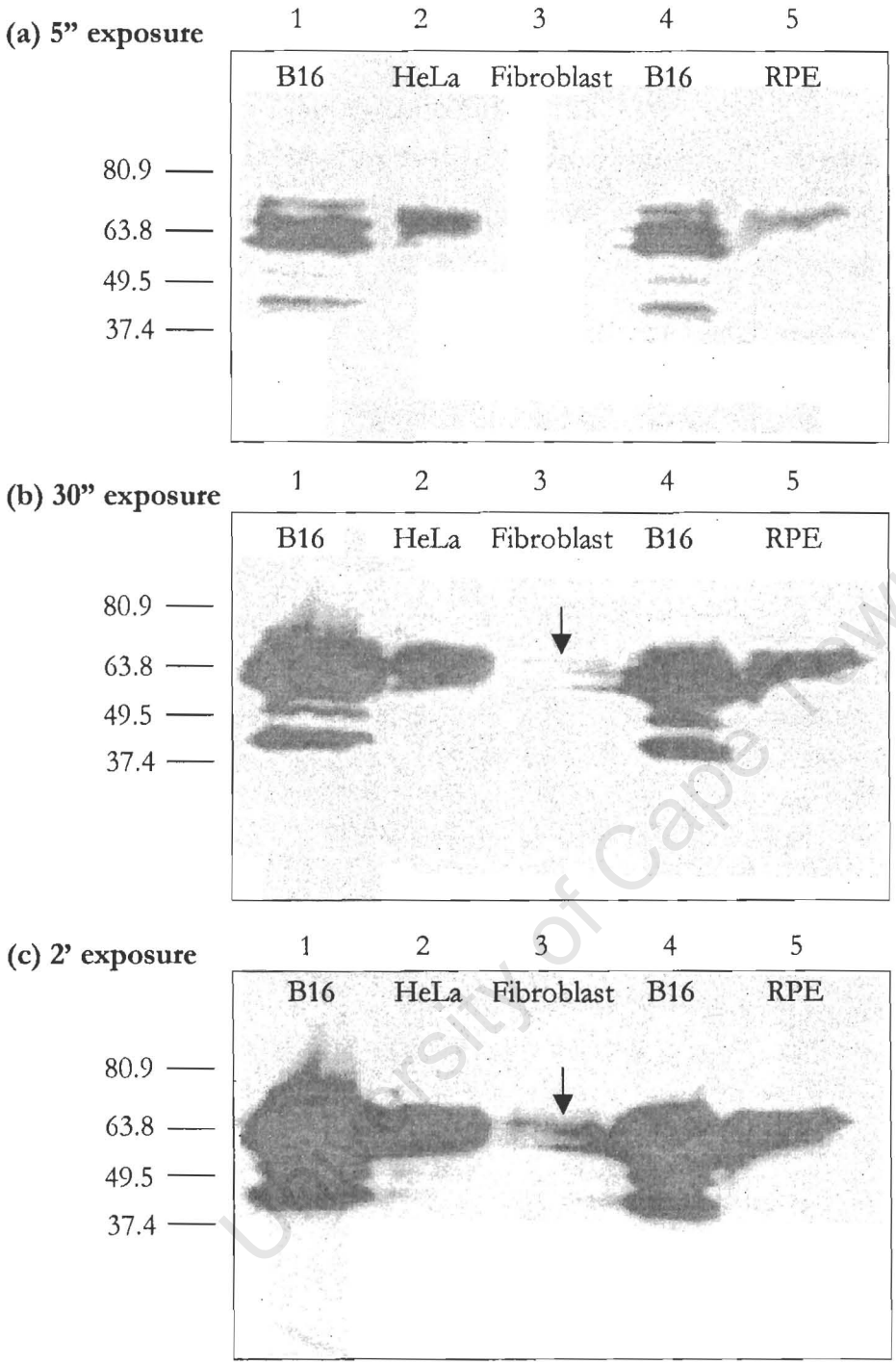

Figure 3.12. Western blotting demonstrating that the mouse anti-Mitf antibody cross-reacts with the chicken Microphthalmia protein. **(a)** A five-second exposure produces the previously described 60-65 kDa melanocyte-type Microphthalmia protein doublet (Takemoto *et al.*, 2002) in mouse B16 melanoma cells (lanes 1 and 4), a doublet of ~ 65-69 kDa in human HeLa cells (lane 2), no protein in chicken embryonic fibroblasts (lane 3), and a doublet of ~ 65-69 kDa in chicken RPE cells (lane 5). **(b)** A thirty second exposure produces a very faint doublet (indicated with arrow) of ~ 65-69 kDa in chicken embryonic fibroblasts (lane 3). **(c)** A two minute exposure intensifies the bands in chicken embryonic fibroblasts (lane 3). The molecular weight marker sizes (kDa; BenchMark Prestained Protein Ladder, Invitrogen) are shown on the left-hand side.

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previously described ~ 60-65 kDa melanocyte-type doublet (Takemoto *et al.*, 2002) is detected in B16 cells, and bands of ~ 65-69 kDa are detected in HeLa and fibroblast cells, although the bands detected in HeLa cells are much stronger than those in fibroblasts.

3.4.3 Diversity in the 3'-UTR region of endogenous microphthalmia transcripts

As described in Section 3.2.4, the length of the *cMI-m* cDNA 3'-UTR differs substantially from the lengths reported for mouse and human cDNA 3'-UTRs, but is very similar to that reported for the *cmi9* cDNA. Thus the question arises: are these two chicken cDNA 3'-UTRs representative of the 3'-UTR of chicken microphthalmia mRNAs present in cells?

As a start to investigating this in a pigmented tissue, RT-PCR was carried out on total eye RNA using a primer that anneals from nts 998-1020 of the *cMI-m* coding region, and an oligo dT_{15} primer. In theory, based on known sequencing results, PCR with this primer pair should amplify a transcript of 1.5 kb (160 bp of the coding region and the 1341 bp 3'-UTR). However, two transcripts are amplified: Products of 1.3 kb and 0.90 kb are amplified from Black Australorp total eye RNA, while products of 1.3 kb and 1.0 kb are amplified from White Plymouth Rock x Pile Game total eye RNA (Fig. 3.13). These results suggest that the 3'-UTR of microphthalmia transcripts is subjected to alternative polyadenylation, a concept further discussed in Chapter Four.

3.5 Expression and functional analyses of chicken microphthalmia cDNAs in transfected cells.

Having established the general and tissue-specific expression patterns of *cMI-m* and *cmi9* isoforms of chicken microphthalmia, the next series of experiments were designed to functionally analyse these two microphthalmia proteins by assessing the ability of each to transactivate the chicken tyrosinase gene promoter in pigmented and non-pigmented cell lines. In order to carry out these experiments, it was first necessary to clone the *cMI-m* insert into a suitable expression vector and establish that mRNA is transcribed and further translated into proteins from the *cMI-m* and *cmi9* cDNAs. The following sections describe the cloning of RCAS/*cMI-m* into RCAS BP(A) as well as the existing RCAS BP(A)/*cmi9* cDNA.

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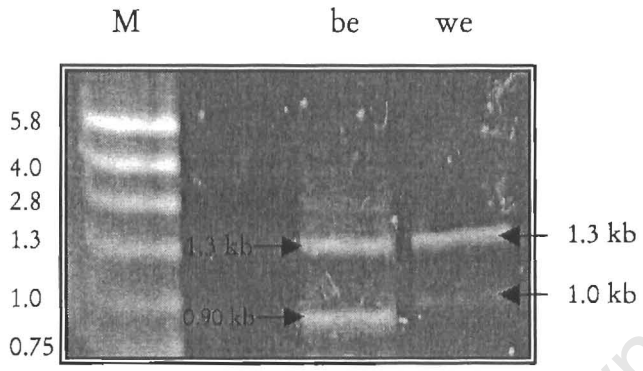

Fig. 3.13. Expression of the 3' UTR of *cMI* in total eye. Shown are products of one-tube RT-PCR of total eye (e) extracted from Black Australorp (b) and White Plymouth Rock x Pile Game (w) chicken embryos. The PCR products obtained with each sample are indicated, along with the sizes of each. The molecular weight marker (M) is Pox/*Eco*RI and the sizes (kb) of the marker fragments are indicated on the left hand side.

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3.5.1 Gene constructs

The transient transfection assays require that a protein be translated from *cMI-m*. Thus, the *cMI-m* cDNA was cloned into the RCAS BP(A) avian retroviral vector, with the intention of using the construct to mediate efficient gene transfer in chicken cells *in vitro* and *in vivo*. This replication-competent retroviral vector requires that foreign DNA be inserted into the *Cla*I restriction enzyme site with minimal 5' and 3'-UTRs, as these sequences have been shown to give rise to abortive protein translation (Morgan and Fekete, 1996).

3.5.1.1 Cloning of RCAS/*cMI-m* and RCAS/*cMI-mr*

Two approaches for the cloning of the *cMI-m* coding region into RCAS BP(A) were initially considered. The first was to isolate the coding region from clone M156 and ligate it into the RCAS BP(A) *Cla*I site. This approach required the identification of restriction enzyme sites on either side of the coding region. However, due to difficulties experienced with restriction enzyme mapping (further detailed in Appendix A), it proved impossible to locate appropriate restriction enzyme sites in clone M156 that would be suitable for the excision of the coding region without extensive 5' or 3' UTRs. The second approach was to PCR amplify the coding region using *Cla*I-tagged primers designed to amplify from the ATG and the TAG codons in clone M156, with subsequent ligation of the PCR fragment into the *Cla*I site of RCAS BP(A). As detailed in Appendix A, difficulties introduced through *dam* methylation at the *Cla*I restriction site resulted in a failed cloning strategy. Thus both of these two simple approaches turned out to be problematic. Although much was learnt through assessing and exploring the viability of these two options, another approach, ultimately successful, was taken.

The full-length *cMI-m* coding region, excluding extensive 5' or 3'-UTR sequences, was obtained by ligation of the *Bam*HI fragment of clone M156 to a PCR fragment of the 3' portion of the coding region. In brief, clone M156 was restriction enzyme mapped and subjected to Southern blotting using the mouse *Mitf* cDNA probe. A positively hybridising *Bam*HI 1.1 kb fragment was subcloned into pBluescript SK- digested with *Bam*HI (Fig. 3.14.a) and the resulting clone was called *cMI*-1.1. Clone *cMI*-1.1 was sequenced manually using a combination of universal vector and custom-designed primers and was found to carry the 5' portion (nts 1- 1042) of the chicken microphthalmia gene.

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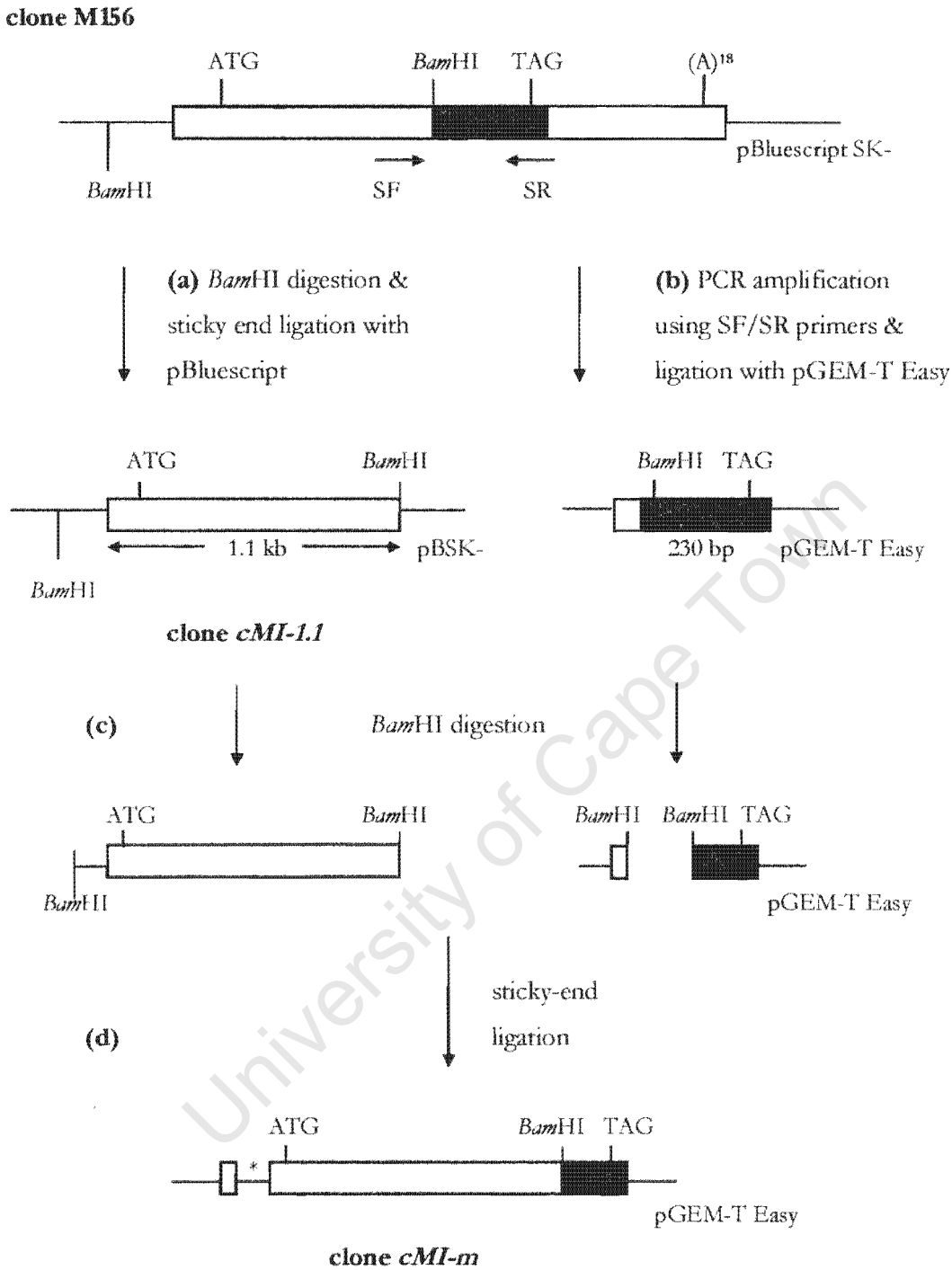

Figure 3.14. Cloning strategies for the generation of *cMI-m*. See text for details. The sequence marked with an asterisk (*) in clone *cMI-m* is pBluescript sequence carried through from cloning strategies used to generate clone *cMI-1.1*. *cMI-m* thus carries, from left to right, 52 bps of sequence from the PCR product (small yellow bar), 19 bps of pBluescript sequence, 34 bps of 5' UTR, the 1158 bp coding region, and 30 bps of 3' UTR (yellow/red bar).

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Next, a 230 bp fragment (nt 992-1222 of the chicken microphthalmia cDNA) was amplified from clone M156 using PCR and the SF and SR primers. The PCR product was A-tailed and ligated into pGEM-T Easy (Fig. 3.14.b). The pGEM-T Easy clone was then digested with *Bam*HI and the excised insert was ligated with the gel purified *Bam*HI fragment from clone *cMI-1.1* (Fig. 3.14.c and 3.14.d). The resulting clone of 1293 bp was called *cMI-m* for chicken microphthalmia-melanocyte type. Automated sequencing confirmed: (a) the presence of an ATG initiation codon and a TAG termination codon; (b) the orientation of the 1.1 kb fragment relative to the PCR fragment; (c) that the *Bam*HI site was correct, and; (d) that mutations had not been introduced during the PCR amplification of the 230 bp fragment. The automated sequencing reaction was performed only once as it verified information obtained from the manual sequencing of the two cDNAs used to generate *cMI-m*.

The next step was to clone the *cMI-m* insert into the RCAS BP(A) retroviral vector. The 1293 bp insert was excised from clone *cMI-m* with *Eco*RI and the protruding 5' overhangs were blunt-ended using Klenow polymerase. Likewise, RCAS BP(A) was digested with *Cla*I and the protruding 5' overhangs were blunt-ended using Klenow polymerase. The *cMI-m* insert was ligated with RCAS BP(A), *E. coli* cells were transformed with the ligation mixture, and resulting colonies screened for orientation of the insert relative to the retroviral genome. Clones of both orientations were desired. The insert in the correct orientation will transcribe microphthalmia using the viral LTR as a promoter and the *cMI-m* ATG as a transcription start point. The insert in the incorrect orientation will not transcribe microphthalmia, and will thus serve as a control for both nonsense translation and the effect of the retroviral vector in transfection assays. Digestion with *Kpn*I digests yielded fragments of approximately 3.1 kb and 9.8 kb, indicating an insert in the correct orientation relative to the retroviral genome, and 2.3 kb and 10.6 kb when the insert is in a reverse orientation relative to the retroviral genome (Fig. 3.15). The resulting clones were called RCAS BP(A)/*cMI-m* for Replication-Competent, Avian leukemia virus LTR, Splice acceptor, Bryan high titer Polymerase, (A)-envelope subgroup/chicken microphthalmia-melanocyte type isoform and RCAS BP(A)/*cMI-mr* for reverse orientation. The RCAS/*cMI-m* clone contains 52 bps of sequence from the PCR fragment, 19 bps of sequence carried through from pBluescript, 34 bps of 5' UTR, the 1158 bp coding region, and 30 bps of 3' UTR (see Fig. 3.14).

The cloning of RCAS BP(A)/*cMI-m* and RCAS BP(A)/*cMI-mr*, and the position of the *cMI-m* and *cMI-mr* inserts relative to the viral genome, are schematically shown in Fig. 3.16.a; Fig. 3.16.b

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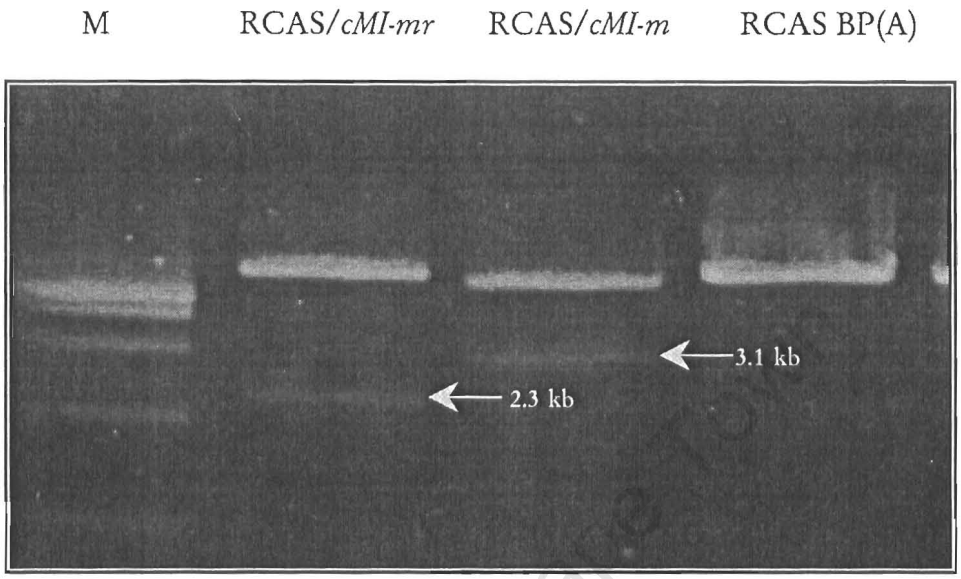

Fig. 3.15. *KpnI* restriction enzyme digestion of RCAS/*cMI-mr* and RCAS/*cMI-m* to confirm the orientation of each insert relative to the genome of the retroviral vector. Arrows indicate the different insert sizes obtained with *KpnI*. These predicted insert sizes were based on a combination of computer-aided restriction enzyme mapping of RCAS BP(A) and that of the chicken microphthalmia cDNA. The final lane is RCAS BP(A) restriction enzyme digested with *Cla*I, which will linearise at 11.599 kb, as a control for the vector size. The molecular weight marker (M) is Pox/*Eco*RI.

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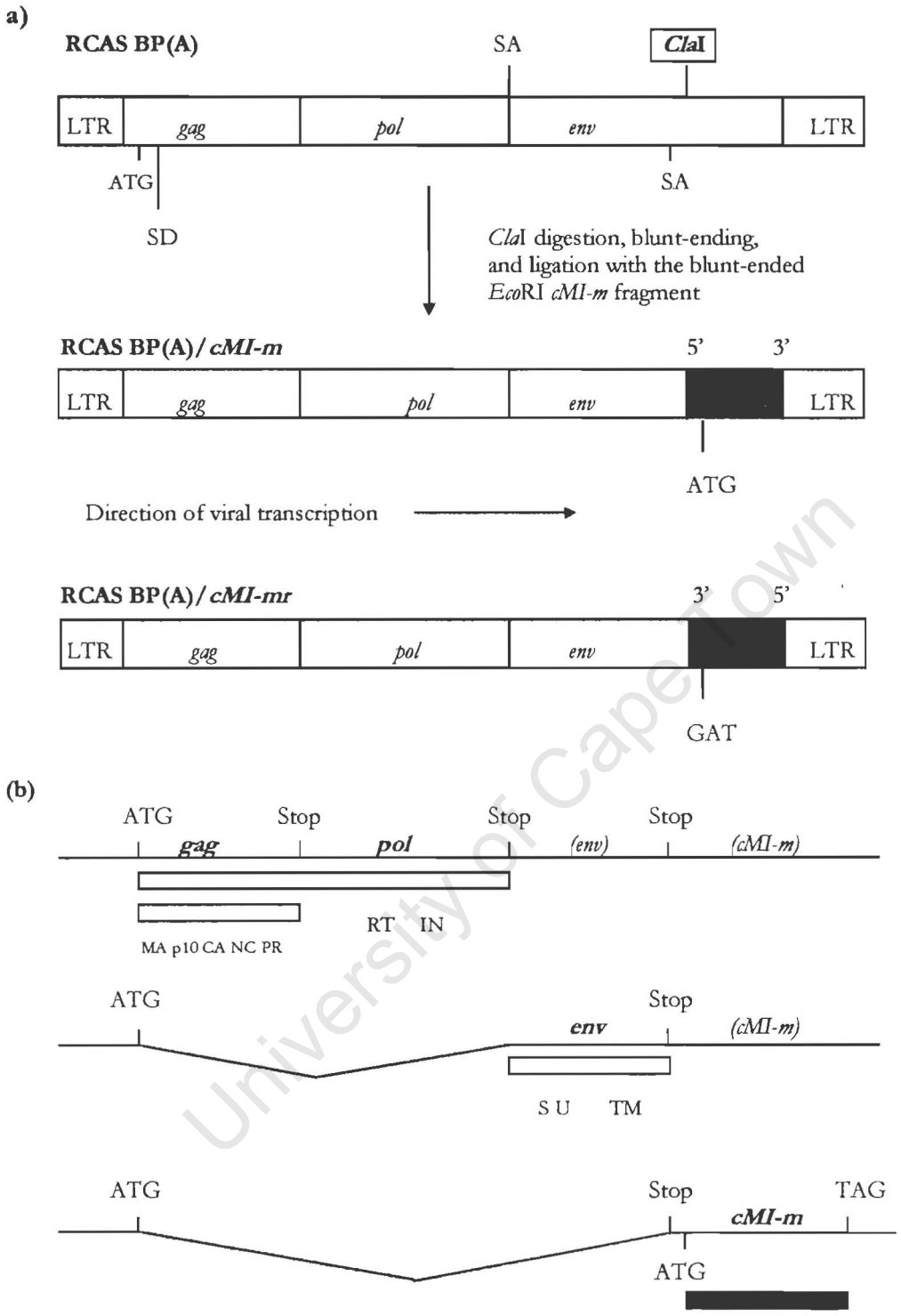

Figure 3.16. **(a)** Schematic representation of the cloning of the coding region of the chicken *cMI* cDNA isolated in the present study into the retroviral vector RCAS BP(A). The coding region (ATG - TAG) was inserted both in the direction of viral genome transcription (RCAS BP(A)/*cMI-m*) and in the opposite direction to that of viral genome transcription (RCAS BP(A)/*cMI-mr*) in the *ClaI* restriction site (boxed). LTR, long terminal repeat; SA, splice acceptor; SD, splice donor. **(b)** Three RNA variants result, with the splicing indicated. Stop refers to the presence of a stop codon. Open bars beneath each mRNA indicate the precursor proteins encoded by each. In most cases the transcript carrying the foreign gene (in this case *cMI-m*) makes up about 20-30% of the virally encoded RNAs. CA, capsid; IN, integrase; MA, matrix; NC, nucleoprotein; PR, protease; RT, reverse transcriptase; SU, surface (receptor binding); TM, transmembrane. Nomenclature according to Leis *et al.* (1988). Adapted from Morgan and Fekete (1996).

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shows the three alternative splice RNA variants expected from RCAS BP(A) transcription. The RNA translated by *gag* and *pol* can be packaged into virions as genomic RNA, or it may be used for translation of the viral core proteins: MA, p10, CA, NC, and PR. The enzymes reverse transcriptase (RT) and integrase (IN) are produced from a translational frame shift of the same mRNA. Alternative splicing generates a shorter transcript, which is used for the translation of the viral envelope proteins, SU and TM. Finally, the shortest alternative splice variant translates the exogenous gene (in this case *cMI-m*). Usually this short transcript makes up about 20-30% of the virally encoded RNAs (Morgan and Fekete, 1996), which places an upper limit on the level of expression that can be expected from the exogenous gene.

3.5.1.2 RCAS/*cmi9*

The plasmid here referred to as RCAS/*cmi9* was a kind gift of Dr. M. Mochii and has previously been described as RCAS*Mitf* (Mochii *et al.*, 1998a). Briefly, Mochii *et al.* amplified the coding region of the *cmi9* cDNA using primers with *Cla*I sites, and ligated the product into the *Cla*I site of the RCAS BP(A) vector. The primers amplified 60 nts of 5' UTR, the 1.404 kb coding region, and 119 nts of the 3' UTR; hence this clone also carries minimal 5' and 3' UTR sequences.

3.5.2 Transcription of RCAS/*cMI-m* and RCAS/*cmi9* in transiently transfected cells

When transfected into cells, the RCAS/*cMI-m* and RCAS/*cmi9* expression vectors should be transcribed from the viral LTR promoters and be translated into proteins. The presence of the proteins may then be verified by western blotting and their activity/function assessed using co-transfection with the tyrosinase promoter-*luciferase* reporter constructs.

To determine whether mRNA is transcribed from the two constructs, RT-PCR analyses were performed. Fibroblasts were transfected with 5 μg of RCAS/*cMI-m* or RCAS/*cmi9* and, twenty-four hours later, total RNA was extracted from the cells. As a negative control, RNA was also extracted from untransfected fibroblasts that had been isolated at the same time and subjected to the same culture conditions as the transfected cells. The three RNAs were reverse transcribed either with the RT enzyme (MuMLV) or, as a control for DNA contamination, without the RT enzyme. The first strand cDNAs were then amplified using PCR (30 cycles) and the bHLH-Zip common primer pair. The results are shown in Fig. 3.17. Lane (a) illustrates that microphthalmia transcripts are detected in untransfected fibroblasts following 30 PCR cycles, confirming the previous finding in this study. The lack of amplification products in lane (b) (the untransfected fibroblast RNAs that were subjected to reverse transcription in the absence of the RT enzyme)

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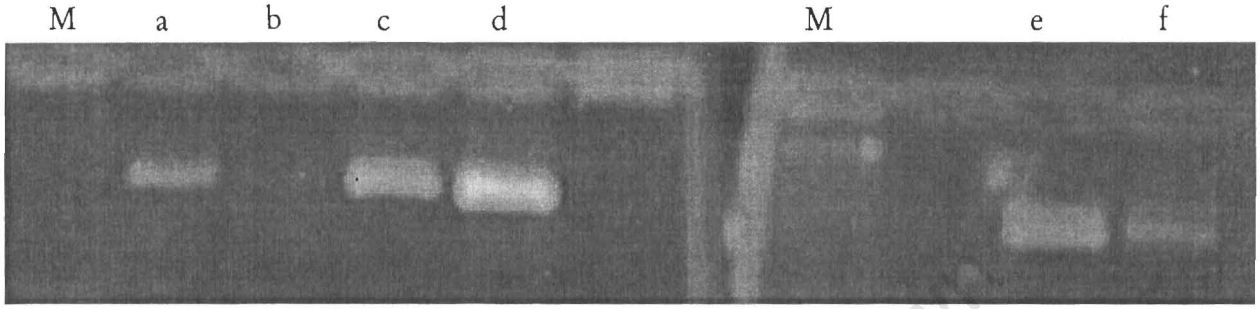

Fig. 3.17. Plasmid DNA interferes with the detection of *cMI-m* and *cmi9* transcripts in transfected fibroblasts. Shown are the products of two-step RT-PCR of chicken fibroblast RNA amplified using the primer pair that amplifies the conserved bHLH-ZIP domains of all microphthalmia transcripts. Lane (a): amplification of untransfected fibroblast RNA in the presence of both RNA and MuMLV RT; Lane (b): amplification of untransfected fibroblast RNA in the presence of RNA but in the absence of MuMLV RT; Lane (c): amplifiction of RNA extracted from fibroblasts transfected with RCAS/*cMI-m* in the presence of both RNA and MuMLV RT; Lane (d): amplification of RNA extracted from fibroblasts transfected with RCAS/*cMI-m* in the presence of RNA but in the absence of MuMLV RT; Lane (e): amplification of RNA extracted from fibroblasts transfected with RCAS/*cmi9* in the presence of both RNA and MuMLV RT; Lane (f): amplification of RNA extracted from fibroblasts transfected with RCAS/*cmi9* in the presence of RNA but in the absence of MuMLV RT. In all cases, reactions were subjected to 30 PCR cycles. The molecular weight marker (M) is Pox/*Eco*RI.

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shows that the amplification products are not the result of genomic DNA contamination. Lane (c) and lane (e) show microphthalmia products in fibroblasts transfected with RCAS/*cMI-m* or RCAS/*cmi9*, respectively. However, the presence of products in the transfected control samples without RT ("mock reverse transcribed") in lanes (d) and (f) demonstrate that in these samples the amplification products are the result of contaminating plasmid DNA (because these products are not amplified from the mock reverse transcribed sample of the non-transfected fibroblasts, and because the primer pair used crosses more than one intron/exon boundary, the source of the contaminating DNA cannot be genomic, but must rather be the plasmid DNA used in the transfections).

Therefore, another approach was needed to determine whether the mRNA is transcribed from the two RCAS constructs. Two additional tactics were necessary: First, a method was required to remove the contaminating plasmid DNA from the transfected fibroblast RNA samples. Secondly, a semi-quantitative and 5' termini-specific PCR approach was crucial in order to distinguish between the endogenous microphthalmia transcripts (previously shown to be *cMI-m* transcripts detectable at 30 PCR cycles) and the exogenously expressed transcripts.

With this in mind, experiments were repeated and total RNA was extracted from non-transfected fibroblasts and fibroblasts transfected with RCAS/*cMI-m* or RCAS/*cmi9*. In an attempt to eliminate the contaminating plasmid DNA, RNA samples were treated with DNAse (Promega) at 37°C for 15 minutes prior to reverse transcription. The first strand cDNAs were then subjected to 20, 25, or 30 PCR cycles using a primer pair that will amplify the unique first exon of *cMI-m*, or a primer pair that will amplify the unique first exon of *cmi9*.

Fig. 3.18.a illustrates the results obtained in fibroblasts transfected with RCAS/*cMI-m*. Microphthalmia transcripts, specific for the 5' terminus of *cMI-m*, were detected after only 20 PCR cycles. Compare this to Fig. 3.18.b, which shows that in untransfected cells, an additional 10 PCR cycles are required to detect *cMI-m* transcripts. In both cases the results represent mRNA and not plasmid DNA, as products are not amplified from the mock reverse transcribed samples (lanes d-f in both cases). Thus the DNAse treatment was successful at eliminating plasmid DNA from RNA samples.

In fibroblasts transfected with RCAS/*cmi9*, 30 PCR cycles are required to detect *cmi9*-specific transcripts (Fig. 3.19.a). In untransfected cells, *cmi9*-specific transcripts are not detectable at these cycle numbers (Fig. 3.19.b), therefore demonstrating that the transcripts are generated as a result

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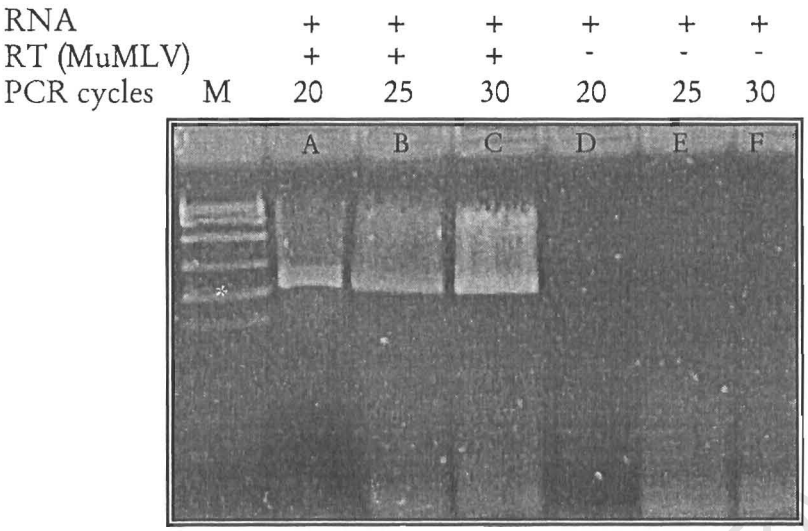
(a) RCAS/*cMI-m* transfected fibroblasts (cMI-m primers)



(b) Untransfected fibroblasts (cMI-m primers)

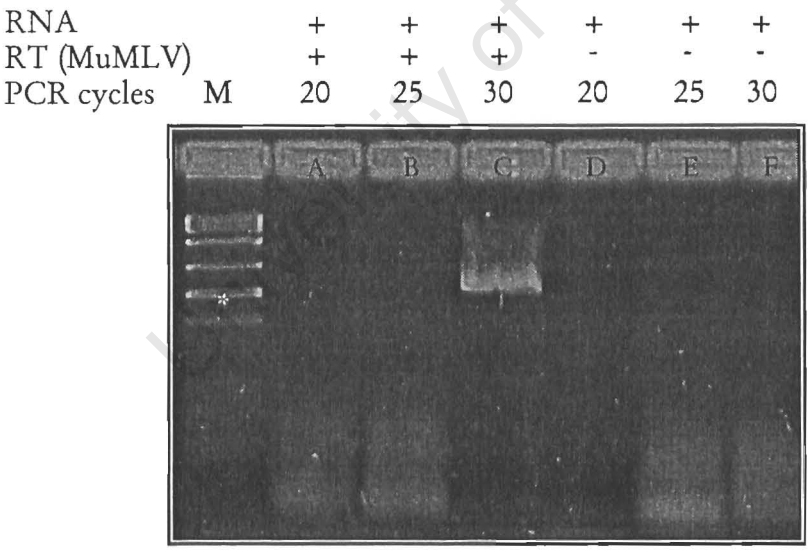


Fig. 3.18. RCAS/*cMI-m* transfected fibroblasts express *cMI-m* transcripts following 20 PCR cycles. Shown are the products of two-step RT-PCR of RNA extracted from (**a**) fibroblasts transfected with RCAS/*cMI-m* or (**b**) untransfected fibroblasts. RNA was subjected to reverse transcription or mock reverse transcription, followed by 20-30 PCR cycles using the primer pair that amplifies the unique first exon of *cMI-m*. (+) indicates the presence of RNA or RT enzyme in each sample and (-) indicates the absence of RNA or RT enzyme in each sample. Numbers above each lane indicate the number of PCR cycles used. The molecular weight marker (M) is Pox/*Eco*RI and the 1.0 kb fragment is indicated with an asterisk (*) in each case.

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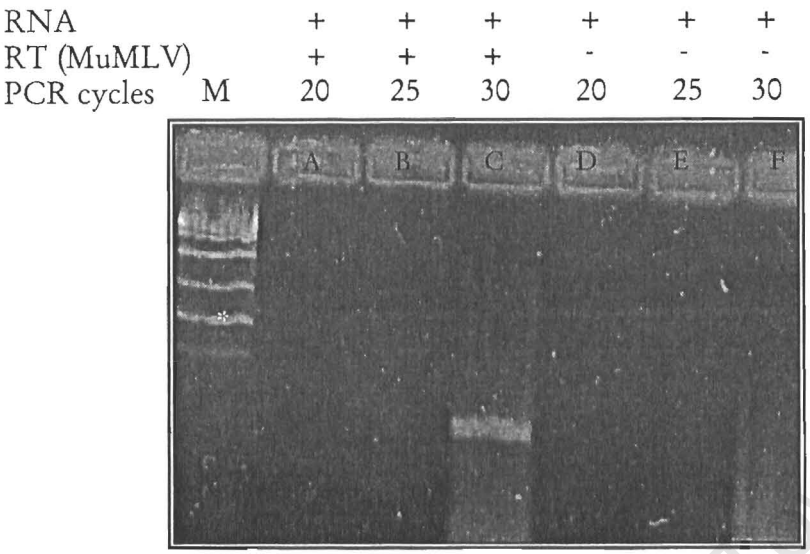
(a) RCAS/*cmi9* transfected fibroblasts (cmi9 primers)



(b) Untransfected fibroblasts (cmi9 primers)

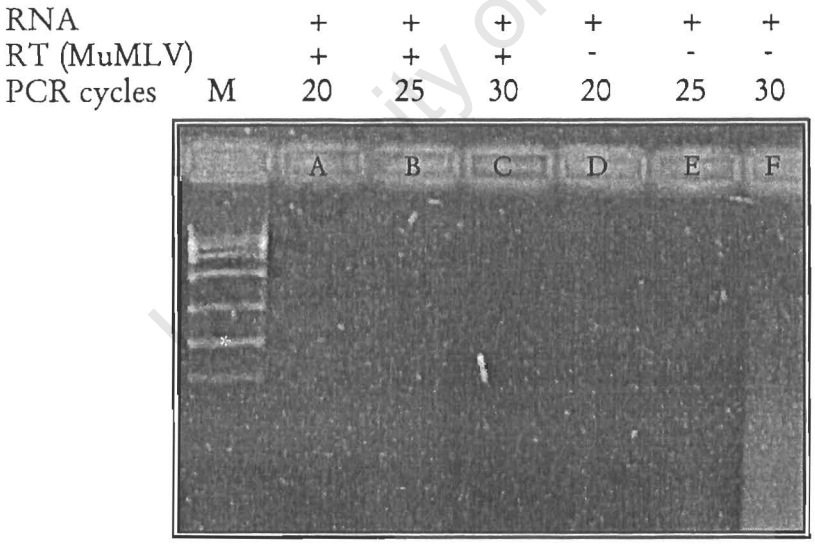


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Fig. 3.19. RCAS/*cmi9* transfected fibroblasts express *cmi9* transcripts following 30 PCR cycles. Shown are the products of two-step RT-PCR of RNA extracted from **(a)** fibroblasts transfected with RCAS/*cmi9* or **(b)** untransfected fibroblasts. RNA was subjected to reverse transcription or mock reverse transcription, followed by 20-30 PCR cycles using the primer pair that amplifies the unique first exon of *cmi9*. (+) indicates the presence of RNA or RT enzyme in each sample and (-) indicates the absence of RNA or RT enzyme in each sample. Numbers above each lane indicate the number of PCR cycles used. The molecular weight marker (M) is Pox/*Eco*RI and the 1.0 kb fragment is indicated with an asterisk (*) in each case.

of transfection with RCAS/*cmi9*. The lack of products in the mock reverse transcribed samples (lanes d-f in both cases) illustrate that the DNAse treatment was again successful at eliminating the plasmid DNA.

To summarise, mRNA is transcribed from both RCAS/*cMI-m* and RCAS/*cmi9*. However, the RCAS/*cMI-m* construct appears to express greater numbers of transcripts than does the RCAS/*cmi9* construct. This difference may indicate slight variations in the two constructs or, possibly, variations in the stability of the transcripts generated from the two cDNA isoforms.

3.5.3 Expression of Microphthalmia proteins in non-pigmented cells transfected with RCAS/*cMI-m* and RCAS/*cmi9*

The next step was to carry out transient transfection/western blot experiments to determine whether proteins are translated from RCAS/*cMI-m* and RCAS/*cmi9*. It was also necessary to determine how much of each construct must be used in order to produce equal amounts of protein. This is important because it is known that different levels of transcripts are detected using 5 μg of each construct and it is therefore possible that the levels of protein produced might also be different.

The aims of the present section are therefore to determine: (i) whether proteins are translated from the two constructs when they are transiently transfected into cells, and (ii) how much of each construct must be transfected into the cells in order to produce approximately equal levels of protein. To accomplish this, RCAS/*cMI-m* and RCACS/*cmi9* were transiently transfected into human non-pigmented HeLa cells and the cell lysates were analysed by western blotting. HeLa cells were chosen because they are readily and very efficiently transfected (using PolyFect; Qiagen PolyFect Transfection Reagent Handbook; Dr. B. Barnes, personal communication). [Note: One possible criticism of the use of HeLa cells lies in the fact that protein is detected in these cells upon western blotting with the mouse anti-Mitf antibody (see Fig. 3.12). This important point was kept in mind as transfections and subsequent western blotting experiments were undertaken.]

For each transfection, HeLa cells at 60-80% confluence were transfected with 0, 5, 6, or 7 μg of: (i) RCAS/*cMI-m* and (ii) RCAS/*cmi9* and cellular protein isolated forty-eight hours later and subjected to western blotting. 5 μg was chosen as a starting point as this was the amount of each construct that was transfected into chicken embryonic fibroblasts for semi-quantitative RT-PCR

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analyses of transcript levels. B16 cell lysates were used as a positive control in each western blot As can be seen in Fig. 3.20.a, the B16 Mitf proteins are seen at ~ 60-65 kDa and in control (mock transfected) HeLa cells, a doublet of ~ 65-69 kDa is visible (as was the case in Fig. 3.12). When HeLa cells are transfected with 5, 6, or 7 μg of RCAS/*cMI-m* two different protein species of ~ 56-63 kDa are detected (Fig. 3.20.a) and there is a slight increase with in protein levels with increasing amount of plasmid. Intriguingly, in these transfected cells, the endogenous doublet of 65-69 kDa is not seen in the transfected cell lysates. These results, which were repeated at least thrice, demonstrate that (i) in Hela cells, RCAS/*cMI-m* is translated into proteins of ~ 56-63 kDa and suggest that (ii) the endogenous expression of MITF is inhibited by exogenous expression (or over-expression) of cMI-m. This is further discussed below.

Proteins of different sizes were detected when cells were transfected with the second construct, RCAS/cmi9, as seen in Fig. 3.20.b. In this Figure, the B16 Mitf protein shows as the characteristic doublet of ~ 60-65 kDa and the HeLa cell Mitf protein shows as the doublet of 65-69 kDa. When HeLa cells are transfected with 5, 6, or 7 μg of RCAS/*cmi9*, proteins the same size as the endogenous HeLa cell proteins were detected (i.e. ~ 65-69 kDa) in increasing amounts. These results suggest, but do not definitively demonstrate, that protein is translated from RCAS/*cmi9* in HeLa cells.

When comparing the levels of protein arising from the two different constructs, it must be noted that the levels of Microphthalmia protein detected do not significantly increase with increasing amounts of the RCAS/*cMI-m* plasmid (Fig. 3.20 (a); compare intensity of the band obtained with 6 and 7 μg of cMI-m). In contrast, the protein levels increase with increasing amounts of transfected RCAS/*cmi9* (Fig. 3.20 (a); compare intensity of the band obtained with 6 and 7 μg of cmi9. Experiments were therefore designed to determine whether increasing the amount of transfected RCAS/*cMI-m* would result in an increased level of protein. However, when higher (8, 9, and 10 μg) amounts of RCAS/*cMI-m* were transiently transfected into HeLa cells, after twenty-four hours post-transfection the cells had lifted from the tissue culture dish and died. The same result was observed with increasing the amount of transfected RCAS/*cmi9*. The cytotoxicity was found not to be caused by the increased amounts of PolyFect required to complex with the higher μg amounts of the constructs (as assessed by removing the PolyFect-containing medium following three hours, a minimum time to allow for transfection; PolyFect Transfection Reagene Handbook; Qiagen), nor was the RCAS BP(A) vector itself the cause of the cytotoxicity (as assessed by transient transfections using the empty vector). These results therefore suggest that

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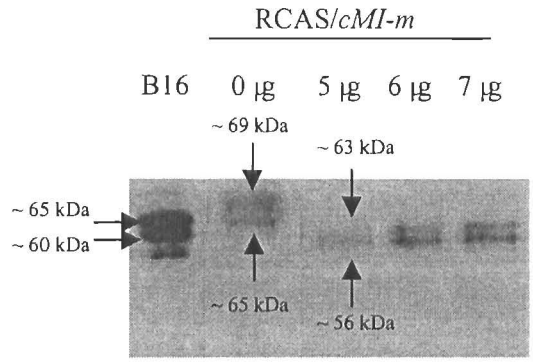
(a) Transfection of HeLa cells with RCAS/*cMI-m*



(b) Transfection of HeLa cells with RCAS/*cmi9*

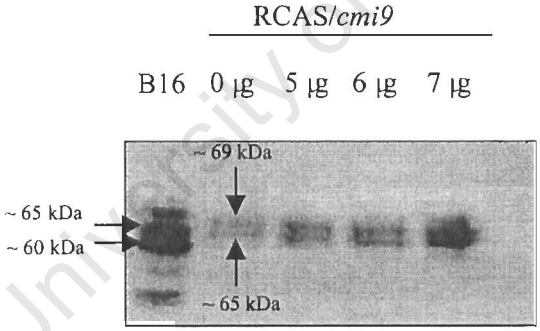


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Fig. 3.20. Western blotting of HeLa cellular proteins following transient transfection of four different amounts (5, 6, 7, and 0 µg) of **(a)** RCAS/*cMI-m* or **(b)** RCAS/*cmi9*. Cellular proteins from B16 cells are shown in the first lane of each western blot and are included for a more precise molecular weight determination than that allowed by a pre-stained molecular weight maker alone. Both western blots were exposed for ~30 seconds. Approximately equal levels of protein are detected in cell lysates following transfection with 6 µg of RCAS/*cMI-m* and 6 µg of RCAS/*cmi9*. The estimated sizes of each protein – as assessed by the molecular weight maker used (kDa; Benchmark Prestained Protein Ladder, Invitrogen) and comparisons with the known molecular weight of B16 proteins (Takemoto *et al.*, 2002) – are indicated.

the over-expression of cMI – be it cMI-m or cmi9 – is not conducive to continued cell viability; this is further discussed in Chapter Four.

To shed more light on the results obtained in HeLa cells, transfections were next carried out in chicken embryonic fibroblasts (shown to express very low levels of Microphthalmia; Fig. 3.12). In these experiments, fibroblasts were "double" transfected to increase expression levels as follows: Fibroblasts were transfected with (i) 6 μg of RCAS/*cMI-m* or (ii) 6 μg of RCAS/*cmi9*, the transfections were allowed to proceed overnight, and then cells were again transfected with an additional (i) 6 μg of RCAS/*cMI-m* or (ii) 6 μg of RCAS/*cmi9* and the transfections allowed to proceed for an additional 12 hours. The results are shown in Fig. 3.21.a and demonstrate that a protein doublet of ~65-69 kDa is translated from RCAS/*cmi9* (lane 2), a protein doublet of ~56-63 is translated from RCAS/*cMI-m* (lane 3). No protein was detected in mock-transfected or untreated fibroblast cells (lanes 4 and 5, respectively) at this level of exposure. These results confirm that both RCAS/*cMI-m* and RCAS/*cmi9* are translated into Microphthalmia proteins when expressed in Hela cells and fibroblasts, and that approximately the same levels of protein are translated from 6 μg of each construct.

Finally, the question of why the endogenous HeLa MITF expression was inhibited when cells were transfected with RCAS/*cMI*-m was addressed. After considering a number of lines of thought, it was hypothesised that the inhibition might be the result of the transfection procedure itself (and not the result of the expression of exogenous cMI). A comparison of endogenous MITF expression in mock transfected (transfection reagent only) versus vector transfected (6 μg of RCAS/*cmi9;* or 6 μg of RCAS/*cMI-m)* HeLa cells was therefore carried out. An additional control was cells in medium only. The results are shown in Fig. 3.21.b. When cells are transfected with RCAS/*cmi9* (lane 1) a doublet of ~ 65-69 kDa is again detected and the same bands are seen in mock-transfected (lane 3) and untreated (lane 4) cells. Of note is the fact that the vector transfected cells contain higher levels of protein than mock transfected cells. When cells are transfected with RCAS/*cMI-m* (lane 2) one doublet of ~ 56-63 kDa is detected. These results demonstrate that the loss of endogenous MITF is not due to the transfection procedure or reagent, and suggest that the inhibition is due to the over-expression of exogenous cMI. This is a very intriguing and surprising result and most certainly warrants further investigation. Due to time constraints on the present study, this has not been further investigated, although it is discussed in Chapter Four.

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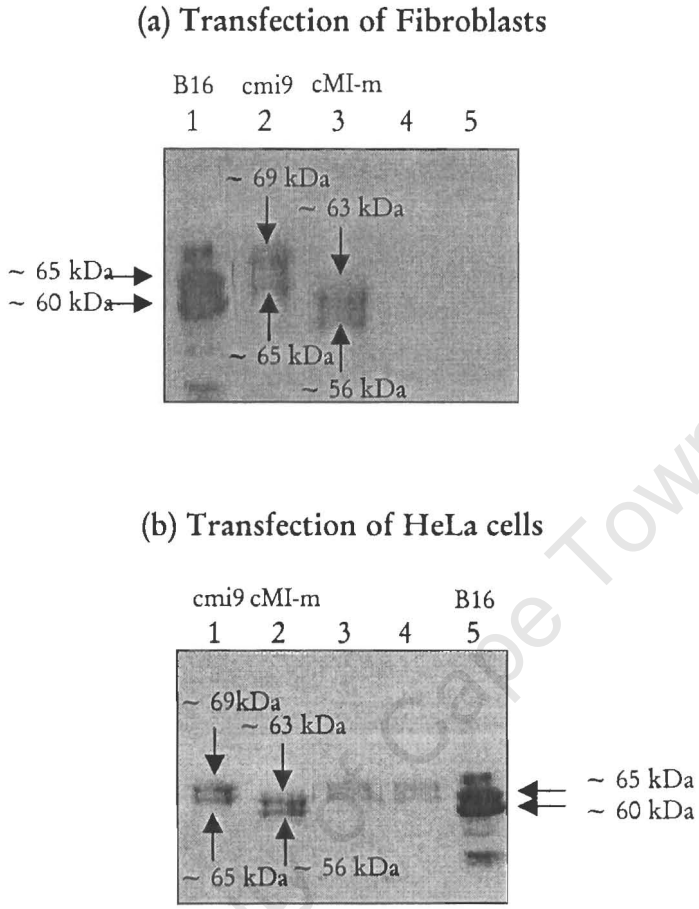

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Figure 3.21. Western blotting of (a) B16 cellular proteins (lane 1); chicken embryonic fibroblast cellular proteins following transfection with 6 μg of RCAS/*cmi9* (lane 2); 6 μg of RCAS/*cMI-m* (lane 3); mock transfection with PolyFect (lane 4); or no treatment (lane 5) and (b) HeLa cellular proteins following transfection with 6 μg of RCAS/*cmi9* (lane 1); 6 μg of RCAS/*cMI-m* (lane 2); mock transfection with PolyFect (lane 3); or no treatment (lane 4); and B16 cellular proteins (lane 5). Both western blots were exposed for ~ 30 seconds. The estimated sizes of each protein – as assessed by the molecular weight marker used (kDa; Benchmark Prestained Protein Ladder, Invitrogen) and comparisons with the known molecular weight of B16 proteins (Takemoto *et al.*, 2002) – are indicated.

To summarise, the 5 μg of the two microphthalmia constructs (RCAS/*cMI-m* and RCAS/*cmi9*) used in the present study generate neither equal levels of transcripts nor equal levels of protein when transiently transfected into non-pigmented cells. This is further discussed in Chapter Four. In any case, at this point of the study it has now been shown that 6 μg of RCAS/*cMI-m* or 6 μg of RCAS/*cmi9* produce the same levels of protein in transfected HeLa cells. Thus, for further co-transfections with tyrosinase reporter promoter constructs, it was decided that 6 μg of each microphthalmia construct would be used.

3.6 Activity of the chicken tyrosinase gene promoter in transiently transfected B16 and HeLa cells

To test and compare the transactivational activities of the cMI-m and cmi9 proteins, cotransfectionss with the RCAS/*cMI-m* and RCAS/*cmi9* plasmids together with tyrosinase promoter-*luciferase* constructs were carried out.

Transfections were carried out in B16 mouse melanoma cells (a pigmented cell line; known to express high levels of Mitf-m as assessed by western blotting in the present study), and two non-pigmented cell lines (human HeLa cells – shown to express moderate levels of MITF as assessed by western blotting in the present study - and chicken embryonic fibroblasts, shown to express very low levels of cMI as assessed by western blotting in the present study). Previous investigators have used B16 and HeLa cells in similar co-transfections experiments (to those described below) with mammalian Microphthalmia protein and tyrosinase gene promoter constructs (Yasumoto *et al.*, 1997; Takemoto *et al.*, 2002; Takeda *et al.*, 2002).

3.6.1 The chicken tyrosinase gene promoter reporter constructs

A 2.1-kb chicken tyrosinase gene promoter and two deletion constructs were previously cloned upstream of the *luciferase* reporter gene (Ferguson, Ph.D. Thesis, 1996). These constructs have been tested for tissue-specific activity by transfection into a variety of pigmented and non-pigmented cell types. Higher levels of activity in pigmented cells such as RPE and mouse melan-a cells (Clarke, M.Sc. Thesis, 2000) and lower levels of activity in HepG2 non-pigmented cells (Clarke, M.Sc. Thesis, 2000) indicated that the full-length promoter was tissue specific in its activity. Clarke's results obtained with the two deletion constructs were less definitive, probably because of low transfection efficiencies in previous studies.

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3.6.2 The chicken tyrosinase gene promoter is pigment cell-specific in B16 cells

Before proceeding with co-transfection studies with the cMI constructs, it was necessary to verify and extend the above results in the cell types used in the present study (B16 melanoma cells, HeLa cells, and chicken embryonic fibroblasts). Thus, transient transfections of the three tyrosinase promoter constructs were carried out in these three cell types. Transfections were carried out at least three times in duplicate (except for some of the chicken embryonic fibroblast transfections). For each experiment, the relative luciferase activity (RLA) was calculated as a percentage of the activity of the pGL2 control expression. The results from individual experiments were then combined and are presented as a mean ± the standard deviation.

As can be seen in Fig. 3.22, the RLA in HeLa cells and in fibroblasts was very low (approximately 5-10) for all three promoter constructs. In B16 cells, the RLA of the full-length construct was approximately 180 and the level of activity dropped to approximately 10 for the two deletion constructs. These results confirm that the full-length tyrosinase construct used in this study does indeed function in a predictable tissue specific fashion. It was further noted that in B16 melanoma cells the deletion of ~ 1.0 kb from the 5'-flanking region of the tyrosinase promoter results in an approximately 9 fold decrease in reporter activity. This indicates that there are clearly important *cis*-acting elements in this region that play a role in controlling tissue specificity.

The very low activity of the three promoter constructs in HeLa cells makes it possible to utilise these constructs and cells to measure the transactivation of the tyrosinase gene promoter by the cMI-m and cmi9 proteins using a co-transfection approach (see below). [Note: the transfection results using chicken embryonic fibroblasts are not elaborated on here because of the relatively low transfection efficiency of the cells. However, as detailed under Appendix B, transfections into these cells did reveal that the trend of promoter activity in fibroblasts was similar to the trend of promoter activity in HeLa cells and the fibroblast transfection results are thus included in Fig. 3.22.]

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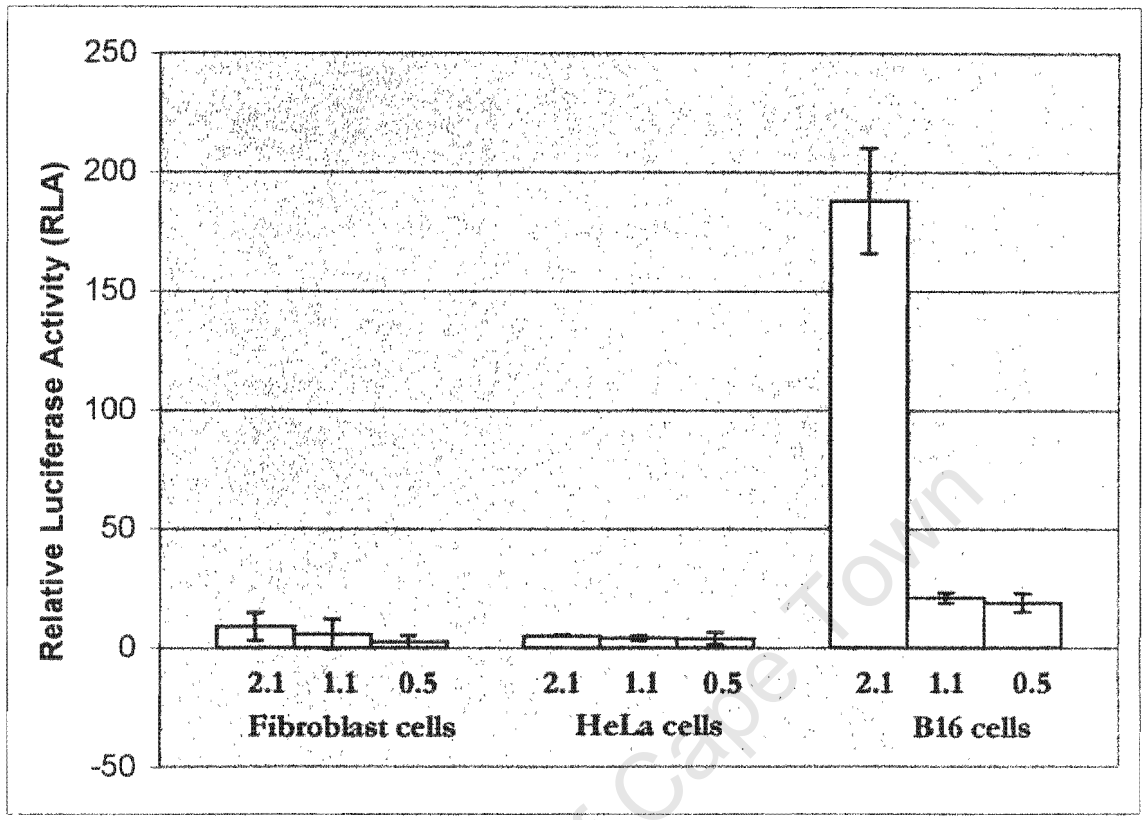

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Fig. 3.22. Activity of the three chicken tyrosinase gene promoter constructs in chicken embryonic fibroblasts, HeLa and B16 cells. Blue bars represent transfections with Tyr2.1-luc (2.1); yellow bars represent transfections with Tyr1.1-luc (1.1); and green bars represent transfections with Tyr0.5-luc (0.5). Data are shown as the means of individual experiments ± standard deviations, and experiments were repeated three times in duplicate. Standard deviations were calculated using the Excel (Microsoft Corporation) standard deviation function. In all cases, results are expressed as a relative luciferase activity (RLA) normalised with respect to *Renilla* activity in the same cell extract and expressed as a percentage of the *luciferase* activity obtained with the positive control vector, pGL2-control. Protein concentrations in all cell extracts were normalised using the BioRad Assay.

3.6.3 Transactivation of the chicken tyrosinase gene promoter by the chicken Microphthalmia protein in transiently transfected B16 and HeLa cells

The next set of experiments were aimed at determining whether each cMI protein is able to transactivate the chicken tyrosinase gene promoter in non-pigmented (HeLa) and pigmented (B16) cells, and to begin to determine which promoter elements, if any, are particularly important for the transactivation by the two different isoforms of the cMI protein. For all co-transfections described below, each experiment was carried out at least three times in duplicate and the RLA was calculated as described above. The raw transfection data for each experiment is detailed under Appendix B.

Duplicate wells (in a 6-well tissue culture plate) of HeLa cells were co-transfected with either: (i) 6 μg RCAS/*cMI-m* and Tyr2.1-luc or (ii) 6 μg RCAS/*cmi9* and Tyr2.1-luc. Results are shown in Fig. 3.23.a. The baseline activity of Tyr2.1-luc in HeLa cells is from Fig. 3.22 and is included for comparative purposes.

Co-transfection of RCAS/*cMI-m* and Tyr2.1-luc in HeLa cells results in a 4-fold increase in the activity of Tyr2.1-luc, from a mean baseline RLA of 4.85 to a mean RLA of 19.78. Likewise, co-transfection of RCAS/*cmi9* and Tyr2.1-luc in HeLa cells results in a 4-fold increase in the activity of Tyr2.1-luc, from a mean baseline RLA of 4.85 to a mean RLA of 20.17. Thus, both cMI-m and cmi9 increase the activity of the full-length tyrosinase gene promoter to equal levels in HeLa cells.

Next, HeLa cells were co-transfected with either: (i) 6 μg RCAS/*cMI-m* and Tyr1.1-luc or (ii) 6 μg RCAS/*cmi9* and Tyr1.1-luc. Results are shown in Fig. 3.23.b. The baseline activity of Tyr1.1-luc in HeLa cells is from Fig. 3.22 and is included for comparative purposes. In HeLa cells, both cMI-m and cmi9 increase the activity of the Tyr1.1-luc promoter construct: From a mean baseline RLA of 4.18, cMI-m increases the activity to a mean RLA of 22.75, and cmi9 increases the activity to a mean RLA of 22.28. Thus, both cMI-m and cmi9 increase the activity of a truncated (Tyr1.1-luc) tyrosinase gene promoter by approximately 5-fold in HeLa cells.

Finally, HeLa cells were co-transfected with either: (i) 6 μg RCAS/*cMI-m* and Tyr0.5-luc or (ii) 6 μg RCAS/*cmi9* and Tyr0.5-luc. Results are shown in Fig. 3.23.c. The baseline activity of Tyr0.5-luc is from Fig. 3.22 and is included for comparative purposes. In HeLa cells, both cMI-m and

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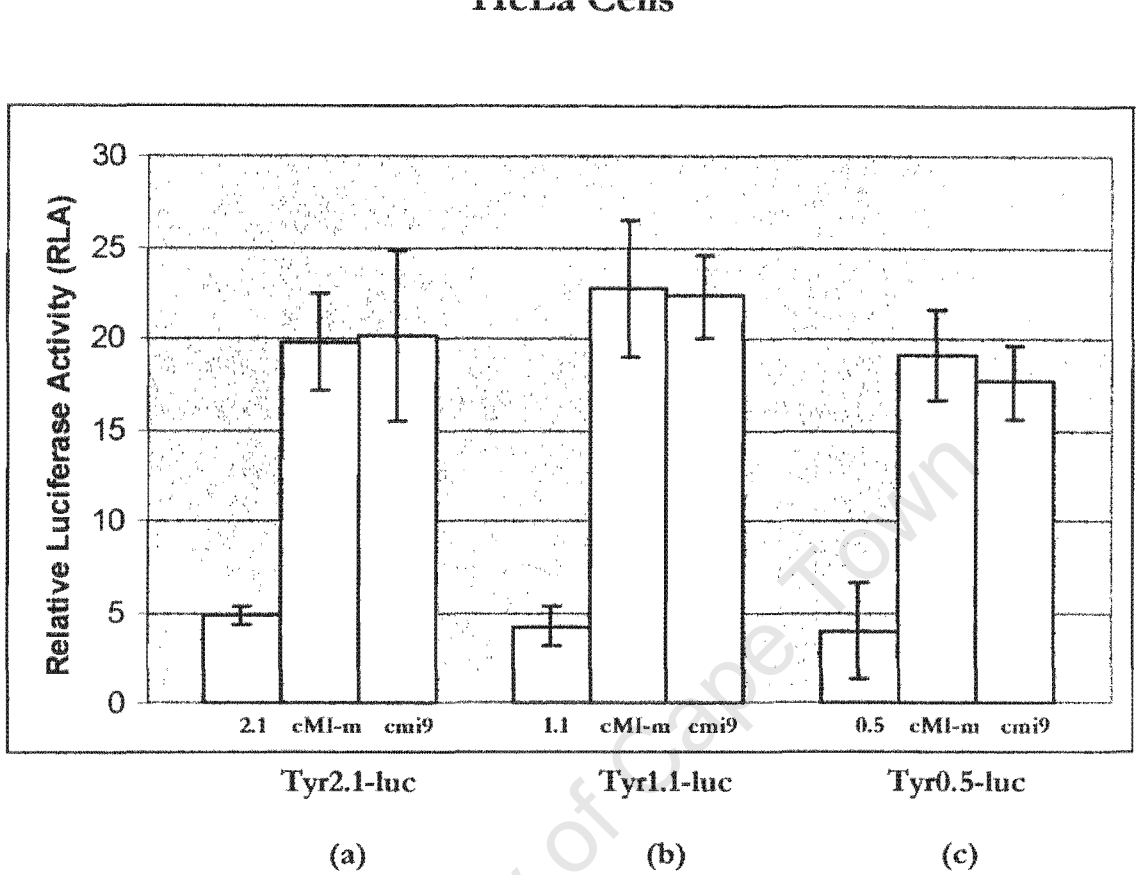

Fig. 3.23. Activity of **(a)** Tyr2.1-luc, **(b)** Tyr1.1-luc, and **(c)** Tyr0.5-luc in HeLa cells following co-transfections with RCAS/*cMI-m* (cMI-m) or RCAS/*cmi9* (cmi9). Blue bars represent co-transfections with Tyr2.1-luc (2.1); yellow bars represent co-transfections with Tyr1.1-luc (1.1); and green bars represent co-transfections with Tyr0.5-luc (0.5). The baseline activity of each promoter construct (first bar in each series) is from Fig. 3.22 and is included for comparative purposes. Data are shown as the means of individual experiments ± standard deviations, and all experiments were repeated three times in duplicate. Standard deviations were calculated using the Excel (Microsoft Corporation) standard deviation function. In all cases, results are expressed as a relative lucierfase activity (RLA) normalised with respect to *Renilla* activity in the same cell extract and expressed as a percentage of the *luciferase* activity obtained with the positive control vector, pGL2-control. Protein concentrations in all cell extracts were normalised using the BioRad Assay.

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cmi9 increase the activity of the Tyr0.5-luc construct: From a mean baseline RLA of 3.92, cMI-m increases the activity to a mean RLA of 19.07, and cmi9 increases the activity to a mean RLA of 17.6. Thus, cMI-m increases the mean activity of a truncated (Tyr0.5-luc) tyrosinase gene promoter by 4.9-fold in HeLa cells, and cmi9 increases the activity of a truncated (Tyr0.5-luc) tryosinase gene promoter by 4.5-fold in HeLa cells.

Next, the ability of the two chicken Microphthalmia proteins to transactivate the chicken tyrosinase gene promoter constructs in B16 cells was assessed. First, B16 cells were co-transfected with either: (i) 6 μg RCAS/*cMI-m* or (ii) 6 μg RCAS/*cmi9* and Tyr2.1-luc. Results are shown in in Fig. 3.24.a. The baseline activity of Tyr2.1-luc in B16 cells is from Fig. 3.22 and is included for comparative purposes.

In B16 cells, both cMI-m and cmi9 increase the activity of the Tyr2.1-luc construct: From a mean baseline RLA of 188, cMI-m increases the activity to a mean RLA of 265, and cmi9 increases the activity to a mean RLA of 238. Thus, cMI-m increases the mean activity of a truncated (Tyr0.5-luc) tryosinase gene promoter by 1.4-fold in B16 cells, and cmi9 increases the activity of a truncated (Tyr0.5-luc) tryosinase gene promoter by 1.3-fold in B16 cells.

Next, B16 cells were co-transfected with either: (i) 6 μg RCAS/*cMI-m* and Tyr1.1-luc or (ii) 6 μg RCAS/*cmi9* and Tyr1.1-luc. Results are shown in Fig. 3.24.b. The baseline activity of Tyr1.1-luc in B16 cells is from Fig. 3.22 and is included for comparative purposes. In B16 cells, both cMI-m and cmi9 increase the activity of the Tyr1.1-luc promoter construct: From a mean baseline RLA of 21, cMI-m increases the activity to a mean RLA of 289, and cmi9 increases the activity to a mean RLA of 276. Thus, both cMI-m and cmi9 increase the activity of a truncated (Tyr1.1-luc) tyrosinase gene promoter by approximately 13-fold in B16 cells.

Finally, B16 cells were co-transfected with either: (i) 6 μg RCAS/*cMI-m* and Tyr0.5-luc or (ii) 6 μg RCAS/*cmi9* and Tyr0.5-luc. Results are shown in Fig. 3.24.c. The baseline activity of Tyr0.5-luc is from Fig. 3.22 and is included for comparative purposes. In B16 cells, both cMI-m and cmi9 increase the activity of the Tyr0.5-luc construct: From a mean baseline RLA of 19, cMI-m increases the activity to a mean RLA of 263, and cmi9 increases the activity to a mean RLA of 243. Thus, both cMI-m and cmi9 increase the activity of a truncated (Tyr0.5-luc) tyrosinase gene promoter by approximately 13-fold in B16 cells.

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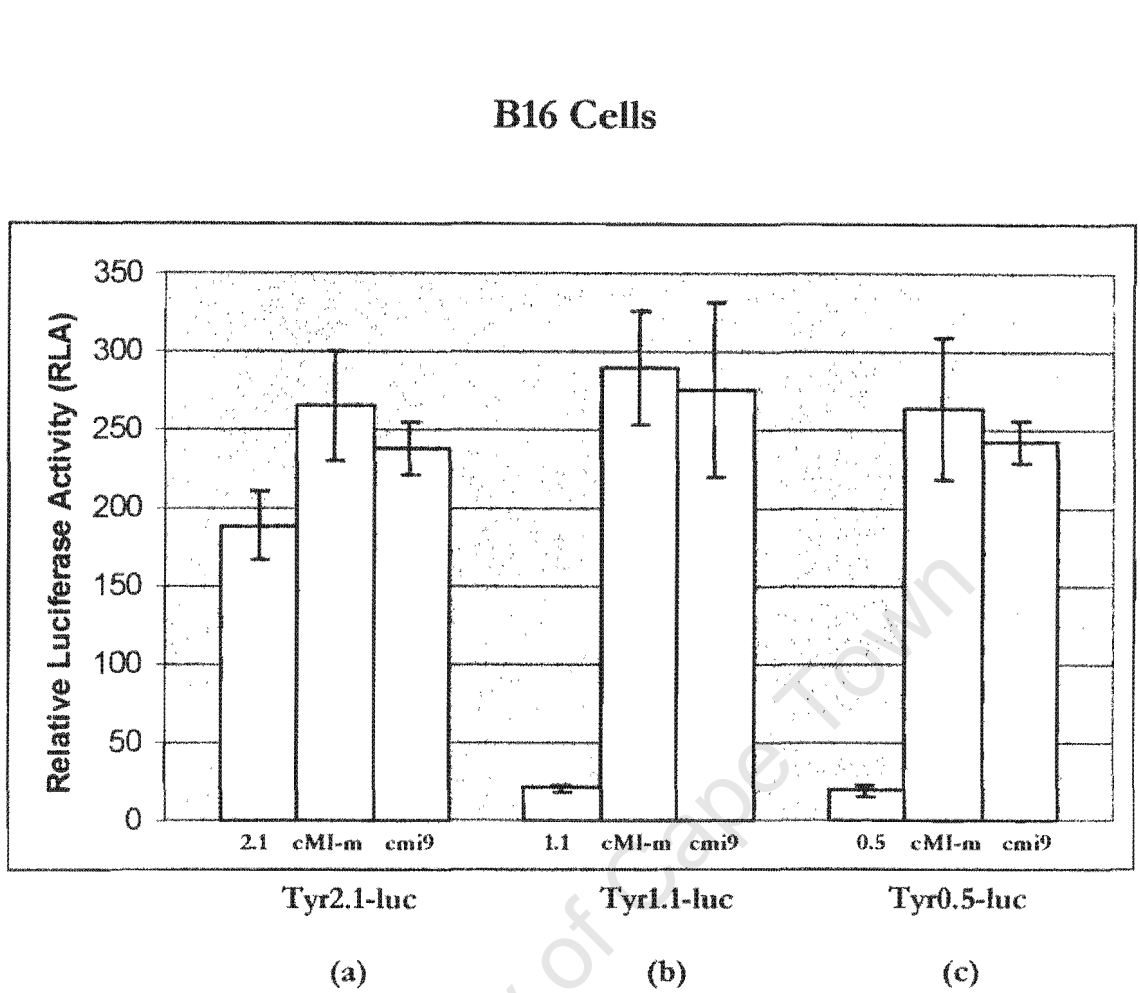

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Fig. 3.24. Activity of **(a)** Tyr2.1-luc, **(b)** Tyr1.1-luc, and **(c)** Tyr0.5-luc in B16 cells following co-transfections with RCAS/*cMI-m* (cMI-m) or RCAS/*cmi9* (cmi9). Blue bars represent co-transfections with Tyr2.1-luc (2.1); yellow bars represent co-transfections with Tyr1.1-luc (1.1); and green bars represent co-transfections with Tyr0.5-luc (0.5). The baseline activity of each promoter construct (first bar in each series) is from Fig. 3.22 and is included for comparative purposes. Data are shown as the means of individual experiments ± standard deviations, and all experiments were repeated three times in duplicate. Standard deviations were calculated using the Excel (Microsoft Corporation) standard deviation function. In all cases, results are expressed as a relative lucierfase activity (RLA) normalised with respect to *Renilla* activity in the same cell extract and expressed as a percentage of the *luciferase* activity obtained with the positive control vector, pGL2-control. Protein concentrations in all cell extracts were normalised using the BioRad Assay.

In summary, in transient co-transfections in B16 cells, both cMI-m and cmi9 increase the activity of the three tyrosinase gene promoter reporter constructs, although the magnitude of increase observed for the full-length promoter (Tyr2.1-luc) is less than that of the increase observed for the two deletion constructs (Tyr1.1-luc and Tyr0.5-luc). The fact that both proteins increase the activity of Tyr0.5-luc as efficiently as they do Tyr2.1-luc (i.e. the mean RLAs ± standard deviations are no different) suggests that those chicken tyrosinase promoter elements important for the transactivation by cMI in pigmented cells are contained within Tyr0.5-luc. Furthermore, the ability of each protein to increase the promoter activity is equal. That is, there appears to be no difference in the ability of cMI-m and cmi9 to transactivate the chicken tyrosinase promoter in pigmented cells *in vitro*.

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Chapter 4 | Discussion

The molecular basis of cell differentiation during embryonic development is extremely complex, and the intricacies of the process are, in general, poorly understood. Studies using melanogenic cells as a model cell type not only contribute new knowledge to the process, but also foster an appreciation for the depth of the complexity. For example, melanogenic cells arise from two distinct populations of embryonic cells: the neural crest and the neuroepithelium. In addition to their differing origins, the cells have different mature functions and morphology. Studies into these differences have revealed that at least one gene, microphthalmia, may be involved as a key regulator in the differentiation, development and function of both melanocytes and RPE cells. It is known that Microphthalmia is absolutely required for the differentiation of epidermal melanocytes and for the correct formation of the RPE. However, microphthalmia must be regulated differently and/or exert different molecular responses in each cell type, as mutations at the microphthalmia locus do not always have the same effect(s) in melanocytes as they do in RPE cells.

Most recently, there have been studies of the upstream regulation of microphthalmia by signalling factors pathways/factors such as Wnt, Sox10, MSH, and Pax3 (Dorsky *et al.*, 2000; Takeda *et al.*, 2000; Lee *et al.*, 2000; Potterf *et al.*, 1999; 2000; Busca and Ballotti, 2000) However, there is still very little known about the precise function(s) that Microphthalmia plays in regulating its downstream target(s). Studies, predominantly using *in vitro* transient transfection assays, have demonstrated that Microphthalmia can target and bind the tyrosinase gene promoter, suggesting a significant functional role for the protein in the regulation of melanogenesis. Yet there are still many unanswered questions about the Microphthalmia protein, some of which have been formally stated by Goding (2000): "...Is Mitf continuously required as suggested by the progressive age-related loss of melanocytes in the vitiligo-associated $Mitf^{Mi\text{-}vit}$ mutant, or only necessary at one or more key stages of development? What genes are targeted by Mitf other than those required for pigmentation? When is Mitf phosphorylated by MAP kinase, Rsk, or GSK-3β during development, and how are the regulatory mechanisms using cells in culture relevant to its function in development?".

Most pertinent to the present discussion is the latter question. Regulatory mechanisms that may have relevance during development have been extensively studied *in vitro* using mammalian microphthalmia gene/protein constructs. In contrast, apart from studies on the classical genetics of domestic fowl pigmentation – particularly as it relates to the commercial poultry industry (Smyth, 1990) – very little is known about the molecular genetics of avian plumage and eye pigmentation. Although there are many similarities between mammalian and avian melanogenesis (Carver and Brumbaugh, 1974; Zimmerman *et al*, 1982; Bowers, 1988), the cell biology and biochemistry of avian

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melanogenesis has not been as well described as that for mammalian melanogenesis, and it is not clear whether chicken pigmentation genes are similar in structure or function to those in other organisms. Indeed, at least three avian pigmentary genes, *MMP115* (Mochii *et al.*, 1988), *pP334* (Agata *et al.*, 1993) and *QNR-71* (Turque *et al.*, 1996) have been cloned that, as yet, have no reported mammalian equivalents. In terms of microphthalmia, there have only been two reports of avian microphthalmia cDNAs (one of which – *cmi9* – also appears to be unique to avians; Mochii *et al.*, 1998a; 1998b) and no extensive analyses of downstream targets of the avian Microphthalmia protein. Therefore, one of the broad aims of the present study is to further the understanding of the role of *cMI*/cMI in avian pigmentation. Specifically, it is of interest to investigate whether the generation of chicken microphthalmia transcript diversity relates to specialised expression patterns and functions of the gene. This Chapter discusses the mechanisms utilised by the chicken microphthalmia gene in the generation of its molecular diversity, and then endeavours to associate the diversity to expression patterns of the gene and the function(s) of its protein.

4.1 Diversity of chicken microphthalmia transcripts

To clone a chicken microphthalmia cDNA, April (Ph.D. Thesis, 1998) screened a chicken neural crest-derived melanocyte cDNA library using a mouse *Mitf* cDNA probe. The single clone (M156) isolated formed the starting point of the current study and was sequenced and found to contain an insert of a 34 bp 5'-UTR, a 1158 bp coding region, and a 1341 bp 3'-UTR. The cDNA encodes a predicted protein with a length of 385 amino acids. Comparison of the deduced amino acid sequence of this clone with microphthalmia cDNAs isolated from other vertebrates reveals a high degree of conservation (on average, 89.5% aa sequence identity; Table 3.2), particularly in the basic/helix-loop-helix/leucine zipper domains. Further comparisons of the deduced amino acid sequence of clone M156 reveals that its 5' region most closely matches the melanocyte-type isoform of the human and mouse microphthalmia genes: The initiation Met of clone M156 is in the same location as that of the mammalian melanocyte-type isoform and the first 11 aas of clone M156 encode a melanocyte-type first exon. This demonstrates that clone M156 encodes the chicken homologue of the human and mouse melanocyte-type microphthalmia isoform, and the clone was renamed *cMI-m*, for chicken microphthalmia-melanocyte type. There are therefore now three known isoforms of the chicken microphthalmia gene: *cMI-m* (present study); *cMI-a* (Mochii *et al.*, 1998a); and *cmi9* (Mochii *et al.*, 1998a), each of which is encoded by a unique first exon. This indicates that the use of alternative first exons is a feature of the microphthalmia gene that occurs in lower and higher vertebrates.

Another feature of the microphthalmia gene is that alternative splicing of the coding region can generate multiple forms of transcripts. Amino acid alignments with previously reported microphthalmia cDNAs demonstrate that *cMI-m*, isolated from neural crest-derived melanocytes, is

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missing exon 3 and exon 6a. A previous report indicates that the deletion of exon 3 also occurs in approximately half of all chicken cDNA clones isolated from RPE cells and *cmi9*, also isolated from RPE cells, is missing exon 6a (Mochii *et al.*, 1998a). In mammals, these alternatively spliced transcripts are also present in both neural crest-derived melanocytes and RPE cells and are known to result from alternative splice-acceptor site utilisation at the intron/exon boundaries. Sequencing of *cMI-m* indicates that it has the same exon boundaries as higher vertebrates, and it is likely therefore that the deletion of exon 3 and exon 6a from chicken transcripts results from the same alternative splice-acceptor site utilisation as the mammalian gene. For example, when considering the deletion of exon 3, the 3' sequence of exon 2 (GAAAAAGAG) and the 5' sequence of exon 4 (ATGGATGAT) of *cMI-m* are the same as those reported for the exon/intron junctions in the mouse genome (Hallsson *et al.*, 2000). The functional significance of these deletions in neural crest-derived melanocytes and/or RPE cells has not been intensively investigated.

A third mechanism known to generate transcript diversity is variation in the 3'-UTR. Differences in the 3'-UTR are known to affect transport of the mRNA from the nucleus (Huang and Carmichael, 1996), and to enhance the translation and stability of mRNA (Sachs *et al.*, 1997; Wickens *et al.*, 1997). To determine if variations in the 3'-UTR are a feature of *cMI*, this study assessed the lengths of the 3'-UTR of transcripts in a pigmented tissue using RT-PCR. RNA was extracted from a pigmented tissue (eye) of two chicken breeds: Black Australorp (BA) chickens, which have a black plumage and pigmented eyes, and White Plymouth Rock x Pile Game (WPR x PG) chickens, which have a white plumage and pigmented eyes (the two breeds are compared wherever possible during the present study, because it is possible that the differences in plumage pigmentation are due to subtle differences in the microphthalmia gene). First strand cDNAs were generated with an oligo dT_{15} primer and microphthalmia-specific transcripts were then PCR amplified using a combination of the oligo dT_{15} primer and a primer specific for the 3' end of the coding region of the *cMI* gene. The PCR products thus obtained will represent the 3'-UTR of the *cMI* transcripts. PCR products of 0.9 kb and 1.3 kb are obtained from the BA breed, and products of 1.0 kb and 1.3 kb are obtained from the WPR x PG breed.

The results of the present study suggest that the most likely explanation for the different sized 3'-UTRs are a combined use of alternative 3' polyadenylation signals and alternative 3' exons. Present in the 3'-UTR of the chicken microphthalmia gene are three putative polyadenylation signals (see Fig. 3.5 and Fig. 3.6). Two of these are consensus signals (AAUAAA; nt positions 760 and 1180 of the *cMI-m* 3'-UTR), and the third is the most common natural variant (AUUAAA; nt position 50 of the *cMI-m* 3'-UTR). If transcription can be terminated at all three sites, then three lengths of chicken microphthalmia 3'-UTRs are possible: one approximately 50 nts long, one approximately 760 nts long, and one approximately 1180 nts long. The two transcripts amplified from the BA eye (0.9 kb

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and 1.3 kb) and the two amplified from WPR x PG eye (1.0 kb and 1.3 kb) may therefore be explained by alternative usage of the latter two polyadenylation sites. The fact that only the latter two sites appear to be used may simply reflect an inability to resolve a 50 nt product using agarose gel electrophoresis, or it may be explained by the sequence of each: Only the last two sites conform to the consensus AAUAAA sequence; the first conforms to the most natural variant (AUUAAA) and may therefore not be used preferentially. The preferential use of certain polyadenylation signals is a mechanism that has been formally recognised (Hook and Kellems, 1988) and, in *cMI*, it does appear that the third polyadenylation signal is the preferred choice. This is evidenced by the fact that the chicken 3'-UTR reported in this study (*cMI-m*) and in a previous study (*cmi9*, Mochii *et al.*, 1998a) are 1.3 kb in length, and that a common 1.3 kb 3'-UTR transcript is amplified from both eye RNA samples.

Puzzling, however, is the fact that a 3'-UTR PCR product of the same length as that seen in the cDNA (*cMI-m*) cloned from the neural crest-derived melanocyte cDNA library (which would generate a ~ 1.5 kb product when using the "preferred" polyadenylation signal) is not detected in total eye RNA (present study) or RPE RNA (Mochii *et al.*, 1998a). It thus seems possible that the chicken microphthalmia gene exhibits differential usage of alternative 3'-exons in the eye versus developing neural crest-derived melanocytes. If this is the case then it may explain the fact that a ~ 1.5 kb PCR product is not obtained during amplification of the 3'-UTR present in the eye. That is, it is possible that there is a ~ 0.2 kb exon that is spliced from the 3'-UTR of microphthalmia transcripts in the eye that is not spliced from the 3'-UTR of microphthalmia transcripts in developing chicken neural crest-derived melanocytes. This would explain the difference in the known maximum sizes of 1.3 kb in eye RNA and 1.5 kb in neural crest-derived melanocytes. Thus, it seems possible that the chicken microphthalmia 3'-UTR utilises both alternative 3' exons *and* alternative polyadenylation signals.

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Finally, it was thought that the different lengths of the 3'-UTR in the two chicken breeds may indicate either the amplification of another member(s) of the MiT subfamily, or non-specific amplification of other gene transcripts. The amplification of the 3'-UTR of other members of the MiT subfamily (TFE3, TFEB and/or TFEC) is very unlikely. The forward primer (cMIF2) used during PCR amplification of 3'-UTR transcripts is specific for the 3' end of the chicken microphthalmia cDNA sequence and will not anneal to *TFE3*, *TFEB*, or *TFEC* (Table 4.1.a). Non-specific amplification of other gene transcripts cannot be excluded based on the results of the current study. However, the forward primer used is specific for chicken microphthalmia transcripts to such an extent that it will not even anneal to microphthalmia cDNA sequences from other species (mouse and human; Table 4.1.b). Hence it is unlikely that it will be specific for a transcript other than microphthalmia.

Table 4.1. An amino acid sequence alignment of mammalian and avian MiT proteins, and corresponding nucleotide sequence, in the region of primer cMIF2 (highlighted).

(a)

Protein/cDNA	Predicted aa sequence	cDNA nt sequence
cMI-m/*cMI-m*	NLGNVTEPTGTYSVPA	AATGTGACTGAACCAACTGGCACT (primer: cMIF2)
mTFE3/*mTFE3*	LDLH~~~~~~~~~~~~	ATC~~~~~~~~~~~~~~~~~~~~~~
mTFEB/*mTFEB*	AQPQSPFHHLDFSHGL	AGTCTCCGTTCCATCACCTGGACT
hTFEB/*hTFEB*	TQPPSPFHHLDFSHSL	AGCCACCGTCCCCATTCCATCACC
mTFEC/*mTFEC*	SDPLSHFTDLSFSAALK	TCTGATCCATTGTCTCACTTCACA
hTFEC-L/*hTFEC-L*	SDPLSYFTDLSFSAALK	TTGTCATACTTCACAGATTTATCA

(b)

Protein/cDNA	Predicted aa sequence	cDNA nt sequence
cMI-m/*cMI-m*	NLGNVTEPTGTYSVPA	AATGTGACTGAACCAACTGGCACT (primer: cMIF2)
cmi9/*cmi9*	NLGNVTEPTGTYSVPA	AATGTGACTGAACCAACTGGCACT
MITF-M/*MITF-M*	NLGTGTEANQAYSVPT	ACTGGGACTGAGGCCAACCAAGCC
Mitf-m/*Mitf-m*	NLGTMPESSPAYSIPR	ACCATGCCGGAGAGCAGCCCGGCC

DNA sequences were translated using the "Translate" programme and were then aligned and compared in a pair wise fashion using the "Gap" programme (GCG) and/or according to Rehli *et al.* (1999). GenBank accession numbers for aligned sequences are as follows: cMI-m, AF145751; mTFE3, S76673; mTFEB, from Rehli *et al.*, 1999; hTFEB, M33782; mTFEC, AF077742; TFEC-L, D43945; cmi9, D88363; MITF-M, Z29678; Mitf-m, NM008601 (prefixes: c, chicken; m, mouse; h, human).

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In summary, the results of the present study demonstrate that the chicken microphthalmia gene is complex. *cMI* appears to generate diversity in its transcripts through the use of alternative first promoters/exons, alternative splicing of the coding region, and through differences in the length of the 3'-UTR. The first two mechanisms have been extensively reported in the literature, as the 5' terminus of the microphthalmia gene has received considerable attention. In contrast, this is the first direct report suggesting that the 3'-UTR may also be a source of diversity in microphthalmia transcripts. In light of this, it will be interesting in the future to perform a profile, using RT-PCR and sequence analyses, of the expression of the 3'-UTR of the microphthalmia gene, both during embryonic development and in mature melanogenic cells.

4.2 Genomic organisation of the chicken microphthalmia gene

Alterations of the genomic sequence of the microphthalmia gene have received a considerable amount of attention. Small molecular lesions in microphthalmia (reviewed by Moore, 1995), as well as larger alterations such as deletions of several kilobases of genomic DNA (Opdecamp *et al.*, 1998) and duplications of the entire microphthalmia-related zebrafish *nacre* gene (Lister *et al.*, 1999; 2001; reviewed by Rawls *et al.*, 2001) have been reported. These alterations produce phenotypic effects ranging from mild to severe variations of pigmentation, deafness, bone and eye defects, or, in the case of the zebrafish *nacre* duplication, a postulated ability to compensate for the loss of the first gene in a pigmented tissue (RPE).

Of particular interest in the present study is the observation that one of the two chicken breeds used exhibits pigmentation patterns that may also be related to alterations of the microphthalmia locus. Unlike the BA breed that has a black plumage and pigmented eyes, the WPR x PG breed has a white plumage and pigmented eyes. This latter phenotype is reminiscent of the mi^{bw} mouse, which exhibits a black eyed/white coat phenotype, and is a result of a large genomic rearrangement that specifically affects the melanocyte-type isoform of microphthalmia (Yajima *et al.*, 1999).

As a very preliminary start to investigating the genomic organisation of the *cMI* locus, Southern blot hybridisation of genomic DNA from the two chicken breeds was carried out. The results demonstrate that there are no gross differences (deletions or rearrangements) between the two breeds at the chicken *cMI* locus. Thus, if the pigmentation differences are due to a mutated microphthalmia gene, then the mutation is not a gross one. This does not, however, negate the potential for either a duplication of the gene or smaller alterations (such as point mutations) in the gene, neither of which will be detected by Southern blot hybridisation.

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A duplication of the chicken microphthalmia gene seems unlikely. The present study shows that the microphthalmia gene spans at least 39 kb of the chicken genome (as demonstrated by the maximum size of bands detected following restriction enzyme digestion and Southern blotting of chicken genomic DNA). This is similar to the reported 50 kb size of the mouse and human microphthalmia genes (Hallsson *et al.*, 2000; Udono *et al.*, 2000). In humans, Southern blot hybridisation analysis of genomic DNA using isoform-specific probes demonstrates that "each isoform-specific exon of the *MITF* gene is present as a single copy in the human genome" (Udono *et al.*, 2000). This in turn suggests that the entire human *MITF* gene is present as a single copy. Because the chicken gene is of a similar estimated genomic size, it is likely that the chicken gene is also present as a single copy.

The one report of a mutation in an avian microphthalmia gene lends further strength to a single gene model for chickens. Mochii *et al.* (1998b) established that the Japanese *silver* quail has two mutations: one in the basic domain, and the second a frame shift causing a premature stop codon following the leucine zipper region. That is, the mutations are in domains that are common to all microphthalmia isoforms. This is unlike the previously described murine mi^{bw} mutation, which specifically affects the melanocyte-type microphthalmia isoform, but is similar to the zebrafish *nacre* mutation, which also affects domains common to all microphthalmia isoforms. However, the *nacre* mutation only results in disruptions of neural crest-derived pigment cells, which led investigators to speculate that a duplication of the gene is compensating for function in the RPE (Lister *et al.*, 1999). On the other hand, the Japanese *silver* quail exhibits defects in both eye development and plumage colour (Homma, 1971; Mochii *et al.*, 1998b). This demonstrates that the mutations affect both pigmented tissues equally and therefore suggests only one copy of the quail microphthalmia gene. Because chickens and quails belong to the same vertebrate class (Aves), it seems likely that the gene is highly conserved between the two species and that the chicken microphthalmia gene is also present as a single copy. This has not, however, been experimentally tested or demonstrated in the current study, and it will be of future interest to more closely examine the genomic structure of the chicken microphthalmia gene.

The possibility that there are small alterations such as point mutations or polymorphisms in the microphthalmia gene of the WPR x PG breed, cannot be excluded based on the results of the present study. One approach in determining whether such differences occur would be to PCR amplify RNA extracted from pigmented cells of WPR x PG and BA breeds. DNA sequence comparisons between the WPR x PG and BA *cMI* cDNA would then distinguish whether there are any small alterations between the two breeds. If there are small alterations of the microphthalmia gene in the WPR x PG breed, then it seems likely that they will lie within the *cMI-m* transcripts and proteins. This is because the only observable difference between the two breeds is the pigmentation; there is no evidence to suggest that other microphthalmia-dependent cell types (for example

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osteoclasts) are affected. Therefore, at the genomic level, if small alterations in microphthalmia are responsible for the pigmentation differences, then such alterations will likely lie in the melanocyte-type first exon. Alterations at any other point across the genomic sequence would likely affect other isoforms of the gene, and hence produce additional phenotypic effects.

4.3 Endogenous expression of microphthalmia mRNAs and proteins

Although it is well established that microphthalmia exists in many different isoforms, it is less clear what the significance(s) of the different isoforms are. For example, are the splice variants and the different amino termini important for the function of the proteins or do they merely represent different embryological and/or developmental histories in various tissues? Are the restricted expression patterns of some isoforms also a feature of lower vertebrates (such as chickens) or have the different isoforms only evolved in higher vertebrates such as mammals? As a start to investigating this in lower vertebrates, the endogenous expression patterns of the chicken microphthalmia gene were here investigated.

The results of the present study and those of Mochii *et al.* (1998a) demonstrate that at least two different isoforms (*cMI-m* and *cmi9)* are transcribed from the chicken microphthalmia gene. To determine whether these different forms are expressed preferentially or exclusively in different pigmented tissues, the expression of *cMI-m* and *cmi9* transcripts were compared by RT-PCR using first exon specific sense primers and RNA isolated from neural crest-derived melanocytes (skin) and RPE cells. It was established that *cMI-m* is expressed only in neural crest-derived melanocytes (skin), while *cmi9* transcripts are only amplified from RPE cells. These results suggest that some chicken microphthalmia isoforms are preferentially expressed in some tissues, as is the case with similar preferential expression patterns of some mammalian isoforms (reviewed by Yasumoto *et al.*, 1998 and Goding, 2000). The expression of *cMI-m* and *cmi9* are further considered below.

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cMI-m Expression

One of the most interesting findings of the present study is the detection by RT-PCR of microphthalmia transcripts in non-pigmented tissues such as heart, brain, and the gastrointestinal tract. Although this is in agreement with a previous report of RT-PCR amplification of microphthalmia transcripts from chicken non-pigmented tissues (Mochii *et al.*, 1998a), it does not reconcile with *in situ* analyses which show that microphthalmia mRNA is restricted to tissues known to require the gene for differentiation (i.e. RPE cells) (Hodgkinson *et al.*, 1993; Mochii *et al.*, 1998a; Nakayama *et al.*, 1998; Amae *et al.*, 1998). The simplest explanation is that RT-PCR is a much more sensitive technique than *in situ* hybridisation and that in both the present study and those of Mochii *et al.*, (1998a), the numbers of PCR cycles used to detect transcripts in non-pigmented tissues were

quite high (35 cycles, Mochii *et al.*, 1998a; and 40 cycles, present study). However, the results of the present study also consistently show that whereas 25 cycles are sufficient to detect microphthalmia transcripts in pigmented tissues, a further 5 cycles are required for the detection of microphthalmia transcripts in non-pigmented tissues.

These results strongly suggest that there is a relatively high level of "illegitimate" transcription of microphthalmia in non-pigmented tissues. Illegitimate transcription was first described over a decade ago and refers to "low-level ubiquitous transcription of tissue-specific genes" (Chelly *et al.*, 1989). Since then many genes, expressed both as spliced transcripts and from alternative tissue-specific promoters, have been detected non-specifically or "illegitimately" in tissues using RT-PCR (Kaplan *et al.*, 1992; Lopez-Guerrero *et al.*, 1997; Kimoto, 1998a, 1998b; Gala *et al.*, 1998). Most significantly, it has been demonstrated that the transcription start sites are the same in specific and non-specific cells types and in many cases it has been shown that the transcripts arise from a low level activity of the normal promoter (Chelly *et al.*, 1991).

Given that the microphthalmia gene has multiple promoters, the question then arises as to which of the promoters is being used "illegitimately". This was tested by determining whether illegitimate transcription could be detected using primers specific for the first exon of *cMI-m* or for the first exon of *cmi9*. The results show that the illegitimate transcription of *cMI* in non-pigmented cells (fibroblasts) appears to result from a low level of activity of the melanocyte-type promoter since transcripts were only amplified using the *cMI-m*-specific primers. These transcripts were verified to be microphthalmia by sequencing the PCR products using a primer that anneals to the melanocyte-type first exon, thus confirming that these PCR products are not the result of non-specific amplification of another MiT sub-family member (such as TFE3 or TFEB).

The fact that only *cMI-m*, and not *cmi9*, appears to be illegitimately transcribed is intriguing. One possible explanation may lie in a hypothesis put forth to explain illegitimate transcription: Chelly *et al.* (1989) suggest that ubiquitous transcription factors may be responsible for illegitimate transcription. Due to chromatin disruptions that occur during cell division, Chelly *et al.* reason that all promoters, including tissue-specific promoters, can be minimally active at all times when ubiquitous transcription factors reach their target elements. Due to the complex promoter region of microphthalmia and the large intronic sizes between first exons/promoters (see Table 1.1), it is possible that the chromatin is disturbed in the region of the *cMI-m* promoter, but not in the region of the *cmi9* promoter. It would therefore be interesting to test for illegitimate transcription of all microphthalmia isoforms using RT-PCR and primers specific for each first exon, to determine whether the relative location of each first exon impacts on transcription in non-pigmented cells.

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Very recently, other investigators have also reported the presence of *Mitf-m* transcripts in both mouse mast cells and heart tissue, although they are present at very low levels as detected by RT-PCR (Oboki *et al.*, 2002). The key issue then is that, in contrast to earlier reports that demonstrated that *Mitf-m* was expressed exclusively in neural crest-derived melanocytes, microphthalmia transcripts have now been amplified from non-pigmented tissues. The next question is whether these microphthalmia transcripts present in non-pigmented cells are translated into proteins; this was investigated in the present study by comparing western blots of untransfected and RCAS/*cMI-m* transfected chicken embryonic fibroblasts and HeLa cells. The results demonstrate that Microphthalmia protein is indeed present in fibroblasts (at very low levels) and in HeLa cells (at slightly higher levels). Estimations of the molecular weights of the Microphthalmia proteins suggest that in fibroblasts, the Microphthalmia protein is not derived from melanocyte-type microphthalmia transcripts: when chicken embryonic fibroblast or HeLa cells are transiently transfected with RCAS/*cMI-m*, a doublet of ~ 56 and 63 kDa is observed upon western blotting. In contrast, the endogenous protein detected upon western blotting of the untransfected cells runs as a doublet at ~ 65-69 kDa. Even when taking into account the deletion of exons 3 and 6a from RCAS/*cMI-m* (which are predicted to cause the protein to migrate ~4.08 kb faster than a protein containing the two exons), the endogenous protein detected in chicken embryonic fibroblasts and HeLa cells is probably too large to represent a melanocyte-type protein. It probably represents a different, as yet uncharacterised, slightly larger isoform or additional post-translational modifications, or even a splice variant not previously described (as is likely the case with the proteins detected by others in additional non-pigmented tissues (Takemoto *et al.*, 2002)).

At least two further questions thus arise: (i) which isoform is detected in these non-pigmented cells?; and (ii) is the protein functional with respect to melanogenesis? Because the endogenous proteins detected in the non-pigmented cells migrate at the same molecular weights as the exogenous proteins translated from RCAS/*cmi9*, it is possible that the endogenous protein carries an amino terminus (first exon) similar to the cmi9 protein. The only isoform identified to date that carries a first exon similar to *cmi9* is *MITF-D* (Takeda *et al.*, 2002). However, it is unlikely that the protein detected in HeLa cells is MITF-D because *MITF-D* transcripts are not detected in HeLa cells, as assessed by RT-PCR (Takeda *et al.*, 2002). Thus, if the protein detected in HeLa cells carries a first exon similar to cmi9, it may represent a novel isoform. A second possible explanation is that the cmi9-encoded isoform itself is present in both human HeLa cells and chicken embryonic fibroblasts. However, this possibility is unlikely because the present study demonstrates that (i) *cmi9* transcripts are not detected in chicken embryonic fibroblasts (see section 3.4) and (ii) the protein detected in HeLa cells is unable to increase the activity of the tyrosinase gene promoter in transfection assays, but co-transfection with RCAS/*cmi9* in the same cells does increase the promoter reporter activity (see section 3.6.3). The very minimal promoter-reporter activity detected in HeLa cells transfected with the tyrosinase

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gene promoter suggests that the endogenous Microphthalmia protein present in HeLa cells does not function to transactivate the tyrosinase gene promoter, at least *in vitro*. Further work shall be required to identify the protein and any functional role for Microphthalmia in non-pigmented cells.

In summary, there appears to be a relatively high level of illegitimate transcription of *cMI-m* in chicken embryonic fibroblasts, but it is not clear whether *cMI-m* is translated into protein and, indeed, it does appear that there may be another form of cMI protein present in the cells. The significance of the illegitimate transcription of *cMI-m* is not clear, but perhaps the explanation is as simple as that put forward for illegitimate transcription of human tissue-specific genes: that it is unlikely that the transcripts play any role but that they rather indicate a basal level of transcription outside of the tissues where they are normally active (Chelly *et al.*, 1989).

cmi9 Expression

Of particular note in the present study is the apparently restricted expression of *cmi9* in RPE cells, as assessed by RT-PCR. Although Mochii *et al.* (1998a) have similarly demonstrated that *cmi9* transcripts are restricted to RPE cells when compared to a number of non-pigmented tissues (neural retina, brain, heart, liver, and gizzard), they did not investigate its expression in different types of pigmented tissues. The finding of the present study is thus important because this is the first identification of an isoform that is apparently restricted in expression to the RPE, at least at the mRNA level. What is less clear is whether cmi9 is the predominant protein present in the RPE: although western blotting detects a single cMI protein species in RPE cells, the size of the endogenous protein (~69 kDa) does not precisely correspond to the known size of exogenous cmi9 (~65-69 kDa). Thus, if the endogenous protein is indeed cmi9 then this difference needs to be explained. One possibility is that the post-translational modifications of the endogeneous and exogenous proteins are not identical.

The expression of *cmi9* transcripts and proteins is of further interest if considered in conjunction with the expression of the so-called RPE isoform (*Mitf-a*), which is enriched in the mammalian RPE (Amae *et al.*, 1998). Although its expression patterns have not been assessed, a chicken RPE-type (partial) cDNA has been cloned (Mochii *et al.*, 1998a; here referred to as *cMI-a*) and it is possible that it will also be enriched in the chicken RPE. If this is the case, it suggests that both the *cMI-a* and *cmi9* microphthalmia isoforms are required for the correct development of the chicken RPE. However, results obtained in the present study suggest that this may not be the case: only one protein is detected upon western blotting of chicken RPE cells and the size of the protein most closely correlates with the predicted size of the cmi9 protein. What is possible is that *cMI-a* is required for the early development of the chicken embryonic RPE but has been downregulated at the developmental stage of 12-14 days (which was the age of the chicken embryos used for the RPE

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western blot analyses in the present study). Evidence for the downregulation of microphthalmia during embryonic eye development comes from the work by Mochii *et al.* (1998a) who demonstrate that, although both *cMI-a* and *cmi9* may be amplified from 9 day-old chicken embryo RPE cells, microphthalmia expression is not detected in the eyes of the hatched chicken. Although the group did not assess the expression of any particular isoform (but rather used an *in situ* probe common to all microphthalmia isoforms), it is possible that *cMI-a* is expressed as late as 9 days old (Mochii *et al.*, 1998a) but is not expressed by developmental days 12-14 (present study). It will therefore be of interest in the future to assess the expression pattern of *cMI-a* and compare it to that of *cmi9* in the developing chicken RPE.

To summarise, it is now known that there are at least two full-length (*cMI-m;* present study, and *cmi9;* Mochii *et al.*, 1998a) and one partial (*cMI-a*; Mochii *et al.*, 1998a) chicken microphthalmia transcripts, which differ significantly only at the amino termini. Thus, the chicken microphthalmia locus generates alternative isoforms in a manner similar to the mammalian microphthalmia locus. In avian pigmented tissues, *cMI-m* is expressed in neural crest-derived melanocytes but not in RPE cells, while *cmi9* is expressed in RPE cells but not in neural crest-derived melanocytes. This apparently restricted expression of *cMI-m* is similar to the previous reports of a restricted expression pattern of the mammalian melanocyte-type isoform, while the apparently restricted expression of an isoform (*cmi9*) to the RPE is a novel finding. Thus is appears possible that the different amino termini may be involved in specifying those tissues in which a given transcript is expressed. The next question examined here is whether the different amino termini of cMI-m and cmi9 proteins have different transactivational properties.

4.4 Analyses of exogenous expression of *cMI-m* and *cmi9 in vitro*

Prior to assessing any functional significance(s) of the different amino termini, it was first necessary to establish whether proteins are expressed from the transfected DNA constructs.

4.4.1 Protein translation from RCAS/*cMI-m* and RCAS/*cmi9*

When HeLa or chicken embryonic fibroblast cells were transfected with RCAS/*cMI-m* a doublet of ~56-63 kDa was detected; when the same cells were transfected with RCAS/*cmi9* a doublet of ~ 65-69 kDa was detected. The presence of the characteristic doublet of the proteins is encouraging and suggests that the chicken melanocyte-type and cmi9 isoforms translated from RCAS/*cMI-m* and RCAS/*cmi9* are phosphorylated in the same manner as the mammalian melanocyte-type isoform (Hemesath *et al.*, 1998). This is an important consideration for the potential activity of cMI, as it has been demonstrated that the phosphorylation of Microphthalmia increases its ability to transactivate the tyrosinase gene promoter (Hemesath *et al.*, 1998).

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It should be noted that the Microphthalmia protein derived from the chicken melanocyte-type isoform (~56-63 kDa) is a different size to that of the Mitf-m protein (~60-65 kDa) present in B16 cells. Furthermore, there have been discrepancies in the published sizes of the B16 protein doublet: sizes of ~54-60 kDa (Hemesath *et al.*, 1998), ~52-56 kDa (King *et al.*, 1999) and ~60-65 kDa (Takemoto *et al.*, 2002) have been reported. This discrepancy in the sizes of the Mitf-m doublet has not been further investigated or explained, but investigators have speculated that this merely represents inconsistencies between different protein molecular weight markers (C. Takemoto, personal communication). However, this cannot explain the difference in sizes between cMI-m and the B16 Mitf protein obtained in the present study (because the same molecular weight marker was used) and a more likely explanation for the different sizes detected in the present study is as follows: The cMI-m protein is translated from the RCAS/*cMI-m* construct. Sequencing has shown that it is missing both exon 3 (28 aas) and exon 6a (6 aas). These deleted 34 aas are predicted to decrease the size of the cMI-m protein by ~ 4.08 kDa (because the average mass of each amino acid is 120 Da). That is, the cMI-m protein may be predicted to migrate faster than a protein carrying both exon 3 and exon 6a. This in turn suggests that the protein detected in B16 cells carries both exon 3 and exon 6a, and that the deletion of both exons from cMI-m decreases the size of the translated protein in a predictable manner. In light of this, it is possible that the previously detailed discrepancies of the human melanocyte-type microphthalmia isoform (Hemesath *et al.*, 1998; King *et al.*, 1999; Takemoto *et al.*, 2002) may also represent differences in alternative splicing of detected proteins, rather than differences in protein molecular weight markers.

4.4.2 Comparison of transcription/translation from RCAS/*cMI-m* and RCAS/*cmi9*

Two important variations between RCAS/*cMI-m* and RCAS/*cmi9* have been observed during the course of the present study: Firstly, when chicken embryonic fibroblasts are transfected with 5 µg of either construct different transcript levels are present, as assessed by RT-PCR. *cMI-m* transcripts were detected following 20 PCR cycles while *cmi9* transcripts were only detected following 30 PCR cycles, suggesting that with equivalent amounts of transfected DNA, there are more transcripts of *cMI-m* than *cmi9*. Confusingly, however, these differences do not correspond to equivalent differences in protein. That is, western blotting of HeLa cells transfected with RCAS/*cMI-m* produce, on the whole, lower levels of protein than do HeLa cells transfected with RCAS/*cmi9*. While there is an increase in the amount of protein translated from RCAS/*cmi9* with increasing amounts of the construct, there appears to be a relatively steady state of protein translation from the RCAS/*cMI-m* construct. These findings of apparently different transcriptional and translational levels are seemingly contradictory. However, it is common for investigators to report such inconsistencies in transfection studies, and the differences are probably the result of a variety of phenomena including different transcript and

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protein half-lives, different translational efficiencies, and/or different post-translational processing. Most important with respect to the current study is that, regardless of the differences in transcription levels between RCAS/*cMI-m* and RCAS/*cmi9*, 6 µg of both constructs produce approximately equal levels of protein, as assessed by western blotting. With this in mind it is recognised that the functional studies discussed below should, as a result of the differences, be interpreted with some caution.

The second important point is that the over-expression of cMI-m appears to repress the endogenous HeLa Microphthalmia protein. It is not clear whether cmi9 has the same effect because the cmi9 protein appears to be the same size as the endogenous protein and therefore any repression could not be deduced from the present study. There have been numerous reports of exogenous transfected genes regulating the expression of endogenous genes (some more recent examples are: Mitchell and El-Deiry (1999); Mitchell *et al.* (2000); Ayroldi *et al.* (2001); Ayroldi *et al.* (2002)). The question is: how and/or why does the over-expression of exogenous cMI-m down regulate the endogenous Microphthalmia protein in HeLa cells? Does this imply that Microphthalmia is self-regulated?

In this vein of thought, it is known that the melanocyte-type Microphthalmia isoform can regulate its own promoter, and it is thought to do so in order to "function as a self-regulator of its own expression in order to maintain a threshold of MITF-M that is required for melanocyte development" (Saito *et al.*, 2002). Perhaps it is the case that the *overall* levels of Microphthalmia are self-regulated and that a threshold of all isoforms (in those cells in which more than one isoform is transcribed/translated) is maintained by one Microphthalmia isoform regulating the expression of another isoform(s). In the experiments described here, the exogenous protein may act to downregulate the endogenous protein promoter activity, but the exogenous protein itself is under the control of the RCAS BP(A) LTR promoter and is thus constitutively expressed and will not be able to self-regulate its expression levels. It would therefore be interesting to carry out experiments to assess the kinetics of the apparent down regulation. One approach would be to clone the *cMI-m* coding region into a tagged expression vector. This expression vector could then be transfected into cells at various concentrations and over various time periods and one could measure its expression levels using an anti-tag antibody. The expression of the exogenous protein and any correlating down regulation of the endogenous protein could be compared by simultaneously tracking the endogenous protein using the anti-Mitf antibody. "Recovery" experiments could also be carried out, where the transfected cells are permitted to grow for longer periods, allowing for the determination of whether the endogenous protein expression returns with time.

A second possible explanation for the apparent suppression of endogenous HeLa protein by cMI-m may lie in the cytotoxicity of the cMI protein. The present study demonstrates that, at 8 µg, cMI-m is

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toxic to HeLa cells. Perhaps the cytotoxic effects are already evident in the molecular/cellular machinery but have not yet resulted in actual cell death when 6 μg of *cMI-m* is transfected into the cells. That is, perhaps the cells are responding to the cMI-induced disturbance by down-regulating non-essential genes, such as the endogenous Microphthalmia protein. This possibility could be further tested by transfecting the cells with varying amounts of the toxic cMI-m protein and assaying for a down regulation of endogenous genes, or increased expression of genes known to be upregulated during induced cell death.

In summary, the mouse anti-Mitf antibody used in the present study does cross-react with the chicken Microphthalmia protein in RPE cells. Endogenous Microphthalmia protein is also detected in two non-pigmented cell lines (HeLa and fibroblasts), although it is not clear from the results of the present study which isoform is being detected. However, the over-expression of cMI-m in HeLa cells appears to function to suppress the endogenous HeLa protein, as assessed by western blotting. Further work will be required to fully explain how and/or why the exogenous cMI-m disrupts the transcription and/or translation of the endogenous HeLa protein.

4.5 Functional analyses of *cMI-m* and *cmi9 in vitro*

The isolation and molecular characterisation of *cMI-m* (this study) and *cmi9* (Mochii *et al.*, 1998a) paves the way for an understanding of the role of *cMI* in avian melanogenesis. One focus of this study was to determine whether the two Microphthalmia proteins with different amino termini have different abilities to regulate the tyrosinase gene promoter. The transactivation activity of each isoform was determined by co-transfections of each with the chicken tyrosinase gene promoter in both non-pigmented (HeLa) and pigmented (B16) cells. However, it was first necessary to determine the baseline level of activity of the chicken tyrosinase gene promoter in the two cell types used here and, most importantly, to demonstrate that the promoter functions in a pigment cell-specific manner.

The results of the present study clearly show that the full-length chicken tyrosinase gene promoter is pigment cell specific in B16 cells. This is in general agreement with a previous study that demonstrated that the promoter is pigment cell-specific in both chicken RPE cells and mouse melan-a cells (Clarke, M.Sc. Thesis, 2000), although the activity of the promoter in pigmented cells in the current study is approximately ten-fold higher than that reported by Clarke. Taken together, the results of the present study and those by Clarke clarify previous results that failed to demonstrate that the tyrosinase gene promoter is pigment cell-specific in its activity (Ferguson, Ph.D. Thesis, 1996). However, the studies by Ferguson where performed in MQTNC cells, which are a transformed quail neural crest-derived cell line with dubious pigmented cell characteristics, and this may explain why Ferguson did not obtain a clear pigment cell-specific activity in transient

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transfections with the tyrosinase gene promoter. It is also possible that the previous studies by Clarke and by Ferguson had comparatively poor transfection efficiencies. The reasons for this may be that different transfection reagents were used in each case: Ferguson transfected cells with the standard calcium phosphate co-precipitation method (Kingston, 1987); Clarke utilised FuGENE (Roche), while the present study utilised PolyFect (Qiagen). It is known that PolyFect is optimised for transfections of HeLa cells (which were used in the present study) and it is possible that PolyFect is also an optimal reagent for transfections of B16 cells (which were also used in the present study). Determining that the different transfection methods/reagents did indeed impact on transfection efficiencies would require comparisons of the same transfections in the same cells using the three different transfection methods.

The present study also clearly shows that when the distal ~1.0 kb of the chicken tyrosinase gene promoter is deleted, there is a decrease in the pigment cell-specific activity of the promoter, suggesting the presence of important *cis*-acting elements/enhancers in the distal promoter. The elements present in the distal promoter include the tyrosinase distal elelment (TDE), two of the p-MSE elements and the proximal core elements. The TDE, first identified in the human tyrosinase gene promoter, is an element that functions as a strong enhancer in directing pigment cell-specific promoter activity in human neural crest-derived melanocytes (Yasumoto *et al.*, 1994). The p-MSEs are M-box-like elements (Yamamoto *et al.*, 1992) that have not been identified in mammalian tyrosinase gene promoters but are present in both the chicken tyrosinase gene promoter (Ferguson and Kidson, 1996) and the quail tyrosinase gene promoter (Akiyama *et al.*, 1994). The core elements include the Sp1 binding site, the M-box, and the Inr.

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When the TDE-like sequence and the two distal p-MSEs are deleted from the chicken tyrosinase gene promoter, the reporter activity is much lower than that obtained with the full-length promoter, although a weak pigment cell-specific activity is retained. These results suggest three things: Firstly, that the chicken TDE is required for a strong pigment cell-specific activity of the tyrosinase gene promoter in B16 cells. This is in agreement with a previous report that the human TDE functions as a strong enhancer in directing pigment cell-specific promoter activity in human neural crest-derived melanocytes (Yasumoto *et al.*, 1994).

Secondly, the two p-MSE elements may also be important in mediating the pigment cell-specific activity of the promoter. Akiyama *et al.* (1994) have reported that at least one of the equivalent quail p-MSE elements is indispensable for conferring tissue-specific expression of the quail tyrosinase gene promoter. Although it does not appear that either of the two chicken p-MSEs are *indispensable* for conferring tissue-specific expression of the chicken tyrosinase gene promoter, they may be involved in directing the strong pigment cell-specific activity of the full-length promoter. Whether the p-MSE

elements are required in addition to the TDE, or whether they themselves are responsible for the strong pigment cell-specific activity of the full-length promoter, is not clear based on the results of the present study. Clarification of this will require mutation analysis of each element with repeated transfections of each mutant construct into B16 and HeLa cells.

Third, the results indicate that the proximal core elements (which include an Inr, Sp1 binding site, and M-box) of the chicken tyrosinase gene promoter are sufficient for a weak pigment cell-specific activity in B16 cells. This contrasts a previous report in which a quail tyrosinase gene promoter deletion construct containing the Inr, Sp1, and the M-box (i.e. Equivalent to the shortest construct used in the present study) did not function in a pigmented cell-specific manner in neural crest-derived melanocytes (Akiyama *et al.*, 1994). Since the chicken and quail promoters are almost identical, this discrepancy is most likely indicative of different methodologies between the two studies. For example, it is possible that Akiyama *et al.* experienced low transfection efficiencies. Indeed, the finding that the core proximal elements are sufficient for a weak pigment cell-specific activity is in agreement with a previous report of a mammalian tyrosinase gene promoter in which a minimal (270 bp) mouse promoter is sufficient to mediate weak pigment cell-specific activity in both human and mouse neural crest-derived cell lines (Klüppel *et al.*, 1991). Taken together, these results suggest that the regulation of the chicken tyrosinase gene promoter activity in neural crest-derived cells is similar to the mouse tyrosinase gene promoter.

In summary, the results of transfections of three chicken tyrosinase reporter promoter constructs in pigmented (B16) and non-pigmented (HeLa) cells demonstrate that the activity of the full-length promoter is strongly pigment cell-specific in neural crest-derived B16 cells. The TDE and/or the two distal p-MSE promoter elements appear to mediate this strong pigment cell-specific activity, although the core promoter elements are still able to mediate a weak pigment cell-specific activity.

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4.5.1 Function(s) of the two chicken microphthalmia isoforms in transfected non-pigmented (HeLa) cells

The generation of diversity at the 5' termini of microphthalmia transcripts in both lower and higher vertebrates suggests that it may have a conserved functional significance. One possible functional implication is that the differing amino termini will regulate the ability of each Microphthalmia isoform to transactivate its downstream target promoter(s). As detailed in Chapter One (section 1.5), the experimental evidence regarding this conjecture is less than clear. In particular, there have been few studies to investigate whether different isoforms of Microphthalmia differ in their ability to transactivate the tyrosinase gene promoter in pigmented and non-pigmented cells. The present study

directly tested this using transient co-transfections of two chicken Microphthalmia isoforms (*cMI-m* and *cmi9)* and the chicken tyrosinase gene promoter in B16 and HeLa cells.

The results from all of the co-transfections of tyrosinase promoter-reporter gene constructs and the *cMI-m-* and *cmi9*-encoding RCAS constructs in non-pigmented (HeLa) cells give a first indication that they may not have different trans-activational properties. In HeLa cells, both cMI-m and cmi9 increase the full-length tyrosinase promoter reporter activity by approximately four-fold (i.e. an induction ratio (IR) of 4.0) above the baseline activity of the promoter. In a similar study using fibroblasts, the activity of the mouse tyrosinase gene promoter is also enhanced by four-fold upon co-transfection with Microphthalmia (Ganss *et al.*, 1994b). These IRs of 4.0 are, on average, much lower than that obtained by other groups where IRs between 11 and 75 were obtained on similar studies in HeLa cells (Yasumoto *et al.*, 1994; IYasumoto *et al.*, 1995; Yasumoto *et al.*, 1997a; Fuse *et al.*, 1999).

One possible explanation for the lower IR obtained in the present study may lie in the fact that avian constructs were transfected into mammalian cell lines, which may not be an optimal molecular environment for stable transcription/translation from RCAS/*cMI-m* and RCAS/*cmi9*. Attempts were made in the earlier stages of the present study to correct for this by using chicken embryonic fibroblasts. However, it was subsequently found that these cells are not readily transfected and further experiments with the cells were abandoned.

A second possible explanation is that the over-expressed exogenous cMI is deleterious to the HeLa cells. Results of the present study demonstrate that cMI-m, and possibly cmi9, appear to inhibit the endogenous expression of MITF in HeLa cells. Perhaps it is the case that the over-expression of cMI inhibits additional endogenous genes that are required as co-factors for the transactivation of the tyrosinase gene promoter in HeLa cells. The result would be an overall lower transactivation of the promoter-reporter construct, and hence a lower IR. It would therefore be of interest in the future to transfect the cells with *lower* amounts of the two cMI constructs to determine whether this will increase the promoter-reporter activity.

As was the case with the full-length promoter, both cMI-m and cmi9 increase the activity of the two deletion promoter constructs by approximately four-fold in HeLa cells. These results shed some light on the potential targets of the Microphthalmia protein. As discussed above, the chicken tyrosinase gene promoter contains five putative target elements for Microphthalmia: the TDE, two of the p-MSE elements, the M-box and the Inr. The TDE and the two p-MSE elements are excluded from both of the deletion constructs. Thus it seems unlikely that one or all three of these elements are required for the promoter transactivation by cMI, at least in non-pigmented cells *in vitro,* but that the

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M-box and/or the Inr is mainly responsible for the transactivation of the promoter by cMI. The results do not negate the possibility that cMI can target and bind the TDE and/or the p-MSEs, but merely suggest that the elements are not absolutely required for transactivation. Clarification of which elements are crucial will require mutation analysis of each with repeated co-transfections of each mutant construct with the cMI proteins in HeLa cells.

A final point of interest is the possible importance of exon 3 for the function of Microphthalmia. RCAS/*cMI-m* is missing exon 3, while RCAS/*cmi9* is not. The fact that both proteins exhibit the same activity in HeLa cells suggests that the deletion of the exon has no effect on the ability of the cMI protein to transactivate its downstream target. Such a result is consistent with one previous report in which the deletion of exon 3 from MITF also has no effect on the ability of the protein to transactivate the tyrosinase gene promoter (Yasumoto *et al.*, 1997a). Information regarding the potential significance of the deletion of exon 6a from Microphthalmia transcripts cannot be gleaned from the results of the present study, as both cMI proteins used are missing the exon.

In summary, the two chicken cMI proteins used in the present study (cMI-m and cmi9) transactivate the three chicken tyrosinase promoter reporter constructs – most likely through the Inr E-box motif - to equal levels in HeLa cells, at least *in vitro*. This strongly suggests that the differing amino termini do not have a functional significance in regulating the activity of the tyrosinase gene promoter in non-pigmented cells, a result which is, on the whole, in agreement with previous reports (Fuse *et al.*, 1999; Takemoto *et al.*, 2002).

4.5.2 Function(s) of cMI-m and cmi9 in transfected neural crest-derived (B16) cells

The next question to be addressed is whether this apparent lack of functional difference between the two cMI isoforms is also evident in a pigmented cell line. Two significant results, common to both isoforms, were obtained in B16 cells: First, cmi9 and cMI-m increased the reporter activity of the full-length chicken tyrosinase gene promoter construct by approximately 1.3– and 1.4-fold, respectively. Secondly, both proteins increased the activity of the two deletion constructs, Tyr1.1-luc and Tyr0.5-luc by approximately 13-fold, to the same level of activity as the full-length promoter. Each of these results is considered separately below.

The 1.3- and 1.4-fold fold-increase in the activity of the full-length promoter in B16 cells is lower than the previously detailed four-fold-increase of the activity of Tyr2.1-luc in HeLa cells. However, such low levels are also evident upon examination of studies reported on the transactivation of the mammalian tyrosinase gene promoter by the mammalian Microphthalmia protein in pigmented and non-pigmented cells: IRs of approximately 5, 5.8, and 3.5 have been observed in MeWo melanoma

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cells (Yasumoto *et al.*, 1994, 1995; Amae *et al.*, 1998) while much higher IRs have been reported in HeLa cells (IR of 50, Yasumoto *et al.*, 1994; IR of 60, Yasumoto *et al.*, 1995; IR of 75; Yasumoto *et al.*, 1997a; IR of 11, Fuse *et al.*, 1999). Yasumoto *et al.* (1994) speculate, "Such a big difference in the magnitude of activation may be at least in part due to the fact that HeLa cells are deficient in MITF". In other words, the suggestion is that cells expressing low (or no) levels of endogenous microphthalmia may result in higher activation of *tyrosinase* gene expression when exogenous microphthalmia is expressed. Although the present study demonstrates that HeLa cells are not in fact deficient in MITF (as assessed by western blotting with the anti-Mitf antibody), it also demonstrates that the endogenous MITF protein does not appear to be functional (as assessed by the lack of *luciferase* activity following transient transfection of the Tyr2.1-luc reporter construct in HeLa cells) and thus the cells are "*essentially*" deficient in MITF with respect to these studies.

In contrast to the relatively low increase in the activity of the full-length (Tyr2.1-luc) chicken tyrosinase gene promoter, co-transfection of Tyr1.1-luc and Tyr0.5-luc with cMI-m or cmi9 strongly enhances the activity of both deletion constructs in B16 cells. Because the results obtained in HeLa cells suggest that the cMI proteins transactivate the chicken tyrosinase promoter through the Inr E-box and/or M-box elements, this result is not unexpected. That is, the result demonstrates that both cMI proteins also transactivate the promoter through elements contained in Tyr0.5-luc (which includes both the Inr E-box and the M-box) in neural crest-derived B16 cells.

The fact that cMI only significantly increased the activity of the deletion constructs (and not the full-length promoter) in B16 cells suggests the presence of a negative regulatory element between –1.1 kb and the ATG of the chicken tyrosinase gene promoter. Such a negative regulatory element is also present in both the human and mouse tyrosinase gene promoter (Bentley *et al.*, 1994; Ganss *et al.*, 1994a) and is located just upstream of the M-box. If the position of this negative regulatory element is conserved in the chicken tyrosinase gene promoter, then it will be included in both of the deletion constructs used in the present study. The increase in promoter activity of the two deletion constructs observed upon co-transfection with cMI-m or cmi9 suggests that the proteins might mediate the removal of a repressor bound to the negative element, thereby increasing the promoter activity. An interesting observation from this is that both isoforms have the same effect. This again suggests that the differences at the 5' termini between the two isoforms do not have a functional role in regulating its downstream target.

Finally, the fact that cmi9 increases the tyrosinase gene promoter in the present *in vitro* studies, but that it does not stimulate the endogenous *tyrosinase* gene in the *in vitro* studies of Mochii *et al.* (1998a) needs to be addressed. As introduced in section 1.7, it was thought possible that the inability of cmi9 to increase the endogenous *tyrosinase* gene – in contrast to the ability of mammalian Microphthalmia

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proteins to increase the activity of exogenous tyrosinase promoter reporter constructs – may reflect differences in the experimental approaches. The results of the present study suggest that this is the case: transiently co-transfected cmi9 and chicken tyrosinase gene promoter reporter constructs do, on the whole, behave much like co-transfections with mammalian constructs. Hence it may be concluded that because the simple over-expression of cmi9 did not lead to stimulation of the endogenous *tyrosinase* gene, the *in vitro* assays detailed in this study do not necessarily reflect physiological regulation.

4.6 Future directions

As introduced under section 1.7, the current study sought - in conjunction with additional lateral investigations - to answer a number of specific questions. The results obtained throughout the present study answer these questions, but they also raise additional ones, as described below. The following discussion is not intended to describe *how* these additional questions may be addressed, but rather serves to highlight potential areas of future research.

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Why are white chickens white?

Two of the chicken breeds used during the present study (White Plymouth Rock x Pile Game (WPR x PG) and White Leghorn (WL)) have pigmented eyes but white plumages. The very preliminary data on the genomic organisation of the chicken microphthalmia gene presented in this thesis demonstrates that there are no large genomic alterations of the gene in WPR x PG chickens. It is not known if the same is true of WL chickens, as this breed was not included in the genomic Southern blot analysis performed in the present study. Despite the lack of any large genomic alterations, it is possible that there are small alterations/mutations of the microphthalmia gene in white chickens. The phenotype of the white chicken breeds is reminiscent of the mi^{bw} mouse, which exhibits a black eye/white coat phenotype that is a result of a large genomic rearrangement that specifically affects the melanocyte-type isoform of microphthalmia (Yajima *et al.*, 1999). This then provides an opportunity to address some biologically meaningful information in white chickens: namely, are the chickens white due to an absence of the melanocyte-type microphthalmia isoform, as in the mi^{bw} mouse? If it is found that the melanocyte-type isoform is absent or altered in these chickens, then the next step will be to more fully characterise the genomic structure of the microphthalmia gene, both in white chickens and in chickens with pigmented plumages. A more detailed characterisation of the genomic structure of the chicken microphthalmia gene is given further consideration below.

The genomic structure of the chicken microphthalmia gene

At least three findings described in this thesis suggest that future consideration should be given to a more detailed characterisation of the genomic structure of the chicken microphthalmia gene. Firstly,

as discussed in Chapter One, a human isoform (MITF-D; Takeda *et al.*, 2002) shows some similarity to the chicken cmi9 isoform. If MITF-D is indeed a human homologue of cmi9, then this is an important point. It would demonstrate that the chicken microphthalmia gene transcribes equivalent isoforms (i.e. in addition to the *cMI-m* isoform isolated in the present study) to those found in mammals, thus further clarifying whether the avian cMI locus does or does not encode species-specific microphthalmia isofoms.

The second result that calls for a more detailed characterisation of the chicken cMI locus is the variations of the 3'-UTRs amplified from eye (both total (present study) and RPE (Mochii *et al.*, 1998a)) RNA, and neural crest-derived melanocyte RNA. It is not clear whether these differences result from alternative 3' exons and/or alternative polyadenylation signals, and a more detailed study of the cMI locus will help to clarify this.

Finally, the question of whether the white chicken breeds used in the present study have white plumages due to cMI genomic alterations, as discussed in the preceding section, could be answered with a more detailed analysis of the cMI locus.

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Alternative splicing of exon 3 from microphthalmia transcripts

The *cMI-m* cDNA isolated from the chicken embryonic neural crest-derived melanocyte cDNA library is missing exon 3, a potentially significant fact considering the size of the exon (28 aas). Similarly, Mochii *et al.* (1998a) have reported that approximately half of the clones isolated from a chicken embryonic RPE cDNA library are missing the exon. It is therefore interesting to question whether the deletion of this exon has some functional/biological significance during embryonic development. This could be addressed in the future through an assessment of the relative abundance of mRNAs containing or lacking this exons in the cells of interest, namely developing melanocytes from the neural crest and developing RPE cells.

Variations in protein translation from RCAS/*cMI-m* and RCAS/*cmi9*

As discussed in section 4.4.2, the levels of translated protein obtained following transient transfection of HeLa cells differ between RCAS/*cMI-m* and RCAS/*cmi9*. Although these differences did not impact on the results of the present study – because both constructs exhibit comparable activity when 6 μg of each is used in co-transfection experiments – they may impact on future studies of the cMI-m and/or cmi9 protein. Hence one possible solution is presented here.

The major variation between the RCAS/*cMI-m* and RCAS/*cmi9* constructs is found in the 5'- and 3'-UTRs (as detailed in Chapter Three). Since (i) there are these notable 5' and 3' differences between the two constructs, and (ii) there appears to be a difference in the levels of transcription/translation

between the two constructs, one approach to determine any correlation between the two concepts would be to standardise the 5'- and 3'-UTRs with respect to each other. Standardisation of the 5'-UTR could be accomplished by using an N-terminal myc tag in each construct. This would impart the same Kozak sequence on each protein, and the nucleotides immediately upstream of the AUG intiation codon (which are critical for ribosome binding, regulation of translation, and hence protein levels) would be the same. Likewise, the use of a standard 3-UTR, such as that from SV40T, would standardise regulation at the level of message stability. Although it was not necessary to take steps such as these during the present study – because transfections with 6 μg of each cMI construct resulted in equal activities of the tyrosinase gene promoter constructs - they may become necessary in future studies of the cMI protein.

4.7 Concluding comments

It is still not clear whether the mechanisms that regulate mammalian melanogenesis are also utilised in the regulation of avian melanogenesis. Although the recent cloning and characterisation of the chicken tyrosinase gene family (April, Ph.D. Thesis, 1998) suggests that the same mechanisms and key genes may be functioning in both higher and lower vertebrates, there are also reports of apparently species-specific genes, such as mammalian *agouti/ASP* (Butlman *et al.*, 1992; Wilson *et al.*, 1995) and *mottled/ATP7A* (Vulpe *et al.*, 1993; Cecchi *et al.*, 1997) genes, and avian *cmi9* (Mochii *et al.*, 1998a), *MMP115* (Mochii *et al.*, 1988), and *QNR-71* (Turque *et al.*, 1996) genes. A key question is whether these genes are indeed species-specific or whether their isolation from only mammals or avians is merely indicative of the relatively sparse knowledge of avian melanogenesis - further investigations into the molecular mechanisms governing avian melanogenic cell fate and/or function are clearly needed.

The present study describes the cloning and preliminary characterisation of the chicken melanocyte-type microphthalmia gene. The molecular characteristics of the gene are the same in avians as they are in mammals (for example, the diversity of transcripts as generated through alternative promoters/first exons and alternative splicing), and comparative analyses with a second chicken isoform, *cmi9*, suggests that the function(s) of the chicken microphthalmia gene do not differ in lower vertebrates. In addition, there seems to be no functional significance to the differing amino termini of chicken microphthalmia isoforms, as assessed through their ability to transactivate the chicken tyrosinase gene promoter. It is still possible that the different amino termini are important for the interaction(s) of each protein with different tissue-specific binding partners that may be important for the full activity of each protein. For example, it is known that the cmi9 protein carries a charged domain (at its amino terminus) that cMI-m does not, and this charged domain is thought to be important for protein-protein interactions (Rehli *et al.*, 1999). In any case, the identification of

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the third microphthalmia isoform (*cMI-m*) in avians should make possible further comparative analyses of the regulatory mechanisms governing melanogenesis in a variety of species.

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Appendix A | Analyses of technical problems encountered

A number of additional technical difficulties were encountered during the course of the present study. Some of the difficulties impacted on the results of experiments while others are of academic interest only; both cases warrant further description.

Restriction enzyme mapping of clone M156 (*cMI-m*)

Restriction enzyme mapping of clone M156 *(cMI-m)* was carried out by Craig April and Toni Wiggins, Department of Human Biology, The University of Cape Town, South Africa, and the analysis of the result formed portions of the current study. Clone M156 was digested with *Ava*I, *Bam*HI, *Kpn*I, *Pst*I, *Sma*I, *Pst*I/*Xho*I, *Sma*I/*Xho*I, and *Eco*RI/*Xho*I and electrophoresed on an agarose gel. The results of this restriction mapping are shown in Fig. 3.1 and an analysis of this result reveals anomalies within the clone. For example, the *Eco*RI/*Xho*I digest should, by virtue of the strategies used to isolate clone M156 from the cDNA library (see section 3.1), excise the insert. Because the sequencing performed in the present study demonstrates that clone M156 carries an insert of 2.6 kb with no *Eco*RI or *Xho*I sites, this double digest should yield two products: the 2.6 kb insert and the 2.96 kb pBluescript SK- vector. The *Eco*RI/*Xho*I digest, however, consistently revealed product sizes of 5.4 kb, 3.9 kb, 2.9 kb, 2.6 kb, 2.1 kb, and 1.5 kb. That is, the digest yielded the expected 2.9 kb product and the expected 2.6 kb product (which, encouragingly, was the only product to hybridise to the mouse *Mitf* cDNA probe upon Southern blot hybridisation of the gel), but also yielded four unexpected product sizes. All other restriction enzyme digests yielded similar anomalies. Despite numerous attempts by many researchers in our laboratory, these anomalies remain unexplained.

One possible explanation, however, is the so-called "ghost plasmid" of pBluescript (Hengen, 1994). It has been reported that the use of Stratagene's pBluescript phagemid vectors in combination with the *E. coli* host cell XL1-Blue (as was the case in the present study) can result in contamination of the plasmid preparation. An extraneous band is observed when cDNAs are digested with restriction enzymes, electrophoresed, and stained with ethidium bromide. The extraneous band usually correlates to approximately 2.0 – 2.2 kb of linear double-stranded DNA, but researchers also observe that the extra band can migrate at

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different positions, depending on the size of the cloned insert and the amount of plasmid DNA that is electrophoresed. Most interesting is that the band(s) is not present in all plasmid preparations, and it is thought possible that modified forms of plasmid DNA can arise from nicking, degradation, or denaturation, depending on the method of plasmid purification used. However, Hengen (1994) also notes that "it is entirely possible that XL1-Blue is contributing a factor(s) for the spontaneous initiation of coding strand replication, and that the ghost band(s) could be any one of a number of replication intermediates or recombination factors". Should this be the case, then the four extraneous product sizes obtained upon *Eco*RI/*Xho*I restriction enzyme digestion of clone M156 may be explained as follows: the 2.1 kb product correlates to the known "ghost band" size, and the other three may indeed be one of a number of replication intermediates or recombination factors.

Solutions for the problem of the "ghost band" have been sought and are presented on-line at ftp://ftp.ncifcrf.gov/pub/methods/TIBS/mar94.txt. The most common means of circumventing the problem is to use another bacterial host strain (HB101 or DH5α seem to be acceptable substitutes) or to transform a fresh batch of competent cells, which appears to eliminate the band for a period of time, prior to further manipulations or long-term storage. This was attempted to some extent during the course of the present study; a number of different preparations of clone M156 were prepared, using either XL1-Blue or JM109 host bacteria. In both cases the extraneous bands persisted. It would be interesting to attempt to transform HB101 or DH5α cells and determine whether this approach will eliminate the extraneous bands observed with restriction enzyme digestion of clone M156.

As reported by Hengen (1994), "the band does not normally interfere with further DNA manipulations and is therefore mostly ignored." This, on the whole, was the case in the present study. The extraneous bands observed with restriction enzyme digestion of clone M156 only gave rise to difficulties while attempting to excise the coding region without extensive 5'- or 3'-UTR for subsequent sub-cloning into RCAS BP(A). It proved impossible to locate suitable restriction enzymes that produced expected product sizes. This necessitated another approach to this cloning problem, and hence the relatively complicated strategy detailed in Section 3.5.1.1 was necessary.

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Cloning of *cMI-m*

In addition to the difficulties introduced by the anomalous restriction enzyme products, further technical difficulties were experienced during attempts to clone the coding region of the chicken melanocyte-type microphthalmia cDNA into RCAS BP(A). RCAS BP(A) is a Rous sarcoma virus that is a member of a large family of related retroviruses, collectively called avian sarcoma and leukemia viruses (ASLVs). The Rous sarcoma virus is unusual in that, in addition to the normal viral gene complement of *gag*, *pol*, and *env*, it also carries *src*, a host-derived oncogene of about 2 kb in length. The Hughes group (National Cancer Institute, Frederick Cancer Research and Development Centre, Frederick, Maryland, U.S.A.; Hughes *et al.*, 1984) recognised that *src* is not necessary for viral replication, and thus removed and replaced it with a *Cla*I restriction enzyme site. The resulting nature of RCAS BP(A) is that foreign DNA (up to 2 kb in length) may be inserted at the *Cla*I restriction enzyme site without disrupting the viral genome. Therefore the cloning strategy of the present study required that *cMI-m* be flanked with *Cla*I restriction enzyme sites.

Therefore, a PCR cloning strategy was initially adopted and *Cla*I-tagged primers (cMIF5 and cMIR4; Table 2.1) were designed to amplify the full-length chicken *cMI-m* coding region (Figure A.1.a). cMIF5 anneals to the 5' terminus sequence, beginning with the 5'-ATG-3' initiation codon of *cMI-m*, while cMIR4 anneals to the 3' terminus of *cMI-m*, beginning with the 3'-GAT-5' termination codon. Thus the PCR product amplified with this primer pair will exclude the 5' and 3' UTRs, an important consideration as these sequences can give rise to abortive translation (Morgan and Fekete, 1996). Clone M156 was PCR amplified using this primer pair and the resulting PCR product was ligated into pGEM-T Easy according to manufacturer's instructions (Promega).

This theoretically created a clone with the full-length *cMI-m* coding region flanked by *Cla*I sites; this in turn could be digested with *Cla*I and the resulting insert ligated into the *Cla*I site of RCAS BP(A). However, when resulting clones were digested with *Cla*I, incorrect product sizes were obtained (Fig. A.1.b). The products linearised at ~4.3 kb, the correct size for the combination of the *cMI-m* insert (~1.3 kb) and the vector, pGEM-T Easy (~3 kb), suggesting a partial digestion with *Cla*I. Repeated preparations of a number of clones resulted in the same linearised products. To test whether a factor in the plasmid preparation

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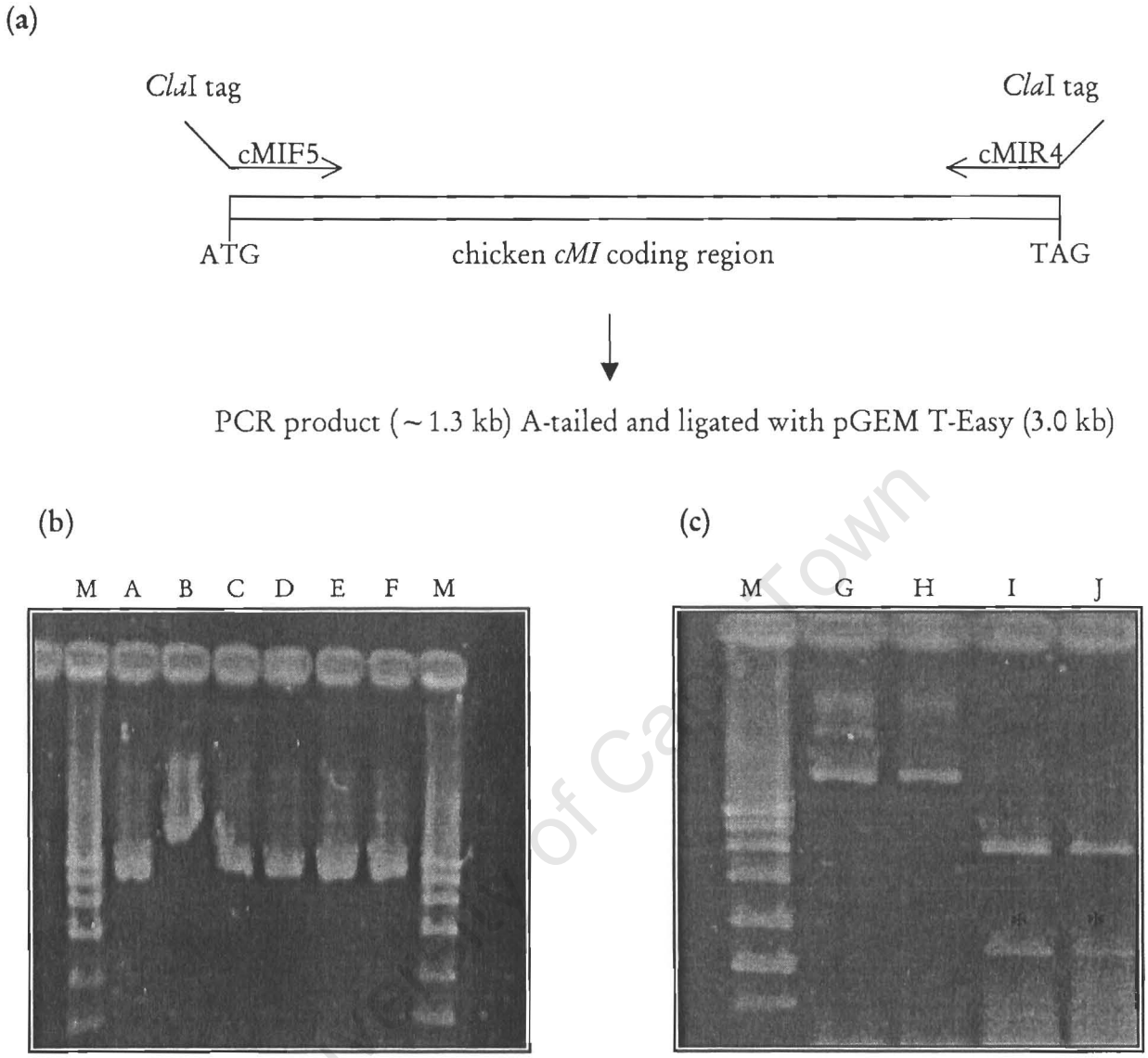

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Fig. A.1. **(a)** Schematic of the PCR strategy used in attempts to clone the coding region of the chicken microphthalmia cDNA into pGEM-T Easy. Primers cMIF5 and cMIR4 amplify from the ATG initiation codon and the TAG termination codon of *cMI-m*, respectively. The PCR product was A-tail ligated with pGEM T-Easy and subjected to restriction enzyme digestion. **(b)** *Cla*I digestion of six "mini-preps" of ligation product from (a). Lanes A-F demonstrate that the cDNA was linearising at ~4.0 kb (lane B is a failed digestion) **(c)** Lanes I and J: *Eco*RI digestion of two "mini-preps" of ligation product from (a). Asterisks indicate the presence of a ~1.3 kb insert. Lanes G and H are undigested samples of the same "mini-preps". The molecular weight marker (M) is Pox/*Eco*RI.

technique was partially inhibiting restriction enzyme digestion of the cDNA, the same samples were digested with *Eco*RI. *Eco*RI flanks the insert through its presence in the polycloning site of pGEM-T Easy and it is known, based on computerised restriction enzyme mapping of *cMI-m*, that there are no *Eco*RI sites within the coding region of the *cMI-m* cDNA. Digestion with *Eco*RI yielded the result that was expected with a *Cla*I digestion: a fragment of 1.3 kb, representing the *cMI-m* insert, and a fragment of 3 kb, representing pGEM-T Easy (Fig. A.1.c). Hence, although the cloning had been successful, as illustrated by the *Eco*RI digests, *Cla*I digestion consistently yielded linearised cDNA. The immediate explanation that seemed feasible for the unexpected results was a flaw in the design of one of the *Cla*I-tagged primers; that is, a sequence error in the *Cla*I restriction enzyme site in one of the primers would explain the linearised cDNA products. However, no sequence error was found in either primer.

An analysis of the sequence of each primer did, however, lead to the answer. It was discovered that the *Cla*I restriction site is sensitive to *dam* methylation when the restriction site overlaps the methylation sequence. *Dam* is a bacterial gene, present in the XL1-Blue and JM109 cells used during the present study, that encodes the dam methylase, an enzyme that methylates at the N6 position of adenine in the sequence 5'-GATC-3' (Hattman *et al.*, 1978). An analysis of the forward primer (cMIF5) used in the present study reveals that it contains two consecutive such sequences (uppercase letters): 5' – cGATCGATCgatgctggaaatgcttgag-3'. These sequences were unfortunately introduced through a combination of the CG "linker" (shown in the primer sequence in bold letters) between the *Cla*I recognition sequence (5'-ATCGAT-3') and the ATG initiation codon of *cMI-m* (underlined in the primer sequence), and the CG "cap" at the utmost 5' end of the primer. As such, a PCR product generated using cMIF5/cMIR4 will have been subjected to *dam* methylation in the *dam* positive *E. coli.* cells (JM109) used during transformations and subsequent plasmid preparations. The result was a PCR product that was restricted with *Cla*I at its 3' end (as the reverse primer cMIR4 does not carry a methylation site overlapping the restriction site), but not at its 5' end, thus explaining the linearised products obtained with all attempts at the PCR-based cloning approach. This problem may readily be overcome through the use of a *dam* negative cell line during transformations and subsequent plasmid preparations.

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However, such a cell line was not readily available at The University of Cape Town, and hence a blunt-end cloning strategy was adopted (with subsequent success; section 3.5.1.1).

Attempts to infect chicken embryonic fibroblasts with RCAS BP(A)

As detailed in Chapter Three, one of the major aims of the current study was to assess the ability of the chicken Microphthalmia protein to translate a functional protein and to transactivate the chicken tyrosinase gene promoter. To this end, *cMI-m* was cloned into RCAS BP(A) with the intention of using this construct to virally infect cells.

The viral surface protein on the surface of a cell exhibits sequence variability that defines five major subgroups of ASLVs, designated envelope subgroups A, B, C, D, and E (Dorner *et al.*, 1985) – the envelope subgroup A was used in the present study. These distinctions are based on which receptors the virus interacts with on host cells and contribute to the tropism of the virus. Related to the viral tropism is the existence of strains of chickens that differ in their susceptibility to different envelope subgroups (Morgan and Fekete, 1996), and such variability has practical implications for retrovirus-mediated gene transfer. To achieve infection and viral spread, the cells must express the appropriate receptor for the envelope subgroup. Thus it was necessary to determine that the fibroblasts here isolated from White Plymouth Rock x Pile Game chicken embryos are susceptible to the infection by the (A) envelope retrovirus.

The fibroblasts were first tested for viral integration and replication by treating transfections as stable. RCAS/*cMI-m* was transfected into fibroblasts using FuGENE (Roche) and cells were tested for viral integration and replication by further introducing the full-length chicken tyrosinase gene promoter (Tyr2.1-luc) seven days later. This time period was chosen because "the entire process of generating a producer stock takes 7-12 days" (Morgan and Fekete, 1996), thus it was assumed that retroviral infection of a dish of cells would be almost complete following seven days. On the same day that Tyr2.1-luc was transfected into "infected" cells, separate dishes of fibroblasts were transfected with only Tyr2.1-luc, to obtain a baseline level of promoter activity in fibroblasts. If the RCAS construct is infecting the cells, it will be expected that a cMI protein will be translated with the retroviral genome, which will have infected neighboring cells, and will result in an increase in the tyrosinase

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gene promoter activity. In contrast, if the cells are not being infected, then the presence of the cMI protein will be minimal after seven days and therefore will not significantly increase tyrosinase gene promoter activity when compared to the baseline. All necessary transfection controls were performed as described in Section 2.9 and cells were assayed as detailed in Section 2.10. Cell lysates were analysed 24 hours following the transfection with Tyr2.1-luc (i.e. a total of eight days following the retroviral "infection").

When the activity of the tyrosinase gene promoter construct was assayed eight days following "infection" with RCAS/*cMI-m*, there was no statistical difference (both in RLA or as assessed through Box and Whisker plots of the results (data not shown)) between the fibroblasts co-transfected with RCAS/*cMI-m* and Tyr2.1-luc and the fibroblast transfected with only Tyr2.1-luc. This fairly conclusively demonstrates that envelope (A) viral integration and replication is poor or non-existent in the fibroblasts used, indicating that White Plymouth Rock x Pile Game chicken embryos are not susceptible to infection by the (A) envelope subgroup retrovirus. However, it was still not clear at this stage of the present study that the RCAS/*cMI-m* construct was capable of translating a protein – that is, a lack of cMI protein could also explain the lack of increase in tyrosinase gene promoter reporter activity.

To confirm that this was not the case and that the chicken embryos are not susceptible to infection by the (A) envelope subgroup retrovirus, attempts were made to transfect the fibroblasts with an additional RCAS BP(A) construct. A previously constructed and functionally tested (B. Hogan, personal communication) RCAS BP(A) construct carrying the coding region of the *green fluorescent protein* (*GFP*) was therefore used. RCAS/*GFP* was transfected into fibroblasts using FuGENE, and assessed for fluorescence using a fluorescent microscope two, four, and seven days following "infection". Signals were not detected at any time point (data not shown), therefore conclusively demonstrating that White Plymouth Rock x Pile Game chicken embryos are not susceptible to infection by the (A) envelope retrovirus. As a result of this, all transfections were performed using FuGENE and were treated as transient.

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Appendix B | Raw Transfection Data

Transient Transfections # 3, 5, 6-9, 11-13, 18: Chicken Embryonic Fibroblasts

Abbreviations: T (transfection); RLU (Relative Light Units); Luc (Luciferase); Ren (Renilla); [prot] (protein concentration); pGL2 (pGL2-control); Tyr2.1 (Tyr2.1-luc); Tyr1.1 (Tyr1.1-luc); Tyr0.5 (Tyr0.5-luc); RCAS (RCAS BP(A)); cMI-m (RCAS/cMI-m); cmi9 (RCAS/cmi9).

All measurements for transient transfections of chicken embryonic fibroblasts were taken using the Bio-Orbit 1253 luminometry system. This luminometer allows for 'zeroing' prior to reading the *luciferase* and *Renilla* RLU; hence no corrections for background RLU are required.

T #	Plasmid(s)	Luc RLU (A)	Ren RLU (B)	[prot] (C)	A/B / C	% of pGL2-control
3	Tyr2.1	0.483	4.234	0.38	0.300	6.8%
	cMI-m + Tyr2.1	0.724	3.75	0.20	0.965	21.2%
	pGL2	18.19	10.27	0.40	4.43	100%
5	Tyr2.1	2.042	9.117	0.31	0.723	9.7%
	Tyr2.1	2.443	10.47	0.42	0.556	7.5%
	cMI-m + Tyr2.1	3.217	4.605	0.28	2.495	33.5%
	cMI-m + Tyr2.1	6.963	5.529	0.39	3.229	43.4%
	cMI-mr + Tyr2.1	1.271	3.904	0.25	1.302	17.5%
	cMI-mr + Tyr2.1	1.468	4.988	0.31	0.949	12.8%
	pGL2	1.513	0.730	0.39	5.31	x=7.44
	pGL2	21.62	7.292	0.31	9.56	(100%)
6	Tyr2.1	0.882	1.729	0.257	1.98	20.2%
	Tyr2.1	0.375	0.721	0.210	2.48	25.2%
	cMI-m + Tyr2.1	1.394	1.449	0.31	3.10	31.6%
	cMI-m + Tyr2.1	1.664	1.810	0.20	4.60	46.8%

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	cMI-mr + Tyr2.1	0.921	1.883	0.215	2.27	23.1%
	cMI-mr + Tyr2.1	0.841	1.588	0.30	1.77	18.0%
	pGL2	20.16	4.912	0.39	10.52	x=9.83
	pGL2	11.97	4.224	0.31	9.141	(100%)
7	Tyr2.1	0.118	1.251	0.30	0.315	7.2%
	Tyr2.1	0.089	0.932	0.34	0.507	11.6%
	cMI-m + Tyr2.1	1.154	3.284	0.31	1.134	25.9%
	cMI-m + Tyr2.1	0.185	1.002	0.38	0.486	11.1%
	cMI-m + Tyr1.1	0.240	2.154	0.32	0.348	7.94%
	cMI-m + Tyr1.1	0.274	2.483	0.29	0.381	8.7%
	cMI-m + Tyr0.5	0.006	0.787	0.39	0.019	0.45%
	cMI-m + Tyr0.5	0.004	0.638	0.32	0.019	0.45%
	pGL2	5.215	2.350	0.35	2.22	x=4.38
	pGL2	1.664	0.706	0.36	6.55	(100%)
8	Tyr2.1	0.045	1.080	0.27	0.154	7.2%
	Tyr2.1	0.052	0.909	0.27	0.212	9.9%
	Tyr1.1	0.018	2.787	0.17	0.0379	1.8%
	Tyr1.1	0.014	1.989	0.18	0.039	1.8%
	Tyr0.5	0.001	0.615	0.19	0.119	5.6%
	Tyr0.5	0.001	0.202	0.19	0.0261	1.2%
	cMI-m + Tyr1.1	0.000	0.002	0.13	0.000	0.0%
	cMI-m + Tyr1.1	0.006	0.357	0.14	0.120	5.6%
	cMI-m + Tyr0.5	0.000	0.245	0.17	0.000	0.0%
	cMI-m + Tyr0.5	0.000	0.268	0.17	0.000	0.0%
	cMI-mr + Tyr1.1	0.022	0.316	0.18	0.387	18.0%
	cMI-mr + Tyr0.5	0.002	0.372	0.19	0.028	1.3%

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	cMI-mr + Tyr0.5	0.003	0.385	0.18	0.043	2.0%
	pGL2	0.110	1.859	0.27	0.219	x=2.15
	pGL2	2.125	2.079	0.25	4.089	(100%)
9	Tyr1.1	0.006	0.069	0.08	1.09	13.1%
	Tyr1.1	0.006	0.071	0.06	1.41	17.0%
	Tyr0.5	0.082	2.208	0.075	0.495	6.0%
	Tyr0.5	0.094	1.94	0.10	0.48	5.9%
	cMI-m + Tyr0.5	0.002	0.760	0.03	0.088	1.1%
	cmi9 + Tyr2.1	0.102	1.710	0.09	0.66	8.0%
	cmi9 + Tyr2.1	0.022	1.031	0.095	0.225	2.7%
	cMI-mr + Tyr1.1	0.082	1.152	0.075	0.949	11.5%
	cMI-mr + Tyr1.1	0.082	1.632	0.080	0.628	7.6%
	cMI-mr + Tyr0.5	0.029	1.245	0.070	0.333	4.0%
	cMI-mr + Tyr0.5	0.037	1.10	0.075	0.448	5.4%
	pGL2	0.495	0.559	0.09	9.83	x=8.27
	pGL2	0.248	0.462	0.08	6.71	(100%)
11	cMI-m + Tyr2.1	0.074	0.056	0.32	4.13	36.4%
	cMI-m + Tyr2.1	0.030	0.030	0.27	3.70	32.7%
	cMI-m + Tyr1.1	0.002	0.018	0.74	0.150	1.3%
	cMI-m + Tyr0.5	0.001	0.086	0.32	0.036	0.3%
	cMI-m + Tyr0.5	0.002	0.045	0.27	0.165	1.5%
	cMI-mr + Tyr1.1	0.002	0.005	0.45	0.889	7.8%
	cMI-mr + Tyr1.1	0.001	0.003	0.33	1.01	8.9%
	cMI-mr + Tyr0.5	0.003	0.093	0.48	0.067	0.6%

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	cMI-mr + Tyr0.5	0.002	0.050	0.26	0.154	13.6%
	pGL2	0.506	0.175	0.33	8.76	x=11.3
	pGL2	0.735	0.196	0.27	13.9	(100%)
12	cMI-m + Tyr2.1	0.025	0.049	0.48	1.06	9.4%
	cmi9 + Tyr2.1	0.003	0.086	0.45	0.078	0.70%
	cmi9 + Tyr2.1	0.001	0.048	0.49	0.043	0.40%
	pGL2	0.506	0.175	0.33	8.76	x=11.3
	pGL2	0.735	0.196	0.27	13.89	(100%)
13	Tyr1.1	0.004	0.042	0.23	0.414	4.9%
	Tyr1.1	0.009	0.072	0.26	0.481	5.7%
	Tyr0.5	0.003	0.109	0.24	0.115	1.4%
	Tyr0.5	0.003	0.106	0.31	0.091	1.1%
	cMI-m + Tyr1.1	0.003	0.037	0.33	0.246	2.9%
	cMI-m + Tyr1.1	0.003	0.032	0.24	0.391	4.6%
	cmi9 + Tyr2.1	0.000	0.030	0.32	0.000	0.0%
	cmi9 + Tyr2.1	0.002	0.023	0.34	0.256	3.0%
	pGL2	0.213	0.101	0.25	8.44	x=8.48
	pGL2	0.142	0.037	0.45	8.53	(100%)
18	Tyr2.1	1.969	8.12	0.28	0.866	12.8%
	Tyr2.1	1.113	5.22	0.32	0.667	9.8%
	cMI-m + Tyr2.1	2.873	3.12	0.30	3.07	45.3%
	cMI-m + Tyr2.1	3.73	4.16	0.34	2.64	38.9%
	cmi9 + Tyr2.1	0.370	9.21	0.40	0.100	14.8%
	cmi9 + Tyr2.1	0.265	8.61	0.39	0.079	11.6%
	cmi9 + Tyr1.1	0.000	0.011	0.34	0.000	0.0%
	cmi9 + Tyr1.1	0.000	0.009	0.31	0.000	0.0%
	cmi9 + Tyr0.5	0.000	0.201	0.27	0.000	0.0%

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	cmi9 + Tyr0.5	0.000	0.200	0.30	0.000	0.0%
	pGL2	1.422	0.720	0.41	4.82	x=6.78
	pGL2	3.069	0.90	0.39	8.74	(100%)

Transient Transfections # 3, 5, 6-9, 11-13, 18: Chicken Embryonic Fibroblasts Summary

Mean RLA ± standard deviation:

Tyr2.1-luc: 11.6 ± 5.91
Tyr1.1-luc: 7.4 ± 6.3
Tyr0.5-luc: 3.5 ± 2.5
Tyr2.1-luc + RCAS/cMI-m: 31.4 ± 12.4 (Tyr2.1-luc + RCAS/cMI-mr: 17.9 ± 4.2)
Tyr1.1-luc + RCAS/cMI-m: 3.92 ± 3.35
Tyr0.5-luc + RCAS/cMI-m: 0.54 ± 0.56
Tyr2.1-luc + RCAS/cmi9: 5.15 ± 5.64
Tyr1.1-luc + RCAS/cmi9: 0.00 ± 0.00
Tyr0.5-luc + RCAS/cmi9: 0.00 ± 0.00

Transient Transfections # 14-16, 19, 21: Melan-a Cells

Abbreviations: T (transfection); RLU (Relative Light Units); Luc (Luciferase); Ren (Renilla); [prot] (protein concentration); pGL2 (pGL2-control); Tyr2.1 (Tyr2.1-luc); Tyr1.1 (Tyr1.1-luc); Tyr0.5 (Tyr0.5-luc); RCAS (RCAS BP(A)); cMI-m (RCAS/cMI-m); cmi9 (RCAS/cmi9).

All measurements for transient transfections of mouse melan-a cells were taken using the Bio-Orbit 1253 luminometry system. This luminometer allows for 'zeroing' prior to reading the *luciferase* and *Renilla* RLU; hence no corrections for background RLU are required.

T #	Plasmid(s)	Luc RLU (A)	Ren RLU (B)	[prot] (C)	A/B C	% of pGL2-control
14	RCAS/ *cMI-m* + Tyr2.1	0.031	0.028	0.50	2.21	62.1%
	RCAS/ *cMI-m* + Tyr2.1	0.035	0.024	0.36	4.05	113.7%
	RCAS/ *cmi9* + Tyr2.1	0.030	0.024	0.42	2.98	83.5%

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	RCAS/ *cmi9* + Tyr2.1	0.018	0.010	0.41	4.39	123.2%
	pGL2	0.043	0.034	0.42	3.011	x = 3.56
	pGL2	0.027	0.016	0.41	4.116	(100%)
15	Tyr2.1	0.001	0.009	0.37	0.300	23.1%
	Tyr2.1	0.002	0.016	0.38	0.329	25.3%
	RCAS/ *cMI-m* + Tyr2.1	0.004	0.008	0.34	1.471	113%
	RCAS/ *cMI-m* + Tyr2.1	0.002	0.006	0.34	0.980	75.4%
	RCAS/ *cmi9* + Tyr2.1	0.002	0.005	0.35	1.14	87.9%
	RCAS/ *cmi9* + Tyr2.1	0.004	0.011	0.48	0.758	58.3%
	pGL2	0.002	0.012	0.33	0.505	x = 1.30
	pGL2	0.007	0.009	0.37	2.10	(100%)
16	Tyr2.1	0.002	0.008	0.36	0.694	120.2%
	Tyr2.1	0.006	0.015	0.54	0.741	128.2%
	Tyr1.1	0.002	0.026	0.56	0.1367	23.7%
	Tyr1.1	0.001	0.025	0.56	00.0714	12.4%
	Tyr0.5	0.001	0.016	0.45	0.139	24.0%
	Tyr0.5	0.002	0.007	0.41	0.697	120.6%
	RCAS/ *cMI-m* + Tyr2.1	0.004	0.017	0.57	0.413	71.5%
	RCAS/ *cMI-m* + Tyr2.1	0.003	0.011	0.54	0.505	87.4%
	RCAS/ *cMI-m* + Tyr1.1	0.002	0.008	0.49	0.510	88.3%
	RCAS/ *cMI-m* + Tyr0.5	0.002	0.008	0.60	0.417	72.1%
	RCAS/ *cMI-m* + Tyr0.5	0.004	0.006	0.62	1.075	186.1%
	RCAS/ *cmi9* + Tyr2.1	0.002	0.011	0.66	0.275	47.7%

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	RCAS/ *cmi9* + Tyr2.1	0.002	0.010	0.57	0.351	60.7%
	RCAS/ *cmi9* + Tyr1.1	0.004	0.009	0.46	0.966	167.3%
	RCAS/ *cmi9* + Tyr1.1	0.003	0.011	0.50	0.545	94.4%
	RCAS/ *cmi9* + Tyr0.5	0.002	0.009	0.70	0.667	115.4%
	RCAS/ *cmi9* + Tyr0.5	0.002	0.006	0.50	0.667	115.4%
	pGL2	0.036	0.112	0.58	0.554	x=0.578
	pGL2	0.043	0.110	0.65	0.601	(100%)
19	Tyr2.1	0.004	0.007	0.46	1.242	166.1%
	Tyr2.1	0.006	0.008	0.55	1.364	182.3%
	Tyr1.1	0.002	0.041	0.45	0.108	14.5%
	Tyr1.1	0.001	0.025	0.55	0.0727	9.7%
	Tyr0.5	0.002	0.020	0.69	0.145	19.3%
	Tyr0.5	0.003	0.027	0.44	0.253	33.8%
	RCAS/ *cMI-m* + Tyr2.1	0.005	0.018	0.47	0.591	79.0%
	RCAS/ *cMI-m* + Tyr2.1	0.004	0.017	0.61	0.386	51.57%
	RCAS/ *cMI-m* + Tyr1.1	0.002	0.009	0.58	0.383	51.22%
	RCAS/ *cMI-m* + Tyr1.1	0.003	0.008	0.63	0.595	79.6%
	RCAS/ *cMI-m* + Tyr0.5	0.004	0.008	0.46	1.087	145.3%
	RCAS/ *cMI-m* + Tyr0.5	0.003	0.010	0.54	0.556	74.3%

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	RCAS/ *cmi9* + Tyr2.1	0.003	0.011	0.46	0.593	79.3%
	RCAS/ *cmi9* + Tyr2.1	0.004	0.012	0.45	0.741	99.0%
	RCAS/ *cmi9* + Tyr1.1	0.004	0.008	0.51	0.980	131.1%
	RCAS/ *cmi9* + Tyr1.1	0.003	0.011	0.44	0.619	82.9%
	RCAS/ *cmi9* + Tyr0.5	0.002	0.007	0.46	0.6211	83.0%
	RCAS/ *cmi9* + Tyr0.5	0.002	0.009	0.46	0.483	64.6%
	pGL2	0.035	0.113	0.45	0.688	x=0.748
	pGL2	0.040	0.110	0.45	0.808	(100%)
21	Tyr2.1	0.002	0.008	0.25	1.00	93.5%
	Tyr2.1	0.004	0.009	0.24	1.85	173.2%
	Tyr1.1	0.003	0.025	0.25	0.48	44.9%
	Tyr1.1	0.001	0.024	0.26	0.160	15.0%
	Tyr0.5	0.003	0.024	0.27	0.654	61.1%
	Tyr0.5	0.001	0.017	0.28	0.210	19.7%
	RCAS/ *cMI-m* + Tyr2.1	0.004	0.013	0.25	1.231	115%
	RCAS/ *cMI-m* + Tyr2.1	0.005	0.011	0.27	1.684	157.5%
	RCAS/ *cMI-m* + Tyr1.1	0.002	0.007	0.27	1.058	98.9%
	RCAS/ *cMI-m* + Tyr1.1	0.003	0.009	0.31	1.075	100.6%
	RCAS/ *cMI-m* + Tyr0.5	0.002	0.007	0.37	0.772	72.2%

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	RCAS/ *cMI-m* + Tyr0.5	0.003	0.008	0.37	1.014	94.8%
	RCAS/ *cmi9* + Tyr2.1	0.002	0.009	0.32	0.694	64.9%
	RCAS/ *cmi9* + Tyr2.1	0.002	0.009	0.30	0.741	69.3%
	RCAS/ *cmi9* + Tyr1.1	0.003	0.010	0.37	0.811	75.8%
	RCAS/ *cmi9* + Tyr1.1	0.002	0.008	0.37	0.676	63.2%
	RCAS/ *cmi9* + Tyr0.5	0.002	0.007	0.37	0.772	72.2%
	RCAS/ *cmi9* + Tyr0.5	0.002	0.008	0.35	0.714	66.8%
	pGL2	0.037	0.114	0.30	1.082	x=1.069
	pGL2	0.041	0.111	0.35	1.055	(100%)

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Transient Transfections # 14-16, 19, 21: Melan-a Cells Summary

Mean RLA ± standard deviation:

Tyr2.1-luc: 113.9 ± 62.9
Tyr1.1-luc: 20.0 ± 13.06
Tyr0.5-luc: 46.4 ± 39.6
Tyr2.1-luc + RCAS/cMI-m: 92.6 ± 31.9
Tyr1.1-luc + RCAS/cMI-m: 83.7 ± 20.1
Tyr0.5-luc + RCAS/cMI-m: 107.5 ± 47.7
Tyr2.1-luc + RCAS/cmi9: 77.4 ± 22.3
Tyr1.1-luc + RCAS/cmi9: 102.5 ± 39.3
Tyr0.5-luc + RCAS/cmi9: 86.2 ± 23.5

Transient Transfections # 17, 20, 22: Chicken RPE Cells

Abbreviations: T (transfection); RLU (Relative Light Units); Luc (Luciferase); Ren (Renilla); [prot] (protein concentration); pGL2 (pGL2-control); Tyr2.1 (Tyr2.1-luc); Tyr1.1 (Tyr1.1-luc); Tyr0.5 (Tyr0.5-luc); RCAS (RCAS BP(A)); cMI-m (RCAS/cMI-m); cmi9 (RCAS/cmi9).

All measurements for transient transfections of chicken RPE cells were taken using the Bio-Orbit 1253 luminometry system. This luminometer allows for 'zeroing' prior to reading the *luciferase* and *Renilla* RLU; hence no corrections for background RLU are required.

T #	Plasmid(s)	Luc RLU (A)	Ren RLU (B)	[prot] (C)	A/B / C	% of pGL2-control
17	Tyr2.1	0.001	0.004	0.18	1.39	104.5%
	Tyr2.1	0.002	0.004	0.18	2.78	209.0%
	Tyr1.1	0.002	0.005	0.19	2.11	158.4%
	Tyr1.1	0.003	0.004	0.19	3.95	297.0%
	Tyr0.5	0.002	0.005	0.21	1.91	143.3%
	Tyr0.5	0.003	0.005	0.21	2.86	215.0%
	RCAS/ *cMI-m* + Tyr2.1	0.014	0.063	0.82	0.271	20.4%
	RCAS/ *cMI-m* + Tyr2.1	0.014	0.060	0.24	0.972	73.2%
	RCAS/ *cMI-m* + Tyr1.1	0.000	0.004	0.22	0.000	0.0%
	RCAS/ *cMI-m* + Tyr1.1	0.000	0.005	0.26	0.000	0.0%
	RCAS/ *cMI-m* + Tyr0.5	0.000	0.005	0.31	0.000	0.0%
	RCAS/ *cMI-m* + Tyr0.5	0.000	0.007	0.32	0.000	0.0%
	RCAS/ *cmi9* + Tyr2.1	0.003	0.006	0.26	1.923	144.7%
	RCAS/ *cmi9* + Tyr2.1	0.004	0.007	0.25	2.29	171.9%

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	RCAS/ *cmi9* + Tyr1.1	0.003	0.006	0.33	1.52	114.0%
	RCAS/ *cmi9* + Tyr1.1	0.002	0.006	0.34	0.98	73.4%
	RCAS/ *cmi9* + Tyr0.5	0.009	0.327	1.21	0.023	1.7%
	RCAS/ *cmi9* + Tyr0.5	0.008	0.278	1.28	0.022	1.7%
	pGL2	0.003	0.007	0.26	1.648	x=1.33
	pGL2	0.003	0.009	0.33	1.010	(100%)
20	Tyr2.1	0.002	0.004	0.25	2.0	138.2%
	Tyr2.1	0.003	0.006	0.24	2.1	144.0%
	Tyr1.1	0.004	0.005	0.25	3.20	221.1%
	Tyr1.1	0.003	0.004	0.26	2.88	199.3%
	Tyr0.5	0.003	0.005	0.27	2.22	153.5%
	Tyr0.5	0.002	0.004	0.28	1.79	123.4%
	RCAS/ *cMI-m* + Tyr2.1	0.012	0.061	0.25	0.787	54.4%
	RCAS/ *cMI-m* + Tyr2.1	0.014	0.068	0.27	0.763	52.7%
	RCAS/ *cMI-m* + Tyr1.1	0.000	0.071	0.27	0.000	0.0%
	RCAS/ *cMI-m* + Tyr1.1	0.000	0.061	0.31	0.000	0.0%
	RCAS/ *cMI-m* + Tyr0.5	0.000	0.004	0.37	0.000	0.0%
	RCAS/ *cMI-m* + Tyr0.5	0.000	0.005	0.37	0.000	0.0%
	RCAS/ *cmi9* + Tyr2.1	0.004	0.006	0.32	2.08	144.0%

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	RCAS/ *cmi9* + Tyr2.1	0.003	0.006	0.30	1.67	115.2%
	RCAS/ *cmi9* + Tyr1.1	0.003	0.006	0.37	1.35	93.4%
	RCAS/ *cmi9* + Tyr1.1	0.002	0.005	0.37	1.08	74.7%
	RCAS/ *cmi9* + Tyr0.5	0.001	0.010	0.37	0.270	18.7%
	RCAS/ *cmi9* + Tyr0.5	0.001	0.012	0.35	0.238	16.5%
	pGL2	0.004	0.008	0.30	1.67	x = 1.45
	pGL2	0.003	0.007	0.35	1.22	(100%)
22	Tyr2.1	0.004	0.008	0.26	1.92	103.4%
	Tyr2.1	0.003	0.10	0.25	0.12	6.4%
	Tyr1.1	0.005	0.004	0.27	1.25	67.2%
	Tyr1.1	0.003	0.007	0.26	1.65	88.6%
	Tyr0.5	0.004	0.005	0.34	2.35	126.5%
	Tyr0.5	0.003	0.004	0.25	3.0	161.2%
	RCAS/ *cMI-m* + Tyr2.1	0.002	0.009	0.35	0.635	34.1%
	RCAS/ *cMI-m* + Tyr2.1	0.003	0.007	0.20	2.14	115.2%
	RCAS/ *cMI-m* + Tyr1.1	0.000	0.011	0.030	0.000	0.0%
	RCAS/ *cMI-m* + Tyr1.1	0.001	0.005	0.25	0.80	4.3%
	RCAS/ *cMI-m* + Tyr0.5	0.000	0.015	0.33	0.000	0.0%
	RCAS/ *cMI-m* + Tyr0.5	0.000	0.015	0.34	0.000	0.0%

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	RCAS/ *cmi9* + Tyr2.1	0.003	0.005	0.30	2.0	107.5%
	RCAS/ *cmi9* + Tyr2.1	0.004	0.006	0.25	2.67	143.3%
	RCAS/ *cmi9* + Tyr1.1	0.007	0.011	0.26	2.45	1.32%
	RCAS/ *cmi9* + Tyr1.1	0.002	0.009	0.19	1.17	62.9%
	RCAS/ *cmi9* + Tyr0.5	0.002	0.012	0.26	.0641	34.5%
	RCAS/ *cmi9* + Tyr0.5	0.001	0.008	0.25	0.50	26.9%
	pGL2	0.008	0.016	0.30	1.67	x=1.86
	pGL2	0.008	0.015	0.26	2.05	(100%)

Transient Transfection #17,20,22: Chicken RPE Summary

Mean RLA ± standard deviation:

Tyr2.1-luc: 117.6 ± 66.7
Tyr1.1-luc: 171.9 ± 85.9
Tyr0.5-luc: 153.8 ± 33.4
Tyr2.1-luc + RCAS/cMI-m: 58.3 ± 33.3
Tyr1.1-luc + RCAS/cMI-m: 0.72 ± 1.76
Tyr0.5-luc + RCAS/cMI-m: 0.00 ± 0.00
Tyr2.1-luc + RCAS/cmi9: 137.8 ± 23.3
Tyr1.1-luc + RCAS/cmi9: 69.9 ± 38.2
Tyr0.5-luc + RCAS/cmi9: 16.7 ± 13.2

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Transient Transfections # 26 – # 28: B16 Cells

Abbreviations: T (transfection); Bkd (Background); RLU (Relative Light Units); Luc (Luciferase); Ren (Renilla); [prot] (protein concentration); pGL2 (pGL2-control); RLA (relative luciferase activity); Tyr2.1 (Tyr2.1-luc); Tyr1.1 (Tyr1.1-luc); Tyr0.5 (Tyr0.5-luc); RCAS (RCAS BP(A)); cMI-m (RCAS/cMI-m); cmi9 (RCAS/cmi9).

All measurements for transient transfections of mouse B16 cells were taken using the Monolight 2010 Analytical Luminescence Laboratory. This luminometer does not allow for 'zeroing' prior to reading the *luciferase* and *Renilla* RLU; hence corrections for background RLU are required.

T #	Plasmid(s)	Bkd RLU	Luc RLU	Ren RLU	Luc RLU - Bkgd RLU (A)	Ren RLU - Bkgd RLU (B)	[prot] (C)	A/B C	% of pGL2-control (RLA)
26	pGL2	115	30203	4930	30088	4815	0.076	82.22	x=76.2
	pGL2	128	28595	5261	28467	5133	0.079	70.20	(100%)
	Tyr2.1	127	65493	5612	65366	5485	0.083	143.5	188%
	Tyr2.1	127	64621	5645	64494	5518	0.072	162.3	213%
	Tyr1.1	117	7550	5100	7433	4983	0.079	18.88	24.8%
	Tyr1.1	126	6121	4799	5995	4673	0.081	15.84	20.8%
	Tyr0.5	123	6532	5002	6409	4879	0.069	19.04	24.9%
	Tyr0.5	117	6912	5239	6795	5122	0.077	17.23	22.6%
	RCAS	132	222	688	90	556	0.069	2.3	3.0%
	RCAS	126	136	587	10	461	0.082	0.26	0.3%
	cMI-m + Tyr2.1	116	94563	4987	94447	4871	0.079	245.4	322%
	cMI-m + Tyr2.1	123	88549	5326	88426	5203	0.084	202.3	265%
	cMI-m + Tyr1.1	125	92688	4648	92563	4523	0.081	252.3	332%
	cMI-m + Tyr1.1	118	95750	4107	95632	3989	0.094	255.0	334%
	cMI-m + Tyr0.5	121	94920	4336	94799	4215	0.088	255.6	335%
	cMI-m + Tyr0.5	118	74707	5763	74589	5645	0.084	157.3	206%
	cmi9 + Tyr2.1	123	83707	5646	83584	5523	0.077	196.5	258%
	cmi9 + Tyr2.1	124	89909	5811	89785	5687	0.085	185.7	243%

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	cmi9 + Tyr1.1	124	93671	5971	93547	5847	0.076	210.5	276%
	cmi9 + Tyr1.1	121	91689	3806	91568	3685	0.085	292.3	383%
	cmi9 + Ty0.5	118	87682	5987	87564	5869	0.078	191.3	251%
	cmi9 + Ty0.5	119	83688	5597	83569	5478	0.078	195.6	257%
	None	143	349	775	206	632	0.081	4.02	5.2%
	None	114	222	681	108	567	0.082	2.32	3.0%
27	pGL2	111	29687	4396	29576	4285	0.078	88.5	x=88.8
	pGL2	122	32201	4569	32079	4447	0.081	89.1	(100%)
	Tyr2.1	120	74956	5421	74836	5301	0.084	168	189%
	Tyr2.1	122	78521	5968	78399	5846	0.073	184	207%
	Tyr1.1	117	7455	5002	7338	4885	0.080	18.7	21%
	Tyr1.1	124	7598	4985	7474	4861	0.078	19.7	22%
	Tyr0.5	123	6523	4758	6400	4635	0.082	16.8	19%
	Tyr0.5	135	5589	5144	5454	5009	0.077	14.1	16%
	RCAS	122	278	500	156	378	0.078	5.29	5.9%
	RCAS	144	348	565	201	421	0.081	5.89	6.6%
	cMI-m + Tyr2.1	121	97977	4838	97856	4717	0.083	249.9	281%
	cMI-m + Tyr2.1	116	92703	5120	92587	5004	0.081	228.4	257%
	cMI-m + Tyr1.1	115	88572	4871	88457	4756	0.075	247.9	279%
	cMI-m + Tyr1.1	123	90581	5368	90458	5245	0.069	249.9	281%
	cMI-m + Tyr0.5	121	88663	5570	88542	5449	0.075	216.6	245%
	cMI-m + Tyr0.5	120	98683	4995	98563	4875	0.085	237.9	268%
	cmi9 + Tyr2.1	122	90376	5246	90254	5124	0.094	187.4	211%
	cmi9 + Tyr2.1	119	91706	5121	91587	5002	0.082	223.3	251%
	cmi9 + Tyr1.1	121	89876	4708	89755	4587	0.080	244.6	275%
	cmi9 + Tyr1.1	121	88665	5245	88544	5124	0.077	224.4	253%
	cmi9 + Ty0.5	119	84697	4976	84578	4857	0.082	212.4	239%
	cmi9 + Ty0.5	124	90245	5269	90121	5145	0.077	227.5	256%

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	None	125	324	754	199	629	0.082	3.9	4.3%
	None	119	297	687	178	568	0.081	3.9	4.3%
28	pGL2	125	45982	5170	45857	5045	0.080	113.6	x = 108
	pGL2	121	38690	5108	38569	4987	0.075	103.1	(100%)
	Tyr2.1	124	69980	5338	69856	5214	0.082	163.4	151%
	Tyr2.1	126	79937	5123	79811	4997	0.082	194.8	180%
	Tyr1.1	125	8579	5007	8454	4882	0.078	22.2	20.6%
	Tyr1.1	122	8086	4997	7964	4875	0.082	19.9	18.4%
	Tyr0.5	125	6967	5139	6842	5014	0.079	17.3	16.0%
	Tyr0.5	127	7084	5112	6957	4985	0.079	17.7	16.4%
	RCAS	119	173	342	54	223	0.085	2.85	2.6%
	RCAS	121	157	442	36	321	0.084	1.34	1.2%
	cMI-m + Tyr2.1	125	97667	4410	97542	4285	0.084	270.9	251% %
	cMI-m + Tyr2.1	124	92668	5147	92544	5023	0.079	233.2	216%
	cMI-m + Tyr1.1	122	93677	4336	93555	4214	0.081	274.1	254%
	cMI-m + Tyr1.1	117	98695	4604	98578	4487	0.081	271.2	251%
	cMI-m + Tyr0.5	116	88373	3567	88257	3451	0.082	311.9	289%
	cMI-m + Tyr0.5	115	89693	4627	89578	4512	0.079	251.3	233%
	cmi9 + Tyr2.1	120	95965	5232	95845	5112	0.075	249.9	231%
	cmi9 + Tyr2.1	119	97781	4694	97662	4575	0.085	251.1	233%
	cmi9 + Tyr1.1	122	90075	4709	89953	4587	0.081	242.1	224%
	cmi9 + Tyr1.1	122	93710	4447	93588	4325	0.082	263.9	244%
	cmi9 + Ty0.5	121	98667	5266	98546	5145	0.078	245.6	227%
	cmi9 + Ty0.5	120	98005	5143	97885	5023	0.079	246.7	228%
	None	119	623	791	504	672	0.089	8.43	7.8%
	None	119	452	994	333	875	0.075	5.07	4.7%

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Transient Transfections # 26-28 (B16) Summary

Mean RLA ± standard deviation:

Tyr2.1-luc:	188 ± 22
Tyr1.1-luc:	21 ± 2.1
Tyr0.5-luc:	19 ± 3.8
Tyr2.1-luc + RCAS/cMI-m:	265 ± 35.1
Tyr1.1-luc + RCAS/cMI-m:	289 ± 36.6
Tyr0.5-luc + RCAS/cMI-m:	263 ± 45.5
Tyr2.1-luc + RCAS/cmi9:	238 ± 16.7
Tyr1.1-luc + RCAS/cmi9:	276 ± 56.0
Tyr0.5-luc + RCAS/cmi9:	243 ± 13.6

Transient Transfections # 29 – # 31: HeLa Cells

Abbreviations: T (transfection); Bkd (Background); RLU (Relative Light Units); Luc (Luciferase); Ren (Renilla); [prot] (protein concentration); pGL2 (pGL2-control); RLA (relative luciferase activity); Tyr2.1 (Tyr2.1-luc); Tyr1.1 (Tyr1.1-luc); Tyr0.5 (Tyr0.5-luc); RCAS (RCAS BP(A)); cMI-m (RCAS/cMI-m); cmi9 (RCAS/cmi9).

All measurements for transient transfections of human HeLa cells were taken using the Monolight 2010 Analytical Luminescence Laboratory. This luminometer does not allow for 'zeroing' prior to reading the *luciferase* and *Renilla* RLU; hence corrections for background RLU are required.

T #	Plasmid(s)	Bkd RLU	Luc RLU	Ren RLU	Luc RLU - Bkgd RLU (A)	Ren RLU - Bkgd RLU (B)	[prot] (C)	A/B C	% of pGL2-control (RLA)
29	pGL2	119	31687	5104	31568	4985	0.075	84.43	x=98.3
	pGL2	123	40224	4649	40101	4526	0.079	112.15	(100%)
	Tyr2.1	122	1145	4709	1023	4587	0.085	2.62	2.7%
	Tyr2.1	121	1106	4106	985	3985	0.083	2.98	3.0%
	Tyr1.1	109	1365	4342	1256	4233	0.076	3.90	4.0%
	Tyr1.1	122	1446	4645	1324	4523	0.079	3.71	3.8%
	Tyr0.5	124	111	4125	987	4001	0.085	2.90	3.0%
	Tyr0.5	125	1240	3817	1115	3692	0.084	3.60	3.7%
	RCAS	128	227	414	99	542	0.078	2.34	2.4%
	RCAS	127	281	460	154	333	0.071	6.5	6.6%
	cMI-m + Tyr2.1	127	7203	4682	7076	4555	0.077	20.17	20.5%

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	cMI-m + Tyr2.1	127	7552	4125	7425	3998	0.080	23.21	23.6%
	cMI-m + Tyr1.1	126	7238	5371	7112	5245	0.074	18.32	18.6%
	cMI-m + Tyr1.1	125	6458	4114	6333	3989	0.085	18.68	19.0%
	cMI-m + Tyr0.5	125	6648	5223	6523	5098	0.076	16.84	17.1%
	cMI-m + Tyr0.5	124	6636	4901	6512	4777	0.076	17.93	18.2%
	cmi9 + Tyr2.1	114	8661	5359	8547	5245	0.075	21.73	22.1%
	cmi9 + Tyr2.1	114	7799	5001	7685	4887	0.079	19.91	20.2%
	cmi9 + Tyr1.1	115	7100	4114	6985	3999	0.085	20.55	20.9%
	cmi9 + Tyr1.1	116	7131	4694	7015	4578	0.081	18.92	19.2%
	cmi9 + Ty0.5	124	6978	5238	6854	5114	0.080	16.75	17.0%
	cmi9 + Ty0.5	124	6669	5132	6545	5008	0.080	16.34	16.6%
	None	123	238	578	115	455	0.092	2.75	2.8%
	None	123	324	670	201	547	0.090	4.08	4.2%
30	pGL2	115	32699	4693	32584	4578	0.071	100.2	x=89.3
	pGL2	112	29977	5001	29865	4889	0.078	78.3	(100%)
	Tyr2.1	122	1274	4379	1152	4257	0.080	3.38	3.8%
	Tyr2.1	142	1166	4127	1024	3985	0.080	3.21	3.6%
	Tyr1.1	126	1001	4714	875	4588	0.078	2.45	2.7%
	Tyr1.1	125	1239	4338	1114	4213	0.079	3.35	3.7%
	Tyr0.5	122	676	4811	554	4689	0.081	1.46	1.6%
	Tyr0.5	122	810	4784	688	4662	0.079	1.81	2.0%
	RCAS	156	607	714	451	558	0.081	9.98	11.2%
	RCAS	154	268	722	114	568	0.086	2.33	2.6%
	cMI-m + Tyr2.1	125	7979	6000	7854	5875	0.075	17.8	19.9%
	cMI-m + Tyr2.1	124	6671	5245	6547	5121	0.085	15.0	16.8%
	cMI-m + Tyr1.1	114	8968	4682	8854	4568	0.080	24.2	27.1%
	cMI-m + Tyr1.1	117	8073	4894	7956	4777	0.084	19.8	22.2%

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	cMI-m + Tyr0.5	116	6784	5438	6668	5322	0.079	15.9	17.8%
	cMI-m + Tyr0.5	119	7264	5263	7145	5144	0.079	17.6	19.7%
	cmi9 + Tyr2.1	121	9876	5010	9755	4889	0.081	24.6	27.6%
	cmi9 + Tyr2.1	122	8110	8246	7988	5124	0.087	17.9	20.1%
	cmi9 + Tyr1.1	121	9008	5350	8887	5229	0.081	20.9	23.5%
	cmi9 + Tyr1.1	120	8674	5118	8554	4998	0.075	22.8	25.6%
	cmi9 + Ty0.5	119	7677	5804	7558	5685	0.086	15.5	17.3%
	cmi9 + Ty0.5	118	9120	5607	9002	5489	0.086	19.1	21.4%
	None	124	234	449	110	325	0.082	4.13	4.2%
	None	123	279	535	156	412	0.079	4.79	5.4%
31	pGL2	121	45976	5244	45855	5123	0.075	119.3	x = 114
	pGL2	120	42891	5164	42771	5044	0.078	108.7	(100%)
	Tyr2.1	119	2331	4117	2212	3998	0.081	6.83	5.9%
	Tyr2.1	121	3910	4233	3789	4112	0.080	11.5	10.1%
	Tyr1.1	125	1913	3693	1788	3568	0.082	6.1	5.4%
	Tyr1.1	124	1878	3913	1754	3789	0.074	6.3	5.5%
	Tyr0.5	125	2134	5137	2009	5012	0.081	4.9	4.3%
	Tyr0.5	125	3910	4693	3785	4568	0.081	10.2	8.9%
	RCAS	119	133	695	14	443	0.074	0.43	0.37%
	RCAS	129	238	695	109	566	0.079	2.44	2.1%
	cMI-m + Tyr2.1	125	9336	5793	9211	5668	0.085	19.1	16.8%
	cMI-m + Tyr2.1	121	9876	4866	9755	4745	0.085	24.2	21.1%
	cMI-m + Tyr1.1	120	9142	3666	9022	3546	0.082	31.0	27.2%
	cMI-m + Tyr1.1	120	8644	4345	8524	4225	0.079	25.5	22.4%
	cMI-m + Tyr0.5	118	6642	4005	6524	3887	0.082	20.5	17.9%
	cMI-m + Tyr0.5	121	7786	3710	7665	3589	0.079	27.0	23.7%
	cmi9 + Tyr2.1	119	9708	5581	9589	5462	0.089	19.7	17.3%
	cmi9 + Tyr2.1	122	6979	5333	6857	5211	0.084	15.7	13.7%

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	cmi9 + Tyr1.1	122	10120	4977	9998	4855	0.079	26.1	22.9%
	cmi9 + Tyr1.1	120	9762	5087	9642	4967	0.079	24.6	21.6%
	cmi9 + Ty0.5	123	9481	5569	9358	5446	0.084	20.5	17.9%
	cmi9 + Ty0.5	123	8379	5535	8256	5412	0.087	17.5	15.4%
	None	125	211	612	86	487	0.078	2.3	1.9%
	None	124	233	238	109	114	0.085	11.2	9.9%

Transient Transfections # 29-31 (HeLa) Summary

Mean RLA ± standard deviation:

Tyr2.1-luc: 4.85 ± 0.51
Tyr1.1-luc: 4.18 ± 1.08
Tyr0.5-luc: 3.92 ± 2.64
Tyr2.1-luc + RCAS/cMI-m: 19.78 ± 2.63
Tyr1.1-luc + RCAS/cMI-m: 22.75 ± 3.75
Tyr0.5-luc + RCAS/cMI-m: 19.07 ± 2.43
Tyr2.1-luc + RCAS/cmi9: 20.17 ± 4.67
Tyr1.1-luc + RCAS/cmi9: 22.28 ± 2.22
Tyr0.5-luc + RCAS/cmi9: 17.6 ± 2.04

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Appendix C | Additional Transient Transfections

This Appendix details the transient transfections of chicken embryonic fibroblasts, RPE cells, and mouse melan-a cells performed during the course of the present study and presented as a major portion of the original version of this thesis. The majority of the following sections are not included in the main body of the revised thesis as it was found that the transfections into the three cell types were not meaningful, as assessed by examination of the raw transfection data presented in Appendix B. The following sections are, however, included here as an Appendix in the interest of honesty and respect to those two examiners who shall not be required to re-examine the thesis.

Characteristics of cell types used in transient transfection assays

The transfection experiments required that pigmented (i.e. *tyrosinase* expressing) cell lines of both neural crest and neuroepithelial origins be cultured. In addition, a non-pigmented (i.e. not expressing *tyrosinase*) cell line is essential as a negative control.

Ideally, an analysis of the ability of the chicken Microphthalmia protein to transactivate the chicken tyrosinase promoter should be carried out using avian cell lines. However, there are no readily available avian sources of neural crest-derived melanocytes, and there are no immortal avian melanocyte cell lines. Melanocytes may be cultured from dissected chicken neural tubes; however, this procedure requires an impossibly large number of neural tubes to obtain pure melanocyte cultures. Thus melan-a cells were selected as the neural crest-derived cell line. Melan-a is a spontaneously immortalised cell line of pigmented melanocytes derived from normal epidermal melanoblasts of C57/BL mice (Bennett *et al.*, 1987), and the cells express high levels of *tyrosinase*, as assessed by northern blotting (Tachibana *et al.*, 1996). Many groups have used this non-tumorigenic and well-characterised cell line for similar studies to those described in the present study.

For cells of a neuroepithelial origin, primary cultures of chicken RPE cells were performed according to the methods of Eguchi and Okada (1973) with minor modifications, as detailed under materials and methods. Primary cultures of chicken RPE cells have previously been shown to express *tyrosinase*, as assessed by northern blotting (Ferguson, Ph.D. Thesis, 1996;

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April *et al.*, 1996). RPE cells used in the present study proliferated well, exhibited the characteristic "cobblestone" morphology, and were visibly pigmented both on the day of transfection and the day of cell harvest.

Primary chicken embryonic fibroblasts were used as a visibly non-pigmented control cell line. Mouse NIH/3T3 fibroblasts have previously been assessed for melanogenic potential. Although visibly non-pigmented, NIH/3T3 cells express *Dct* when tested using northern blotting or RT-PCR (40 cycles) (Tachibana *et al.*, 1996). However, additional melanogenic markers, including microphthalmia, were not detected upon northern blot hybridisation of NIH/3T3 RNA. In contrast, the chicken embryonic fibroblasts used in this study were found to express detectable melanocyte-type microphthalmia transcripts upon 30 PCR cycles. To get some idea of whether these microphthalmia transcripts might be functional (i.e. transactivating a target downstream gene) or indicative of melanogenic potential, RT-PCR of chicken fibroblast RNA was carried out using primers specific for the chicken *tyrosinase* gene and the chicken *Dct* gene. As a positive control tissue, total eye RNA was also subjected to RT-PCR with the same primers. As illustrated in Fig. C.1, *tyrosinase* and *Dct* transcripts are only detected in the positive control RNA (eye tissue), demonstrating that neither *tyrosinase* nor *Dct* is expressed in chicken embryonic fibroblasts. Despite this uncertainty of a potential functional role of the microphthalmia transcripts, the chicken primary embryonic fibroblasts do not express *tyrosinase* or *Dct*, and were therefore chosen as an appropriate non-melanogenic cell line for functional analyses.

Establishing transfection procedures

As a start, calcium phosphate co-precipitation transfections (according to the methods of Kingston, 1987) of primary chicken embryonic fibroblasts using the pGL2-control vector were performed. It was found that fibroblasts could not be transfected using this method. Numerous transfection experiments were performed and several parameters, including the amount of plasmid and length of time that the cells were exposed to the precipitate both with and without glycerol shocks, were tested without success. The fibroblasts were found to be refractile, in the process of lifting from the tissue culture dishes, or dead, the day following the transfection.

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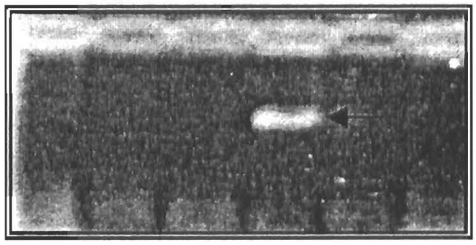
(a) **Tyrosinase primers**

Fibroblast RNA	+	+	-
Total eye RNA	-	-	+
RT (MuMLV)	+	-	+
PCR cycles	M 35	35	35



(b) **Dct primers**

Fibroblast RNA	+	+	-
Total eye RNA	-	-	+
RT (MuMLV)	+	-	+
PCR cycles	M 35	35	35

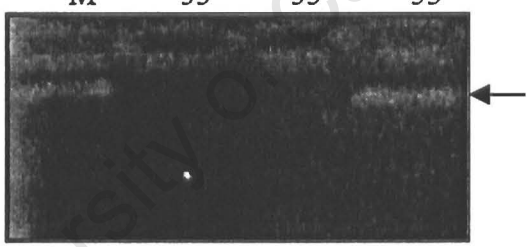


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Fig. C.1. Chicken embryonic fibroblasts do not express *tyrosinase* or *Dct*. Shown are the products from two-step RT-PCR of chicken fibroblast RNA and chicken total eye RNA using (a) primers specific for the chicken *tyrosinase* gene, or (b) primers specific for the chicken *Dct* gene. (+) indicates the presence of RNA or RT in each sample and (-) indicates the absence of RNA or RT in each sample. Numbers above each lane indicate the number of PCR cycles used. Arrows indicate the amplification products. The molecular weight marker (M) is Pox/*Eco*RI.

Similar observations have been made in this laboratory in attempts to transfect melan-a cells and primary RPE cultures using the calcium-phosphate co-precipitation method. The morphology and growth of both melan-a and RPE cells is disturbed by the precipitate and, in extreme cases, the cells die (Clarke, M.Sc. Thesis, 2000). Hence, for all cell types, an alternative transfection method was utilised.

FuGENE is a lipidic, non-liposomal transfection reagent which has been shown to mediate high levels of transfection with minimal cytotoxicity to cells (Boehringer-Mannheim; Roche). Test transfections of pGL2-control into primary chicken embryonic fibroblasts demonstrated that the morphology and growth of the cells is not adversely affected by FuGENE. The morphology and growth of melan-a and primary chicken RPE cultures are also not adversely affected by FuGENE (Clarke, M.Sc. Thesis, 2000).

Thus all transfections described in the following sections were performed using FuGENE according to the manufacturers' instructions (Roche). In all experiments, all necessary transfection controls were performed as described in materials and methods, and experiments were performed at least thrice, with duplicates for each transfection. Cell lysates were analysed 24 hours post-transfection, and the relative luciferase activity (RLA) obtained in each was calculated as detailed in materials and methods.

Activity of chicken tyrosinase gene promoter constructs in pigmented and non-pigmented cells[1]

Prior to assessing the ability of the chicken Microphthalmia protein to transactivate the chicken tyrosinase gene promoter, it was crucial to demonstrate that the 5' flanking sequence of the chicken *tyrosinase* gene functions as a promoter in the cell types used here. Specifically, it is desirable to show that the promoter functions in a pigment cell-specific manner. Although similar experiments had previously been performed in this laboratory (Ferguson, Ph.D. Thesis, 1996; Clarke, M.Sc. Thesis, 2000), it was necessary to repeat these experiments for two reasons: First, the previous analyses of the chicken tyrosinase promoter activity in

[1] The results detailed in this section, which represented a large portion of the original version of this thesis, do not form part of the results discussed in Chapter Four of this revised thesis. They are, however, included in this revised thesis in the interest of both honesty and scientific integrity with respect to the original thesis.

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non-pigmented cells had been carried out in human liver cells (HepG2). In the current study chicken fibroblasts were initially chosen as the non-pigmented cell line, and so it was necessary to assay promoter function in these cells. Secondly, the previous comparative transfection analyses of melan-a and RPE cells performed by Clarke had encountered a number of difficulties, not least of which was a very low tyrosinase promoter activity when compared to the positive control vector, pGL2-control, and it was therefore necessary to repeat all previous transfections.

Thus, transient transfections of Tyr2.1-luc, Tyr1.1-luc, and Tyr0.5-luc into chicken embryonic fibroblasts, melan-a cells, and RPE cells were performed. The means of individual results, plus and minus the standard deviations, are shown in Fig. C.2.

In fibroblasts, the following results were obtained:

1. The highest mean activity was obtained with the full-length (Tyr2.1-luc) promoter construct.
2. However, the mean RLA obtained with Tyr2.1-luc (11.6 ± 5.91) was not different (as assessed by the standard deviation bars) from the mean RLA obtained with Tyr1.1-luc (7.4 ± 6.3) or Tyr0.5-luc (3.5 ±2.5).

In melan-a cells, the following results were obtained:

1. The highest level of activity was obtained with the full-length (Tyr2.1-luc) promoter construct. Furthermore, a comparison of the activity of Tyr2.1-luc in melan-a cells and fibroblasts suggest that the full-length chicken tyrosinase promoter is pigment cell-specific in neural crest-derived melanocytes.
2. The activity obtained with Tyr1.1-luc was, on average, 5.6-fold lower than the activity of the full-length construct. In addition, the mean RLA obtained with Tyr1.1-luc in melan-a cells was not different (as assessed by the standard deviation bars) from the mean RLA of Tyr1.1-luc in fibroblasts, suggesting a loss of the pigment cell-specific activity of the promoter when elements between –2.1 and –1.1 are deleted.
3. The activity of Tyr0.5-luc was also lower (2.5-fold) than the activity of the full-length construct. In addition, the mean RLA obtained with Tyr0.5-luc in melan-a cells was not different (as assessed by the standard deviation bars) from the mean RLA of Tyr1.1-luc

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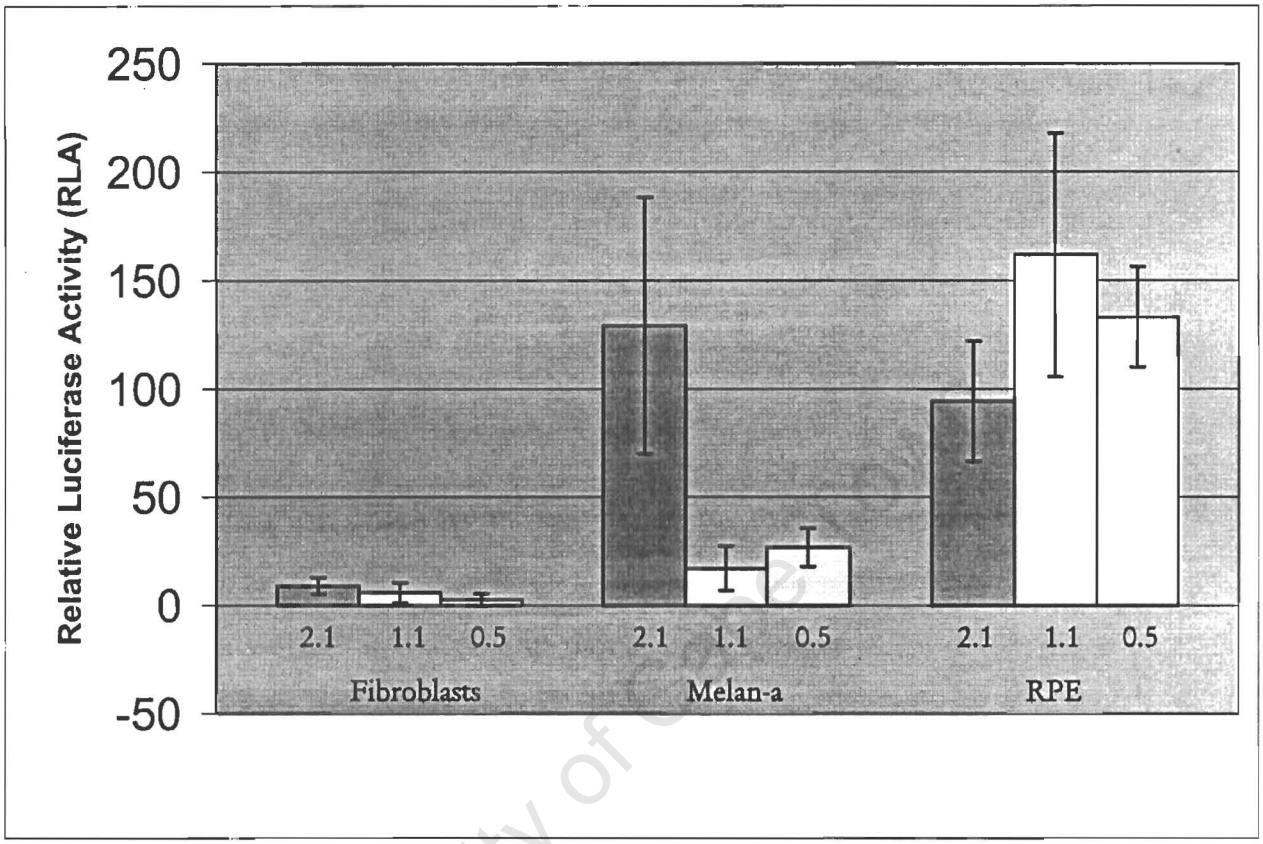

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Fig. C.2. Activity of the three chicken tyrosinase gene promoter constructs in fibroblasts, melan-a cells, and RPE cells. Bars represent transfections with Tyr2.1-luc (2.1); Tyr1.1-luc (1.1); and Tyr0.5-luc (0.5). Data are shown as the means of individual experiments. Standard deviations were calculated using the Excel (Microsoft Corporation) standard deviation function. In all cases, results are expressed as a relative luciferase activity (RLA) normalised with respect to *Renilla* activity in the same cell extract and expressed as a percentage of the *luciferase* activity obtained with the positive control vector, pGL2-control. Protein concentrations in all cell extracts were normalised using the BioRad Assay.

4. in fibroblasts, suggesting a loss of the pigment cell-specific activity of the promoter when elements between –2.1 and –0.5 are deleted.

A somewhat different pattern of activity was observed upon transfection of the promoter constructs in RPE cells:

1. The levels of reporter activity with all three promoter constructs were similar and, in each case, were more active in RPE cells than in fibroblasts.
2. Both Tyr1.1-luc and Tyr0.5-luc retained pigment cell-specific activity in neuroepithelial-derived RPE cells when compared to their activity in fibroblasts.
3. The Tyr1.1-luc and Tyr0.5-luc constructs were, on average, almost 5 times more active in RPE cells than they were in melan-a cells.

Thus, none of the three promoter constructs were active in the chicken embryonic fibroblasts. The very low levels of promoter activity in fibroblasts made it possible to utilise these constructs and cells to measure the transactivation by the cMI protein using a co-transfection approach. The full-length promoter was pigment cell-specific in both melanocytes and RPE cells, although the results of transfections with the deletion constructs suggested that the mechanisms that initiate *tyrosinase* transcription may differ between the two cell types.

Transactivation of chicken tyrosinase gene promoter constructs in non-pigmented cells (fibroblasts)[2]

The next series of experiments were aimed at establishing whether the transcripts discussed above are translated into active proteins. This was performed through an assessment of the ability of the two chicken Microphthalmia proteins to transactivate the chicken tyrosinase gene promoter constructs in non-pigmented cells (fibroblasts).

Duplicate dishes of fibroblasts were co-transfected with either (i) RCAS/*cMI-m* and Tyr2.1-luc or (ii) RCAS/*cMI-mr* and Tyr2.1-luc, or (iii) RCAS/*cmi9* and Tyr2.1-luc. The pooled

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results of individual experiments are shown in Fig. C.3.a. The baseline activity of Tyr2.1-luc in fibroblasts is from Fig. C.2 and is included for comparative purposes.

Co-transfection of RCAS/*cMI-m* and Tyr2.1-luc in fibroblasts resulted in increased activity of Tyr2.1-luc by 2.7-fold, from a baseline RLA of 11.6 to a mean RLA of 31.4. Co-transfection of fibroblasts with RCAS/*cMI-mr* and Tyr2.1-luc resulted in a mean RLA of 17.9 ± 4.2, a value not different from the baseline Tyr2.1-luc RLA of 11.6 ± 5.91. cmi9 did not transactivate the full-length tyrosinase gene promoter in fibroblasts; the mean RLA value of 5.15 ± 5.64 is not different from the baseline Tyr2.1-luc RLA of 8.9 ± 3.7.

Next, fibroblasts were co-transfected with either (i) RCAS/*cMI-m* and Tyr1.1-luc or (ii) RCAS/*cmi9* and Tyr1.1-luc. The pooled results of individual experiments are shown in Fig. C.3.b. The baseline activity of Tyr1.1-luc in fibroblasts is from Fig. C.2 and is included for comparative purposes.

In fibroblasts, cMI-m did not increase the activity of the Tyr1.1-luc promoter construct. The baseline activity of Tyr1.1-luc (7.4 ± 6.3) is not different from the activity in the presence of cMI-m (3.92 ± 3.35). Co-transfection with RCAS/*cmi9* resulted in a mean RLA of zero (illustrated by the absence of a bar).

Finally, fibroblasts were co-transfected with either (i) RCAS/*cMI-m* and Tyr0.5-luc or (ii) RCAS/*cmi9* and Tyr0.5-luc. The pooled results of individual experiments are shown in Fig. C.3.c. The baseline activity of Tyr0.5-luc in fibroblasts is from Fig. C.2 and is included for comparative purposes. In fibroblasts, neither cMI-m nor cmi9 increased the already weak baseline activity of Tyr0.5-luc.

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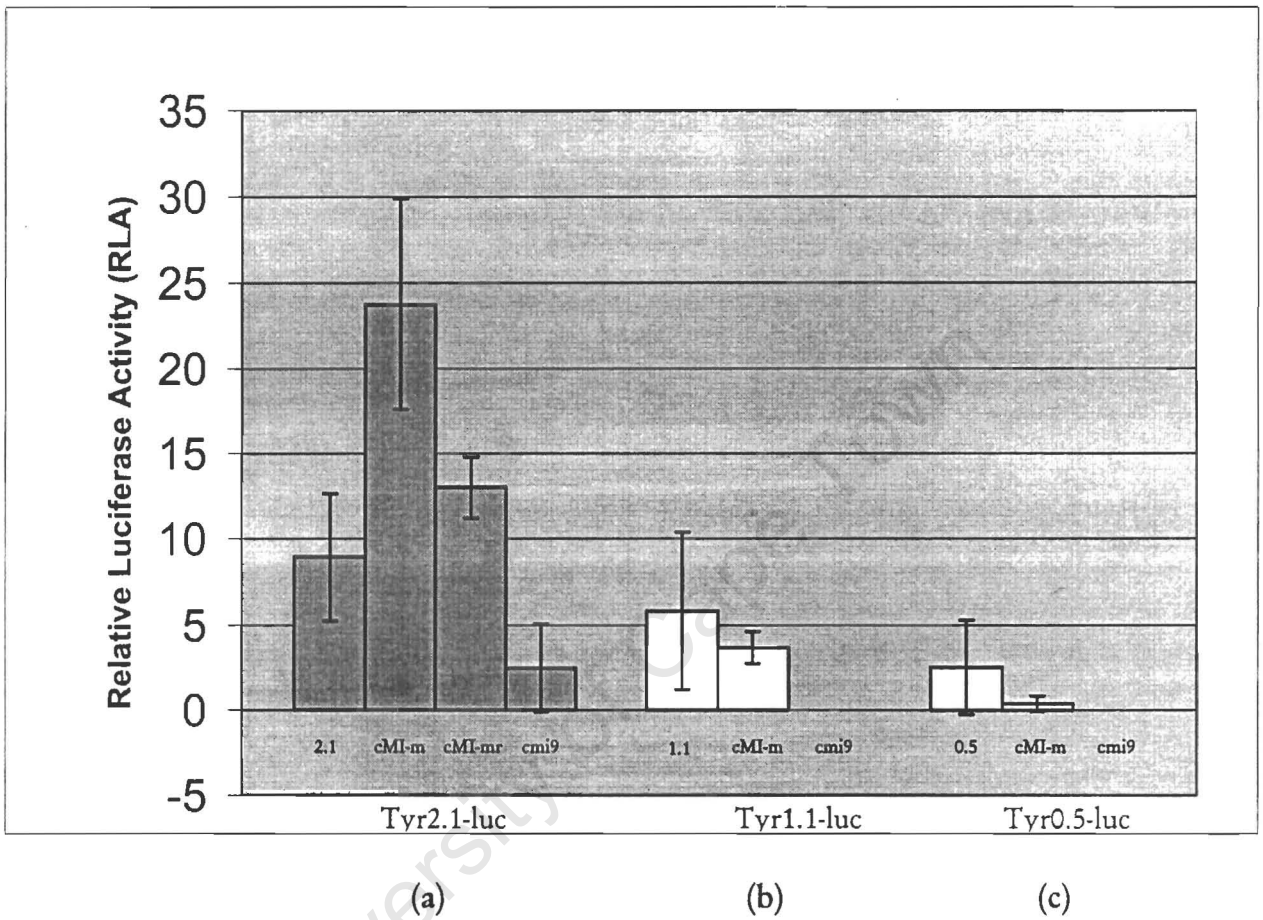

Fig. C.3. Activity of **(a)** Tyr2.1-luc, **(b)** Tyr1.1-luc, and **(c)** Tyr0.5-luc in fibroblasts following co-transfections with RCAS/*cMI-m* (cMI-m); RCAS/*cMI-mr* (cMI-mr); or RCAS/*cmi9* (cmi9). Bars represent co-transfections with Tyr2.1-luc (2.1); Tyr1.1-luc (1.1); and Tyr0.5-luc (0.5). The baseline activity of each promoter construct (first bar in each series) is from Fig. C.2 and is included for comparative purposes. Data are shown as the means of individual experiments. Standard deviations were calculated using the Excel (Microsoft Corporation) standard deviation function. In all cases, results are expressed as a relative luciferase activity (RLA) normalised with respect to *Renilla* activity in the same cell extract and expressed as a percentage of the *luciferase* activity obtained with the positive control vector, pGL2-control. Protein concentrations in all cell extracts were normalised using the BioRad Assay.

Transactivation of chicken tyrosinase gene promoter constructs in neural crest-derived melanocytes (melan-a cells)[3]

Next, the ability of the two chicken Microphthalmia proteins to transactivate the chicken tyrosinase gene promoter constructs in neural crest-derived melanocytes was assessed. First, melan-a cells were co-transfected with either (i) RCAS/*cMI-m* and Tyr2.1-luc, or (ii) RCAS/*cmi9* and Tyr2.1-luc. The pooled results of individual experiments are shown in Fig. C.4.a. The baseline activity of Tyr2.1-luc in melan-a cells is from Fig. C.2 and is included for comparative purposes.

Co-transfection of RCAS/*cMI-m* and Tyr2.1-luc in melan-a cells resulted in a mean RLA of 92.6 ± 31.9, a value not different from the baseline RLA of Tyr2.1-luc in these cells. Likewise, co-transfection of RCAS/*cmi9* and Tyr2.1-luc in melan-a cells results in a mean RLA of 77.4 ± 22.3, a value again not different from the baseline Tyr2.1-luc activity in the cells. Thus, neither cMI-m nor cmi9 increased the activity of the full-length chicken tyrosinase gene promoter in neural crest-derived melanocytes (melan-a cells).

Next, melan-a cells were co-transfected with either (i) RCAS/*cMI-m* and Tyr1.1-luc, or (ii) RCAS/*cmi9* and Tyr1.1-luc. The pooled results of individual experiments are shown in Fig. C.4.b. The baseline activity of Tyr1.1-luc in melan-a cells is from Fig. C.2 and is included for comparative purposes. Both cMI-m and cmi9 increased the activity of Tyr1.1-luc in melan-a cells. cMI-m increased the promoter activity from a baseline RLA of 20.0 ± 13.1 to a mean RLA of 83.7 ± 20.1, while cmi9 increases the activity to a mean RLA of 102.5 ± 39.9. Hence both cMI-m and cmi9 transactivated the Tyr1.1-luc promoter in melan-a cells.

Similar results are obtained with the shortest promoter deletion construct, Tyr0.5-luc. Melan-a cells were co-transfected with either (i) RCAS/*cMI-m* and Tyr0.5-luc, or (ii) RCAS/*cmi9* and

[3] The results detailed in this section, which represented a large portion of the original version of this thesis, do not form part of the results discussed in Chapter Four of this revised thesis. They are, however, included in this revised thesis in the interest of both honesty and scientific integrity with respect to the original thesis.

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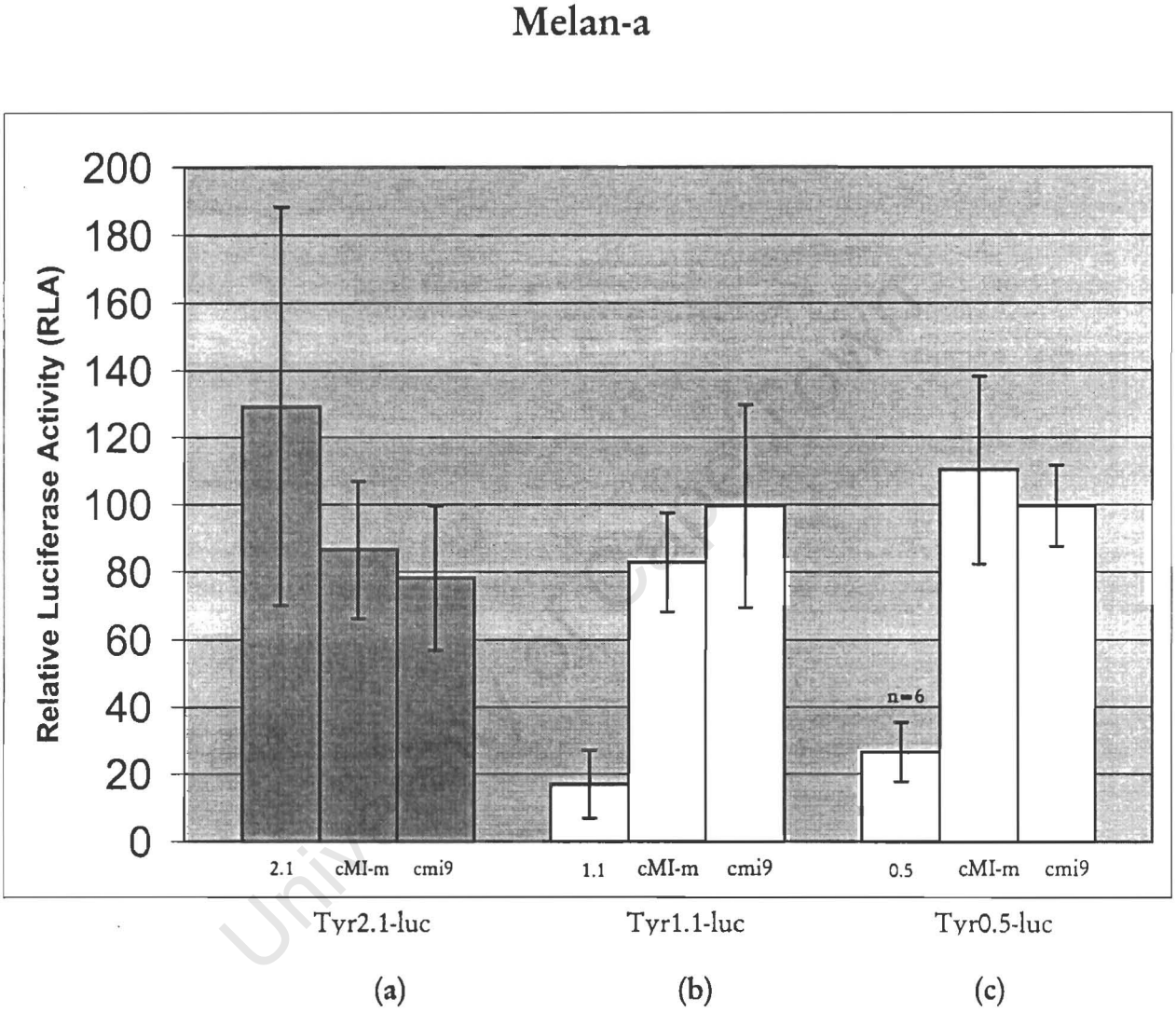

Fig. C.4. Activity of **(a)** Tyr2.1-luc, **(b)** Tyr1.1-luc, and **(c)** Tyr0.5-luc in melan-a cells following co-transfections with RCAS/*cMI-m* (cMI-m); or RCAS/*cmi9* (cmi9). Bars represent co-transfections with Tyr2.1-luc (2.1); Tyr1.1-luc (1.1); Tyr0.5-luc (0.5). The baseline activity of each promoter construct (first bar in each series) is from Fig. C.2 and is included for comparative purposes. Data are shown as the means of individual experiments. Standard deviations were calculated using the Excel (Microsoft Corporation) standard deviation function. In all cases, results are expressed as a relative luciferase activity (RLA) normalised with respect to *Renilla* activity in the same cell extract and expressed as a percentage of the *luciferase* activity obtained with the positive control vector, pGL2-control. Protein concentrations in all cell extracts were normalised using the BioRad Assay.

Tyr0.5-luc. The pooled results of individual experiments are shown in Fig. C.4.c. The baseline activity of Tyr0.5-luc in melan-a cells is from Fig. C.2 and is included for comparative purposes. From a baseline RLA of 46.4 ± 39.6, cMI-m increased the activity to a mean RLA of 107.5 ± 47.7, and cmi9 increased the activity to a mean RLA of 86.2 ± 23.5. Hence both cMI-m and cmi9 transactivated the Tyr0.5-luc promoter in melan-a cells.

Together, these results suggested that neither chicken Microphthalmia protein is able to increase the activity of the full-length tyrosinase promoter in melan-a cells, but that both are sufficient to increase the activity of the two promoter deletion constructs. This suggested that the core tyrosinase promoter elements are sufficient for transactivation by Microphthalmia in neural crest-derived melanocytes.

Transactivation of chicken tyrosinase gene promoter constructs in neuroepithelial-derived (RPE) cells[4]

Finally, the ability of cMI to transactivate the chicken tyrosinase promoter constructs in neuroepithelial-derived cells was assessed by transient co-transfections into RPE cells. First, RPE cells were co-transfected with either (i) RCAS/*cMI-m* and Tyr2.1-luc, or (ii) RCAS/*cmi9* and Tyr2.1-luc. The pooled results of individual experiments are shown in Fig. C.5.a. The baseline activity of Tyr2.1-luc in RPE cells is from Fig. C.2 and is included for comparative purposes. Neither cMI-m nor cmi9 transactivated the Tyr2.1-luc promoter in RPE cells: the baseline activity of Tyr2.1-luc in RPE cells was 117.6 ± 66.7, which is not different from the activity measured following co-transection with RCAS/*cMI-m* (58.3 ± 33.3) or RCAS/*cmi9* (137.8 ± 23.3).

Next, RPE cells were transfected with (i) RCAS/*cMI-m* and Tyr1.1-luc, or (ii) RCAS/*cmi9* and Tyr1.1-luc. The pooled results of individual experiments are shown in Fig. C.5.b. The baseline activity of Tyr1.1-luc is from Fig. C.2 and is included for comparative purposes. Unexpectedly, co-transfection with cMI-m resulted in no Tyr1.1-luc promoter activity in

[4] The results detailed in this section, which represented a large portion of the original version of this thesis, do not form part of the results discussed in Chapter Four of this revised thesis. They are, however, included in this revised thesis in the interest of both honesty and scientific integrity with respect to the original thesis.

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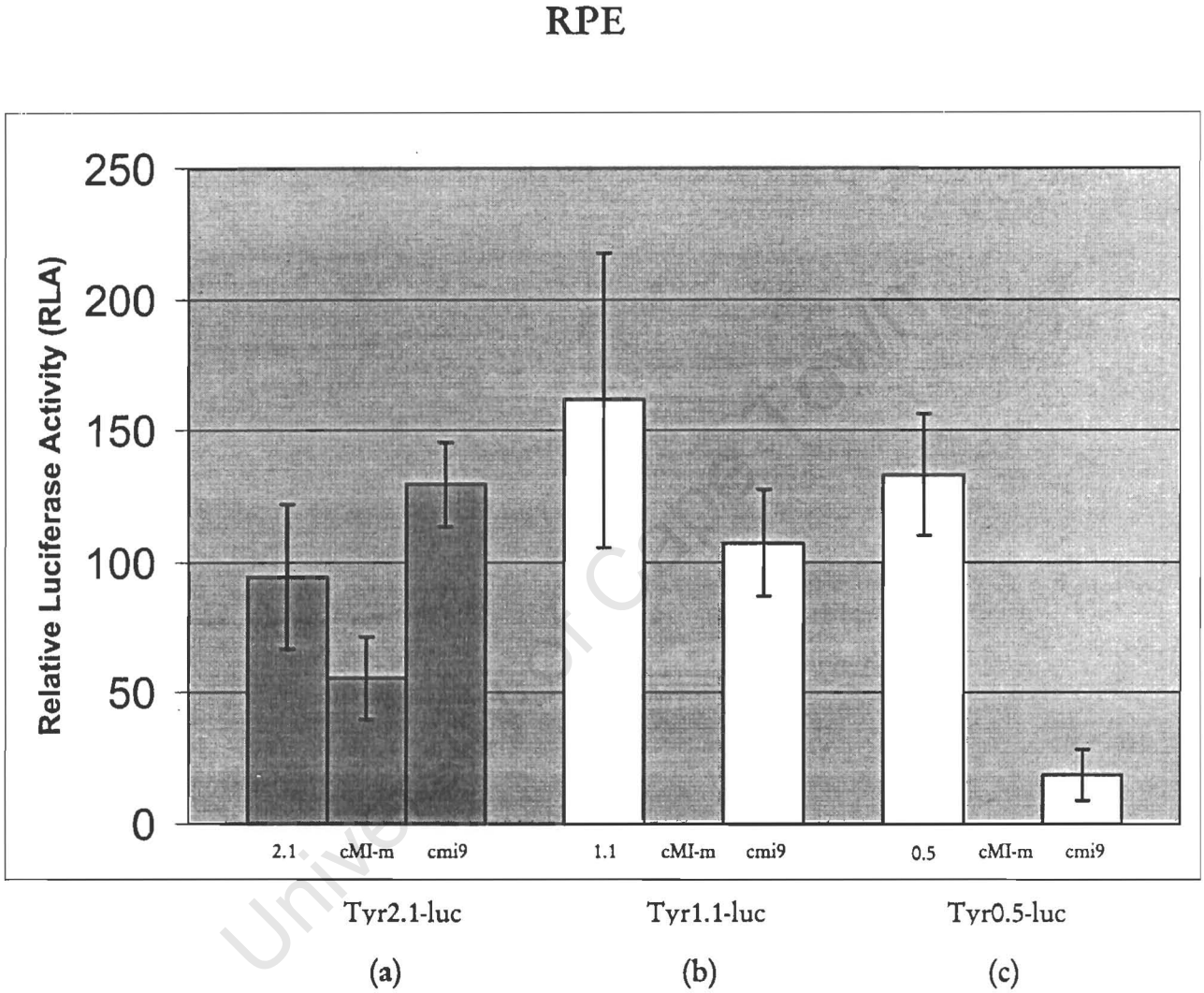

Fig. C.5. Activity of (a) Tyr2.1-luc, (b) Tyr1.1-luc, and (c) Tyr0.5-luc in RPE cells following co-transfections with RCAS/*cMI-m* (cMI-m); or RCAS/*cmi9* (cmi9). Bars represent co-transfections with Tyr2.1-luc (2.1); Tyr1.1-luc (1.1); and Tyr0.5-luc (0.5). The baseline activity of each promoter construct (first bar in each series) is from Fig. C.2 and is included for comparative purposes. Data are shown as the means of individual experiments. Standard deviations were calculated using the Excel (Microsoft Corporation) standard deviation function. In all cases, results are expressed as a relative luciferase activity (RLA) normalised with respect to *Renilla* activity in the same cell extract and expressed as a percentage of the *luciferase* activity obtained with the positive control vector, pGL2-control. Protein concentrations in all cell extracts were normalised using the BioRad Assay.

RPE cells. From a baseline RLA of 171.9 ± 85.9, this co-transfection reduced the RLA to zero. On the other hand, co-transfection with cmi9 had no effect on the activity of Tyr1.1-luc: the baseline RLA activity is not different from that obtained with co-transfection of RCAS/*cmi9* (69.9 ± 38.2).

Lastly, RPE cells were transfected with (i) RCAS/*cMI-m* and Tyr0.5-luc, or (ii) RCAS/*cmi9* and Tyr0.5-luc. The pooled results of individual experiments are shown in Fig. C.5.c. The baseline activity of Tyr0.5-luc is from Fig. C.2 and is included for comparative purposes. As was the case with the Tyr1.1-luc promoter, co-transfection with cMI-m resulted in no promoter activity of Tyr0.5-luc (from a baseline RLA of 153.8 ± 33.4 to and RLA of zero). The results of co-transfection with cmi9 were slightly different: co-transfection with cmi9 reduced the activity of the Tyr0.5-luc promoter, from the baseline RLA of 153.8 ± 33.4 to a weak RLA of 16.7 ± 13.2, but did not result in a complete absence of Tyr0.5-luc activity.

Thus, the functions of the chicken Microphthalmia proteins appeared to be much different in RPE cells than in melan-a cells. On the whole, the over-expression of Microphthalmia in RPE cells appeared to have a detrimental effect on the activity of the tyrosinase gene promoter.

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