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CLONING, SEQUENCING AND FUNCTIONAL ANALYSIS OF THE CHICKEN TYROSINASE GENE PROMOTER

by

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Thesis presented for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Anatomy and Cell Biology in the

Faculty of Medicine

UNIVERSITY OF CAPE TOWN

June 1996

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ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr Sue Kidson. Her enthusiasm, friendship and her gift of enabling students to experience science to the full, have greatly enriched my life. I am very grateful for the privilige of having spent time in her lab, in the company of fine scientists and good friends:

"Such life here through such lengths of hours,
such miracles performed in play..."
Robert Browning.

I would especially like to acknowledge Craig April, Sharon Prince and Philippa Hulley for so very kindly helping with the figures and proof-reading of this thesis. I am also indebted to Toni Wiggins for her assistance with the multitudes of minipreps and RPE cell cultures, and to Henry Fortuin and Elise Fuller for assistance with photography.

I would like to thank Fernanda da Silva Tatley, Gay Elisha, and Janet Hapgood, from the Departments of Biochemistry, Medical Microbiology and Chemical Pathology, for helpful discussions.

To both my families, absent and present, who have encouraged me to succeed and patiently endured my enthusiasm for science, thank you. I am grateful to my sister, Hazel, and close friend, Shaun, for their entertaining conversations that broke the hours of isolation in the last few months.

Finally, I am grateful to Roger (to whom I would dedicate this thesis but for the enormous role he has had in it) for his love and for being there.

ABSTRACT

Cloning, sequencing and functional analysis of the chicken tyrosinase gene promoter.

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The differentiation of melanocytes from multipotential neural crest cells is an ideal system for studying the processes underlying lineage determination in development. Tyrosinase is a key enzyme in melanin biosynthesis and the activation of the tyrosinase gene is characteristic of differentiated melanocytes. In order to study the mechanisms underlying activation of melanocyte-specific genes during differentiation in chick embryos, a chicken genomic DNA library was screened for tyrosinase-encoding sequences using a mouse tyrosinase cDNA probe. Two identical hybridizing clones were identified. Restriction mapping and sequencing revealed that both clones contained a 4.3 kb genomic DNA fragment, CTYR4.3, that included 2125 nt of the 5′ flanking region, the first exon and part of the first intron of the chicken tyrosinase gene.

The 5′ flanking sequence of CTYR4.3, which is the most extensive to be reported for a lower vertebrate tyrosinase gene to date, was analysed further using computer-aided homology searches and primer extension. Alignment of the promoter sequences of CTYR4.3 with those of the human, mouse, quail and turtle tyrosinase genes revealed two evolutionary conserved regions. These regions may be functionally significant as they contain regulatory elements previously reported to play a role in melanocyte-specific expression of the tyrosinase gene in mammals. These include an initiator region and an associated SP1-binding site, the M-box and an upstream enhancer element, TDE. In addition, other potential transcription factor binding motifs were identified, including an AP-1-binding site, a UV-responsive element and glucocorticoid-responsive elements.

Although several TATA box motifs were identified, they were situated more than 200 bp upstream of the transcription start sites mapped by primer extension

analysis and therefore are unlikely to function as TFIID-binding sites. Transcription initiation appears to occur at heterogeneous start sites, and given the absence of a functional TATA box, may be mediated via the conserved initiator region and SP1-binding site.

To test the ability of the 5′ flanking sequence of CTYR4.3 to drive transcription and to begin to assess the functional significance of the various conserved elements, transient transfection assays were carried out. Constructs were generated in which 2.1 kb, 1.1 kb, 0.5 kb and 0.2 kb fragments of the 5′ flanking sequence were linked to a luciferase reporter gene. These constructs were introduced into cultures of chicken retinal pigment epithelial cells (RPE), immortalised quail neural crest cells (MQTNC), and human liver cells (Hep G2) by calcium phosphate-mediated transfection. Transfections with all constructs resulted in luciferase activities significantly greater than those that were observed with the promoterless luciferase construct, thus confirming that the 5′ flanking sequence of CTYR4.3 does possess promoter activity. However, the level of expression from the various constructs differed markedly in the different cell types. In the tyrosinase-negative Hep G2 cells, low levels of expression were observed with all constructs. In the tyrosinase-positive RPE cells, a high level of luciferase activity was obtained specifically with the smallest (0.2 kb) promoter construct. Since the 0.2 kb promoter fragment does not include the conserved initiator region, SP1-binding site, or M-box, the role of these elements in tissue-specific transcription initiation of the chicken tyrosinase gene is now questionable. These results suggest the existence of transcription regulatory mechanisms that are unique to avians and possibly other lower vertebrates. In contrast to the results obtained for RPE cells, the highest luciferase activity was obtained with the full length 2.1 kb promoter construct in the immortalised quail neural crest-derived cells. These results may have developmental significance since they suggest that the chicken tyrosinase gene promoter is regulated differently in RPE cells and neural crest-derived cells.

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CHAPTER ONE

GENERAL INTRODUCTION AND AIMS

Tyrosinase is a key enzyme involved in the biosynthesis of pigment in vertebrates. The enzyme not only initiates the pathway of melanin synthesis by converting the amino acid substrate, tyrosine, to dihydroxyphenylalanine (DOPA), as its name suggests, but catalyses two further steps in the pathway that eventually result in the production of melanin. In the absence of tyrosinase activity, no melanin is produced. Tyrosinase is the founder member of a family of proteins, known as the tyrosinase-related protein family, encoded by genes that are expressed exclusively within the pigment producing-cells (melanocytes) of the vertebrate body. Thus the mechanisms controlling tyrosinase gene expression represent an important level of regulation in the process of melanin synthesis. Moreover, the onset of expression within melanoblasts (precursors to melanocytes) of the genes encoding tyrosinase and its family members represents an important step in vertebrate melanogenesis. Research into the mechanisms underlying melanocyte-specific gene expression in mammals has been extensive over the last eight years, whereas that of lower vertebrates has only recently been initiated.

The purpose of this review is to outline the role of tyrosinase in the process of melanogenesis and to introduce key areas of research. Section 1.1. includes a brief introduction to the processes involved in establishing the pigmentary system during embryonic development of vertebrates. Section 1.2. is dedicated to the tyrosinase-related gene family. Section 1.3. will review current literature on the regulation of transcription of the tyrosinase gene in mammals and lower vertebrates.

1.1. DEVELOPMENTAL BIOLOGY OF VERTEBRATE MELANOCYTES

Melanogenic cells in the vertebrate adult are found primarily in the epidermis of the skin and its derivatives, in the choroid and iris, collectively known as the uvea, and in the retinal pigment epithelium (RPE) of the eye. Melanocytes in the skin function to distribute melanin to the keratinocytes, which then shields these cells and underlying tissues from the mutagenic effects of UV radiation. It is also thought that melanin interacts with and inactivates the potentially harmful free radicals which are frequently produced in epithelial cells (Sarna et al, 1986). Melanin produced by ocular melanocytes serves a similar role, but also functions to absorb scattered light and thereby eliminate the interference of background light at the retina.

Melanocytes are derived from neural crest cells, pluripotent progenitor cells that arise during neuralation from the closing folds of the neural tube. The exception are the RPE cells that develop from the neuroepithelium of the optic cup. Neural crest cells migrate out from the dorsal surface of the neural tube at or after closure of the tube, along well characterised pathways and give rise to a diverse array of cell types. The neural crest cell migration pathways and derivatives are summarised in Fig. 1.1 and Table 1.1. In mammals and avians, neural crest cells that are destined to become melanocytes embark upon the dorsolateral pathway, proliferate and disperse in the subectodermal space (reviewed by Weston, 1970; Le Douarin, 1982; Erickson, 1986; Newgreen and Erickson, 1986) and then invade and colonise the ectoderm (epidermis) where they may become localised in hair follicles or feather germs. Despite extensive research (reviewed by Erickson et al, 1993), it has still not been established at which stage neural crest cells become committed to the melanocyte lineage, that is, lose their pluripotency to become melanoblasts. Melanoblasts also migrate to sites other than skin and eye, for instance, to the Harderian gland (a lachrymal gland) and in mammals, to the cochlea of the inner ear (Deol, 1970; Steel and Barkway, 1989). Interestingly, extracutaneous pigmentation in the Silkie fowl is extensive, with the mesenteries and many of the internal organs populated by neural crest-derived melanocytes (Eastlick and Wortham, 1946).

The process of melanogenesis in neural crest-derived and RPE cells is initially the same. The cells begin to synthesize melanin which is laid down in organelles known as melanosomes. The process of melanin biosynthesis involves a complex biochemical pathway (See Fig. 1.2.) in which tyrosine is converted to either eumelanin, the black and brown pigment, or phaeomelanin which is a yellow-reddish pigment. Melanosomes contain an inner matrix which acts as a scaffold on which melanin is deposited. They are thought to be closely related to lysosomes as some of their structural membrane proteins are identical (Zhou et al, 1993). Four stages of melanosome maturation may be described (Fitzpatrick et al, 1969). Stages I and II are used to describe unmelanised, immature premelanosomes. A melanoblast synthesises only melanosomes of these two initial stages. Fully differentiated melanocytes also contain stages III and IV melanosomes, in which melanin is progressively accumulating. In epidermal melanocytes, fully melanised stage IV melanosomes are transported down the dendritic processes of the melanocytes and transferred to neighboring keratinocytes (Fig. 1.3).

Table 1.1

Some derivatives of the neural crest

Derivative	Cell type or structure derived
Peripheral nervous system (PNS)	Neurons, including sensory ganglia, sympathetic and parasympathetic ganglia, and plexuses Neuroglial cells Schwann cells
Endocrine and paraendocrine derivatives	Adrenal medulla Calcitonin-secreting cells Carotid body type 1 cells
Pigment cells	Epidermal pigment cells
Ectomesenchymal derivatives	Facial and anterior ventral skull cartilage and bones
Connective tissue (ectomesenchymally derived)	Corneal endothelium and stroma Tooth papillae Dermis, smooth muscle, and adipose tissue of skin of head and neck Connective tissue of salivary, lachrymal, thymus, thyroid, and pituitary glands Connective tissue and smooth muscle in arteries of aortic arch origin

After Gilbert (1994), based on multiple sources.

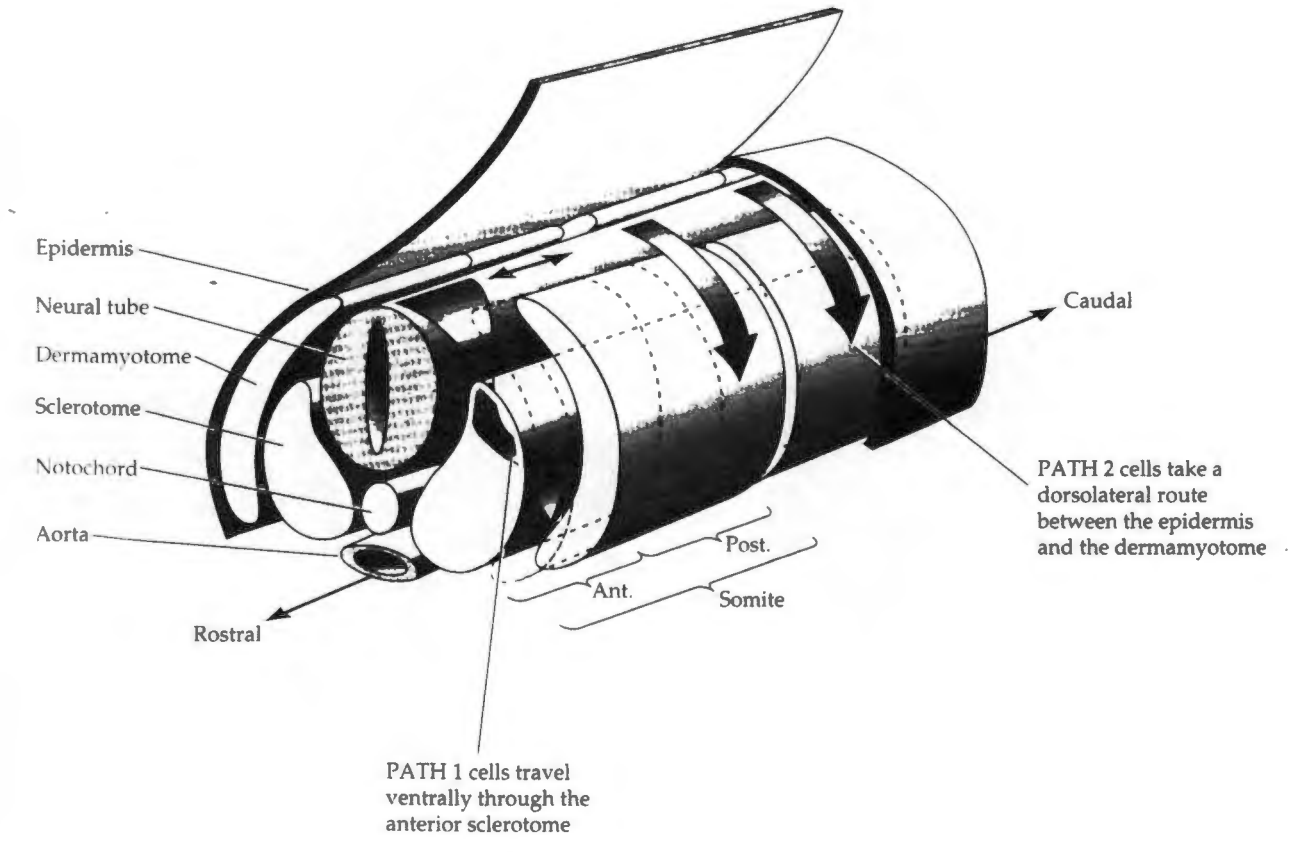

Fig.1.1: Neural crest cell migration in the trunk of the chick embryo. Path 1: Cells travel ventrally through the anterior of the sclerotome (that portion of the somite that generates vertebral cartilage). Those cells initially opposite the posterior potions of the sclerotomes migrate along the neural tube until they come to an opposite anterior region. These cells contribute to the sympathetic and parasympathetic ganglia as well as to the adrenal medullary cells and dorsal root ganglia. Path 2: Somewhat later, cells enter a dorsolateral route beneath the ectoderm. These cells become pigment-producing melanocytes. After Gilbert (1994).

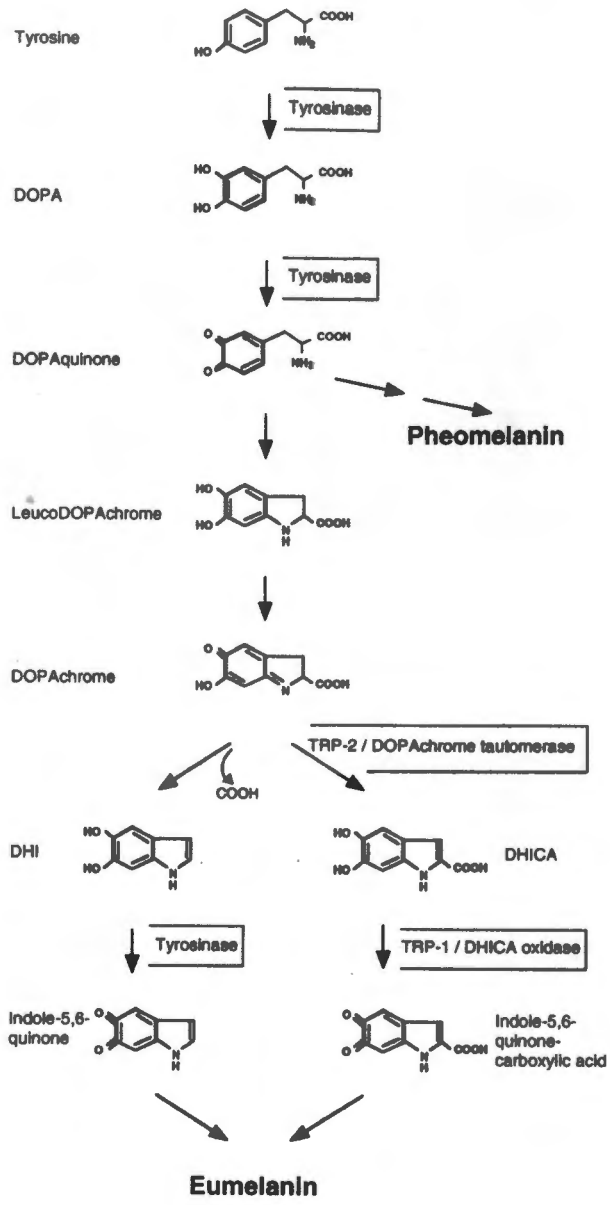

Fig. 1.2: The melanin biosynthesis pathway from tyrosine. Enzymatic reactions attributed to tyrosinase, TRP-1 and TRP-2 are indicated. DOPA, 3,4-dihydroxyphenylalanine; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; DHI, 5,6-dihydroxyindole. After del Marmol and Beermann (1996).

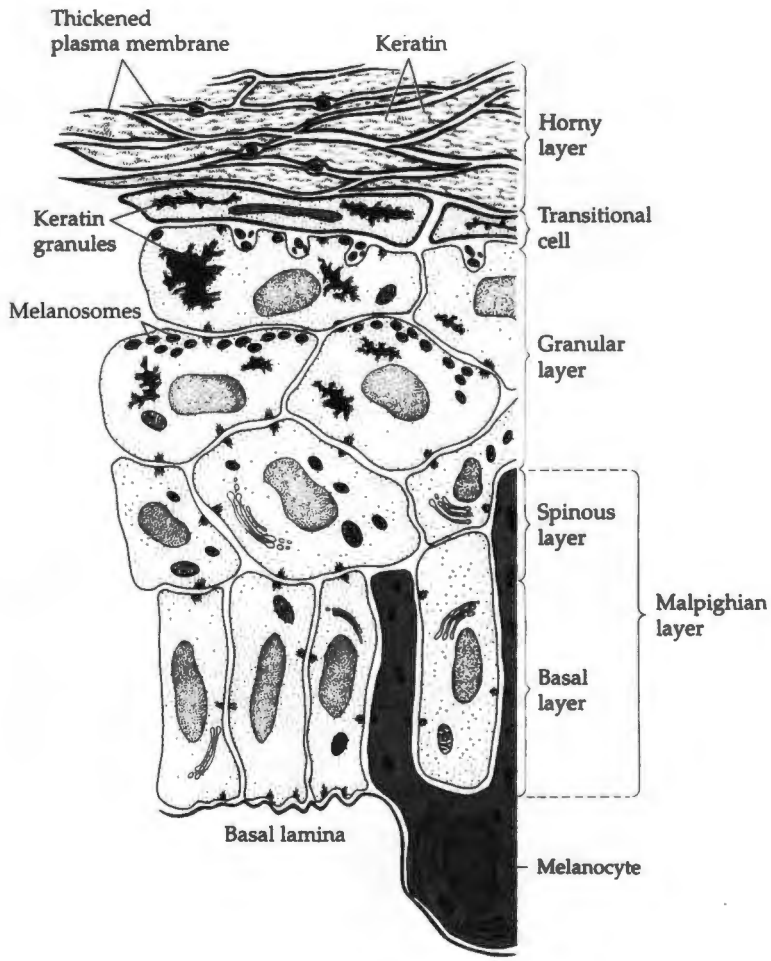

Fig. 1.3: The position and role of melanocytes in the epidermis. The layers of the epidermis are shown. The basal epidermal cells are mitotically active, whereas the fully keratinised cells characteristic of external skin are shed off. The keratinocytes obtain pigment from the transfer of melanosomes from the processes of melanocytes that reside in the basal layer. After Gilbert (1994).

Analyses using electron microscopy (Hirobe, 1982) have shown that the structural components of the melanosome (i.e. unit membranes and inner matrices) are assembled in the Golgi or endoplasmic reticulum. The melanogenic enzymes take a separate route via the Golgi where they are post-translationally modified. The mature enzymes are then delivered to stage I and II melanosomes in coated vesicles (vesicular globular bodies), within which melanogenesis is thought to have been initiated. These fuse, after which pigment deposition begins and melanosome maturation ensues (Fig. 1.4.).

Although epidermal melanocytes and RPE cells share a common melanogenic process, they differ not only in embryonic origin but also in their morphology and functioning. Unlike neural crest-derived melanoblasts, RPE precursor cells do not migrate and therefore are not susceptible to mutations that affect the homing and survival of migrating neural crest cells. Epidermal melanocytes lie on a basement membrane and extend dendritic processes between the layers of keratinocytes. In humans, each melanocyte is associated with 30 to 40 keratinocytes (Lucky and Nordlund, 1985) to which they transfer their melanosomes. These melanocytes are therefore sparsely distributed in the basal layer of the epidermis compared with RPE cells which occur as a continuous layer of pigmented cuboidal cells, making up the outermost layer of the retina (see Figs 1.3 and 1.5). Neither RPE cells nor uveal melanocytes transfer their melanosomes. Thus ocular melanocytes cease melanogenesis when they become congested with melanosomes (Boissy et al, 1988; Hu and Mah, 1979). Finally, certain unique functions have been ascribed to RPE cells. They are responsible for the phagocytosis and disposal of membranous discs from the rods and cones of the adjacent retinal photoreceptor cells. As an epithelial cell layer, the cells are tightly connected to one another by junctional complexes and thus they form an effective blood-retina barrier. Furthermore, it was recently discovered that the RPE cell layer plays an essential role in the establishment of the multi-layered neural retina during embryonic development, and its subsequent maintenance (Raymond and Jackson, 1995). Interestingly, extracutaneous melanocytes have also been shown to play a role in morphogenesis of the inner ear, where their presence is essential for normal development of the stria vascularis (Steel and Barkway, 1989).

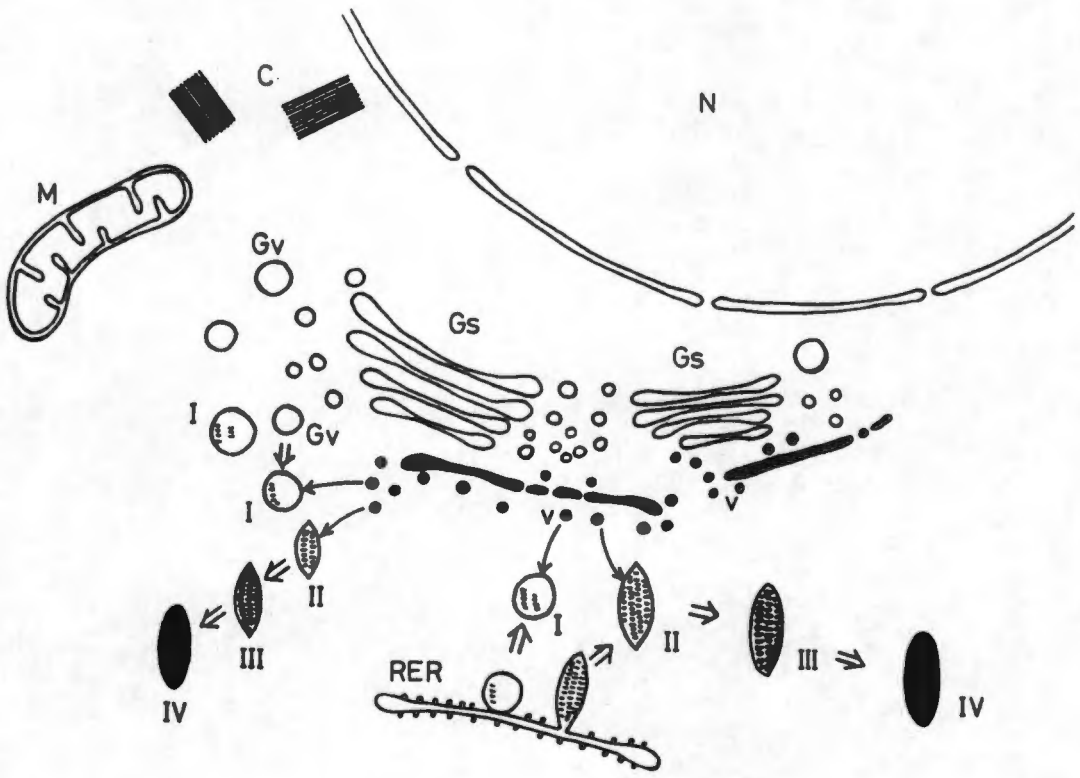

Fig. 1.4: Melanosome formation in epidermal melanocytes. Stage I and II melanosomes (I, II) originate from Golgi vacuoles (Gv) or from rough endoplasmic reticulum (RER). Tyrosinase is transferred via vesicles (v) derived from Golgi saccules (Gs) into these melanosomes, which then mature to become stage III and IV melanosomes (III, IV). N, nucleus; M, mitochondrion; C, centrioles. After Hirobe (1992).

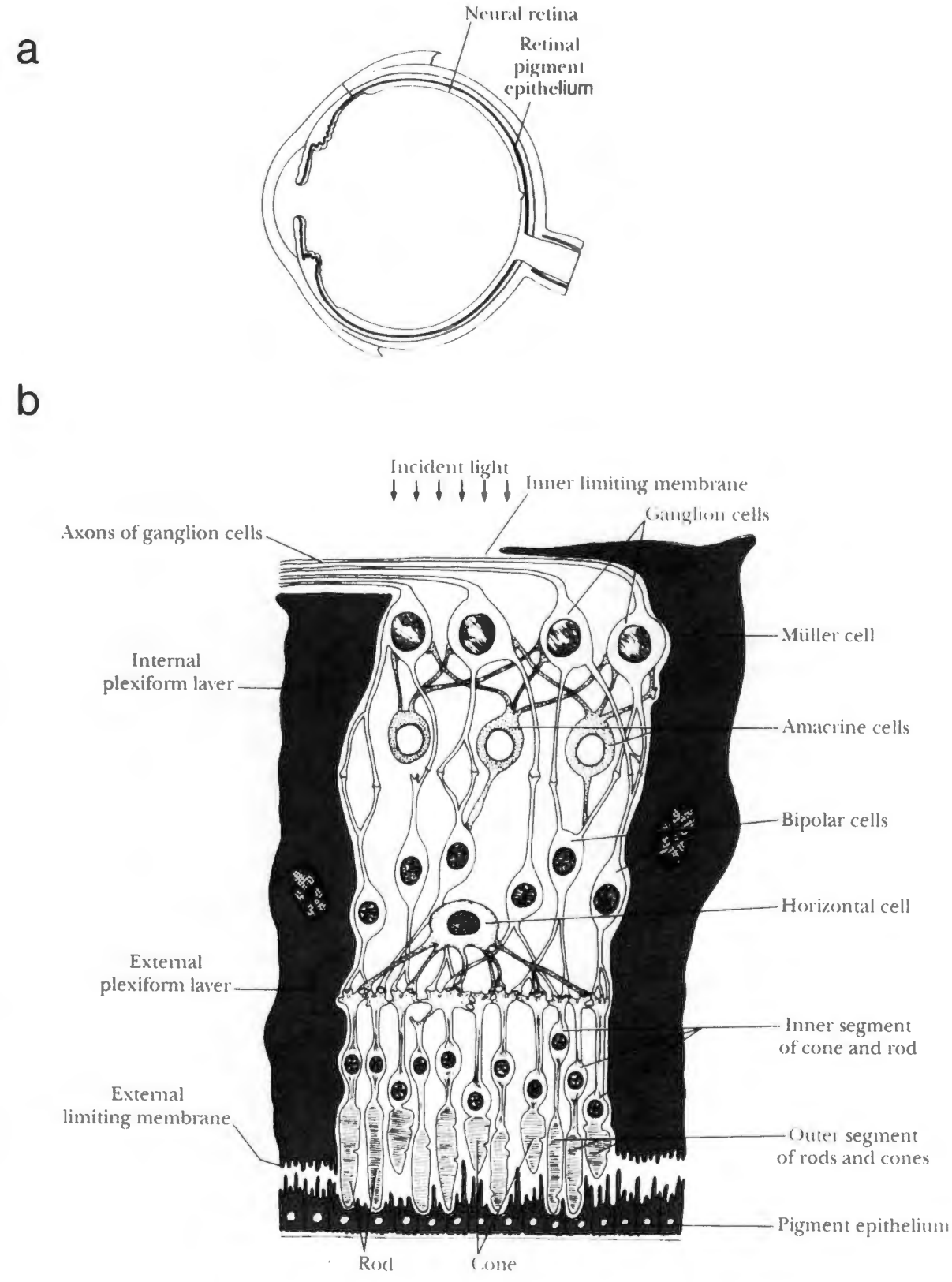

Fig. 1.5: The position of RPE cells in the eye. **(a)** The layers of the eye. The retinal pigment epithelium forms part of the retina, which is the innermost layer of the eye. **(b)** The layers of the retina. RPE cells comprise a simple cuboidal epithelial cell layer which underlies the photosensitive layer of the neural retina. After Ross and Romrell (1989).

1.2. THE TYROSINASE GENE FAMILY

Evidence of a tyrosinase gene family first came to light when attempts to isolate the mammalian cDNA sequence encoding tyrosinase led to the identification of three related sequences that mapped to different chromosomal loci. Each family member encodes an enzyme involved in melanin biosynthesis. These include the prototype, tyrosinase, and two related proteins, tyrosinase-related protein (TRP)-1 and dopachrome tautomerase or TRP-2. As reviewed by Jackson et al (1994), these proteins share an amino acid homology of 40%, they are of similar length (529, 537 and 519 amino acids for tyrosinase, TRP-1 and TRP-2, respectively) and have a number of structural features in common, which are detailed in Fig. 1.6A

Over the last decade, the genes encoding the three family members have been isolated from human and mouse cDNA and genomic DNA libraries. Details of the mutant phenotypes and the enzymatic functions proposed for the proteins encoded by each family member, are discussed below. That there are only three members in the tyrosinase gene family has been confirmed by PCR using degenerate primers designed by I. Jackson and J. Horn (Jackson et al, 1994). These primers recognise DNA sequences required for amino acid identity among the family and enable the simultaneous amplification of all family members (Fig. 1.6B). Only three species of DNA corresponding to the three family members have been amplified from mouse and human cDNA. Comparison of gene structures and DNA sequences, has revealed a greater homology between TRP-1 and TRP-2 genes than between tyrosinase and the TRP sequences (Budd and Jackson, 1995; Morrison et al, 1994; Sturm et al, 1995).

1.2.1. Tyrosinase

Tyrosinase is encoded at the albino locus (c-locus) on chromosome 7 in the mouse and (TYR locus) on chromosome 11 in humans (Barton et al, 1988). A variety of mutations at the albino locus results in hypopigmentation in the coat and eyes of mice (Silvers, 1979), and a condition in humans referred to as tyrosinase-negative oculocutaneous albinism (OCA). The link between OCA and a mutation in the tyrosinase gene was first demonstrated by Tomita et al (1989), and to date more than 30 distinct mutations in the tyrosinase gene have been shown to result in tyrosinase-negative OCA (reviewed by Oetting et al, 1994).

a

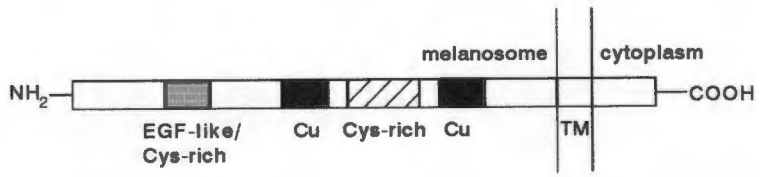

Fig. 1.6. Characteristic features of the proteins encoded by the tyrosinase gene family. **(a)** Schematic representation of the tyrosinase family protein domains which include a signal sequence at the N-terminus and a hydrophobic melanosome transmembrane (**TM**) domain near the C-terminus (Shibahara et al, 1991; Tsukamoto et al, 1992); two copper-binding sites (**Cu**) that are conserved in similar positions to sites in tyrosinases of lower vertebrates and prokaryotes and in arthropod haemocyanins (Müller et al, 1988); and two cysteine-rich (**cys-rich**) sequences that flank the first copper-binding site. One of these sequences has an epidermal growth factor-like (**EGF**) motif, the significance of which is unknown except that this motif is found in a variety of proteins that are membrane-bound or secreted through a membrane, and may participate in protein-protein interactions (Davis, 1990). (Figure adapted from del Marmol and Beermann, 1996).

b

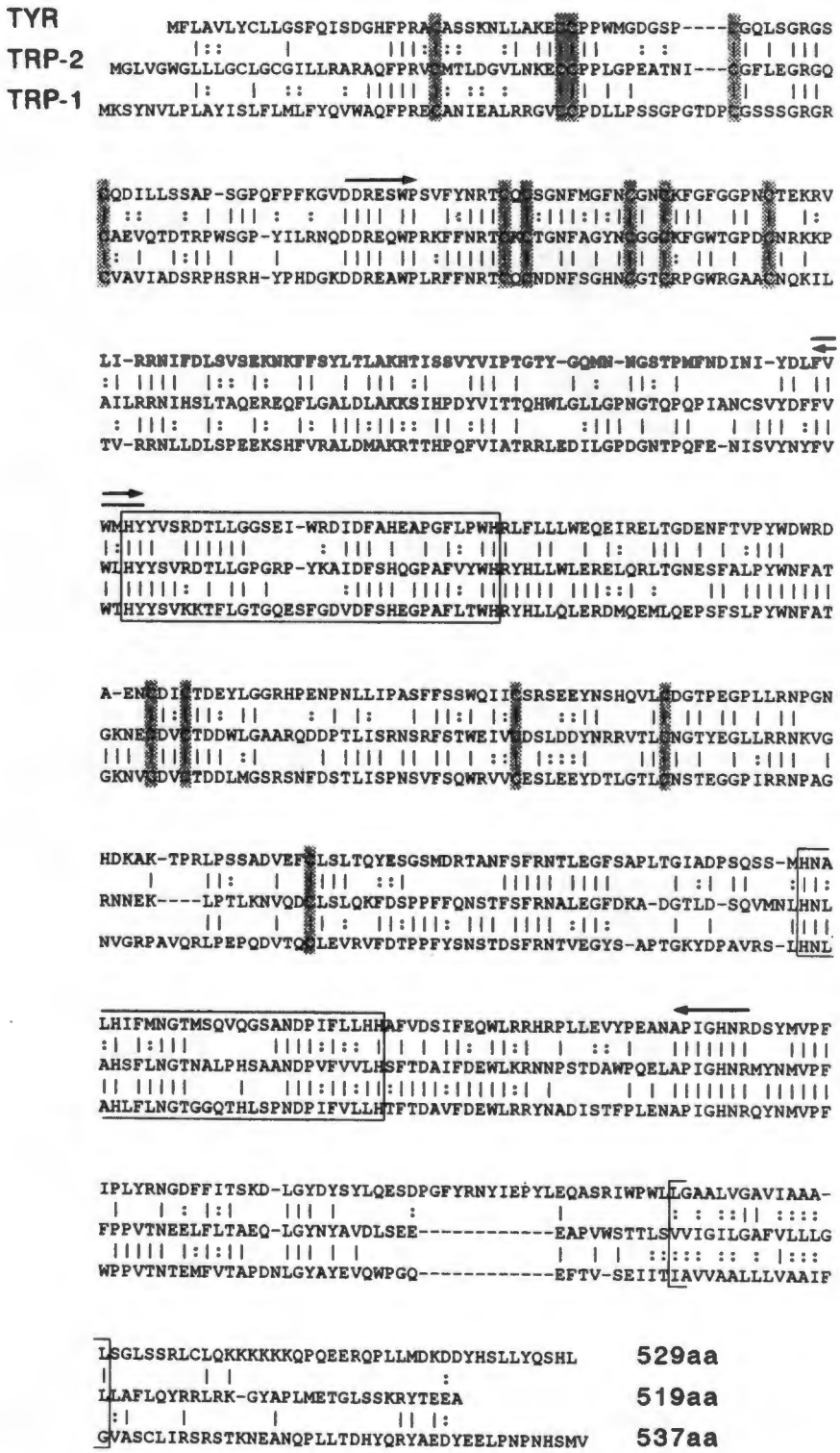

Fig. 1.6: (b) Amino acid sequence alignment demonstrating the sequence similarity between mouse tyrosinase (top row), TRP-2 (middle row) and TRP-1 (bottom row). Identical amino acids are indicated by | and amino acids that are chemically similar by :. The 15 conserved cysteine residues are shaded, the two copper-binding sites are boxed and the transmembrane domain is enclosed by brackets. The relative position of the Jackson-Horn degenerate PCR primers are indicated by arrows. (Figure adapted from Jackson et al, 1992).

Tyrosinase has been found to be responsible for three enzyme activities in the melanin biosynthesis pathway (see Fig.1.2). Tyrosine hydroxylase and DOPA oxidase activities of tyrosinase, first described by Lerner and his colleagues (1949), are utilized in the first two steps of eu- and phaeomelanin biosynthesis and represents the rate-limiting activity of the pathway. A third activity has been assigned more recently to tyrosinase, namely DHI oxidase, and is utilised in a subsequent step in the pathway leading specifically to the formation of eumelanin (proposed by Körner and Pawelek, 1982; confirmed by Tripathi et al, 1992).

1.2.2. Tyrosinase-related protein 1

The gene encoding mouse TRP-1 was the first of the tyrosinase gene family to be isolated (Shibahara et al, 1986) and maps to the brown locus (b-locus) on chromosome 4 in the mouse (Jackson, 1988) and (CAS-2 locus) on chromosome 9 in humans, (Chintamaneni et al, 1991). Mouse b-locus mutants have brown coats instead of the wild-type black, implying that a functional TRP-1 enzyme is required for the synthesis of black melanin rather than brown (Zdarsky et al, 1990; Bennett et al, 1990). The exact catalytic function of TRP-1 in the melanin biosynthesis pathway, however, is still in question. Several functions have been proposed and disputed, including a low level of tyrosinase activity (Jiménez et al, 1989), a catalase/hydrogen peroxidase (Halaban and Moellmann, 1990), a DOPAchrome tautomerase (Winder et al, 1993), DHICA conversion activity as well as interacting with and stabilizing the enzyme, tyrosinase (Kobayashi et al, 1994). Interestingly, del Marmol et al (1993) have shown that for cultured melanogenic cells, TRP-1 expression is restricted to those that produce either eumelanin or mixed melanins, as opposed to phaeomelanin only. Finally, TRP-1 may play a structural role in the melanosome, as the melanocytes of b-mutant mice are abnormal i.e. they are spherical rather than ovoid in shape and contain an unusually large number of membranes that appear to be tangled, rather than arranged in a simple rolled manner (Rittenhouse, 1968).

1.2.3. Tyrosinase-related protein 2

The gene encoding TRP-2 is the most recently identified member of the tyrosinase gene family and maps to the slaty (slt) locus on chromosome 14 of the mouse (Jackson et al, 1992) and human-chromosome 13 (Bouchard et al, 1994; Sturm et al, 1994). Homozygote slaty mice have coats that are dark grey-brown rather than

black. The TRP-2 gene encodes an enzyme with dopachrome tautomerase activity (Tsukamoto et al, 1992; Kroumpouzos et al, 1994; Yokoyama et al, 1994). In the presence of this enzyme, dopachrome, which usually converts spontaneously to 5,6 dihydroxyindole (DHI), is converted to a carboxylated form of DHI, namely DHICA. DHICA-melanins have been found to differ from DHI-derived melanins in several properties: whereas DHI-melanin is black and insoluble, DHICA-melanin is golden brown, soluble and stable when subjected to a variety of extreme conditions such as boiling or lyophilisation.

Interestingly, although TRP-2 is involved at a later step than tyrosinase in the melanin biosynthesis pathway, Steel et al (1992) have found using *in situ* hybridization on sections of mouse embryos, that TRP-2 is the first melanocyte-specific gene to be expressed in cells destined to become melanogenic and is expressed both in migrating differentiating neural crest cells and in the optic vesicle. It can therefore be used as an early marker to detect melanoblasts that have not yet begun to form pigment. Tyrosinase and TRP-1 genes are expressed several days later in melanoblast development.

1.2.4. The tyrosinase gene family in lower vertebrates

DNA sequences encoding tyrosinase have been cloned from many vertebrates including partial genomic sequences from the quail, snapping turtle (Yamamoto et al, 1992), and frog (Miura et al, 1995), and cDNA sequences from the frog (Takase et al 1992), chicken (Mochii et al, 1992; April et al, 1996), and medaka fish (Inagaki et al, 1994). In contrast, much less is known about the TRP genes in other vertebrates. This was the subject of discussion in a workshop report on the evolution and expression of tyrosinase-related proteins by Jackson (1994). TRP-1 cDNA sequences have been cloned for goldfish (Peng et al 1994). Whilst neither of the TRP gene sequences have yet been reported for avians, Austin and Boissy (1995) have recently shown that avian homologues do exist and are actively transcribed in avian melanocytes. Using chickens that are affected with "vitiligo-like depigmentation" (a disorder that appears to involve autoimmune reactions), they demonstrated that a species of autoantibody generated in chickens, cross-reacts with mammalian TRP-1. Furthermore they showed that anti-mouse TRP-1 and -2 antibodies recognise chicken proteins and that using mammalian TRP-1 and -2 probes chicken TRP mRNAs could be identified on Northern blots. Most promising is that two novel DNA species have been amplified from a chicken melanocyte cDNA library using the Jackson-Horn degenerate PCR primers for

the tyrosinase gene family (C.S. April, personal communication). These may well represent TRP-1 and -2 gene sequences. Recently, in a similar attempt to clone the gene family from axolotl using the degenerate primers, Mason and Mason (1995) obtained only one species of DNA, which on sequencing, was identified as a partial cDNA sequence encoding TRP-1.

1.2.5. The Pmel 17 gene family, a second family of pigment cell-specific genes

The Pmel 17 gene, which maps to the silver locus (Kwon et al, 1995) was thought to be a fourth member of the tyrosinase gene family as it is expressed exclusively in melanocytes and was initially cloned by screening a melanocyte cDNA library with polyclonal antibodies against tyrosinase (Kwon et al, 1987). Subsequent amino acid sequence analysis of Pmel 17 has revealed very little homology to the deduced proteins of the tyrosinase gene family and has prompted Kwon (1993) to propose a second family of genes that regulates melanin biosynthesis, namely the pmel 17 gene family. A chicken melanosomal matrix protein, MMP115 (Mochii et al, 1991), is thought to be a second member of this family, by virtue of the similarity in primary protein structure to that of Pmel 17 gene product. MMP115, however, is not the chicken homologue of the human Pmel 17 protein. It is not yet clear just how this second family of genes regulates melanin biosynthesis. The localisation of these proteins to the melanosome matrix and/or membrane (Kwon et al, 1995) suggests a structural role. In addition, since the Pmel 17 gene product has been suggested to promote the conversion of 5,6-dihydroxyindole-2-carboxylic acid to melanin (Chakraborty et al, 1994; Hou et al, 1994), this family has been proposed to play a role in the more distal steps of the melanin synthesis pathway than those involving the tyrosinase gene family (Kwon, 1993).

1.3. FACTORS REGULATING TRANSCRIPTION OF THE TYROSINASE GENE

Since the first report of the human (Kikuchi et al, 1989) and mouse (Yamamoto et al, 1989) tyrosinase genomic sequences, the 5′ flanking regions of the mammalian tyrosinase genes have been extensively analysed for potential regulatory elements (Klüppel et al, 1991; Ponnazhagan and Kwon, 1992; Ganss et al, 1994; Porter and Meyer, 1994; Shibata et al, 1992; Bentley et al, 1994; Yasumoto et al, 1994). However, to date, there has been very little reported on the regulation of tyrosinase gene expression in lower vertebrates. Partial genomic clones for quail, snapping turtle and frog tyrosinase have been isolated (Yamamoto et al, 1992; Miura et al, 1995) and include 519, 699 and 750 nt of 5′ flanking sequence upstream of the ATG translation start site, respectively. Preliminary analyses of the regulatory potential of these 5′ flanking sequences have only recently begun to emerge (Akiyama et al, 1994; Miura et al, 1995).

The control of tyrosinase gene expression is a complex process in which many factors have been identified to play a role. For the purposes of this review, it is necessary to dissect this complex process in order to detail both the individual cis-acting DNA elements and the trans-acting regulatory proteins involved. These factors have been grouped according to the regulatory function that they have been found to mediate. It should be made clear at the outset, however, that certain factors may have more than one regulatory function and that these groups of factors do not act in isolation. The wild-type level of tyrosinase gene expression found *in vivo* is the result of interplay between such groups of factors.

Five classes of regulatory factors may be identified. Firstly, the basal promoter elements, that is, the elements within the 5′ upstream sequence that determine the site of transcription initiation for RNA polymerase II. Secondly, the elements and DNA-binding factors that mediate tissue-specific expression: expression of the tyrosinase gene family is melanocyte-specific and thus these genes are particularly suited to studying the mechanisms underlying tissue-specific restriction of gene expression. Direct sequence comparisons of the promoter regions of these genes have revealed conserved sequences. Analyses of the regulatory potential of these 5′ flanking sequences within both pigment-producing and non-pigment-producing cell types have been carried out to establish the significance of any common elements. The factors controlling the time of onset of expression during embryonic development of the tyrosinase gene,

represent a third class. For instance, there may be specific elements that must be included in the promoter of a transgene to achieve the correct onset of expression in a transgenic mouse. Having established a minimum promoter sequence containing the elements necessary for the correct temporal and spatial expression, still further elements may be required to achieve wild-type levels of gene expression. Finally, specific elements may be required to enable modulation of tyrosinase gene expression in response to humoral factors or external stimuli that are known to influence the amounts of melanin synthesized by fully differentiated melanocytes, such as melanocyte-stimulating hormone and UV irradiation.

1.3.1. DNA sequences essential to transcription initiation of tyrosinase

To identify the transcription start site for RNA polymerase II in the mammalian tyrosinase sequence, the 5′ ends of 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 has revealed several (two to four) transcription initiation sites, with a common site positioned at 80 nt upstream from the ATG translation start site. Akiyama et al (1994) report a transcription start site for quail tyrosinase at the CACT sequence 81 nt upstream of the ATG, although they do not indicate whether the location of this site was derived empirically or by extrapolation from the data derived for the human and mouse tyrosinase genes. The 5′ ends of turtle and frog tyrosinase transcripts have not yet been determined.

It is well established that the binding of a TATA element by transcription factor TFIID serves to direct accurate transcription initiation by RNA polymerase II at a site approximately 30 bp downstream of the TATA element. TATA-like sequences (TATATA) have been identified in both the human and mouse 5′ flanking sequences at approximately 32 nt upstream of the start site at -80 (Ruppert et al, 1988; Yamamoto et al, 1989; Kikuchi et al, 1989; Giebel et al, 1991; Ponnazhagan et al, 1994), but these promoters lack a canonical TATA motif i.e. TATAAA.

It has recently become apparent from the studies of Smale and coworkers, that a transcriptional initiator (Inr) element may be used as an alternative to the TATA box in mammalian gene promoters (O′Shea-Greenfield and Smale, 1992; Javahery et al, 1994; Kaufmann and Smale, 1994). The Inr has been defined as a sequence that

overlaps the transcription initiation site and determines the promoter strength. According to a report by Javahery et al (1994) almost every functional Inr that has been described fits the highly degenerate consensus sequence PyPyAN$^{T}/_{A}$PyPy, with the nt A positioned at +1 and usually a C positioned at -1.

Interestingly, the findings in a recent report by Bentley et al (1994), suggest that in the absence of a canonical TATA box, the location of the transcription start site in the tyrosinase promoter may be determined by both the binding of transcription factors to an Inr-containing region and SP1-binding at an adjacent upstream site. The Inr region was identified as a sequence within the human tyrosinase promoter that was protected in a DNAse I footprinting assay and is conserved in the mouse, quail and turtle tyrosinase promoters. This conserved region overlaps the major transcription start site for the human and mouse tyrosinase genes and includes at its 3′ end, a sequence (from -2/+5) that fits the degenerate consensus sequence for the Inr element with a perfect match in the mouse sequence (TCACTCC), but with one mismatch in the human sequence (TCACTGT). In addition, the Inr region includes a CATGTG motif, which fits the E-box consensus, CANNTG, and a non-consensus octamer element, GTGATAAT, which overlap each other. When mutations are introduced into either element, the level of transcription driven by the promoter is drastically reduced indicating that these sequences are essential for human tyrosinase promoter function (Bentley et al, 1994). Studies have shown that the Inr E-box is recognised by two different bHLH-ZIP transcription factors (Bentley et al, 1994): USF, a ubiquitously expressed protein and the microphthalmia protein (Mi), which plays an essential role in pigment cell development (discussed in section 1.3.2.1.). The octamer element has been shown to bind the POU domain transcription factors, Oct-1 (Bentley et al, 1994) and Brn-2/N-Oct3 (Eisen et al, 1995). Mi is able to transactivate the tyrosinase gene promoter via the E-box motif (Bentley et al, 1994). Brn-2/N-Oct3, however, appears to down-regulate the tyrosinase gene promoter and furthermore can negate transactivation via the Inr E-box by Mi (Eisen et al, 1995). Interestingly, as binding at the two elements within the Inr by Brn-2/N-Oct3 and bHLH-ZIP proteins appears to be mutually exclusive, Brn-2 may achieve its inhibitory effects by displacing bHLH-ZIP proteins or preventing their binding (Eisen et al, 1995).

A non-consensus SP1-binding site (GGTGGGAGTGGT) was identified initially as a protected region using DNase I footprinting. It is situated 43 nt upstream of the Inr in the human promoter, and functions to enhance human tyrosinase gene

expression. While the SP1 motif is not conserved in other vertebrates, they show using gel mobility shift assays that sequences in the same position relative to the Inr in mouse (AGGGGAGTGGT) and quail (AGGGGGTGGGA), are able to compete with the human sequence for SP1-binding. An analogous turtle sequence (AGGTGCAGGAGT), however, is not able to prevent SP1 binding to the human sequence. Bentley et al (1994) speculate that since SP1-binding has not been shown to directly mediate tissue-specific expression from other gene promoters, it is unlikely to determine melanocyte-specific tyrosinase expression. However, they raise the possibility that in the absence of a canonical TATA box, SP1 may play a role in the basal transcription initiation of tyrosinase by facilitating the binding of TFIID to the Inr. This function of SP1 was recently demonstrated for a TATA-less gene promoter by Kaufman and Smale (1994).

Ganss et al (1994) have analysed the proximal **mouse** tyrosinase promoter region from -270/-80 nt upstream of the transcription start site. Thus the mouse sequence homologous to the human tyrosinase promoter region containing the SP1-binding site and Inr motif was not directly analysed. However, the promoter region analysed by DNase 1 footprinting extended to approximately -30 nt upstream of the transcription start site, and Ganss et al observed a protected site at -40 to -30 which is in the vicinity of the SP1-binding site (see Fig. 1.7) and overlaps with one of the non-canonical TATA boxes identified for the mouse tyrosinase sequence. Given the importance of the Inr motif in the functioning of the human tyrosinase promoter and the sequence similarity of the homologous mouse sequence to the human Inr motif (13/15 bp match), the putative mouse Inr sequence may also play a role in basal transcription of the mouse tyrosinase gene. Furthermore, the fact that similar results were obtained when testing human tyrosinase promoter sequences in both human and mouse melanoma cells suggests that Inr-binding factors must be present in the mouse cells.

Apart from testing the sequences from lower vertebrate tyrosinase genes homologous to the human tyrosinase SP1-binding site for their ability to compete for SP1-binding (Bentley et al, 1994), no further functional analyses of potential core elements in these promoters have been carried out.

1.3.2. Factors that mediate pigment cell-specific expression of the tyrosinase gene

In the quest for cis-acting elements that mediate melanocyte-specific gene expression, a comparison of the promoter regions of the tyrosinase, TRP-1 and TRP-2 genes would reveal common motifs, which given the proposed great age of the family may well have been conserved because of their functional significance. One such element has been discovered in the 5′ sequences of all the family members cloned to date. This 11 bp motif, termed the M-box by Lowings et al (1992) or p-MSE by Yamamoto et al (1992) is positioned between 40 and 160 nt upstream of the major transcription start site in the mammalian genes (Table 1.2) and slightly further upstream in the promoters analysed from lower vertebrate tyrosinase genes (quail, snapping turtle and frog). As is the case with the Inr, the M-box contains a central CATGTG motif. Functional analyses in mammals have shown the M-box to be an important component of the minimum promoter sequences required for melanocyte-specific expression of the tyrosinase gene family members.

Another element, termed a pigment cell-specific promoter, has appeared consistently in reports by Shibahara and his colleagues on their studies of the mouse and human TRP-1 and human TRP-2 promoters. This element was so named because its sequence was first detected (at positions -33/-24 nt from the transcription start point) within a minimum TRP-1 promoter (-38/+154) that was found to be sufficient to direct transcription in melanoma (and not HeLa) whole cell extracts. In each case, they have located this motif approximately -30nt upstream of the start of transcription where it overlaps with a non-canonical TATA-box. As this motif, unlike the M-box, is not strictly conserved in these promoters and has not been detected in similar comparisons by other investigators, it may possibly represent nothing more than partially conserved sequences in the vicinity of a functionally important TATA-like sequence.

In addition to computer-aided sequence comparisons, investigators have used a combination of promoter deletion and linker scanner analyses (mutagenesis), DNase I footprinting, and gel mobility shift assays to determine the ability of specific promoter sequences to function as tissue-specific mediators.

Using the above-mentioned techniques, the M-box has been independently identified by several groups as a positive regulatory element in both the mouse and human tyrosinase promoters (Bentley et al, 1994; Ganss et al, 1994;

Table 1.2.

Comparison of M-box sequences in mouse and human

-52	AGGG	**AGTCATGTGCT**	GCCT	-34	Mouse TRP-1[a]
-52	AGGG	**AATCATGTGCT**	GATC	-34	Human TRP-1[b]
-111	AGTT	**AGTCATGTGCT**	TTGC	-93	Mouse tyrosinase[c]
-108	AGTC	**AGTCATGTGCT**	TTTC	-90	Human tyrosinase[d]
-154	TTAG	**GGTCATGTGCT**	AACA	-136	Mouse TRP-2[e]
-139	TTTG	**GGTCATGTGCT**	AATG	-126	Human TRP-2[f]

Note: The M-box is in boldface. Numbering is from the start of transcription. After Budd and Jackson (1995).

[a] Jackson et al (1991); Shibahara et al (1991)

[b] Shibata et al (1992)

[c] Yamamoto et al (1989)

[d] Takeda et al (1989)

[e] Budd and Jackson (1995)

[f] Yokoyama et al (1994)

Yasumoto et al, 1994). Bentley et al, (1994) have identified five elements that regulate the **human** tyrosinase promoter extending to -300 nt upstream of the start of transcription (summarised in Fig. 1.7). Most importantly, they have shown that the ability to confer tissue specificity resides in the proximal 115 nt of this promoter sequence. In addition to the previously discussed SP1-binding site (-45/-37) and an Inr motif, this proximal region contains the M-box (-104/-94). By using linker-scanner mutation analyses, they found that mutation of the M-box in the context of the intact tyrosinase 5′ flanking sequence, resulted in a fifty-fold decrease in promoter activity.

It has long been established that as little as 270 bp of the **mouse** tyrosinase promoter is sufficient for cell type-specific expression in both cultured cells and in transgenic mice (Klüppel et al, 1991). Ganss et al (1994) analysed this promoter region (from -270/-80 nt upstream of the start of transcription) for cis-acting elements and they too identified the M-box as a positive regulatory element (see Fig. 1.7).

Interestingly, when the function of the 270 bp mouse promoter and a human promoter sequence of similar length (-209/+56 relative to the start of transcription) was assessed using transient expression analysis, the human promoter sequence was shown to be significantly weaker than the mouse promoter, despite its sequence similarity to the mouse counterpart. Furthermore, human promoter fragments of 616 bp, 906 bp or 1.8 kb, were also not able to increase reporter gene expression to the level obtained using the 270 bp mouse promoter. Only constructs containing human promoter fragments longer than 1.8 kb could mediate a significant increase in reporter gene activity and this increase was achieved specifically in pigment-producing cells. The element responsible for this tissue-specific enhancer activity was identified within a 39 bp fragment, situated approximately 1.86 kb upstream from the transcription start site in the human tyrosinase promoter (Shibata et al, 1992). This element has now been termed the tyrosinase distal element (TDE) by Yasumoto et al (1994), who have subsequently shown that the enhancer function is encoded by a 20 nt sequence that contains an E-box motif (CATGTG) at its centre. Using mutation analysis, the CATGTG motif has been shown to play a critical role in both protein-binding by the TDE and pigment cell-specificity of the enhancer activity. The homologous region in the mouse 5′ flanking sequence was unable to enhance promoter activity in melanoma cells, despite its sequence similarity (16 out of 20 nt match). This

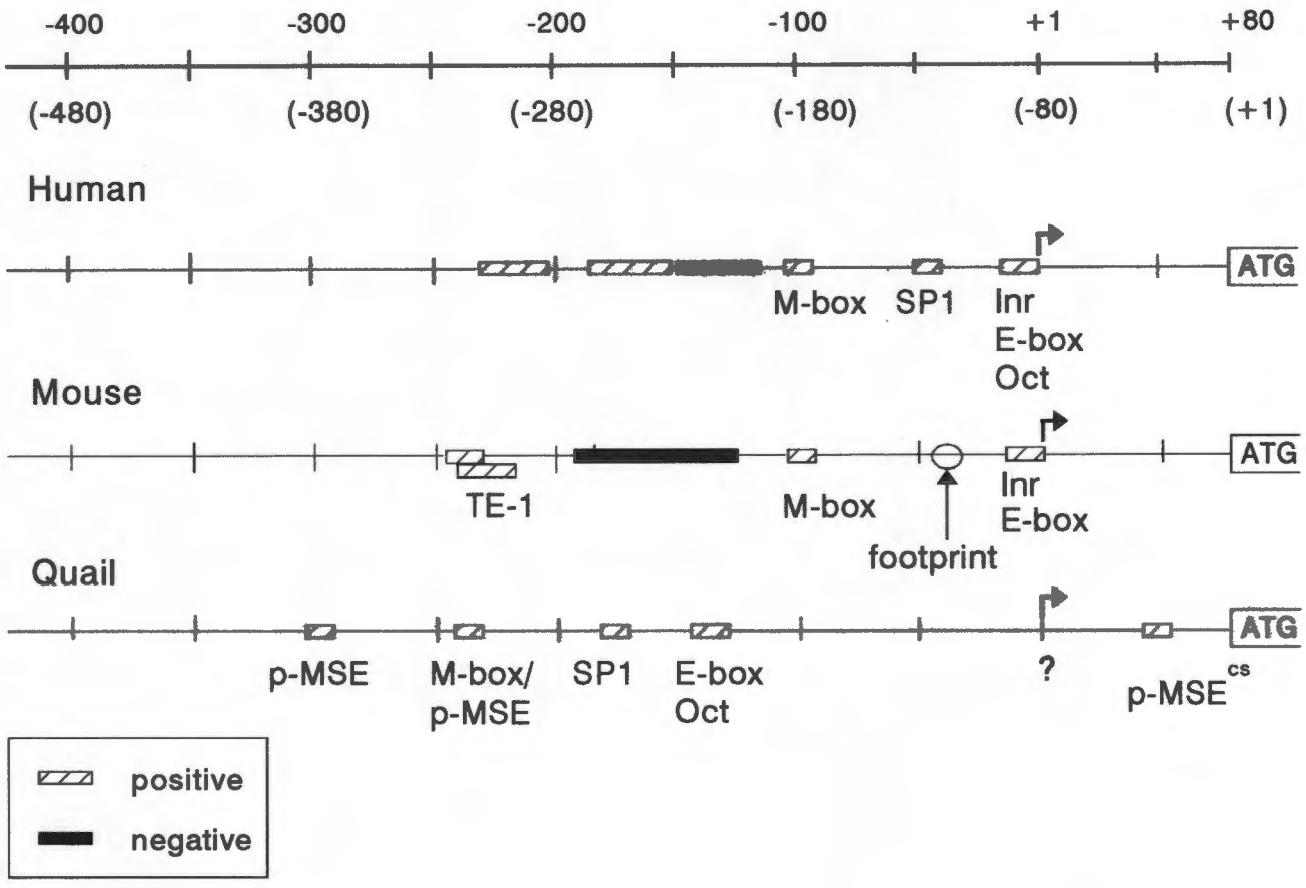

Fig. 1.7: Relative positions of regulatory elements identified in the proximal promoter regions of the human, mouse and quail tyrosinase genes. The diagram is a synopsis of results from the promoter analyses reported by Bentley et al (1994), Ganss et al (1994), Ponnazhagan and Kwon (1992), Yamamoto et al (1992) and Yasumoto et al (1995). Note that except for the SP1-binding site, the functional significance of the elements presented for the quail gene has not been tested. ^{cs}, element is present in the complementary strand.

is consistent with the fact that the mouse minimum promoter is able to activate expression of a reporter gene at a level which may be attained by the minimum human promoter only in the presence of the TDE.

The CATGTG motif is thus found within the TDE, the M-box and the Inr sequence. The relative roles of these three elements in the human tyrosinase promoter have been assessed independently by two groups: Yasumoto et al (1994) analysed the roles of the TDE and the M-box, and Bentley et al (1994) analysed the Inr and the M-box. All three elements are recognised and bound *in vitro* by the microphthalmia protein (Mi), an E-box-binding protein which is known to be involved in melanocyte differentiation. Both groups have found that the relative role of the M-box is not as significant as those of the Inr and TDE. Whilst the E-box motifs present in the Inr and the TDE are essential for the activation of tyrosinase expression by Mi, the M-box only enhances this activation and it alone is not capable of mediating tissue-specific expression of human tyrosinase. Results from a more recent report by Yasumoto et al (1995), show that a **mouse** tyrosinase promoter fragment (-82/+6) containing the Inr E-box but not the M-box, is sufficient for pigment cell-specific gene expression and for transactivation by Mi. They suggest therefore that as for the human promoter, the Inr E-box plays a more important role than the M-box in the mouse tyrosinase promoter.

Sequences similar to the M-box have been identified in the promoters of lower vertebrate tyrosinase genes (Yamamoto et al, 1992; Miura et al, 1995). The M-box was initially referred to as a proximal melanocyte-specific element (p-MSE) by Yamamoto et al (1992), who identified it as an element common to mouse tyrosinase and TRP-1 5′ flanking sequences. They then discovered sequences in the promoters of quail and turtle tyrosinase genes similar to p-MSE. More recently, Miura et al (1995) identified two p-MSE-like sequences in the frog tyrosinase promoter. These lower vertebrate homologues are not strictly conserved with the M-box over its 11 nt and some lack the central CANNTG motif. The functional significance of the quail p-MSE-like sequences has been indirectly investigated (Akiyama et al, 1994). In this report it is shown that 519 bp of the quail tyrosinase 5′ flanking sequence (i.e. the entire 5′ flanking sequence reported thus far for quail tyrosinase) is able to drive the expression of a mouse tyrosinase cDNA sequence exclusively in chicken melanocytes. According to the reports of Bentley et al (1994) and Yamamoto et al (1992), this quail promoter contains the following mammalian tyrosinase promoter homologues (see Fig. 1.7): the Inr, SP1-binding site, the M-box and two further p-MSE-like sequences. A truncated quail

promoter of 369 bp which included only one p-MSE-like sequence, the Inr, SP1-binding site, and M-box allows expression of the downstream gene in chicken melanocytes as well as in fibroblasts and hepatocytes, that is, the truncated promoter no longer directs gene expression tissue-specifically. Therefore, in contrast with the human tyrosinase promoter, a quail sequence containing the Inr, SP1-binding site and M-box, is insufficient to direct tissue-specific expression, and requires additional elements found within the adjacent 150 nt of 5′ flanking sequence, one of which could be the upstream p-MSE-like sequence. Clearly, a more detailed analysis of the quail tyrosinase promoter is required to determine the region that constitutes the minimum essential promoter.

Apart from the M-box, SP1-binding site and Inr, additional positive and negative regulatory regions have been found further upstream in the proximal promoters of the mammalian tyrosinase genes (see Fig. 1.7). For human tyrosinase, these elements are located upstream of the essential -115/+1 promoter region and would thus function to modulate the level of tyrosinase gene expression (Bentley et al, 1994).

An upstream activator element situated at nt positions -245/-230 in the mouse tyrosinase promoter, was identified by Ganss et al (1994) (see Fig. 1.7). This overlaps the position of a putative melanocyte-specific enhancer element (-240/-216) termed tyrosinase element-1 (TE-1) reported by Ponnazhagan and Kwon (1992). Interestingly, they were able to identify TE-1 by DNAse I footprinting using nuclear extracts of B16 melanoma cells, but did not detect DNA-binding at the M-box under the same conditions. TE-1 shares sequence similarity with the 39 bp human enhancer element (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). A sequence similar to TE-1 was found in the quail tyrosinase gene promoter 106 nt upstream of the ATG site (Akiyama et al, 1994), but has not been specifically tested for its ability to act as a melanocyte-specific enhancer of avian tyrosinase gene expression.

A large negative regulatory element (-193/-125) was identified in the mouse 270 bp minimum promoter (Ganss et al, 1994)(see Fig. 1.7). Interestingly, mutagenesis at various sites within this 70 bp region abolished negative regulation within melanoma cells but had no effect on expression in NIH3T3 fibroblasts. This suggests that the tyrosinase gene is under negative control in pigment-producing cells. Ganss et al suggest a purpose for this puzzling

repression: induction of expression by an external factor such as UV or α-MSH, may be mediated by removal of a repressor.

1.3.2.1. The role of the E-box motif and the microphthalmia protein in melanocyte-specific gene expression

The E-box motif, CANNTG, is the consensus sequence of the binding site of a family of transcription factors distinguished by their basic/helix-loop-helix/leucine zipper (bHLH-ZIP) structure (reviewed by López, 1995). This family includes amongst others, the proto-oncogene, *myc*, the regulators of *myc* function, Mad, Max, and Mxil, as well as USF (upstream stimulatory factor) which is an adenovirus major late transcription factor, and myoD-related factors. These proteins depend on dimerisation for DNA-binding and exert their effects as homodimers or heterodimers. Dimerisation occurs via the HLH and ZIP domains and DNA binding via the basic domains.

Given the significant role of the E-box motif in the regulatory elements identified for tyrosinase, TRP-1 and TRP-2 gene promoters, it was thought that this motif might provide a binding site for a melanocyte-specific transcription factor. A potential candidate is the product of the microphthalmia (*mi*) locus in mice, which was recently identified as a novel member of the bHLH-ZIP transcription factor family (Hodgkinson et al, 1993; Hughes et al, 1993). All mutations at the mouse *mi* locus affect melanocytes and in some cases other cell types including RPE cells, which are defective or absent, depending on the allele that is mutated. The most severe mutations result in mice that are microphthalmic, that is the eyes are poorly developed, anatomically reduced in size, and unpigmented. They are also deaf and have unpigmented coats due to a lack of melanocytes in the inner ear and skin. In addition, these mice are deficient in mast cells and have defective osteoclasts (Grüneberg, 1953; Silvers, 1979). *In situ* hybridisation analyses on sections of mouse embryos show that *mi* gene expression is restricted to several cell types including melanoblasts of the developing eye, skin and inner ear (Hodgkinson et al, 1993). Alternative splicing gives rise to two *mi* gene products, one that includes an insert of 6 amino acids (the plus form) and another isoform (the minus form) from which these amino acids are missing (Steingrímsson et al, 1994). Interestingly, the plus isoform binds DNA with 20% higher affinity than the minus form (Hemesath et al, 1994) and appears to be unique to melanocytes (Hodgkinson et al, 1993). Thus the enhanced affinity may serve a function specific to melanocytes (López, 1995).

Cloning of the cDNA sequences encoding mouse Mi and the human homologue of Mi, termed microphthalmia-associated transcription factor (MITF) (Hodgkinson et al, 1993; Hughes et al, 1993; Tachibana et al, 1994) has enabled investigation of the potential of the Mi protein to function as a melanocyte-specific transcription factor *in vitro*. Cotransfection assays demonstrated that reporter gene expression driven by either a tyrosinase, TRP-1 or an M-box-containing SV40 promoter, is increased in the presence of a Mi/MITF expression plasmid. Mutation of the M-box, Inr or TDE in the tyrosinase promoter or deletion of the M-box from the TRP-1 promoter significantly reduced this transactivation (Bentley et al, 1994; Ganss et al, 1994; Hemesath et al, 1994; Yasumoto et al, 1994; Yasumoto et al, 1995; Yavuzer et al, 1995).

Dedifferentiation and a co-ordinated down-regulation of tissue-specific genes is often associated with cellular transformation. Given the potential of the microphthalmia protein to act as a melanocyte-specific transcription factor, Yavuzer et al (1995) investigated the role of microphthalmia, if any, in the downregulation of melanocyte-specific gene transcription in melanocytes that had been transformed using the adenovirus E1A proteins. Using quantitative PCR they found that Mi expression is reduced up to 50-fold in these cells as compared with untransformed controls. This is likely to be responsible for the concomitant repression of tyrosinase and TRP-1 gene promoter activities, which is shown to be reversed in the presence of ectopic Mi expression. E1A may also disrupt Mi activity by competing with Mi for binding of the retinoblastoma protein, p105Rb. The interaction of another bHLH protein, MyoD, with p105Rb was found to be essential for its ability to transactivate other promoters and Mi has been shown to interact with p105Rb *in vitro*.

Not all experimental data to date, however, support the role of Mi as the melanocyte-specific factor that binds the E-box motifs within the tyrosinase-related gene promoters. For instance, several investigators have independently shown using gel mobility shift assays of nuclear extracts from pigmented cells, that it is USF, the ubiquitously expressed transcription factor, and not Mi that binds *in vitro* to the E-box-containing promoter elements of tyrosinase and TRP-1 (Bentley et al, 1994; Yasumoto et al, 1994; Yavuzer et al, 1994). Several theories have been put forward to account for this surprising result. Firstly, the binding specificity of E-box motifs is known to be influenced by flanking sequences and thus may restrict access to certain b-HLH-ZIP proteins *in vivo*.

Hemesath et al (1994) argue that even if ubiquitous factors such as USF are able to bind these E-box motifs *in vivo*, it is possible that their transactivation domains may not be functionally equivalent to that of Mi and this could be important for tissue-specificity. This is supported by an observation of Lowings et al (1992), who found that tissue-specific expression could be obtained only if the M-box was placed upstream from the basal transcription elements of the TRP-1 gene, but not if it was placed upstream of the thymidine kinase promoter. Alternatively, the conditions used in these assays were not sensitive enough to detect Mi DNA-binding activity, which may have been masked by binding of the more abundant USF. The only report to date of DNA-binding activity of Mi/MITF as analysed by gel mobility shift assays, is that of Yasumoto et al (1995). They used purified recombinant MITF protein (glutathione-S-transferase-MITF produced in *E. coli*) as opposed to nuclear extracts from pigmented cells. The MITF fusion protein was shown to bind the CATGTG motif of an oligonucleotide probe containing the tyrosinase M-box.

1.3.3. Are there promoter elements essential for the correct temporal patterns of tyrosinase gene expression?

The time of onset of expression of the tyrosinase gene during embryonic development has been analysed for the mouse using *in situ* hybridisation (Beermann et al, 1992; Steel et al, 1992). Pigment first appears in the retina at day 11.5, but at approximately day 16.5 in the hair follicles. Expression of the tyrosinase gene which precedes the appearance of melanin has been detected in the pigmented epithelium of the retina at embryonic day 10.5 and later in migratory melanocytes at day 14.5. Beermann et al (1992) have shown that this temporal pattern of expression may be largely reproduced in transgenic mice carrying minigenes that contain 270 bp of the proximal tyrosinase promoter sequences previously shown to be sufficient for pigment cell specific expression (see Fig. 1.8). Thus the 270 bp mouse tyrosinase promoter sequence contains the cis-acting elements that when bound by the pertinent transcription factors, function to activate gene expression in the eye and skin at the appropriate time in development. The earlier onset of expression in the eye versus the skin may be explained by one of two scenarios: either different or additional elements/DNA-binding proteins may be required in the different tissues or possibly the same factors are used, but are accessible in the eye at an earlier stage than in the skin. As the minimum promoter contains the M-box and Inr CATGTG motifs it will be interesting to see whether a transcription factor such as Mi is expressed

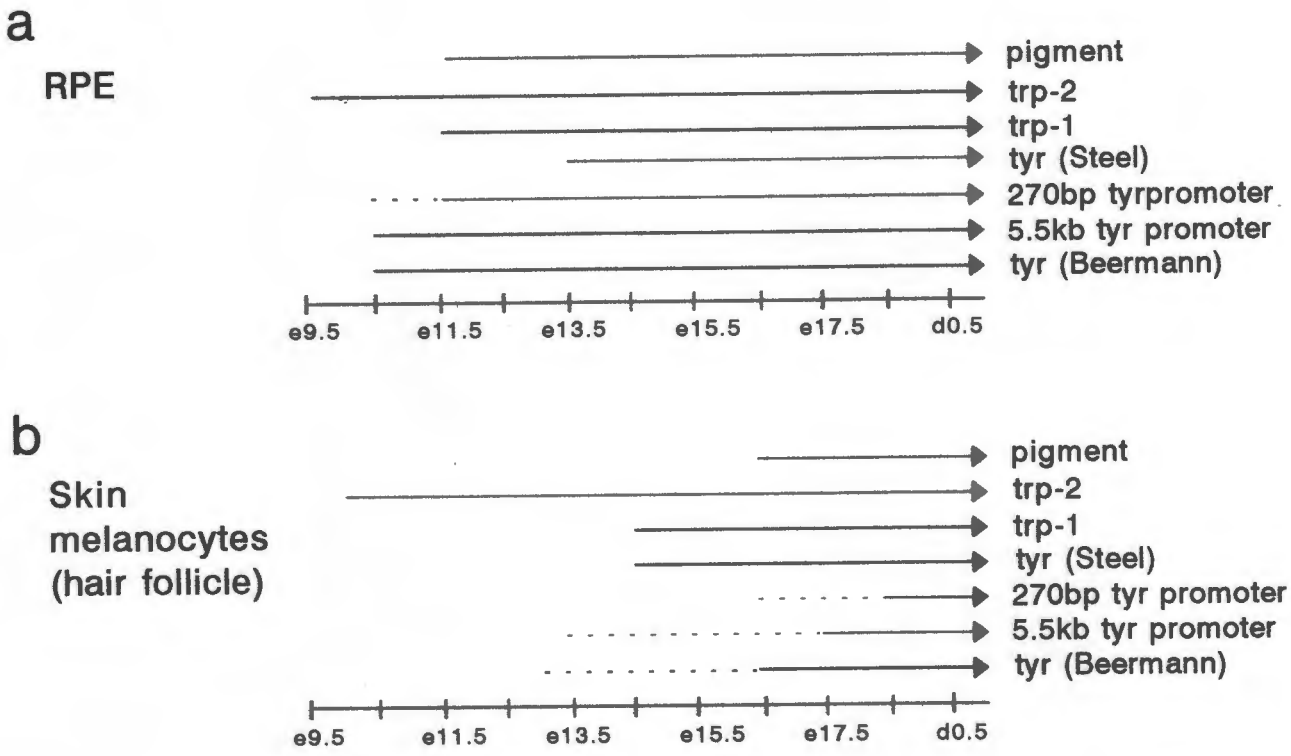

Fig. 1.8: Time of earliest expression of melanocyte-specific genes in **(a)** the retinal pigment epithelium and **(b)** melanocytes of the skin. The data shown represents the results reported by Beermann et al (1992) and Steel et al (1992). **Beermann et al** studied the expression of the tyrosinase gene (**tyr**) during embryonic development and found that the pattern of expression could be largely reproduced in mice carrying transgenes that contain either **5.5 kb** or **270 bp** of proximal tyrosinase promoter sequences. **Steel et al** determined the time of earliest expression of tyrosinase, TRP-1 and TRP-2, as well as the stage at which pigment was first detectable. Note that Steel et al failed to detect tyrosinase mRNA in the RPE at a time when pigment was detectable. They reason that this was most probably due to very few transcripts being present. The temporal pattern of expression of tyrosinase, TRP-1 and TRP-2 in the mouse is similar for RPE cells and skin melanocytes in that TRP-2 is expressed first in both tissues, followed a few days later by the activation of tyrosinase and TRP-1 expression. Days of gestation are given as e9.5, 10.5, etc. (Figure adapted from Beermann et al, 1993.)

earlier in the eye than in skin. Whether elements distinct from those mediating tissue-specific expression are required for correct temporal expression remains to be seen.

1.3.4. Elements that mediate position-independent expression of the endogenous tyrosinase gene and tyrosinase transgenes

Evidence from transgenic mouse studies has brought to light a further level of gene regulation involving elements that mediate position-independent expression of randomly integrated transgenes. A transgene is said to be position-independent when its expression is proportional to the number of copies integrated into the host genome, that is the construct contains the elements required to establish an open chromatin structure and to insulate the transgene from regulatory influences of the neighboring chromatin.

Expression of tyrosinase transgenes containing up to 5.5 kb of 5′ flanking sequences (and the elements required for correct temporal and spatial expression) has been shown to rescue the albino phenotype in transgenic mice (Beermann et al, 1990; Yokoyama et al, 1990; Tanaka et al, 1990). The transgenic mice are pigmented but the level of pigmentation varies according to the level of transgene expression, which is in turn affected by the site of integration in the host genome. The poor expression of these transgenes which failed to reach the same level as that of the endogenous tyrosinase gene, is most probably due to the absence of strong regulatory elements and/or position effects exerted by neighboring chromatin on the randomly integrated transgenes. A yeast artificial chromosome (YAC) carrying a mouse genomic fragment including 80 kb of the tyrosinase gene coding region and 155 kb of 5′ flanking sequence , however, was able to express tyrosinase at wild-type levels (Schedl et al, 1993), indicating that the regulatory elements required for full expression of the tyrosinase gene are located between 5.5 and 155 kb upstream of exon 1.

Further evidence for important regulatory elements situated far upstream in the tyrosinase 5′ flanking sequence , was reported by Porter et al (1991). They found that in mice with the chinchilla-mottled mutation, a rearrangement in the 5′ flanking sequence located more than 5 kb upstream of the tyrosinase gene causes aberrant expression of the endogenous tyrosinase gene. The rearrangement results in separation from the tyrosinase gene of a DNase I hypersensitive site and a potential nuclear matrix attachment region located at approximately -15 kb

(but subsequently recalculated to be at -12 kb by Ganss et al (1994)). Interestingly, this leads to a reduced expression of the tyrosinase gene, the genomic sequence of which is otherwise intact. Furthermore, expression is not homogeneously reduced in all melanocytes, as the coats of homozygous chinchilla-mottled mice have stripes of light and dark grey, resulting from a variable ability of different melanocyte clones to achieve the appropriate chromatin conformation at the tyrosinase locus.

To test the functional ability of the -12 kb region, a 3.6 kb fragment containing this region was linked to the smaller tyrosinase transgene constructs. It was found to both enhance expression from these constructs in transgenic mice, and to insulate the transgenes from some, but not all position effects (Ganss et al, 1994; Porter and Meyer, 1994). The DNase I hypersensitive site has been shown to be specific to pigment-producing cells. Furthermore, a 200 bp element coincides with the DNase I hypersensitive site and analysis in transient transfection assays has shown that it functions as a melanocyte-specific transcriptional enhancer (Ganss et al, 1994).

1.3.5. Cis-acting elements and trans-acting factors that mediate a transcriptional response to extracellular stimuli

A number of agents have been found to regulate melanin synthesis in mammalian melanocytes or melanoma cells *in vitro* and *in vivo* by modulating tyrosinase activity. They may act at a transcriptional level to modulate the levels of tyrosinase mRNA levels and/or may activate pre-existing pools of inactive tyrosinase, for instance at a post-transcriptional level by regulating the rate of glycosylation which is required for tyrosinase maturation (Imokawa and Mishima, 1986; Park et al, 1993).

This section is limited to a review of both those melanogenesis regulatory agents that act (directly or indirectly) to cause a change in tyrosinase mRNA levels, and the relevant cis-acting elements within the tyrosinase 5′ flanking sequence that may mediate the transcriptional response. These agents include α-MSH, a physiologically relevant effector which acts via cAMP (Hoganson et al, 1989; Kwon et al, 1988) as well as other cAMP-elevating chemicals such as forskolin, isobutylmethylxanthine (IBMX), theophylline (Fuller and Lebowitz, 1980), dibutyryl cAMP (Johnson and Pastan, 1972) and cholera toxin (Kreiner et al, 1973;

O'Keefe and Cuatrecasas, 1974), UV irradiation (Friedman and Gilchrest, 1987), retinoic acid, glucocorticoids (Ito et al, 1991) and phorbol esters such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (Fuller et al, 1990).

1.3.5.1. The stimulation of melanogenesis by cAMP-elevators

There appear to be several mechanisms by which raised intracellular levels of cAMP can increase gene expression. Three types of cis-acting elements present in gene promoters have been found to mediate the response to cAMP: the cAMP-responsive element (CRE), the AP-1-binding site and the AP-2 binding site (see Table 1.3 for consensus sequences). Usually, the cAMP response is mediated via a CRE. A transcription factor, CREB (CRE-binding protein), is activated through phosphorylation by Protein Kinase A (Karin, 1994) and then binds to a CRE. Alternatively, the AP-2 site has also been found to mediate responsiveness to cAMP, as well as to TPA and retinoic acid (Imagawa et al, 1987; Miller et al, 1995). In addition, the cAMP response effect may be mediated through binding at an AP-1 site (also known as a TPA-responsive element). Recently, Englaro et al (1995) demonstrated that the treatment of B16 mouse melanoma cells with cAMP-elevators such as forskolin, IBMX and α-MSH could stimulate melanogenesis via phosphorylation of a member of the mitogen-activated protein (MAP) kinase family. The MAP kinase was shown to translocate to the nucleus where it is known to activate several transcription factors (Pulverer et al, 1991). Englaro et al (1995) showed that with cAMP elevators, the transcription factor AP-1 is activated. AP-1 may then bind the tyrosinase gene promoter at one of the AP-1-like binding sites (see below) and thereby upregulate transcription of tyrosinase. The AP-1 transcription factor is a complex consisting of either homodimers of the jun family of proteins, or heterodimers of fos and jun components. Given that AP-1 also mediates a response to TPA distinct from cAMP-elevators, the function of AP-1 may reside in the specific combination of fos and jun components comprising the AP-1 complex. Englaro et al (1995) show that the AP-1 complex activated by cAMP-elevators consists mostly of JunD and Fra-2 (Fos-related antigen-2), whereas a TPA-activated AP-1 complex contains predominantly JunD and cFos.

Sequence analysis of the mouse and human tyrosinase promoters has revealed the presence of a number of sequences with close homology to the CRE, AP-1- and AP-2-binding sites (Giebel et al, 1991; Kikuchi et al, 1989; Ponnazhagan et al, 1994; Ponnazhagan and Kwon, 1992; Miller et al, 1995). Recently, Miller et al (1995)

showed that 0.769 kb of 5′ flanking sequences of the mouse tyrosinase gene contained the elements necessary to mediate increased expression of an interleukin-2 (IL-2) reporter cDNA sequence in cultured B16 mouse melanoma cells, in response to treatment with theophylline. This 0.769 kb mouse sequence lacks AP-2-binding sites (which are found further upstream in the 5′ flanking sequence) but contains two non-consensus CRE-like elements and a sequence similar to an AP-1 binding site (see Fig. 1.9). It is tempting to speculate that these elements may be the functional cAMP-responsive elements. However, these three putative elements are found within the minimum 270 bp mouse promoter that was shown to be unable to confer cAMP-inducibility to a CAT reporter gene when tested in B16 melanoma cells treated with forskolin (Ganss et al, 1994). Thus, the as yet unidentified elements that are required to respond to cAMP must reside between nt positions -769 and -270 upstream of the transcription start site.

1.3.5.2. The regulation of melanogenesis by retinoic acid and glucocorticoids

Reports in the literature about the melanogenic effects mediated by retinoids in human and mouse cell lines are contradictory, in that both inhibitory and stimulatory effects have been reported (summarized in Miller et al, 1995). Miller et al (1995) used the mouse tyrosinase promoter/IL-2 reporter gene system to test RA for its potential to regulate transcription from the tyrosinase promoter in B16 melanoma cells. They found that RA increases gene expression driven by the 0.769 kb mouse tyrosinase promoter in B16 cells. To account for the opposing effects of RA reported in the literature, they propose that in cell lines where melanogenesis is inhibited by RA, tyrosinase gene expression may be increased but downstream events in melanogenesis may be inhibited by RA. RA is known to mediate its effects by binding to a nuclear RA receptor (RAR) which then functions to modulate gene transcription by binding a RA-responsive element (RARE) in the 5′ flanking sequence of the gene. A RARE half-site has been identified within the 0.769 kb mouse tyrosinase promoter which may mediate the stimulatory effects of RA (Fig. 1.9).

As with RA, glucocorticoids (steroid hormones) exert their effects by binding to and activating a specific nuclear hormone receptor, which then interacts directly with a glucocorticoid response element (GRE: 5′-GGTACANNNTGTTCT) within a target gene to modulate the rate of transcription of that gene (Beato, 1989). Glucocorticoids have been found to stimulate tyrosinase activity in melanoma

Table 1.3.

Consensus sequences		
CRE	TGACGTCA	
	TGACGTAA	(Montminy et al, 1986)
AP-1	TGA[G/C]TCA	(Lee et al, 1987; Bohmann et al, 1987)
URE	TGACAACA	(Ronai and Weinstein, 1990)
AP-2	CCCCAGGC	(Imagawa et al, 1987)

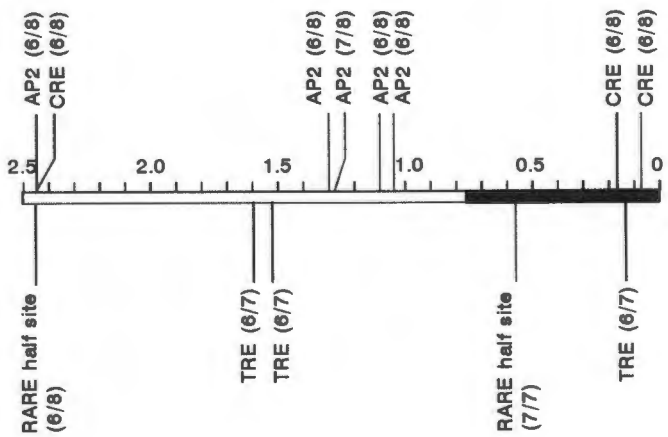

Fig. 1.9: Positions of potential responsive-elements in the human tyrosinase gene promoter sequence. A map is shown of the human tyrosinase promoter fragments tested by Miller et al (1995) for responsiveness to treatment with theophylline, retinoic acid and TPA. The fragments included a "full-length" 2496 bp promoter sequence (unshaded region) and a truncated 769 bp promoter sequence (shaded region). Note that both the full-length and the truncated sequences possess CRE, RARE and TRE motifs but only the full-length promoter has AP-2-binding sites. Consensus sequences used in the study were as follows: AP-2 = CCCCAGGC; CRE = TGACGTCA; RARE = GGTTCAC; TRE = TGAGTCA. After Miller et al (1995).

cells and when applied topically, cause repigmentation in the skin of patients with vitiligo. Ito et al (1991) suggest that this may be achieved through transcriptional control as glucocorticoid receptors have been found in mouse and human melanoma lines and the treatment of B16 melanoma cells with dexamethasone (a synthetic glucocorticoid) resulted in an increase in tyrosinase mRNA when analysed by Northern blot hybridisation. Glucocorticoids may act to increase tyrosinase gene expression directly via GRE-binding as GRE-like sequences have been identified in the 5′ flanking sequences of the mouse and human tyrosinase genes (Ito et al, 1991; Ponnazhagan and Kwon, 1992; Ponnazhagan et al, 1994). Alternatively, Ito et al (1991) propose that glucocorticoids may modulate tyrosinase gene expression indirectly by inhibiting AP-1 activity. This is based on the reports by several investigators that glucocorticoid receptors suppress AP-1 activity by direct protein-protein interaction.

1.3.5.3. The down-regulation of melanogenesis by TPA

TPA is a non-physiological agent that has been routinely used as a mitogen for the establishment of long-term cultures of mammalian and avian melanocytes (Glimelius and Weston, 1981; Sieber-Blum and Sieber, 1981; Eisinger and Marko, 1982). TPA has been found to inhibit melanogenesis partly by down-regulating tyrosinase mRNA levels (Fuller et al, 1990; Ando et al, 1995). Although TPA is a non-physiological agent it is still relevant to study the mechanism underlying TPA-induced inhibition of tyrosinase gene expression because it is generally recognised that artificial agents mimic the effects of certain (possibly unidentified) physiological effectors (Swope et al, 1995).

Two elements found in the 5′ flanking sequences of certain genes have been reported to mediate the effects of TPA: the TPA-responsive element (TRE) and the AP-2 binding sequence. The TRE was discovered to be an AP-1 binding site (Lee et al, 1987) and thus certain effects of TPA appear to be mediated via DNA-binding of AP-1 complexes. As mentioned previously (in section 1.3.5.1.), putative AP-1 and AP-2 target sequences have been identified in the mouse and human tyrosinase 5′ flanking sequences, within 2.5 kb from the ATG translation start site.

The ability of these sequences to modulate tyrosinase gene promoter activity in response to TPA has recently been tested. As has been described for cAMP and RA, Miller et al (1995) used their mouse tyrosinase promoter/IL-2 reporter gene system to establish the response to TPA of two different lengths (2.5 kb or 0.769

kb) of tyrosinase gene promoter. After treating B16 melanoma cells (that were transfected with either construct) with TPA, they observed no change or at most a slight decrease in the levels of IL-2 gene expression. Thus the AP-1 and AP-2 sites are non-responsive to TPA under these conditions. Possibly the concentrations of TPA used (2nM, 200nM and 2μM) were not optimal, as the slight decrease in the levels of IL-2 gene expression was observed only after treatment with 2nM TPA. In contrast, tyrosinase mRNA levels were markedly decreased in cells treated with 25nM TPA (Ando et al, 1995). Alternatively, functional TPA-responsive elements may positioned further upstream (i.e. more than 2.5 kb upstream of the ATG site) in the tyrosinase gene promoter.

Englaro et al (1995) have tested the ability of TPA to activate the AP-1 and AP-2 transcription factors in B16 melanoma cells. Using gel mobility shift assays with labelled oligonucleotide probes containing the AP-1 or AP-2 site, and nuclear extracts from TPA-treated cells, they showed that AP-1 is activated to bind DNA, and not AP-2. This result at least confirms the proposal by Miller et al (1995), that AP-2 sites in the tyrosinase gene promoter do not seem to mediate TPA effects.

1.3.5.4. The mechanisms involved in UVB-induced hyperpigmentation

It has long been established that one of the primary functions of melanin is protection of the organism from damage by ultraviolet light and other forms of radiation. In fact, increased melanogenesis may be considered to be part of the SOS response in skin to UV irradiation (Eller et al, 1996). Increased melanin production in response to UVB irradiation is due to an increased activity of tyrosinase and other enzymes involved in the melanogenic pathway, and an increase in melanocyte proliferation (Jimbo and Uesugi, 1982). Northen blot analysis of tyrosinase mRNA levels in UV-irradiated human melanocytes *in vivo*, and both mouse melanoma cells and normal human melanocytes in culture has confirmed that UV-induced tyrosinase activity is due in part to an increase in tyrosinase gene expression (Imokawa et al, 1995; Eller et al, 1996).

Just how UV light is able to elicit a melanogenic response has been the subject of investigation by several investigators. Reports by Gilchrest and colleagues (Gilchrest et al, 1993; Eller et al, 1994; Eller et al, 1996) have shown that UV-induced DNA damage and/or repair is an important signal in this response. Treatment of unirradiated pigment cells in culture with either DNA damaging

agents such as restriction enzymes or methyl methanesulfonate (MMS), or with thymine dinucleotides, the by-products of DNA excision repair, was sufficient to increase the melanin content and upregulate tyrosinase gene expression in these cells. UV irradiation of Cloudman S91 melanoma cells has been found to enhance their response to MSH by increasing the number of MSH receptors on their cell surfaces (Bolognia et al, 1989; Chakraborty et al, 1991). Accordingly, MMS and thymine dinucleotides also increased the response of these cells to the stimulating effects of α-MSH, further mimicking the action of UV. Thus one mechanism by which tyrosinase gene expression may ultimately be upregulated in response to UV light is via increased binding of MSH (which is secreted by epidermal keratinocytes *in vivo*, and is most probably present in the foetal calf serum which is included in culture medium) at cell surface receptors, followed presumably by elevated levels of intracellular cAMP (described in section 1.3.5.1.).

An independent study by Imokawa et al (1995) has revealed a further melanogenic factor produced by human keratinocytes when exposed to UVB irradiation. This factor, endothelin-1 (ET-1), was originally found to be a vasoconstrictor produced and secreted by endothelial cells (Yanagisawa et al, 1988). It is now known that ET-1 belongs to a family of small peptides that are ligands for the G-protein coupled heptahelical receptors, endothelin-A and -B (Rubanyi et al, 1994; Yanagisawa, 1994). ET-1 has been shown to be a mitogen for human epidermal melanocytes (Yada et al, 1991). Imokawa et al (1995) have subsequently demonstrated that ET-1 also possesses melanogenic potential. They showed that conditioned medium from keratinocytes exposed to UV light could upregulate melanin synthesis in human melanocytes in culture, but this activity was abolished when the conditioned medium was precleared using an anti-ET-1 antibody. Accordingly, the addition of exogenous ET-1 to the culture medium resulted in the stimulation of tyrosinase activity, melanin synthesis and tyrosinase mRNA synthesis. Furthermore, they provided evidence that ET-1 may well be a mediator of the UV-induced melanogenic response *in vivo*, as expression of both ET-1 and tyrosinase increases in skin after exposure to UVB irradiation. However, the mechanisms that link upregulated ET-1 ligand-receptor interaction and increased tyrosinase gene expression are yet to be established.

Another mechanism by which gene expression may be induced via UV irradiation and DNA damage implicates a DNA damage-responsive element (DDRE). Such an element has been discovered in the 5′ flanking sequence of the yeast gene encoding a DNA

repair enzyme, photolyase (Sebastian and Sancar, 1991). Under normal conditions this 39 bp element is bound by a repressor protein, photolyase regulatory protein (PRP), but as a result of UV irradiation PRP is lost from yeast cells and therefore no longer represses photolyase gene expression. Eller et al (1996) report that sequences within the mouse and human tyrosinase 5′ flanking regions (AGAAGATAAAAG; AGAGGATGAAAG) share homology with a portion of the yeast DDRE (AGGGGTGAAAG). Functional analyses of these homologous sequences must still be carried out to assess their role in UV-induced upregulation of tyrosinase expression. That tyrosinase gene expression may be upregulated by removal of a repressor is an attractive proposal, given the observation made by Ganss et al (1994) that the tyrosinase gene is under negative control in pigment-producing cells (mentioned in section 1.3.2.).

A second responsive element distinct from the DDRE, has been described (Ronai and Weinstein, 1990). This element termed the UV-responsive element (URE), was discovered as a protected sequence in the regulatory region of the polyoma virus genome, during an attempt to identify the factors from UV-irradiated host cells that enabled activation of transcription and replication of viral sequences. Interestingly, this DNase I-protected sequence 5′-TGACAACA-3′, which has since been found in the 5′ flanking sequences of several eukaryotic genes, shares homology with the consensus sequences for the CRE and AP-1 binding site (See Table 1.3). However, the use of competitive binding assays has revealed that some of the proteins that bind the URE are distinct from those that bind the CRE and AP-1 sequence. Ponnazhagan and coworkers have identified two URE-like motifs in the 5′ flanking sequence of both the mouse and human tyrosinase genes (Ponnazhagan and Kwon, 1992; Ponnazhagan et al, 1994), however, whether these motifs are functional remains to be elucidated.

1.4. AIM OF THIS STUDY

By virtue of its melanocyte-specificity, activation of tyrosinase gene expression is an ideal marker of melanocyte differentiation. The aim of the present study was to clone, sequence and characterise the promoter region of the chicken tyrosinase gene. This would provide a useful tool with which to investigate the mechanisms that underlie activation of melanocye-specific genes in precursor cells during determination of the melanocyte lineage in developing chicken embryos.

As is reflected in the literature review, very little is known about the regulatory mechanisms that control expression of the tyrosinase gene in lower vertebrates. Nevertheless, information gleaned from the extensive analyses carried out on the promoter sequences of mammalian tyrosinase genes could be used to identify potential key regulatory elements in the 5′ flanking sequences of the chicken gene. Therefore, the specific aim of this research was to use this information and methods such as primer extension and transient transfection assays, to establish the promoter regions that are required for tyrosinase gene expression in pigment-producing cells of the chicken and to determine whether there is any conservation of regulatory mechanisms between lower and higher vertebrates.[1]

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[1] Some of the results included in this thesis have been published in GENE (Ferguson and Kidson, 1996).

CHAPTER TWO

THE ISOLATION AND SEQUENCING OF CHICKEN GENOMIC DNA CLONE CTYR4.3

2.1. INTRODUCTION

The aim of the first part of this study was to isolate 5′ flanking sequences of the chicken tyrosinase gene. The most commonly used method to isolate the genomic DNA of a desired gene involves screening a genomic DNA library with a probe derived from the corresponding cDNA sequence. When this study was initiated the chicken tyrosinase cDNA sequence had not been isolated, nor had any other members of the tyrosinase gene family from lower vertebrates. Therefore, several approaches were taken concurrently to obtain a suitable probe with which to screen a chicken genomic DNA library for sequences encoding tyrosinase. This chapter presents the eventual success in cloning and sequencing a genomic DNA fragment that contains the 5′ flanking sequence of chicken tyrosinase, and touches on some of the technical problems that were experienced.

2.2 MATERIALS AND METHODS

2.2.1. Genomic DNA isolation

Genomic DNA was prepared from the livers of chick embryos (Black Australorp X New Hampshire Red breed) and newborn mice (a BALB/c albino breed, which has no gross rearrangements or deletions or insertions of DNA segments in the tyrosinase gene (Ruppert et al, 1988; Yokoyama et al, 1990)) as well as from B16 mouse melanoma cells according to a rapid DNA isolation method (Davis et al, 1986 pp 42-43). Samples were subsequently treated with 40 μg/ml of DNase-free RNase A for 30 min at 37°C, to get rid of contaminating RNA. The RNase was removed by extraction with phenol: chloroform:isoamyl alcohol. The DNA was precipitated, resuspended in TE (10 mM Tris, pH 7.6; 0.1 mM EDTA, pH 8.0) and quantified.

2.2.2. P.C.R.

2.2.2.1. Description of PCR primers

A 126 nt sequence that lies within exon 3 of tyrosinase cDNA encodes an amino acid sequence that is highly conserved between tyrosinases of different species (Müller et al, 1988). PCR primers were therefore synthesized using exon 3 sequences from mouse tyrosinase (see Fig. 2.1) as follows:

Forward primer (TSK-1): 5′-CACAATGCCTTACATATC-3′
(at nt positions +1169 to +1186)
Reverse primer (TSK-2): 5′-TCCACAAAAGCATGGTGAAG-3′
(complementary to nt positions +1244 to +1264)

The primers were synthesized by Prof. I. Parker (Dept Medical Biochemistry, UCT).

2.2.2.2. PCR amplification

A reaction mix was set up in a total volume of 25 μl as follows:

2.5	μl	10 x amplification buffer (Promega Corporation, Madison, WI, USA): 50mM KCl, 100mM Tris.HCl (pH 9), 15mM $MgCl_2$, 0.1% gelatin (w/v), 1% Triton X-100
1.25	μl	4 mM dNTP mix
2.5	μl	20 μM each primer
3.125	μl	10% formamide
2	units	Taq DNA polymerase (Promega Corporation)

Template DNA, diluted in TE buffer, was added to the mix as follows: 1.65 μg of genomic DNA (prepared as in section 2.2.1.), 0.35 μg mouse tyrosinase cDNA plasmid (positive control) or TE buffer (negative control). Reaction mixtures were overlaid with a drop of mineral oil to prevent evaporation, heated to 95°C for 5 min and subjected to 30 cycles of amplification in a Techne thermal cycler (Laboratory and Scientific Instruments, SA) programmed as follows:

Cycles 1-29	94°C for 1 min	(to denature the DNA)
	50°C for 2 min	(to anneal the primers)
	72°C for 3 min	(to extend the annealed primers)
Cycle 30	94°C for 1 min	
	50°C for 2 min	
	72°C for 12 min	(to ensure completion of the final extension step)

PCR products were resolved on 3% agarose (Sigma) gels in TBE buffer (45 mM Tris-borate, 1mM EDTA) and stained with 0.01% ethidium bromide. The plasmid pBR322 digested with *Hin*fI (pBR322/*Hin*fI) was run alongside PCR products as a molecular weight marker. Southern transfers onto nylon membranes and membrane hybridisations were carried out as described in section 2.2.4. Blots were probed using the 1.9 kb *Eco*RI fragment of mouse tyrosinase cDNA from clone pmcTYR1, radiolabelled as in section 2.2.3.

2.2.3. Preparation of mouse tyrosinase and tyrosinase-related cDNA probes

The mouse tyrosinase cDNA clones, pmcTYR1 and pMTY811C were gifts from Günther Müller (Heidelberg, Germany) and Byoung S. Kwon (Indianapolis, Indiana, USA), respectively. The mouse cDNA clone encoding TRP-1, pMT4, was a gift from Shigeki Shibahara (Sendai, Japan). A 1.9 kb EcoRI fragment from pmcTYR1, a

1.96 kb EcoRI/HindIII fragment from pMTY811C and a 1.6 kb HindIII fragment from pMT4 were gel purified. The cDNA sequences were then radiolabelled with [α-^{32}P] to a specific activity of > 1 x 10^7 cpm/μg DNA by nick translation (see Appendix I).

2.2.4. Southern blot hybridisations of genomic DNA

Genomic DNA samples were digested with a total of 2 units restriction enzyme/μg DNA at 37°C. Steps were taken to ensure even digestion of the high molecular weight DNA, as suggested by Sambrook et al (1989, pp 9.32). Digested DNA was separated on a 0.7% agarose gel in TBE buffer, run overnight at 6.5 V/cm. The DNA was denatured and transferred onto a nylon membrane (Hybond-N+, Amersham) by capillarity according to the manufacturer's instructions. Hybridisations were carried out according to Sambrook et al (1989, pp 9.52-9.55) in heat sealable plastic bags placed on a shaking platform in a shallow, heated waterbath. Blots were prehybridised for 1 h. Denatured probe was added to a final concentration of 5 x 10^5 cpm/ml and hybridisation was carried out at 60°C overnight. Post-hybridisation washes included a low stringency wash in 2 x SSC, 0.1% SDS at RT for 10 min, followed by a higher stringency wash in 0.1 x SSC, 0.1% SDS at 50°C for 10 min. Blots were then exposed to autoradiographic film (AGFA CURIX RP1) at -80°C.

2.2.5. Screening of a chicken genomic DNA library

A chicken genomic DNA library was generated from partial *Hae*III and *Alu*I digests of reticulocyte DNA, cloned into lambda Charon 4A bacteriophage (Dodgson et al, 1979). An aliquot of the library was a gift from J.D. Engel and colleagues, via Professor Albrecht E. Sippel (Heidelberg).

The titre of the bacteriophage library was checked as it had been stored at 4°C for several years and transported to South Africa at RT. The library stock originally contained 10^9 pfu/ml. The number of plaques obtained by plating a series of 10-fold dilutions of phage supernatant are presented below.

Table 2.1.

Dilution factor	No. plaques after 8h incubation
10^{-3}	completely confluent
10^{-4}	plaques just merging
10^{-5}	480
10^{-6}	40

Thus the titre, calculated to be 4.5×10^7 pfu/ml, had dropped by 100 fold in storage.

2.2.5.1. Plaque hybridisation

The method of Benton and Davis (1977) was used with modifications according to Sambrook et al (1989, pp 2.108-2.111, 2.114-2.117). To screen the genomic DNA library 4.5×10^5 pfu were plated on 40 agar plates. For each dish, plating bacteria (*E. coli* strain LE392) were infected with bacteriophage stock containing approximately 1.125×10^4 bacteriophage particles. Molten top agarose was added and the mixture was poured onto a 90-mm agar plate. After 8 h of incubation at 37°C, distinct bacteriophage plaques were detected, the outer edges of which were just touching each other. Phage DNA from each plate was transferred to a nitrocellulose membrane (Schleicher and Schuell BA85), and denatured. Membranes onto which denatured phage DNA had been fixed, were prewashed at 42°C for 1.5 h to ensure removal of all bacterial debris from the surfaces of the membranes. Hybridisation was carried out in a small plastic container placed on a shaking platform in a shallow water bath set at 42°C. The membranes were prehybridised for 1 h in a solution containing 1% dextran sulphate (Sigma), 6 x SSC, 20 mM Tris (pH 7.4), 1 x Denhardt's solution, 40% deionised formamide and 100 µg/ml denatured salmon sperm DNA. A volume of 3.25 ml of solution/membrane was used. Denatured probe was added to the container at a concentration of 5×10^5 cpm/ml of prehybridisation solution. Filters were hybridised at 42°C overnight for at least 16 h. Post hybridisation washes included four washes of 5 min each in 2 x SSC, 0.1% SDS at RT followed by one wash in 1 x SSC, 0.1% SDS at 55°C for 1 h. Damp membranes were placed between 2 layers of thin polyethylene plastic.

Autoradiography was performed at -80°C, using preflashed AGFA CURIX RP1 film with intensifying screens. To strip radioactive signals from nitrocellulose membranes, damp membranes were immersed in a boiling solution of 0.5% SDS. The solution was allowed to cool to RT. Membranes were then re-exposed to autoradiographic film to check that all radioactive signals had been removed. The process was repeated if membranes were not sufficiently stripped.

2.2.5.2. Collection of hybridising plaques and preparation of phage DNA

Agar plugs containing phage plaques corresponding to hybridisation signals were removed from petri dishes by suction into a pasteur pipette. Each plug was placed in 1 ml SM diluent (0.1M NaCl, 10mM $MgSO_4$, 1M Tris (pH 7.5), 0.01% gelatin) plus one drop of chloroform. Phage particles were allowed to diffuse from the agar into the diluent whilst standing the tube at RT for 1-2 h. For large-scale stocks of phage supernatant, plate lysate stocks of plaque-purified phage were prepared exactly according to protocol II of Sambrook et al (1989, pp 2.66).

To isolate DNA from positive bacteriophage, a combination of the methods reported by Yamamoto et al (1970) and Sambrook et al (1989, pp 2.73-2.81) was used. Details of the modified method may be found in Appendix I.

2.2.6. Subcloning of CTYR4.3 into a plasmid vector

CTYR4.3 was subcloned using a shotgun approach as follows: DNA from recombinant phage 23.1 was subjected to double digestion by *Eco*RI and *Bam*HI. This gives rise to four fragments with both BamHI and EcoRI sticky ends, including CTYR4.3, as well as 1.58 kb, 5.1 kb and 14.1 kb fragments (see the map of lambda CG23.1, Fig. 2.7.c). The mixture of restriction fragments was ligated with *Eco*RI/*Bam*HI-cut pUC19 vector DNA using T4 DNA ligase (Boehringer Mannheim). Competent *E. coli* LKIII cells were prepared according to Davis et al (1986, pp 90-92) and transformed with the heterogeneous mixture of recombinant plasmids. Plasmid DNA was prepared from transformed colonies using the mini-lysate technique (Sambrook et al, 1989, pp 1.29-1.31). To identify CTYR4.3-containing plasmids, the DNA was digested with *Eco*RI and *Bam*HI and the sizes of the released inserts estimated on agarose gels. Large

amounts of recombinant CTYR4.3-pUC19 plasmid DNA were prepared using the alkaline lysis maxi-preparation procedure and purified by centrifugation in cesium chloride-ethidium bromide gradients according to Sambrook et al (1989, pp 1.38-1.39; 1.42).

2.2.7. Sequencing strategy

2.2.7.1. Generation of a nested set of deletions with Exonuclease III

Exonuclease III digestion of CTYR4.3-pUC19 was carried out according to Sambrook et al (1989, pp 13.39-13.41) with a few modifications. The plasmid DNA was digested with restriction enzymes that generated an Exonuclease III-susceptible end adjacent to the insert DNA (*Sal*I, Promega) and a 3′ overhang to protect the vector DNA (*Sph*I, Boehringer Mannheim) (Henikoff, 1984). Double-digested plasmid was purified by extraction with phenol-chloroform and resuspended in Exonuclease III buffer. A reaction mixture containing 10μg restricted DNA in 60 μl Exonuclease III buffer was preincubated at 37°C for 5 min prior to adding 150 units Exonuclease III (Boehringer Mannheim)/pmol DNA. Aliquots of the reaction mixture (5 μl) were removed at 1 min intervals for 12 min. On removal, each aliquot was placed at 70°C for 10 min to inactivate the enzyme and then stored on ice. To remove remaining single-stranded tails, each aliquot was treated with 5 units S1 nuclease (Promega Corporation) at 30°C for 30 min after which S1 stop buffer was added and the enzyme heat-inactivated at 70°C for 10 min. The deleted plasmids were blunt-ended by treating with Klenow DNA polymerase I (Boehringer Mannheim): 20 μl of each sample were transferred to 37°C and 0.2 units of enzyme in Klenow buffer was added. After a 5 min incubation period, a dNTP mixture was added to a final concentration of 0.50 μM and incubation continued for 15 min at RT. The deletion-containing plasmids were then recircularised by blunt-end ligation using T4 DNA ligase (Boehringer Mannheim) at RT for 2 h and introduced into competent *E. coli* LKIII cells. Exonuclease III-treated pUC19-CTYR4.3 plasmid DNA was prepared from transformed LKIII colonies by minilysate preparation, digested with *Eco*RI and *Hin*dIII (to release the progressively shortened CTYR4.3 inserts from pUC19) and analysed on agarose gels.

2.2.7.2. Preparation and sequencing of double stranded DNA templates

The success of double-stranded DNA sequencing was found to be largely dependent on the quality of plasmid DNA used as template. The plasmid mini-preparation method (outlined in Appendix I) was strictly adhered to and only freshly prepared DNA templates were used for sequencing.

Dideoxy chain termination sequencing was carried out using a Sequenase version 2.0 kit (United States Biochemical). Template DNA (approximately 4-5 μg of Exonuclease III-derived plasmid) was denatured in the presence of 1M NaOH for 5 min at RT, precipitated with 4 μl of 3M sodium acetate and 100 μl isopropanol at -70°C for 10 min, and pelleted by centrifugation. Denatured DNA template was annealed to 1 pmol primer (reverse M13/pUC universal primer: 5′-GTCCTTTGTCGATACTG-3′; Promega) by incubating the mixture at 65°C for 5 min, at 37°C for 10 min and finally at RT for at least 5 min. Sequencing reactions were carried out according to the manufacturers' instructions, in the presence of 10% DMSO using 10 μCi/reaction of [α-^{35}S]-dATP (Amersham). The reaction mixes were subjected to denaturing polyacrylamide gel electrophoresis (6% acrylamide, 8M urea). The gel was transferred to Whatman 3MM paper and dried at 80°C for >1 h. Sequencing ladders were visualised after 36 h exposure to autoradiographic film.

2.2.7.3. Nucleotide sequence data analysis

Overlapping nucleotide sequences were assembled and analysed using the Genetics Computer Group Inc. (Madison, WI, USA) Sequence Analysis Software Package Version 8.0. DNA sequence alignments were obtained using the alignment search program of Altschul et al (1990). The nucleotide sequence data derived for CTYR4.3 was submitted to the international DNA sequence data collection and has been assigned the accession number L46805.

2.3. RESULTS

2.3.1. Preparation of a tyrosinase probe for screening a chicken genomic DNA library

At the time at which this study was initiated, the chicken tyrosinase cDNA sequence had not been isolated. Therefore, two approaches were taken concurrently to obtain a suitable probe with which to screen the genomic library for sequences encoding tyrosinase. Firstly, PCR was performed on chicken genomic DNA using mammalian primers to generate a chicken tyrosinase probe. Secondly, to determine whether a mouse tyrosinase cDNA sequence would cross-hybridise sufficiently to chicken tyrosinase to be used to screen the library, Southern blot hybridisations were carried out on chicken genomic DNA using mammalian tyrosinase and TRP-1 cDNA probes under various stringency conditions.

2.3.1.1. Attempts to amplify a chicken tyrosinase cDNA sequence using PCR

The information used to design PCR primers was derived from a report by Müller et al (1988). They compared the derived amino acid sequences of tyrosinases from the mouse, human, *Neurospora crassa*, *Streptomyces glaucescens* and *Streptomyces antibioticus*, and mouse tyrosinase-related protein-1, and identified two regions that are clearly conserved (see Fig. 2.1). Homologous region II (Fig. 2.1.b), the most highly conserved sequence among the tyrosinases, contains one of the two copper-binding sites found in the tyrosinase enzyme. Furthermore, deletion studies have shown that this region is essential to obtain a functional tyrosinase enzyme. The sequences used to generate the synthetic primers for PCR (TSK-1 and TSK-2) were chosen such that they fall within the cDNA sequence encoding homologous region II (indicated by horizontal arrows in Fig. 2.1.b), and would possibly anneal to an equally highly conserved chicken sequence. The primer sequences were derived directly from the mouse tyrosinase cDNA sequence, where they flank a 96 bp sequence in exon 3.

To amplify a 96 bp chicken tyrosinase gene sequence, PCR was performed using the above primers and chick genomic DNA as template. The PCR conditions outlined in section 2.2.2.2. were found empirically to give the most successful results. The parameters that were altered in order to optimise the

reaction included the concentrations of DNA templates, primers, formamide and bovine serum albumin, as well as the thermal cycler programme settings.

The PCR products obtained from a typical experiment are shown in Fig. 2.2.a. As expected, the positive control PCR reaction in which mouse tyrosinase cDNA was used as template (lane 1), resulted in a single product of 96 bp. Several products were amplified in the PCR reaction using chicken genomic DNA as template (lane 3), including a 96 bp fragment (indicated by arrow). Most alarming was that the negative control reaction from which template DNA was excluded, also gave rise to a faint product of approximately 96 bp (Fig 2.2.a, lane 6). To determine whether this unexpected product was a concatamer of primer sequences or a contaminant, the gel was Southern blotted and hybridised to the 1.9 kb pmcTYR1 mouse cDNA probe.

The resulting autoradiograph is shown in Fig. 2.2.b. In the negative control sample (lane 6) a single faint signal was detected corresponding to the 96 bp fragment. The primer-dimer products did not hybridise to the probe under the conditions, indicating that the mysterious product was not simply a concatamer of primer sequences, but a tyrosinase PCR product amplified from template DNA contaminating the negative control sample. Given that mouse tyrosinase plasmid was used as a positive control instead of mouse genomic DNA (in which tyrosinase gene sequences would be greatly diluted) and that the primers were designed using the mouse tyrosinase sequence, the most likely source of the contaminating template was the mouse plasmid DNA or "amplicons" of mouse tyrosinase PCR product in the air which may have contaminated the samples whilst being set up (Kwok and Higuchi, 1989).

It is very likely that the chicken genomic DNA sample would also have been contaminated by mouse template. The 96 bp product (Fig. 2.2.b, the hybridising band in lane 3) would therefore have included sequences amplified from both chicken and mouse tyrosinase templates. However, since the primers were mouse-specific, the contaminating mouse DNA would most probably have been favoured as a template. It should be noted that none of the additional products that were amplified in the chicken DNA sample cross-hybridised to the probe. Therefore these were most probably non-specific PCR products which may have resulted from the low annealing temperature (50°C) used to facilitate binding of the mouse primers to chicken genomic DNA.

Attempts were made to eliminate contaminating mouse DNA from both negative control reactions and those in which chicken genomic DNA templates were used. Subsequently, however, it was decided to abandon further attempts at cloning a chicken tyrosinase fragment by PCR and to proceed directly with screening a chicken genomic DNA library using mouse tyrosinase cDNA probes, as progress had been made in optimising the conditions under which a mouse probe would cross-hybridise to chicken genomic DNA on Southern blots.

2.3.1.2. Detection of chicken tyrosinase DNA on Southern blots using mouse tyrosinase cDNA probes

To determine whether a mouse tyrosinase cDNA sequence could cross-hybridise sufficiently to be used as a probe to screen a chicken genomic DNA library for tyrosinase sequences, Southern blots of chicken genomic DNA were hybridised to probes generated from different mouse cDNA clones. Several mouse melanocyte-specific cDNA sequences were assessed as probes including the tyrosinase clones pmcTYR1 (Müller et al, 1988) and pMTY811C (Kwon et al, 1988) and a clone, pMT4, encoding tyrosinase-related protein-1 (Shibahara, 1986). The pmcTYR1 cDNA probe resulted in the clearest signals and lowest levels of non-specific hybridisation (e.g. to molecular weight markers) using the low stringency conditions described below.

The mouse cDNA probes were labelled using the nick-translation method. The "multi-prime" method, an alternative that was tried, generates probe of higher specific activity, but this caused a very high level of background hybridisation under the low stringency conditions employed. To further reduce background hybridisation, nick-translated probes at a concentration of 5×10^5 cpm/ml of hybridisation buffer was used instead of the 10^6 cpm/ml advised in most protocols. Hybridisation was performed at 60°C, followed by a "high stringency" post-hybridisation wash using 0.1 x SSC at 50°C. (A high stringency wash at 68°C is usually employed for hybridisation to highly homologous DNA sequences.) Even with these low stringency conditions, autoradiographs had to be exposed to the blots for up to 8 days.

A typical autoradiograph of a Southern blot hybridised to the 1.9 kb pmcTYR1 cDNA probe can be seen in Fig 2.3.b. In all attempts only one faint band of approximately 4 kb was detectable in the chicken genomic DNA sample,

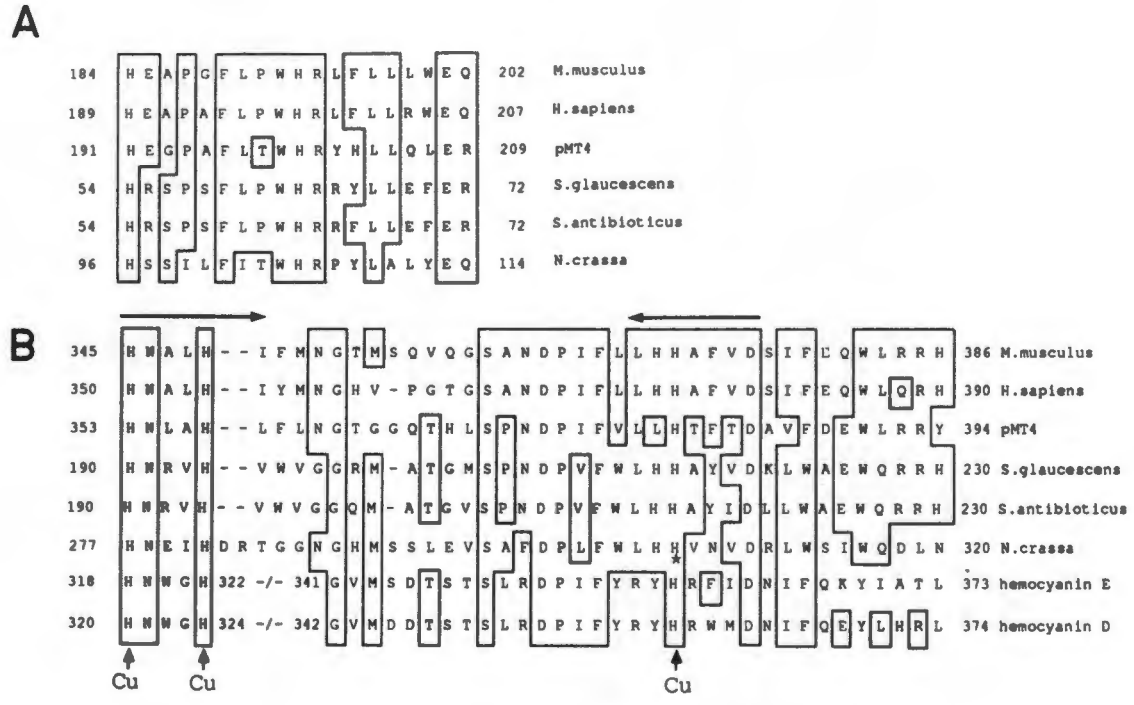


Fig. 2.1. Homologous regions between tyrosinases of different species, the TRP-1 encoded by pMT4 and haemocyanins. **(A)** Homologous region I. **(B)** Homologous region II. Conservation of amino acid sequences is shown by alignment of protein sequences of the tyrosinases of mouse, man, the tyrosinase-related protein encoded by clone pMT4, the tyrosinases of S. glaucescens, S. antibioticus, the fungus N. crassa and subunits E and D of haemocyanin of the spider E. californicum. The numbers indicate the position of the amino acids in the respective protein sequence. Sequences which are conserved by ≥50% between all proteins listed or all tyrosinases, are boxed. The amino acid sequence is given in single letter code. The histidine (H) at position 306 of N. crassa (indicated by an asterisk) has been shown to be part of the catalytic centre. One of the copper-binding sites of haemocyanin is aligned in homologous region II. The positions of the copper-binding histidines are indicated by Cu. Relative positions of the primers used for PCR in this study, are indicated by horizontal arrows. (Figure adapted from Müller et al, 1988).

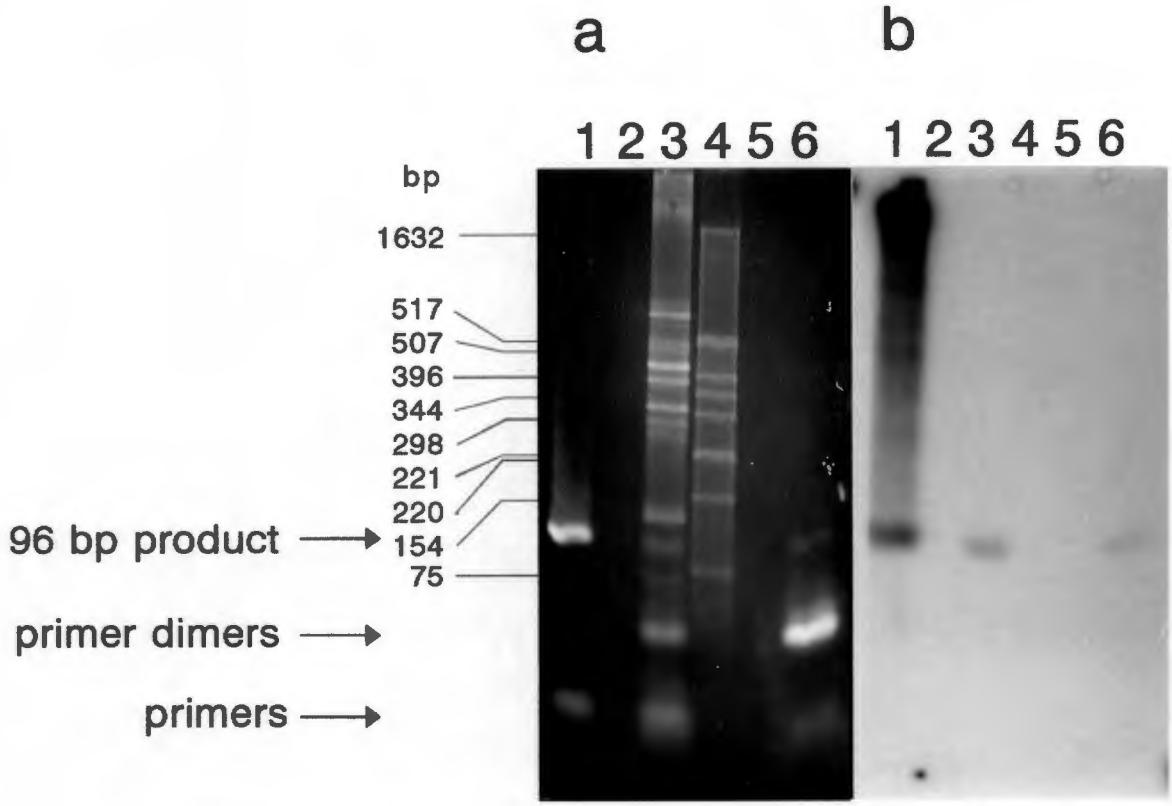


Fig. 2.2. Tyrosinase-encoding PCR products amplified from chicken and mouse DNA. **(a)** Electrophoretic analysis of PCR products amplified from mouse tyrosinase cDNA plasmid (lane 1), chicken genomic DNA (lane 3) and TE buffer (lane 6). Plasmid pBR322 digested with *Hin*fI was used as a molecular weight marker (lane 4). **(b)** Southern blot hybridisation analysis of the PCR products shown in (a). The 1.9 kb *Eco*RI fragment of mouse tyrosinase cDNA clone pmcTYR1 which was used as probe cross-hybridised only to the 96 bp PCR product.

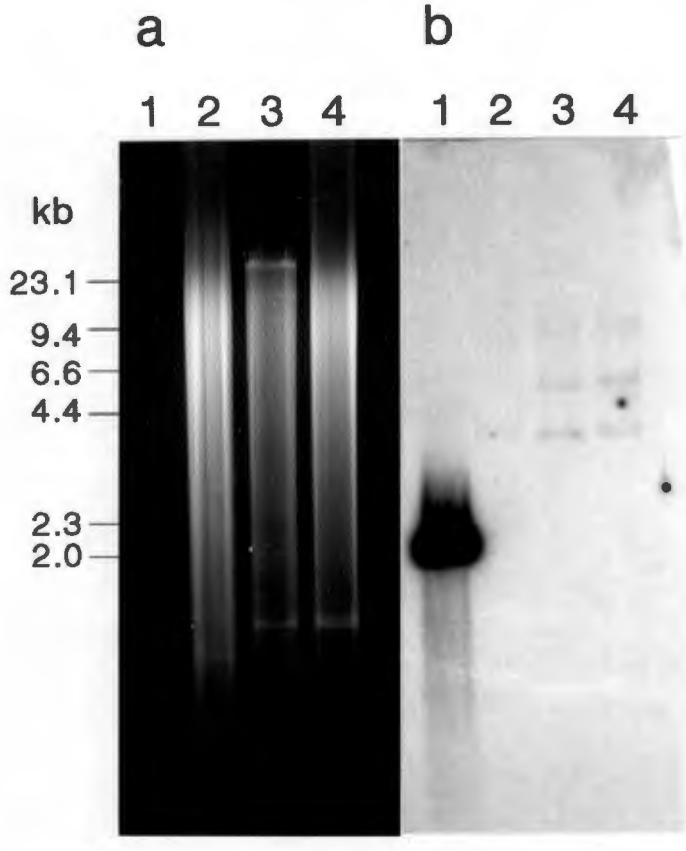


Fig. 2.3. Detection of chicken tyrosinase genomic DNA on a Southern blot hybridised to radiolabelled mouse tyrosinase cDNA probe. **(a)** 20μg of chicken genomic DNA (lane 2), B16 mouse melanoma DNA (lane 3) and BALB/c mouse DNA (lane 4) were digested with *Eco*RI and separated on a 0.7% agarose gel. 50 ng of cold pmcTYR1 sequence was used as an internal positive control for the Southern blot hybridisation procedure (lane 1, band not visible). **(b)** Autoradiograph corresponding to the gel shown in (a). The 1.9 kb *Eco*RI fragment of mouse tyrosinase cDNA clone pmcTYR1 was used as probe and the autoradiograph was developed after 7 d exposure to the Southern blot. The position of the hybridising fragment in the chicken genomic DNA sample (lane 2) corresponds with that of the smallest band in the mouse genomic DNA samples.

corresponding to the smallest of the three bands detected in the mouse genomic DNA samples (lanes 3 and 4).

Since cross-hybridisation of chicken sequences even at low stringency was very weak, it was thought that the mass screening of the genomic library might prove to be very difficult. Therefore, to improve the probability of detecting a tyrosinase-containing sequence, attempts were made to generate a sub-genomic DNA library. A fragment containing the hybridising 4 kb chicken genomic DNA band was isolated from a duplicate agarose gel, the DNA was eluted and ligated directly with a suitable plasmid vector. Competent *E. coli* cultures were transformed with the recombinants and the resulting colonies screened. However, no hybridising colonies were detected, probably due to the loss of DNA sequences during elution and ligation, and therefore incomplete representation of sequences in the mini-library.

Although this method of cloning was unsuccessful, optimal conditions were established under which the 1.9kb pmcTYR1 probe cross-hybridises to chicken genomic DNA on Southern blots and therefore it was possible to proceed with screening the chicken genomic DNA library using this heterospecific tyrosinase probe.

2.3.2. Isolation of hybridising clones from the chicken genomic DNA library

The strategy adopted in order to isolate a clone containing exon 1 and 5′ flanking sequences of tyrosinase, involved two rounds of screening. A primary screening was conducted using the full-length 1.9 kb mouse pmcTYR1 cDNA sequence as probe to identify library phage recombinants that contain tyrosinase exonic sequences. The membranes on which hybridising recombinants were identified, were stripped and reprobed in a second round of screening with a shortened probe. The probe used in this case was the 0.57kb *Eco*RI-*Hin*cII fragment of mouse pmcTYR1 cDNA, which includes only a part of the sequence comprising exon 1 plus approximately 100 bp of 5′ flanking sequence. Both probes are shown in Fig. 2.4. It was hoped that the recombinant phage DNA identified in the second round of screening would include sufficient 5′ flanking sequence to enable the characterisation of the minimal tyrosinase promoter. The results are discussed in detail below.

To screen a library of mammalian DNA (genome complexity 3 x 10^9 bp), several hundred thousand recombinant plaques should be examined (Sambrook et al, 1989). Therefore approximately 4.5 x 10^5 pfu were plated over 40 plates at a density of 1.125 x 10^4 pfu/plate. Phage DNA was transferred to nitrocellulose membranes and hybridised to the 1.9 kb full-length pmcTYR1 mouse cDNA probe using the procedure optimised for Southern blot hybridisation (section 2.3.1.2.) with a few modifications. To prolong the life of the nitrocellulose membranes, hybridisation was performed at 42°C in the presence of formamide. A post-hybridisation high stringency wash was carried out for 1 h (Sambrook et al, 1989) at 55°C using 1 x SSC.

Using these hybridisation conditions, a total of six strongly positive signals were obtained as well as smaller, fainter positive signals (see Fig. 2.5). Large agarose plugs including 4 or 5 plaques were removed from the hybridising areas of the plates and stored at 4°C for further analysis. The six membranes each containing a strongly positive plaque, were stripped and rescreened using the 0.57 kb 5′ mouse tyrosinase cDNA probe. Positive signals appeared in the same positions on the membranes as the signals obtained using the full length 1.9 kb probe.

Since the two strongest signals were found on membranes 11 and 23 in both rounds of screening, the hybridising phage were eluted from the agar plugs that were picked from plates 11 and 23, and plaque-purified.

2.3.3. Characterisation of hybridising library clones

DNA was extracted from two pure bacteriophage clones, termed lambdaCG11.1 and lambdaCG23.1, and analysed by restriction enzyme digestion. As can be seen in Fig. 2.6.a, the patterns of restriction fragments obtained for clones 11.1 and 23.1 indicate that the genomic DNA inserts are identical. The inserts were released from the Charon 4A vector by *Eco*RI digestion (lanes 1 and 2). The left and right arms of the Charon 4A vector are 19.6 kb and 11 kb, respectively. The remaining two fragments of approximately 9.3 kb and 7.5 kb in size, represent a chicken genomic DNA insert of 16.8 kb, which falls within the size range of fragments (15-21 kb) used to generate the library (Dodgson et al, 1979). Southern blot hybridisation shows that only the 9.3 kb *Eco*RI

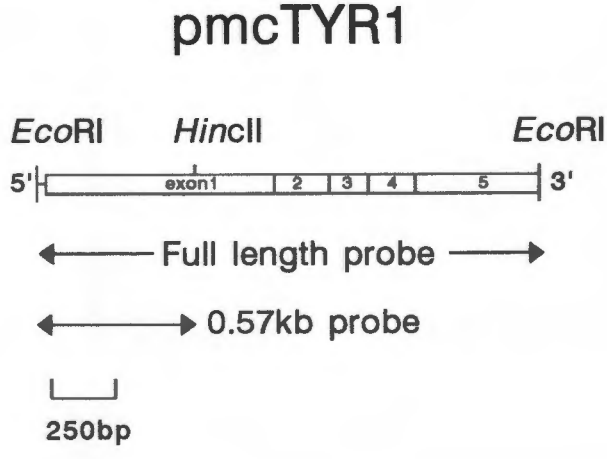


Fig. 2.4: The mouse tyrosinase cDNA sequences from pmcTYR1 (Müller et al, 1988) that were used as probes to screen the chicken genomic DNA library. The 1.9 kb *Eco*RI fragment of pmcTYR1 containing the full-length tyrosinase cDNA was used to probe for library recombinants containing chicken tyrosinase gene exons. The 0.57 kb *Eco*RI/ *Hinc*II fragment of pmcTYR1 was used to detect tyrosinase sequences comprising exon 1 and the adjacent 5′ flanking region.

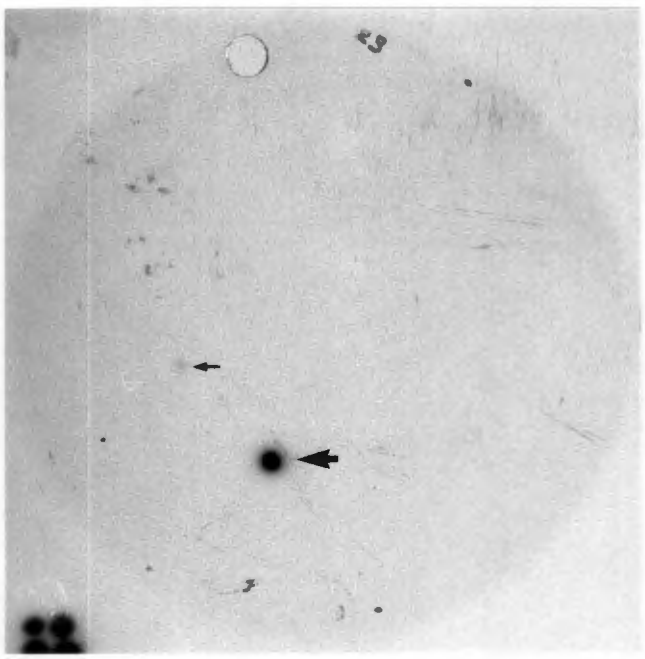


Fig. 2.5: Hybridisation signals resulting from plaque blot number 23 of the genomic DNA library probed with the 1.9 kb EcoRI pmcTYR1 fragment. A strong signal which represents the phage recombinant lambdaCG23.1, is indicated by the big arrow. A smaller, fainter positive signal is shown by a small arrow.

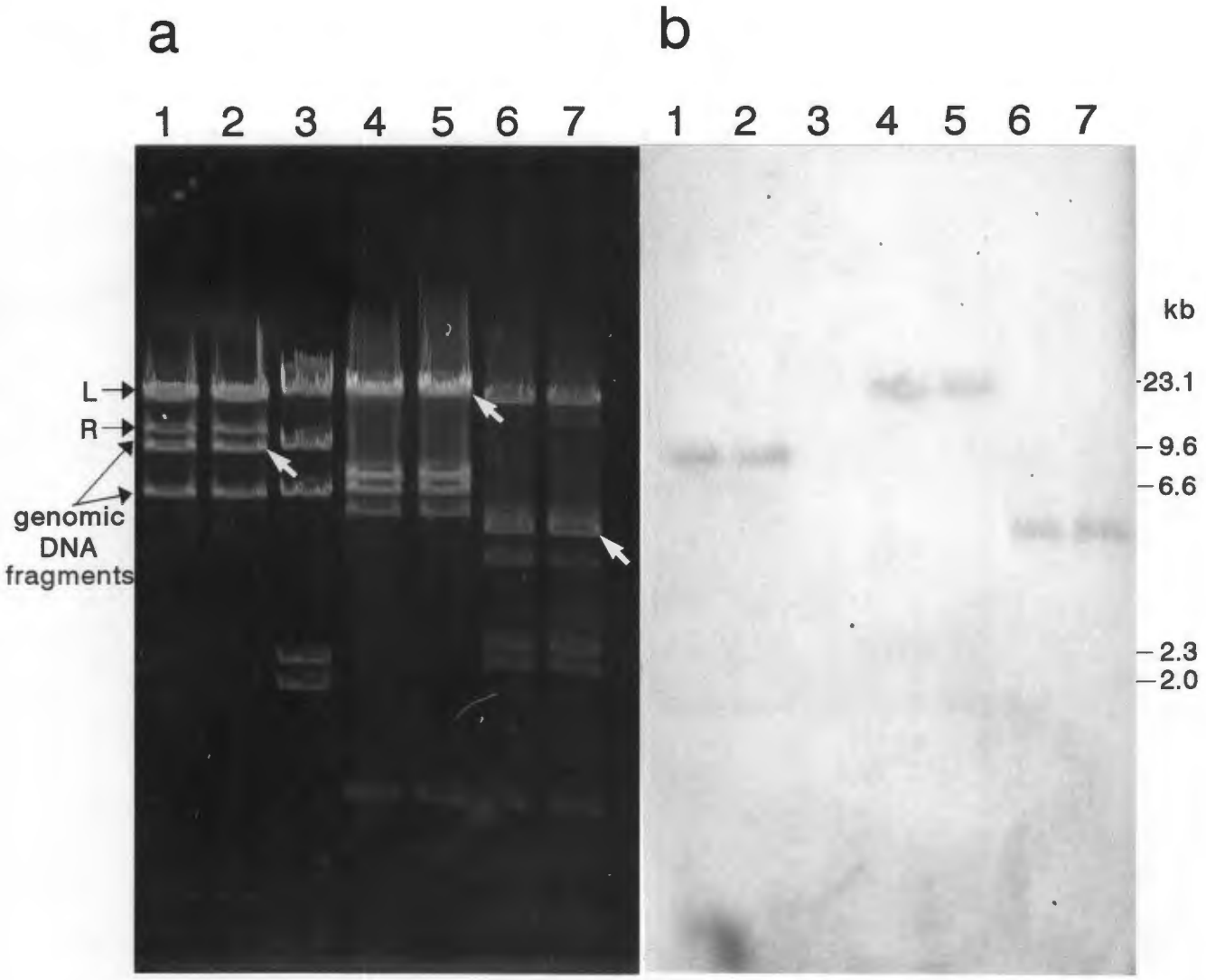


Fig. 2.6. Restriction enzyme analysis of phage DNA extracted from hybridising library recombinants, lambdaCG11.1 and lambdaCG23.1. **(a)** Electrophoretic analysis of the digested phage DNA. 10μg of DNA from each sample were digested with either *Eco*RI (lanes 1 and 2), *Hin*dIII (lanes 4 and 5) or both *Eco*RI and *Hin*dIII (lanes 6 and 7). LambdaCG11.1 DNA is in lanes 1, 4 and 6. LambdaCG23.1 DNA is in lanes 2, 5 and 7. Lambda DNA cut with *Hin*dIII was used as a molecular weight marker (lane 3). The fragments that subsequently cross-hybridised to the mouse tyrosinase probe are indicated by arrows. L, left arm; R, right arm of lambda Charon 4A vector. **(b)** Southern blot hybridisation analysis of the digested phage DNA shown in (a). The probe used was the 0.57 kb *Eco*RI-*Hin*cII fragment of pmcTYR1. Data derived from this analysis was used to construct the partial restriction enzyme map of lambdaCG11.1 and 23.1, shown in Fig. 2.7.c.[1]

[1] Details used for restriction mapping are as follows: digestion of phage DNA with HindIII results in a large hybridising fragment of approximately 25.2 kb, comprising both the entire left arm of the vector plus a portion of the genomic DNA insert (lanes 4 and 5). Since only the 9.3 kb EcoRI fragment hybridises to the 0.57 kb tyrosinase probe (lanes 1 and 2), it should be positioned on the left, so that it overlaps with the hybridising 25.2 kb HindIII fragment. This was confirmed by double digestion of the phage DNA with EcoRI and HindIII. EcoRI cleaves the 25.2 kb HindIII fragment, such that it releases the left arm of the vector (19.6 kb) leaving a single hybridising EcoRI-HindIII fragment of 5.6 kb in size (lanes 6 and 7).

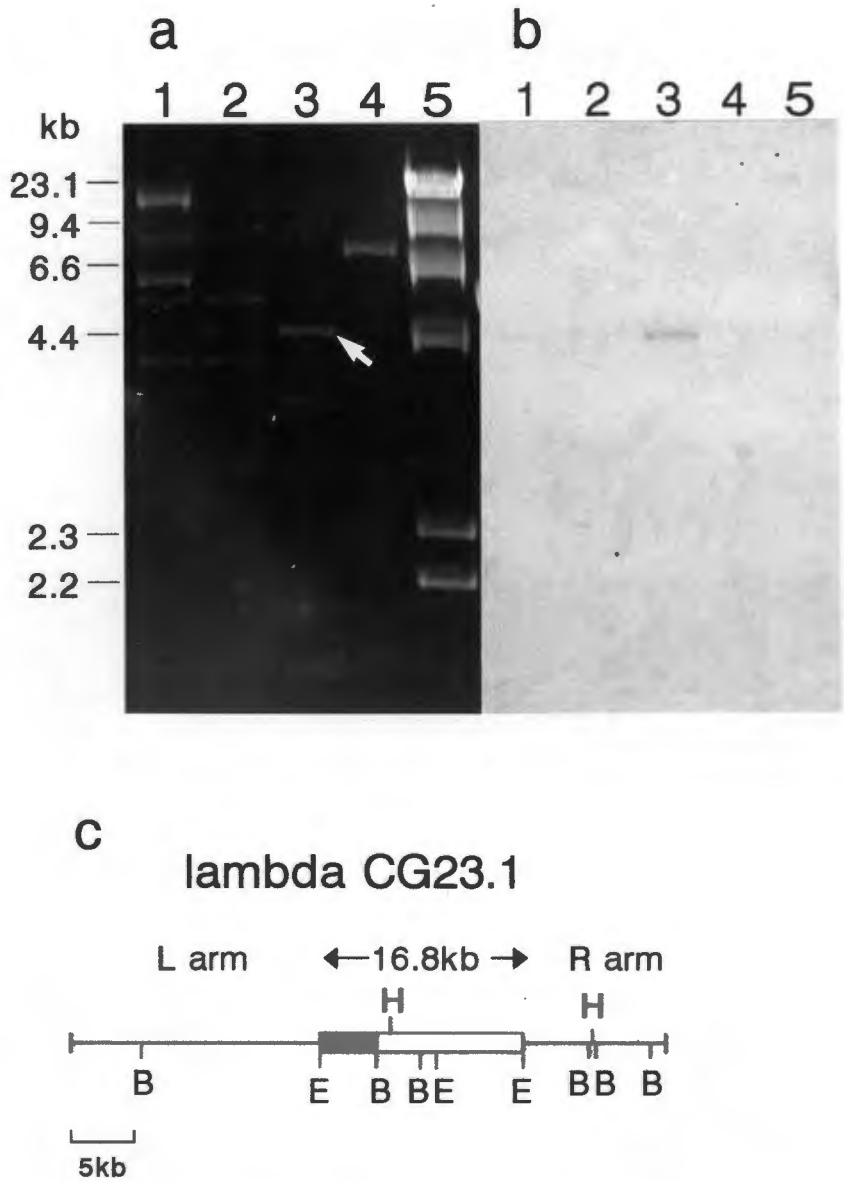


Fig. 2.7.: Restriction mapping of the genomic clone lambdaCG23.1. The four *Eco*RI restriction fragments (shown in Fig. 2.6.a) were excised from a gel, electro-eluted and each fragment subjected to digestion by *Bam*HI. **(a)** Electrophoretic analysis of the resulting fragments from the 19.6 kb L arm of the vector (lane 1), the 11 kb R arm of the vector (lane 2), the 9.3 kb hybridising *Eco*RI fragment of the genomic DNA insert (lane 3) and the 7.5 kb non-hybridising *Eco*RI fragment of genomic DNA insert (lane 4). Lambda DNA cut with *Hin*dIII was used as a molecular weight marker (lane 5). The 4.3 kb fragment that subsequently cross-hybridised to the mouse tyrosinase probe is indicated by an arrow. **(b)** Southern blot hybridisation analysis of the digested phage DNA shown in (a). The probe used was the 0.57 kb *Eco*RI-*Hin*cII fragment of pmcTYR1. **(c)** Partial restriction enzyme map of lambdaCG23.1. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII.[2]

[2] Mapping details are as follows: The 7.5 kb EcoRI fragment remained intact (lane 4). The 9.3 kb fragment was cleaved into three smaller pieces of approximately 4.3 kb, 3.4 kb and 1.6 kb (lane 3). Since only the 4.3 kb fragment cross-hybridises to the mouse cDNA probe, it must be positioned on the 5′ end of the chicken genomic DNA insert such that it also forms part of the following previously identified hybridising fragments: the 9.3 kb EcoRI, the 25.2 kb HindIII and 5.6 kb EcoRI-HindIII fragments. The two remaining fragments that make up the 9.3 kb EcoRI fragment i.e. the 3.4 kb and 1.6 kb fragments, represent BamHI-BamHI and BamHI-EcoRI fragments, respectively. The sizes of the fragments derived from the L (14.1 kb and 5.5kb) and R arms (5.12 kb, 3.93 kb, 1.49 kb and 0.5kb) of the vector agree with the sizes predicted from the Charon 4A map (shown in Appendix II).

fragment of the genomic DNA insert cross-hybridised to the 0.57 kb 5′ mouse probe (Fig. 2.6.b., lanes 1 and 2).

The position of the hybridising sequences within the 16.8 kb genomic DNA insert was determined by restriction enzyme mapping using *Eco*RI, *Bam*HI and *Hin*dIII, followed by Southern blot hybridisation to identify which of the restriction enzyme fragments contain sequences that cross-hybridise to the mouse pmcTYR1 probe (see Figs 2.6 and 2.7, detailed explanations in figure legends). Analysis of the fragments derived by double digestion with *Eco*RI and *Bam*HI revealed a single hybridising fragment of 4.3 kb (shown by an arrowhead in Fig. 2.7.a and b, lane 3). A map of lambdaCG23.1 is shown in Fig. 2.7.c. The shaded box in this diagram represents the 4.3 kb *Eco*RI-*Bam*HI fragment which was mapped to the extreme 5′ end of the 16.8 kb genomic DNA insert. It was hoped that this fragment, termed CTYR4.3, would contain sufficient 5′ flanking sequence to characterise the tyrosinase gene promoter.

2.3.4. Analysis of the chicken genomic DNA subclone, CTYR4.3.

At this stage, all that was known about CTYR4.3 was simply that it cross-hybridised to a 0.57 kb pmcTYR1 cDNA probe containing exon 1. No sequences encoding tyrosinase or the TRPs had yet been reported for a lower vertebrate and therefore it was important to sequence CTYR4.3 to establish whether the sequence represented chicken tyrosinase or a cross-hybridising chicken TRP gene sequence. Assuming that CTYR4.3 did encode chicken tyrosinase and that the first exon was of a similar size to exon 1 of the mammalian tyrosinase gene (0.9-0.95 kb), it was necessary to determine whether the remaining 3.4 kb of CTYR4.3 represented 5′ flanking sequence and/or intronic sequences. Because the genomic DNA library was generated in a non-directed manner, the 5′-3′ orientation of these sequences within CTYR4.3 was unknown.

CTYR4.3 was therefore subcloned into a pUC19 plasmid vector for further characterisation by restriction mapping and sequence analysis.

2.3.4.1. Determining the orientation and a partial restriction map of CTYR4.3

A map of CTYR4.3 is shown in Fig.2.8.b. During the process of restriction enzyme mapping, Mochii et al (1992) reported the sequence of a chicken

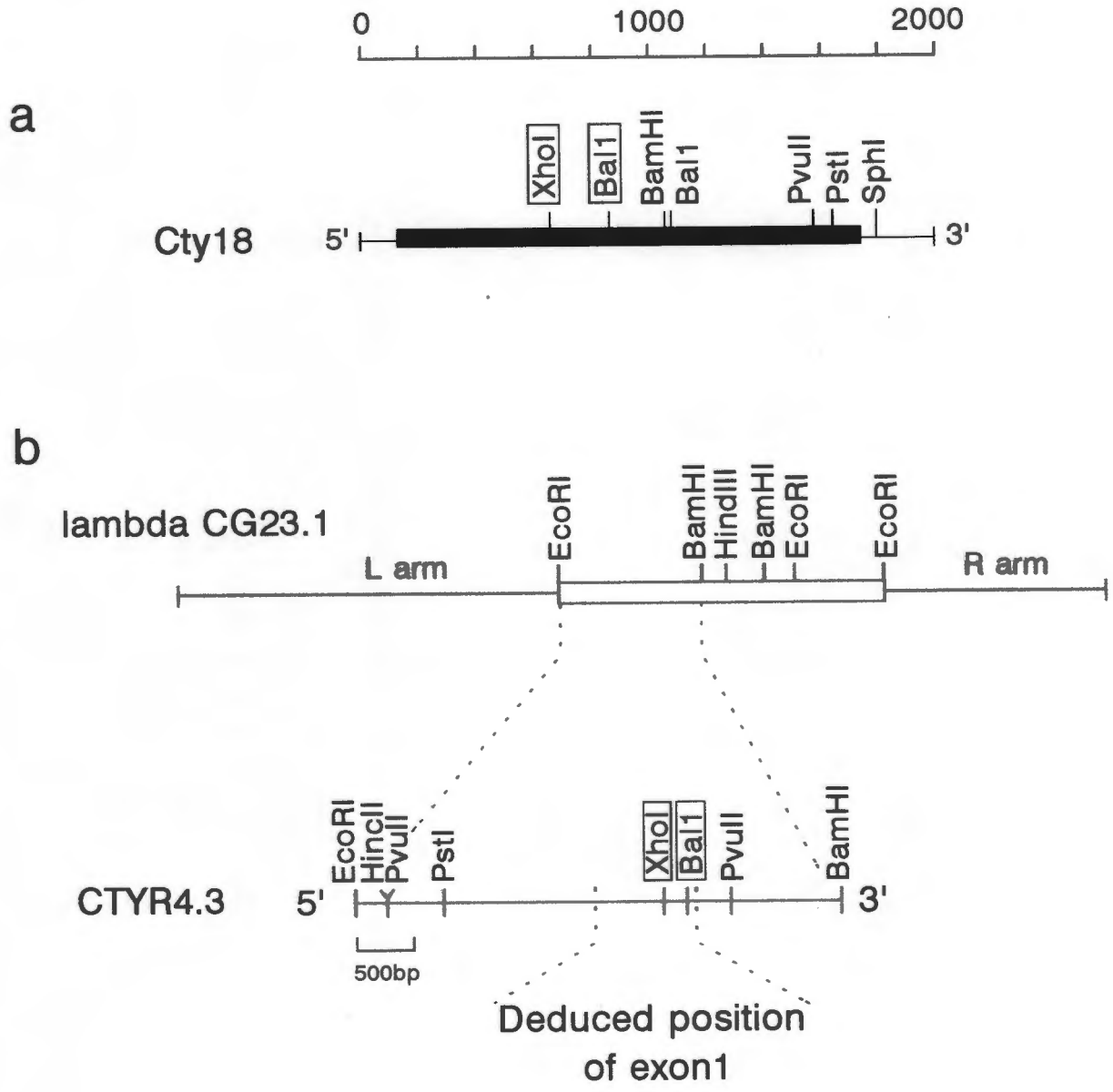


Fig. 2.8: Comparison of the partial restriction enzyme maps of **(a)** Cty18 and **(b)** CTYR4.3. The position of the *Xho*I and *Bal*I sites (which are boxed) in Cty18 made it possible to determine the 5′-3′ orientation of the genomic DNA sequences in CTYR4.3.

tyrosinase cDNA clone, which they named Cty18. A partial restriction map of Cty18 (Fig. 2.8.a) indicated the presence of recognition sites for *Xho*I and *Bal*I at nt positions +660 and +870 respectively. Assuming that exon 1 sequence of chicken tyrosinase approximates the mammalian exon 1 of 0.9 kb in size, it was deduced that these sites should fall within exon 1, and thus should be present in CTYR4.3. Mapping revealed the presence of single *Xho*I and *Bal*I sites in CTYR4.3 approximately 200 bp apart. The position of the *Xho*I site was found to be more proximal to the *Eco*RI terminus of CTYR4.3 than the *Bal*I site.

These results proved almost conclusively that CTYR4.3 contains chicken tyrosinase genomic DNA and furthermore, enabled the orientation of the genomic components within CTYR4.3. Exon 1 is flanked by promoter sequences at the *Eco*RI terminus and intron 1 sequences at the *Bam*HI terminus of CTYR4.3. The position within CTYR4.3 of additional restriction sites that were verified by similar mapping procedures, are also indicated.

2.3.4.2. Generation of a nested set of CTYR4.3 deletions for sequencing

A directed sequencing approach was adopted for CTYR4.3 which involved generating an overlapping set of deletions using Exonuclease III. A resulting set of CTYR4.3 deletions ranging in size from 3.5 kb to 0.25 kb is shown in Fig. 2.9. Each successive time point yielded a collection of subclones containing deletions extending further into the original CTYR4.3 insert.

It was hoped that the resulting deletion clones could subsequently be used for promoter analysis to identify functional domains within the 5′ flanking region. By virtue of its position within the *Eco*RI and *Bam*HI sites of the pUC19 vector (see Appendix II), CTYR4.3 could only be manipulated from its *Bam*HI terminus, adjacent to which 7 unique restriction enzyme recognition sites were available to produce Exonuclease III-resistant and -susceptible ends. As can be seen in Fig 2.10 this resulted in Exonuclease III derivatives that were progressively deleted from their 3′ ends. While these constructs enabled sequencing of CTYR4.3 in one direction, they have limited use for functional analyses of the promoter.

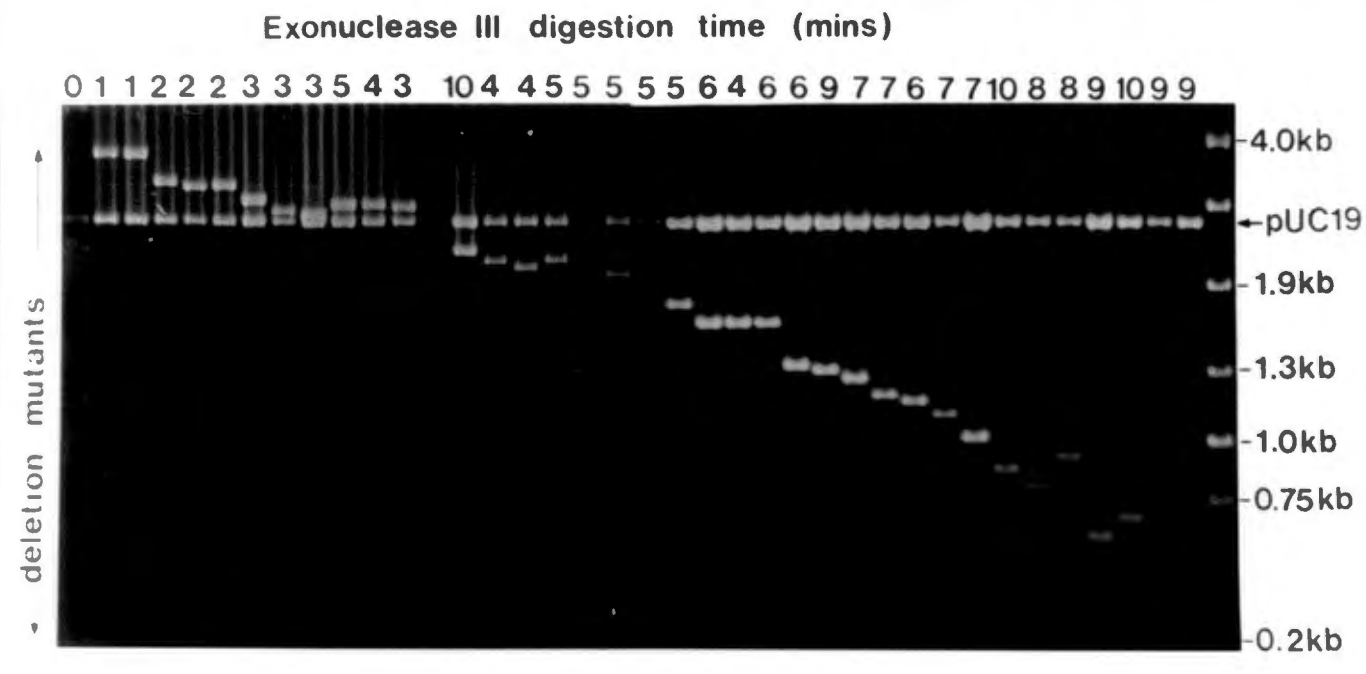


Fig. 2.9: Electrophoretic analysis of CTYR4.3 fragments derived by progressive Exonuclease III digestion.

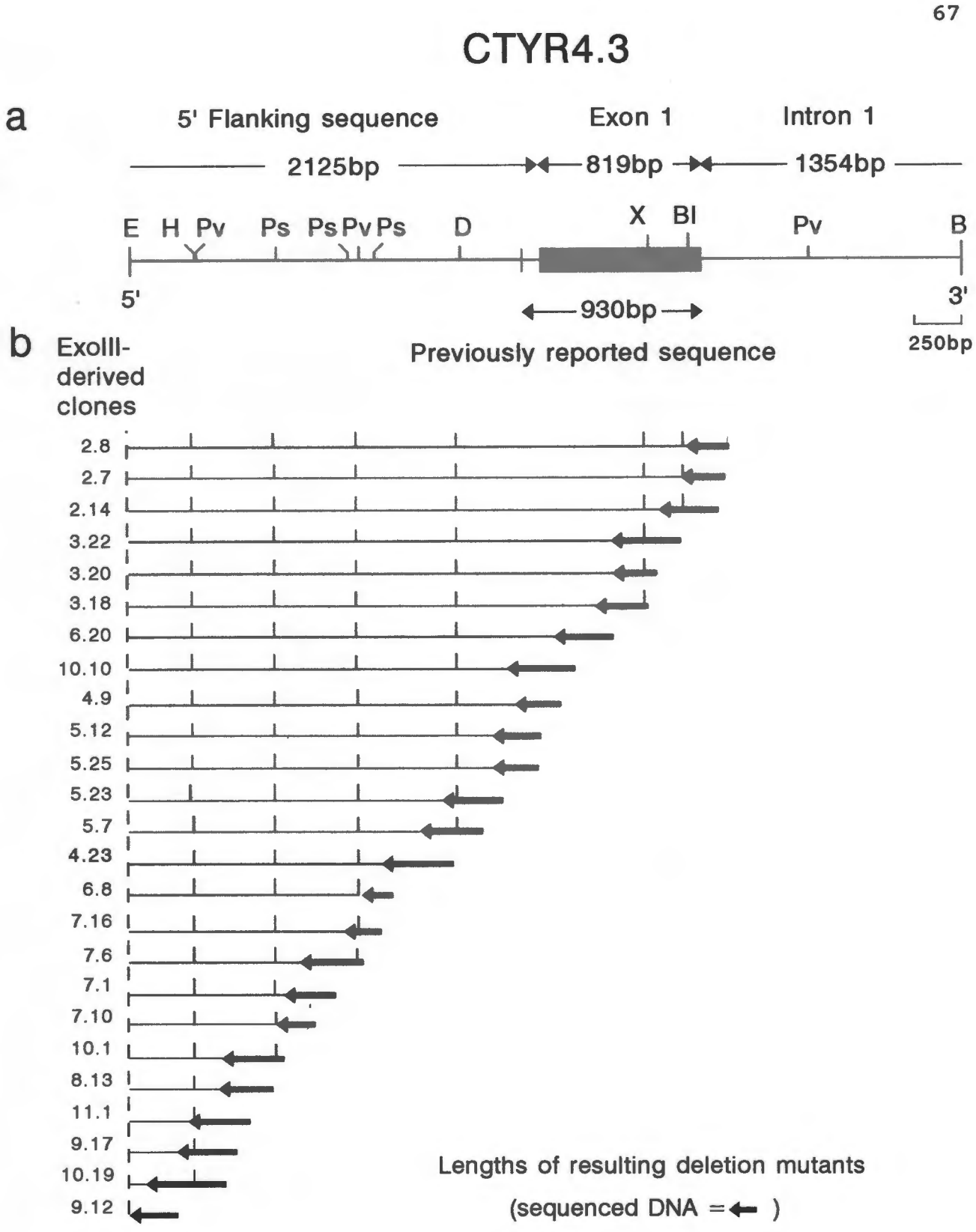


Fig. 2.10: Strategy showing the sequencing of CTYR4.3 from the 3′ end using overlapping Exonuclease III-derived clones. Restriction maps of **(a)** CTYR4.3 and **(b)** overlapping deletion mutants are included. E, *Eco*RI; H, *Hinc*II; Pv, *Pvu*II; Ps, *Pst*I; D, *Dra*I; X, *Xho*I; Bl, *Bal*I; B, *Bam*HI.

Exonuclease III derivatives of CTYR4.3 were used as templates for double stranded sequencing by the dideoxy chain termination method of Sanger (1977). Overlapping sequences derived from twenty-five deletion clones (see Fig. 2.10) were used to assemble the 3122 nt sequence which is shown in Fig. 2.11.

2.3.4.3. Nucleotide sequence of CTYR4.3 and deduced amino acid sequence of exon 1.

The nucleotide sequence of CTYR4.3 provides conclusive evidence that this genomic DNA clone contains sequences that encode chicken tyrosinase and not one of the closely related TRPs. A comparison of CTYR4.3 with Cty18 (Mochii et al, 1992) in the region that they overlap (930 bp shown in Fig. 2.10), showed that these sequences are 100% homologous. Initially, however, the sequence derived for CTYR4.3 differed from Cty18 at two positions in exon 1 (see Fig. 2.12.a). Three nucleotides were missing from CTYR4.3 at these sites, which would have given rise to a substantially altered amino acid sequence, causing a frameshift and the loss of an amino acid. It was subsequently found that this deviation was due to compressions in the sequencing ladder that were not fully resolved during synthesis and electrophoresis. The deviating region was resequenced substituting dITP and ddITP for dGTP and ddGTP, analogues that destabilize the DNA secondary structure associated with dC and dG residues (Barnes et al, 1983; Gough and Murray, 1983), and the sequence was found to be identical to Cty18 (see Fig. 2.12.b). Although it would be difficult to detect errors in the 5′ flanking sequence, the physical manipulations of CTYR4.3 based on sequence data, have shown it to be accurate thus far. For instance, the positions of restriction enzyme recognition sites identified within the nt sequence using the MAP function of GCG, have been shown to be authentic for all enzymes that were physically tested.

The ATG codon placed at nt position +1 in Fig. 2.11 is the presumed start codon of exon 1 based on the alignment of CTYR4.3 with the sequences reported for the chick, quail, snapping turtle and human tyrosinase. To find the 3′ boundary of exon 1, the genomic sequence of CTYR4.3 was compared to the published cDNA sequence (Mochii et al, 1992). At nt position +820 (nt position +927 in the cDNA), homology ceases. Nucleotides +817 CAG:GTAAG +824 fit the donor splice site consensus sequence MAG:GTRAGT, where M=A/C, R= A/G, and the colon denotes the site of cleavage (Breathnach and Chambon, 1981).

Thus, exon 1 ends at nt +819, and encodes the first 273 amino acids of chicken tyrosinase. The nt sequence derived for CTYR4.3 therefore includes 2125 nt of the promoter region, 819 nt of exon 1 and 178 nt (of the approximately 1345 nt included in CTYR4.3) of intron 1. A detailed sequence analysis of the 5′ flanking upstream sequence will be presented in chapter 3.

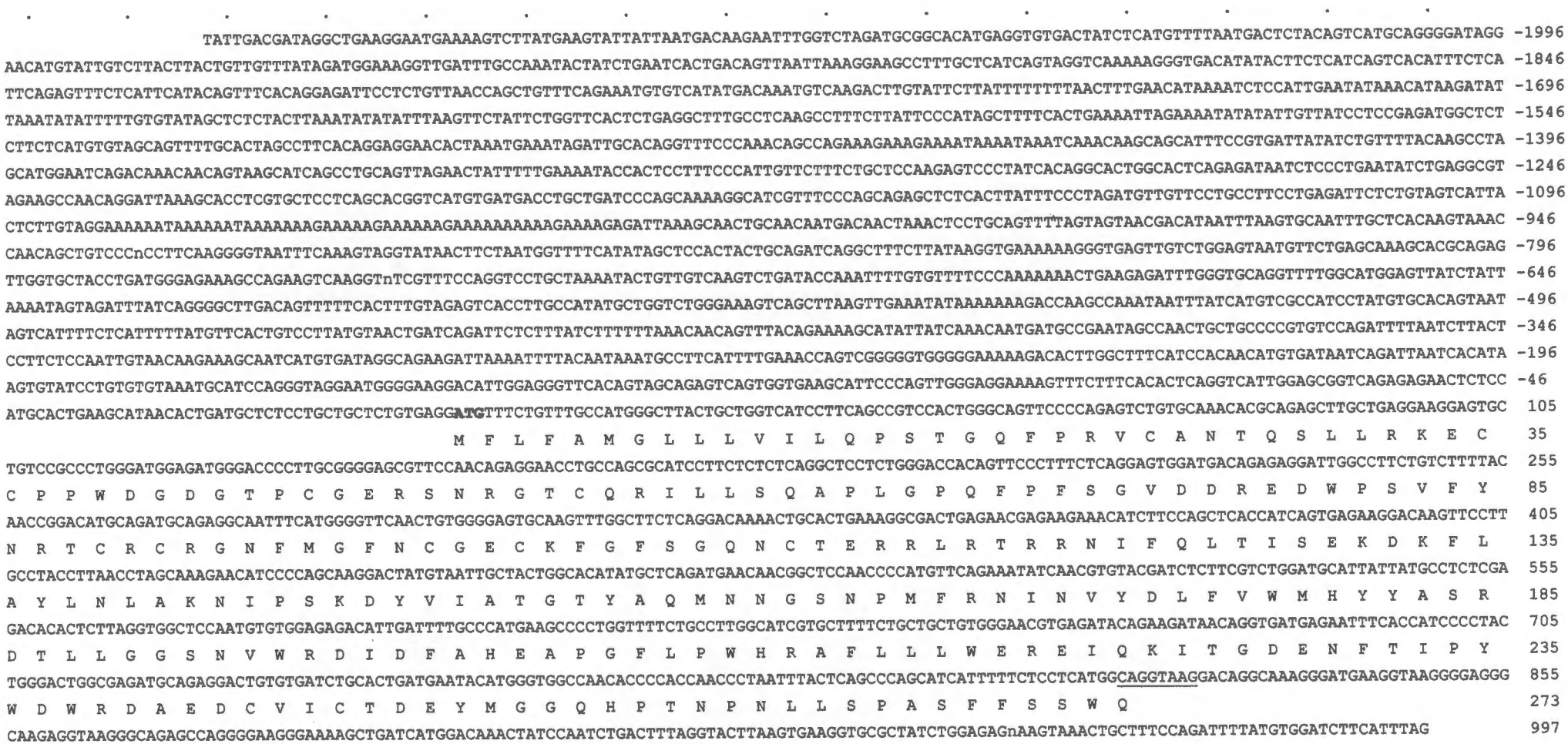
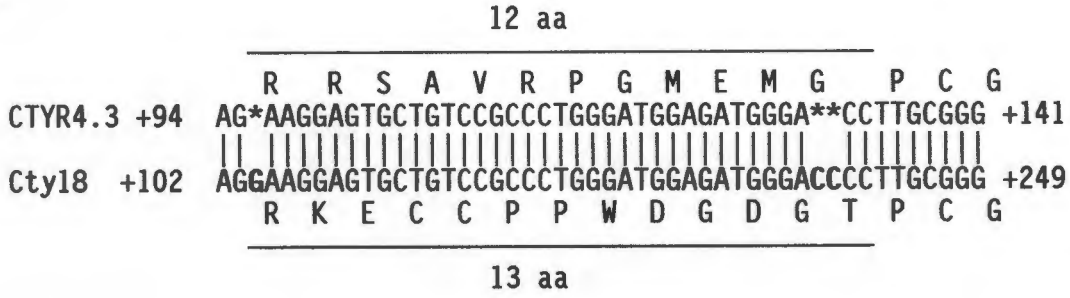


Fig. 2.11: Nucleotide sequence of the 5′ flanking region and exon 1 of CTYR4.3. The deduced amino acid sequence encoded by exon 1 is also indicated. The first letter of the presumed translation start codon (ATG; printed in boldface) has been assigned the nt number +1. The splice site consensus sequence is underlined.

a



b

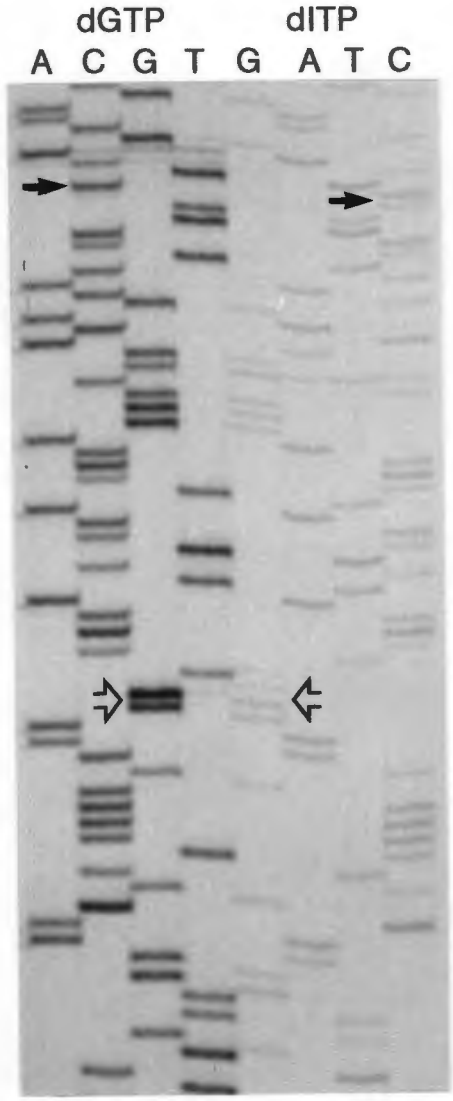


Fig. 2.12. The use of dITP to resolve compressions during dideoxy chain termination sequencing of CTYR4.3. **(a)** A comparison of the nucleotide sequences derived for CTYR4.3 and Cty18 before compressions were resolved. A total of three nt were missing from CTYR4.3. This caused a frameshift in the predicted amino acid (aa) sequence and the loss of one aa, so that 12 different aa were substituted in the genomic sequence for the 13 aa in the published cDNA sequence. **(b)** A portion of the sequence ladder illustrating the complementary strand of nt +86 to +155 in Fig. 2.11. The positions of the resolved compressions are indicated by arrowheads.

2.4. DISCUSSION

This chapter presents a chronological account of the successful isolation and characterisation of a chicken tyrosinase genomic DNA fragment, CTYR4.3. This proved not to be a simple exercise as a number of technical problems were experienced. These problems are presented and discussed in the latter part of this section.

2.4.1. CTYR4.3 contains 5′ flanking sequences of the chicken tyrosinase gene

The aim of the present study was to isolate 5′ flanking sequences of the chicken tyrosinase gene. Therefore, a 0.57 kb fragment from the 5′ end of the mouse tyrosinase cDNA clone, pmcTYR1, was used to screen a chicken genomic DNA library. Two strongly hybridising clones were isolated and found to contain identical genomic DNA inserts of approximately 16.8 kb. The hybridising region of these positive phage recombinants was shown to lie within a 4.3 kb *Eco*RI-*Bam*HI fragment which was termed CTYR4.3 and characterised further. Sequencing confirmed that the genomic subclone CTYR4.3 encodes chicken tyrosinase and includes 2125 nt of 5′ flanking sequence, the first exon and a part of the first intron.

The isolation of a genomic clone makes it possible to begin to determine the genomic structure of chicken tyrosinase. A comparison of CTYR4.3 with the cDNA sequences reported for chicken tyrosinase by Mochii et al (1992) and more recently April et al (1996) reveals that exon 1 is 819 nt in length and encodes an amino acid sequence of 273 residues. The size of exon 1 is consistent with that found for the human, mouse, quail and snapping turtle tyrosinase genes (Giebel et al, 1991; Ruppert et al, 1988; Yamamoto et al, 1992). The exon 1 sequence included in a genomic clone reported recently for the Japanese pond frog, however, encodes an amino acid sequence of 277 residues (Miura et al, 1995). Further inconsistencies have been found in the amphibian and in the Medaka fish tyrosinase gene sequences when compared to the sequences from the higher vertebrates. These are elaborated on in chapter 3.

There are no untranslated exons in the 5′ flanking region of CTYR4.3, as the sequences immediately upstream of the ATG site in the cDNA and CTYR4.3 match directly. This is consistent with the absence of untranslated exons in the mammalian tyrosinase genes (Giebel et al, 1991). Since the 2125 nt of CTYR4.3

positioned 5′ to the ATG site do not represent intronic sequences, one would expect to find the transcription start site(s) within this region. Interestingly, the members of the mammalian tyrosinase gene family differ markedly in their 5′ gene structure. While the tyrosinase gene contains approximately 130 nt of 5′ untranslated sequence between the most distal transcription start site and the ATG site, TRP-2 RNA transcripts transcripts contain approximately 700 nt of 5′ untranslated sequence (Budd and Jackson 1995; Sturm et al, 1995) and TRP-1 transcripts include an untranslated exon (Jackson et al, 1991; Shibata et al, 1992).

The genomic clones that have been reported for tyrosinase genes of lower vertebrates to date, contain limited 5′ flanking sequences. The quail clone includes 519 nt of 5′ flanking sequence while genomic sequences cloned from the Japanese snapping turtle and pond frog include 699 nt and 748 nt of 5′ flanking sequences, respectively (Yamamoto et al, 1992; Miura et al, 1995). Thus, the 2125 nt of 5′ flanking sequence present in CTYR4.3 is the most extensive to be reported for a lower vertebrate tyrosinase gene to date. The functional analysis of this 2125 nt region will be presented in detail in the following chapter.

2.4.2. An analysis of technical problems encountered

Unexpected technical difficulties were experienced during attempts to generate a series of nested deletions that could be used to facilitate sequencing and for subsequent promoter analysis. CTYR4.3 was originally subcloned into the M13mp18 vector and samples subjected to progressive deletion using Exonuclease III. Gel analysis of linearised Exonuclease III derivatives revealed a series of plasmids of expected size ranging from ±11.3 kb to 7.5 kb (representing 4.1-0.3 kb of CTYR4.3 plus 7.2 kb of M13 vector). Sequencing, however, revealed several anomalies. Using the primer supplied with the Sequenase kit, nearly identical sequences were obtained for all Exonuclease III derivatives that were sequenced. This was unexpected as sequences primed from this site should represent the progressively digested DNA. Furthermore, a computer-aided DNA homology search of GenBank showed that the derived sequence corresponded to a portion of the M13mp18 vector (nt positions +578 to +372) situated more than 5732 nt downstream of the priming site. These anomalies were specific to the Exonuclease III derivatives because sequencing of a

CTYR4.3 template that had not been treated with Exonuclease III, gave rise to a unique sequence. This sequence was unrelated to the M13 vector and represented a bona fide chicken tyrosinase intron 1 sequence.

A possible explanation for these results could be that unusual recombinations in the M13 vector may have taken place whilst generating Exonuclease III-susceptible and -protected ends with *Sph*I and *Sal*I or during the first minute of Exonuclease III digestion. Evidence of recombination was not apparent from the sizes of the *Hin*dIII-linearised Exonuclease III derivatives as derived from the gel. But subsequent attempts to excise the deleted CTYR4.3 inserts from their M13 vectors by digestion with *Eco*RI and *Hin*dIII were unsuccessful, indicating that the *Eco*RI recognition site had been lost.

Similar phenomena were reported by da Silva Tatley (1992) and Saul et al (1989) whose attempts to determine nt sequences by M13-sequencing were thwarted by inserts not being stably maintained in the M13 phages. The decision was made to abandon further attempts at M13-sequencing, and to subclone CTYR4.3 into the pUC19 plasmid vector (subsequently used by da Silva Tatley, 1992) and generate a new series of nested deletions which would then be sequenced by double stranded sequencing methods.

To ensure that the pUC19 Exonuclease III derivatives were intact, the CTYR4.3 sequences were excised from prospective clones by digestion with *Eco*RI and *Hin*dIII and analysed by gel electrophoresis. A nested set of intact deletion clones was derived from this experiment, which enabled the successful sequencing of CTYR4.3. It was surprising to find, however, that once again DNA recombination had occurred in a large proportion of the prospective clones analysed, thus preventing the excision of CTYR4.3 inserts by *Eco*RI/*Hin*dIII digestion. Therefore, up to 34 clones from each time point aliquot had to be analysed to obtain sufficient undamaged clones for sequencing. Damage to the vector DNA of these clones may have been due to inefficient protection of the vector DNA by the *Sph*I-derived 3′ overhang, although some protection must have been offered by this protruding 3′ end otherwise the DNA would have been equally susceptible to Exonuclease III digestion from both ends which would have resulted in substantially shortened clones.

As was shown in the results, the Exonuclease III derivatives that were eventually used for sequencing, were progressively deleted from their 3′ ends

and therefore have limited use for functional analyses of the promoter. To be able to manipulate CTYR4.3 from its 5′ (*Eco*RI) end, cloning procedures were undertaken that reversed the orientation of CTYR4.3 relative to the *Sal*I and *Sph*I sites in the multiple cloning cassette of pUC19. After several unsuccesful attempts to generate nested deletions of this "reversed" construct, it was decided not to pursue these endeavours. It was reasoned that the information derived from the nt sequence (i.e. positions of both restriction enzyme recognition sites and evolutionarily conserved tyrosinase promoter motifs) would enable the construction of a set of appropriate promoter-deletions.

Finally, a word concerning the approaches taken to screen a chicken genomic DNA library. In the absence of a chicken TYR cDNA sequence, clones containing CTYR4.3 were identified from within a library of chicken genomic DNA fragments by their ability to cross-hybridise to a mouse tyrosinase cDNA probe ie. a heterospecific probe. Attempts to generate a homospecific probe ie. a chicken tyrosinase chicken cDNA probe by PCR were unsuccessful. This can be largely attributed to the primers that were used. These primers were designed to anneal to and amplify a portion of the tyrosinase sequence encoding an amino acid sequence that is both essential for a functional tyrosinase enzyme and the most highly conserved region evolutionarily. However, the primer design did not allow for differences between the chicken and mouse nucleotide sequences encoding this region. A better approach would have been to use a set of degenerate primers (White et al, 1989) which might also have decreased the chances of amplifying a contaminating mouse sequence, as they would no longer be "mouse-specific".

CHAPTER THREE

CHARACTERISATION OF THE CHICKEN TYROSINASE PROMOTER SEQUENCE IN CLONE CTYR4.3

3.1 INTRODUCTION

As a first step towards determining the requirements for melanocyte-specific tyrosinase gene expression in a lower vertebrate, the proximal 5′ flanking sequence of the chicken tyrosinase gene was cloned (as described in the previous chapter). This chapter details the further analyses that were undertaken to gauge the significance of this 5′ flanking sequence as a potential promoter, and the search for evidence of regulatory mechanisms in common with those of higher vertebrate tyrosinase gene promoters.

Characterisation of the 5′ flanking sequence cloned in CTYR4.3 involved three types of analyses. Firstly, with the nucleotide sequence of CTYR4.3 in hand, it was possible to conduct computer-aided searches and sequence alignments to identify potential transcription factor binding sites including those resembling key regulatory elements that have been identified in the mouse and human tyrosinase gene promoters. Secondly, to determine whether the 2125 nt of the 5′ flanking untranslated sequence in clone CTYR4.3 does contain information necessary for transcription of chicken tyrosinase, it was important to show that transcription start sites are present within this sequence. The position of such a site(s) would represent the 3′ boundary of the gene's regulatory region. Therefore, primer extension analysis was used to map the 5′ ends of RNA transcripts of chicken tyrosinase to reveal the positions of transcription start sites. Finally, it was of primary importance to test the ability of the 5′ flanking sequence to drive expression of a downstream gene. Reporter gene expression assays were used to determine the functional ability of the 5′ sequences of CTYR4.3 in transiently transfected cells. The activity of four constructs containing fragments of the 5′ flanking sequence linked to the luciferase reporter gene were assessed in both pigment-producing and non-pigment-producing cell types, in an attempt to establish the minimum promoter sequence required for tissue-specific expression of the chicken tyrosinase gene.

3.2. MATERIALS AND METHODS

3.2.1. Nucleotide sequence data analysis

All computer analyses were performed using the Genetics Computer Group Inc. (Madison, WI, USA) Sequence Analysis Software Package Version 8.0. Direct comparisons between the 5′ flanking sequence of CTYR4.3 and promoter sequences from other vertebrate tyrosinase genes were performed using the BESTFIT function. The search for consensus transcription factor binding sites was performed using the FINDPATTERNS function of GCG and a file provided by GCG, TFsites, containing transcription factor binding sites drawn from different datasets (see Appendix III for details and references of these datasets). Potential primer sequences for use in primer extension experiments were analysed for self-complementarity using the FOLD and SQUIGGLES functions of GCG.

3.2.2. Primer extension analysis

3.2.2.1. Preparation of RNA templates

Embryonic tissues from which template RNA was extracted, were obtained as follows: fertilized chicken eggs (Black Australorp X New Hampshire Red or White Plymouth Rock X Pile Game breed) were incubated in a humidified incubator set at 37°C. After 9-10 days embryos were removed and eye, skin, brain and musculo-skeletal tissues were dissected in Ca^{2+}/Mg^{2+}-free Hanks Balanced Salt Solution (pH 7.4). RPE tissue was obtained from dissected eyes by mechanical detachment from the neural retina, followed by careful separation from the choroid. RPE tissue isolated in this manner was examined using light microscopy to confirm that it was free of choroidal tissue.

Cytoplasmic RNA (referred to below as total RNA) from tissues or cell cultures was isolated exactly according to Sambrook et al (1989, pp 7.12-7.13). The RNA was checked for signs of degradation by electrophoretic analysis on 1.1% denaturing formaldehyde agarose gels. Where indicated, samples were enriched for poly(A^+) RNA by oligo-dT cellulose affinity chromatography as described in Sambrook et al (1989, pp 7.26-7.29).

3.2.2.2. Primer design and synthesis

The criteria used to select the primer sequence were adapted from a set of rules prescribed by Innes and Gelfand (1990) for the design of PCR primer sequences, as follows:

1. Primers should be 17-28 bases in length;
2. Base composition should be 50-60% (C+G);
3. Primers should end (3′) in a C or G, or CG or GC which prevents "breathing" of ends and increases efficiency of priming;
4. Runs of three or more Cs or Gs at the 3′-ends of primers may promote mispriming at G- or C-rich sequences (because of stability of annealing), and should be avoided;
5. Primer self-complementarity i.e. the ability to form secondary structures such as hairpins, should be avoided.

A 20-mer oligonucleotide sequence, 5′-GTGGACGGCTGAAGGATGAC-3′, complementary to the exon 1 sequence found at nt positions +50/+31 downstream of the ATG site within CTYR4.3 and complying with the above criteria, was synthesised by E. Botes (Dept of Biochemistry, UCT). Before proceeding with the primer extension experiments the oligonucleotide was tested for its ability to prime a DNA sequencing reaction using a CTYR4.3 DNA template.

3.2.2.3. Primer extension reactions

The amounts of RNA used as template for primer extension depended on whether the samples were enriched for mRNA or not, and on the abundance of melanocytes present in the starting material, that is, 1 μg of mRNA from whole eye (containing both pigmented choroid and RPE layers), 10 μg of mRNA from skin (melanocytes at 1 in 40 keratinocytes), or 40 μg of total RNA from RPE layers (100% pigmented cells). Corresponding amounts of body mRNA or brain total RNA were used as negative controls. Primer extension reactions were carried out according to Sambrook et al (1989, pp 7.80-7.83) with a few modifications: 10 pmoles of unlabelled primer was hybridised to template RNA at 24°C overnight in annealing buffer. The primer was extended using 25 units Stratascript Reverse transcriptase (Stratagene) in Reverse transcriptase buffer (50mM Tris HCl, pH 8.3; 75mM KCl; 3mM $MgCl_2$; 10mM DTT; 0.6mM dGTP, dATP, dTTP; 10 units RNase Inhibitor (Boehringer Mannheim); 1 μl (10 μCi) [α^{32}P]-dCTP (Amersham)) at 37°C for 120 min or 42°C for 90 min (Ausubel et al, 1987). The RNA templates were

removed by digestion using 50 μg/ml RNase A at 37°C for 30 min and phenol-chloroform extraction. Products were then run on 6% polyacrylamide/8M urea gel alongside a sequencing reaction in which the same primer was extended using CTYR4.3 DNA as a template.

3.2.3. Construction of promoter-reporter gene recombinants

Chicken tyrosinase 5′ flanking sequences of varying length were inserted into the multiple cloning site of the promoterless luciferase reporter plasmid, pGL2-Basic (Promega Corporation; Appendix D). The constructs described below are depicted in Fig. 3.5. To generate **TYR2.1-LUC**, a 5′ *Eco*RI- 3′ *Hin*dIII fragment containing 2123 nt of 5′ flanking sequence of CTYR4.3 was excised from Exonuclease III deletion derivative 5.25 (previously shown in Fig. 2.10), in which the 3′ end of the insert is 3 nt upstream of the ATG site. Deletion clone 5.25 was first cut with *Eco*RI and the *Eco*RI terminus of the 2123 bp insert was made blunt by a "filling in" reaction using Klenow polymerase. The 2123 bp fragment was then released from the pUC19 vector by digesting with *Hin*dIII and ligated to a *Sma*I-*Hin*dIII-cut pGL2-Basic vector. To generate **TYR1.1-LUC**, a 1149 bp fragment of the 5′ flanking sequence of CTYR4.3 was excised from TYR2.1-LUC by digestion with *Sac*I (5′) and *Hin*dIII (3′), and inserted into *Sac*I-*Hin*dIII-cut pGL2-basic vector. The 504 bp fragment of 5′ flanking sequence that was used to construct plasmid **TYR0.5-LUC**, was obtained by cutting TYR2.1-LUC with *Alw*44I, blunt-ending the *Alw*44I terminus and then releasing the 504 bp fragment by digesting with *Hin*dIII. The 5′blunt-3′*Hin*dIII fragment was ligated into a *Sma*I-*Hin*dIII-cut pGL2-Basic vector, as before. The plasmid **TYR0.2-LUC** was generated by partial digestion of TYR2.1-LUC using *Asn*I. This enzyme cuts the plasmid at 4 sites, 2 of which fall within the 2123 bp insert, and are 1875 nt apart. Partial digestion was achieved by removing samples prematurely from a digestion reaction (that is, 10-20 mins after addition of *Asn*I) and terminating the reaction by adding gel-loading buffer. Digested DNA was separated on an agarose gel to identify the band of plasmid DNA that was cut only at the 2 sites within the 2123 bp insert, thus releasing a 1875 bp fragment. The resulting 5799 bp fragment contained 5′ flanking sequences consisting of 203 bp proximal to the ATG site plus 45 bp derived from the extreme 5′ terminus of CTYR4.3, as well as intact pGL2-Basic sequences (5553 bp) (see Fig. 3.1). The 5799 bp fragment was excised from the gel and the DNA was recircularised by ligation of the *Asn*I sites.

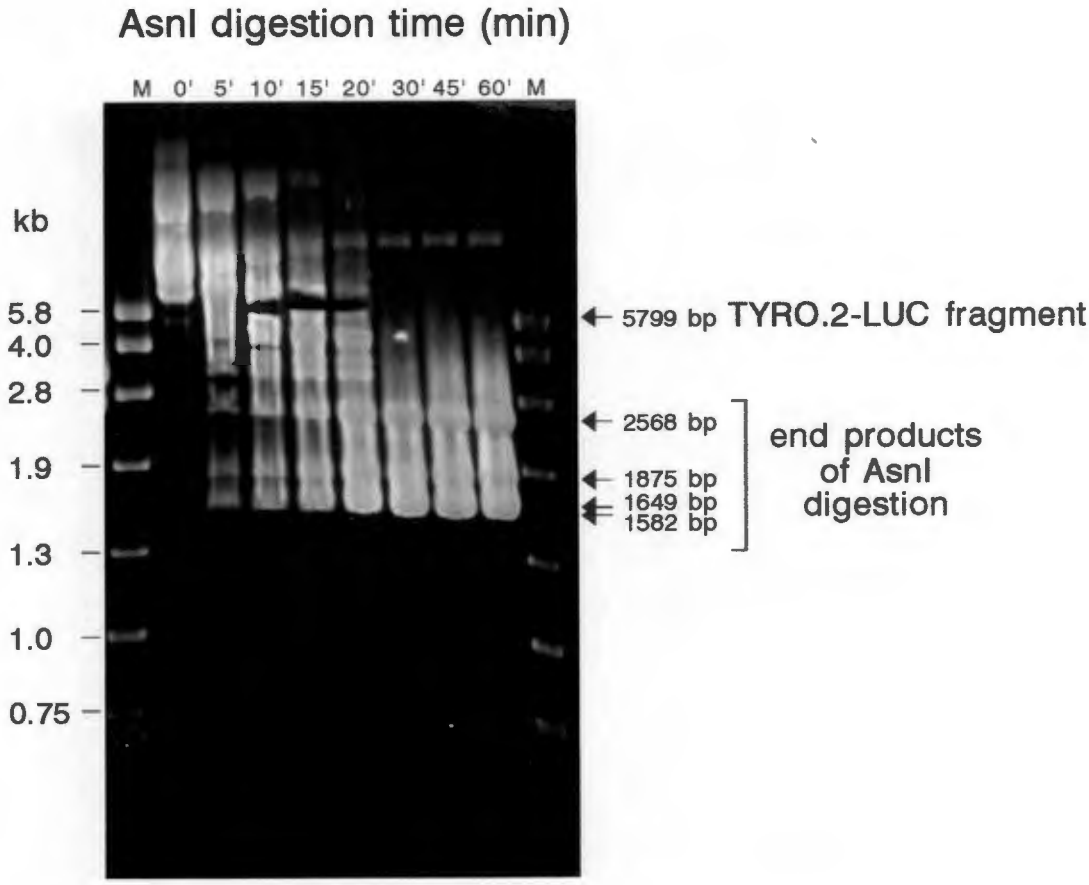


Fig. 3.1: Partial *Asn*I digestion of TYR2.1-LUC to generate TYRO.2-LUC. A 1% low gelling temperature agarose gel showing the fragments derived by partial digestion of TYR2.1-LUC DNA with *Asn*I. Complete digestion by *Asn*I results in four fragments of 2568, 1875, 1649 and 1582 bp. A band containing the partially digested 5799 bp fragment (comprising the 2568, 1649 and 1582 bp sub-fragments) has been excised from the lanes containing samples digested for 10-20 min. This fragment was recircularised to generate TYRO.2-LUC. M, *Eco*RI digest of the molecular weight marker, pAT15 (a plasmid containing a *Hin*dIII fragment of pox DNA [K.R. Dumbell, University of Cape Town, S.A.]).

The digested fragments used to construct the TYR-LUC plasmids were electrophoresed on gels consisting of 1% Nusieve GTG low melting point agarose (FMC Bioproducts). Gel fragments containing the desired pieces of DNA were excised and used directly for "in-gel" ligation and transformation of *E. coli* XL1-blue cells (Stratagene) according to FMC Bioproducts' instructions. Diagnostic restriction enzyme digests were performed to verify the composition of the promoter-reporter gene plasmids. Prior to transfection, plasmid DNA was prepared by the alkaline lysis maxi-preparation procedure and purified by centrifugation in cesium chloride-ethidium bromide gradients (according to Sambrook et al, 1989, pp 1.38-1.39; 1.42).

3.2.4. Cell Culture

MQTNC cells [MC29 immortalised quail trunk neural crest cells (Fauquet et al, 1990) obtained from Dr Mireille Fauquet (Paris, France)] were grown in Dulbecco's modified Eagle's medium (Highveld Biological, SA) containing 10% heat-inactivated foetal calf serum (Delta Bioproducts) and 100 µg/ml each of penicillin and streptomycin (Highveld Biological, SA). A human hepatoblastoma cell line, Hep G2 (ATCC no. HB 8065) was grown in Dulbecco's modified Eagle's medium containing 10% foetal calf serum (which had not been heat-treated) without antibiotics. Primary cultures of chicken retinal pigment epithelial (RPE) cells were established according to Eguchi and Okada (1973). Briefly, RPE tissue was dissected from the pigmented eyes of chicken embryos (at embryonic day 5 or 6) as described in section 3.2.2.1. Isolated RPE sheets were incubated with 0.25% trypsin in Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution (Sigma) for ±45 min at 37°C with gentle agitation. A cell suspension was obtained by trituration of the trypsinised tissue using a fine-bore polished pasteur pipette. Cells from four eyes were plated in 2 x 100 mm diameter dishes (Corning) in 10 ml Eagle's MEM (Sigma) containing 10% heat-inactivated foetal calf serum with antibiotics as above. To ensure that subcultures consisted primarily of RPE cells, pigmented foci from confluent dishes were picked using a tungsten needle, trypsinised, replated and expanded.

All cells were grown at 37°C in a humidified incubator with 5% CO_2. Manipulation of cells was conducted in a laminar flow tissue culture hood. Media

and additives that were not purchased as sterile solutions, were sterile-filtered using 0.22 μm filters or autoclaved before use.

3.2.5. Northern blot hybridisation analysis

Cytoplasmic RNA was extracted from confluent dishes of RPE, MQTNC and Hep G2 cells according to Sambrook et al (1989, pp 7.12-7.13). The RNA was separated on a 1.1% denaturing formaldehyde agarose minigel at 5 V/cm for 1 h, stained for 2.5 min with ± 0.01% ethidium bromide and photographed on a Spectroline transilluminator. The gel was rinsed twice in 10xSSC for 20 min and then the RNA transferred onto a nitrocellulose membrane (Hybond C, Amersham) by capillarity using 10xSSC as blotting buffer. The gel was blotted overnight, after which the towelling was replaced and the blotting continued for a further 3 h. Transfer onto the membrane was checked by transillumination and the position of the rRNA bands marked with pin-pricks. The RNA was then fixed onto the membrane by baking at 80°C for 2 h. Hybridisation was carried out according to Davis et al (1986) in a hybridisation oven (Hybaid). Blots were prehybridised for 16 h. A 1.5 kb *Eco*RI-*Pst*I fragment of chicken tyrosinase cDNA (April et al, 1996) was labelled with [α-^{32}P]-dCTP using a Random-primed DNA labelling kit (Boehringer Mannheim) for use as probe. Radiolabelled probe was separated from unincorporated label on a Sephadex G-50 column. Denatured probe was added to the hybridisation bottle (at a final concentration of 1 x 10^6 cpm/ml) and hybridisation carried out for 16 h. Blots were then washed under moderate stringency conditions (final wash at 55°C in 1xSSC, 1% SDS for 10 min) and exposed to autoradiographic film at -80°C.

3.2.6. Transient Transfections

MQTNC and Hep G2 cells were seeded at 3 x 10^5 cells/60 mm diameter dish 24 h before transfection. RPE cells were seeded at 1 x 10^6 cells/60 mm diameter dish 48-72 h before transfection, because of their poor plating efficiency and relatively slow growth. Culture medium was replaced with 4.5 ml of fresh medium 3-7 h before transfection. Cells in duplicate dishes were co-transfected with luciferase and pRSV-ß-gal plasmids using the calcium phosphate precipitation method that utilises HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered saline (HBS), according to Gorman et al (1982) and Kingston (1987). Briefly, fine, milky calcium phosphate-DNA precipitates were prepared

by mixing a solution containing DNA-$CaCl_2$ with 2 x HBS-sodium phosphate (pH 7.1) under a continuous stream of air bubbles. The precipitate was allowed to stand at RT for 30 min, after which it was mixed with vigorous pipeting and added dropwise (500 µl/dish) over the surface of the medium covering the cells. The cells were exposed to the precipitate overnight for 16 h (RPE cells) or 18 h (MQTNC and Hep G2) after which the medium containing the precipitate was removed, the cells were rinsed twice using phosphate-buffered saline (PBS) and fed with 5 ml fresh medium. At 48 h post-transfection, cells were washed twice in PBS, and lysed *in situ* by incubating the cells in 400 µl of reporter lysis buffer (Promega Corporation) for 15 min. All visible cell debris was then scraped to one edge of the dish using a rubber policeman, and transferred to an eppendorf tube. Cell lysates were centrifuged in a microfuge at 10000 g for 2 min at 4°C to remove nuclei and insoluble proteins. The supernatant fluid was transferred to a fresh tube after which it was either assayed directly for reporter gene activity or frozen at -80°C.

Controls: In all experiments transfections were performed in duplicate for each luciferase plasmid to be tested. To control for efficiency of uptake of DNA in different dishes in a single experiment, the luciferase plasmids were cotransfected with pRSV-ß-gal, a plasmid containing the ß-galactosidase (ß-gal) reporter gene under the control of a Rous sarcoma virus promoter (Edlund et al, 1985). To control for transfection efficiency between different cell types, one set of duplicate dishes in each transfection experiment was transfected with a pGL2-Control vector (Promega Corporation, Appendix D), which contains both SV40 promoter and enhancer sequences. To control for background luciferase expression due to the pGL2-Basic vector backbone, one set of duplicate dishes in each transfection experiment was transfected with the pGL2-Basic vector.

3.2.6.1. Reporter gene expression assays

Protein concentrations were determined using either the Biuret reaction according to Bradshaw (1966) or a BioRad protein assay kit (when a more sensitive detection method was required). BSA made up in reporter lysis buffer was used as a protein standard. Equal amounts of protein from each dish were assayed for luciferase activity using a luciferase assay kit (Promega) and a luminometer (Bio-Orbit 1253 luminometry system). ß-gal activity in cell lysates was assayed according to Herbomel et al (1984) and used as a measure of relative transfection efficiency. Luciferase activity (number of light units/mg protein)

was normalised by dividing by ß-gal activity (OD_{420}/hr/mg protein). To enable comparison of results from different experiments and using different cell types, each result was finally expressed as a relative luciferase activity, that is, relative to the activity obtained using the pGL2-Control plasmid, thus, the mean of the normalised luciferase activity obtained using a particular luciferase plasmid expressed as a percentage of the mean value obtained using pGL2-Control.

3.2.6.2. Transfections using equimolar versus equal microgram amounts of DNA

Cells were initially transfected with equal microgram amounts of each promoter-luciferase construct, that is 7 μg of construct plus 2 μg of pRSV-ß-gal per dish of cells (results of which are indicated in Fig. 3.8 by solid bars for transfection experiments 12-18). However, because of the difference in size between constructs (for example, TYR2.1-LUC (7674 bp) and TYR0.2-LUC (5798 bp)), the cells transfected with the bigger constructs would receive fewer copies of the potentially transcribable DNA than cells transfected with the smaller construct (1.38 pmoles versus 1.83 pmoles, respectively). Subsequent transfection experiments were performed using equimolar amounts of each construct (1.38 pmoles) made up to 7 μg with carrier plasmid (non-transcribable, promoterless pBLCAT3; Luckow and Schütz, 1987) (Fig. 3.8; hatched bars shown for experiments 18-22). As can be seen from the results of experiment 18 (Fig. 3.8.b) where transfections with equimolar versus equal microgram amounts of DNA were compared within one experiment, slight differences in expression could be detected. For instance, the relative expression of TYR2.1-LUC was slightly increased and TYR0.2-LUC slightly decreased when using equimolar amounts of DNA. In general, however, the variation in expression of luciferase obtained using these slightly different amounts of DNA was no more striking than the inter-experimental variation obtained using "equal microgram" amounts of DNA and therefore considered to be negligible.

3.3. RESULTS

3.3.1. Sequence Analysis

Two types of sequence analysis were undertaken to identify putative cis-acting regulatory elements in the 5′ flanking sequence. The first entailed searching for regions of homology between the 5′ flanking sequence of CTYR4.3 and other vertebrate tyrosinase genes that might thus contain functionally significant regulatory elements common to all tyrosinase gene promoters. The second type of sequence analysis involved screening CTYR4.3 for any potential consensus transcription factor binding sites found in the transcription factors datasets represented in TFsites.

3.3.1.1. Alignment of the 5′ flanking sequences of vertebrate tyrosinase genes.

The search for homologous tyrosinase promoter sequences involved a BESTFIT comparison of CTYR4.3 to 5′ flanking sequences of human, mouse, quail, turtle, and frog tyrosinase genes (see Table 3.1). A cDNA sequence encoding Medaka fish tyrosinase was recently isolated, but a genomic DNA sequence encoding a fish tyrosinase gene has not yet been cloned. Nevertheless, the cDNA sequence represents an RNA transcript that includes 321 nt of 5′ untranslated sequence between the ATG site and its transcription initiation site (Inagaki et al, 1994). Although most of the important transcription regulatory sequences are usually found upstream of the transcription start site, for the purposes of completion, attempts were made to align this 321 nt of 5′ untranslated sequence with the tyrosinase 5′ flanking sequence from other vertebrates.

Two regions in CTYR4.3 were found to be homologous to the tyrosinase gene promoters of other vertebrates. These highly conserved sequences will be referred to as the proximal and distal conserved regions. The positions of these regions within CTYR4.3, are highlighted in Fig. 3.2 (shaded boxes). An alignment of the most highly conserved sequences within the two above mentioned regions, with the corresponding tyrosinase promoter sequences from other vertebrates is shown in Fig. 3.3. A 129 nt stretch positioned at nt -337 to -209 in CTYR4.3 constitutes the proximal conserved region (Fig. 3.3.a). Here, CTYR4.3 shares 63% homology with the mouse and 60% homology with the human sequence.

Table 3.1.

Vertebrate species	Gen Bank/EMBL/DDBJ Accession No.	Length of 5′ flanking sequence	Reference
Human	X16073	985 nt	Kikuchi et al, 1989
Human	U03039	2307 nt	Ponnazhagan et al, 1994
Mouse	D00439	2545 nt	Yamamoto et al, 1989
Japanese Quail	S56788	519 nt	Yamamoto et al, 1992
Japanese Snapping turtle	S56789	699 nt	Yamamoto et al, 1992
Japanese Pond frog	D37779	750 nt	Miura et al, 1995
Japanese Medaka fish	D29686	321 nt	Inagaki et al, 1994

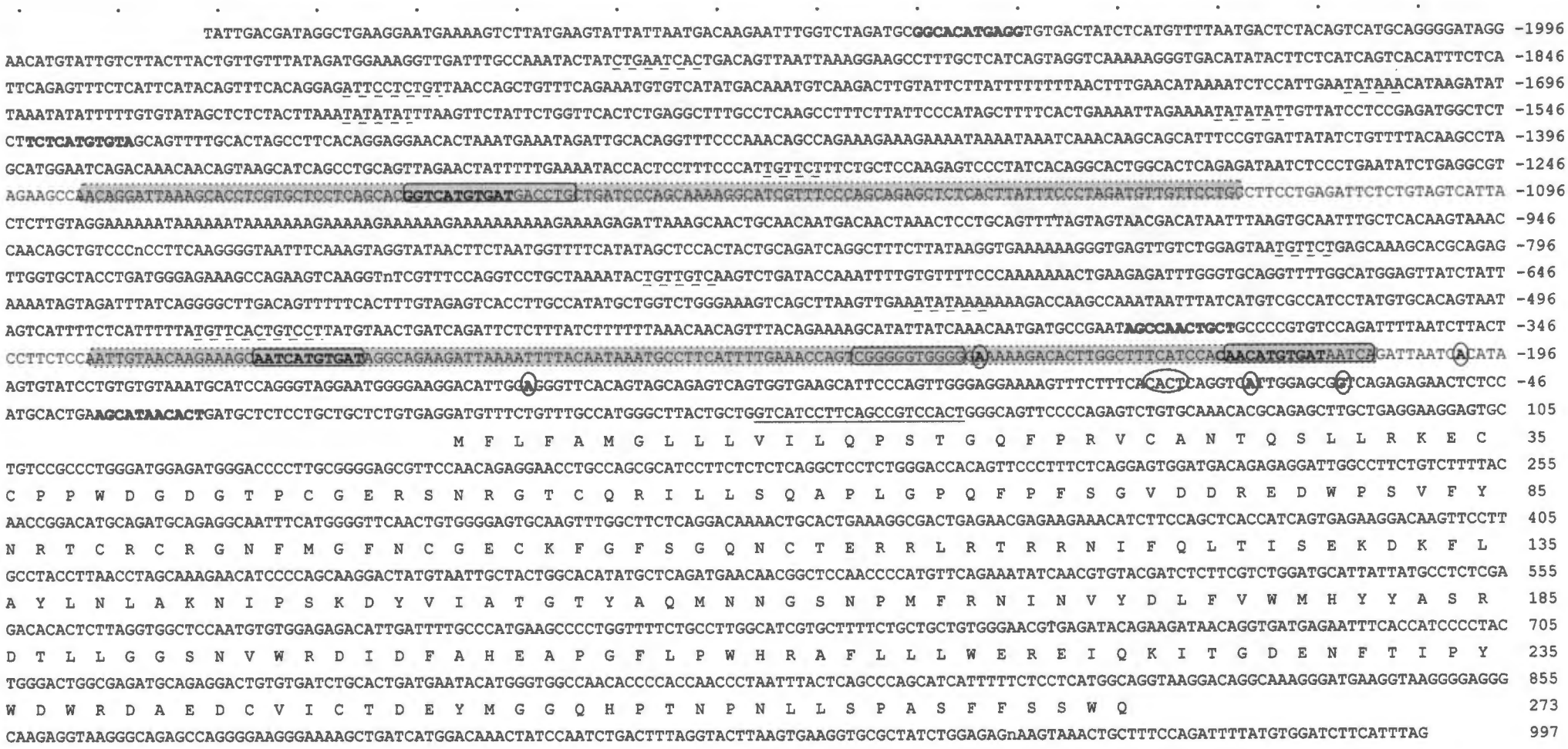


Fig. 3.2: The position of evolutionarily conserved regions and potential transcription factor-binding sites in the 5′ flanking sequence of CTYR4.3. The proximal and distal conserved regions for vertebrate tyrosinase gene promoters are in shaded boxes. The potential Inr, SP-1-binding site, M-box and TDE-like element are shown as boxed sequences within the shaded regions. The p-MSE-like motifs are printed in boldface. Dotted lines indicate the positions of TATA box, AP-1, URE and GRE motifs. The sequence complementary to the synthetic 20-mer used for primer extension is underlined and the nucleotides corresponding to the five putative transcription start sites identified for chicken tyrosinase, are ringed. The transcription start site (CACT) reported for quail tyrosinase by Akiyama et al (1994) is circled.

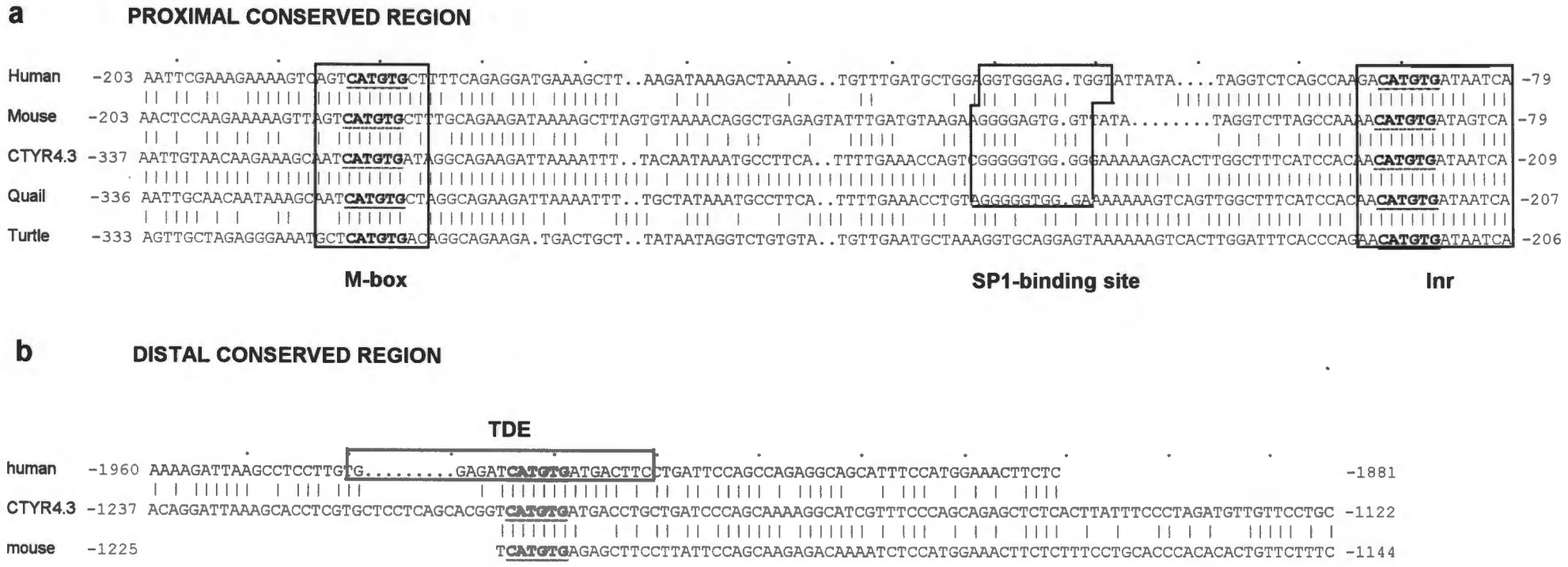


Fig. 3.3: Conserved regions found in vertebrate tyrosinase gene promoters. Sequences derived from the human, mouse, and where possible, quail and snapping turtle tyrosinase genes, were aligned with the 5′ flanking sequence of CTYR4.3 using the BESTFIT function of GCG. Two regions of homology were identified (a) the proximal conserved region and (b) the distal conserved region. Sequences comprising the upstream enhancer (TDE), M-box, SP1-binding site and Inr are boxed. The E-box motif, CATGTG, is underlined and the degenerate octamer component of the Inr is overlined. The positions of the conserved regions are given as the distance in nt upstream of the ATG site.

Homology to the quail and turtle sequences over this region is higher and extends beyond 129 nt. Homology between CTYR4.3 and the entire 519 nt of the quail promoter was 92%. The region of "bestfit" between CTYR4.3 and turtle promoter sequences, however, showed only 67.5% homology and was found over a stretch of 480 nt proximal to the ATG site. The Inr sequence is included at the 3′ end of the proximal conserved region and interestingly, the homology between mammalian and lower vertebrate tyrosinase promoters ends abruptly immediately downstream of the Inr. The second conserved region within the vertebrate tyrosinase 5′ flanking sequences was found at a more distal position (nt -1237 to -1122 in CTYR4.3; Fig. 3.3.b). Here CTYR4.3 shares 70% homology with a sequence at approximately -1900 nt in the human tyrosinase promoter and 60% homology with a mouse sequence beginning at nt -1225. The 5′ flanking sequences reported for the quail and turtle do not extend as far upstream as their mammalian counterparts, therefore homology to this second region could not be ~~found~~ compound within these sequences. Surprisingly, neither the frog nor the Medaka fish tyrosinase 5′ flanking sequences could be aligned with the above sequences due to insufficient homology.

Interestingly, the human, mouse and quail promoter sequences aligned in Fig. 3.3 contain elements that have been previously reported to play a role in the regulation of tyrosinase gene transcription. These include the Inr, SP1-binding site and M-box in the proximal conserved region, and TDE enhancer of the human sequence in the distal conserved region (Yamamoto et al, 1992; Ganss et al, 1994; Bentley et al, 1994; Yasumoto et al, 1994). The positions and sequences of the putative chicken homologues are indicated in Fig. 3.3 (boxed sequences) and in Fig. 3.2 (boxed sequences within shaded areas).

The M-box, an 11-bp motif (AGTCATGTGCT) common to mammalian tyrosinase promoters, was termed the proximal melanocyte-specific element (p-MSE) by Yamamoto et al (1992). They found three similar motifs in the 5′ flanking sequence of both the quail and snapping turtle tyrosinase genes. An analysis of CTYR4.3 revealed three p-MSEs at similar positions to those in the quail promoter i.e. at nt positions -320, -383, and in the complementary strand at -36 (Fig. 3.2, sequences in bold print). The p-MSE at nt position -320 lies within the proximal conserved region (Fig. 3.3.a) and its position corresponds exactly to the mammalian M-box.

Because of the significant role of the CATGTG motif in the functioning of the TDE, Inr and M-box and its presence in some of the reported p-MSE sequences (see Table 3.2), CTYR4.3 was screened for further CATGTG motifs. Two were identified, one of which is situated at nt position -1543 and the other in the complementary strand at nt position -2054. These motifs and their flanking sequences also represent p-MSE-like sequences (see Table 3.2).

3.3.1.2. Identification of potential transcription factor binding sites in the 5′ flanking sequence of CTYR4.3

The search for transcription factor binding sites in CTYR4.3 using consensus sequences from the TFsites datasets, revealed many putative sites. However, the results presented below are restricted to include only those that may be involved in basal transcription or in the response to humoral factors mentioned in chapter one. The significance of the roles of the remaining sites in tyrosinase gene transcription remains to be determined and is beyond the scope of this project. The positions of these potential sites in the 5′ flanking sequence of CTYR4.3 and the transcription factors that recognise them are listed in Appendix III.

Four potential TATA box motifs were identified in the 5′ flanking sequence, one of which is found at nt position -553 and three are far upstream at nt positions -1574, -1661 and -1711. The most proximal TATA box appeared four times in the FINDPATTERNS results, fitting the following consensus sequences: TATAAA, TATAAAA, TATA$^{A}/_{T}A^{A}/_{T}$, ATATAA. One putative AP-1-binding site was identified at nt position -1933 which fits the consensus sequences TGANT$^{A}/_{C}$A and CTGAATCAC. A sequence identical to the URE (TGACAACA) consensus was found in the complementary strand at nt position -731. Several motifs (ATTCCTCTGT, TGTTCT, TGTCCT, TGTTCACT) that have been reported to form part of glucocorticoid receptor-binding sites (in other words, GREs), were identified in CTYR4.3 at nt positions -471, -476, -818, -1319 and -1811. The positions within CTYR4.3 of the abovementioned elements are indicated in Fig. 3.2 (sequences underscored with a dashed line). No consensus sequences for AP-2- or SP1-binding sites, RARE, DDRE, CRE or CCAAT boxes could be found in the 5′ flanking region.

Table 3.2.

Comparison of p-MSE sequences reported for vertebrate tyrosinase gene promoters.

Mouse	-186	AGT**CATGTG**CT	-176		M-box
Quail	-26	AGTGTTATGCT	-36	cs	
	-321	AAT**CATGTG**CT	-311		M-box
	-382	AGACAACTGCT	-372		
Turtle	-49	ATTCATGCACT	-39		
	-293	AGTCATCTTCT	-303	cs	
	-463	AGCAAATTTCT	-473	cs	
Frog	-89	GAA**CATGTG**AT	-79		
	-171	ATTCCTGTGAT	-161		
CTYR4.3	-26	AGCATAACACT	-36	cs	Q-homologue
	-223	CAA**CATGTG**ATAATCA	-209		Inr
	-320	AAT**CATGTG**AT	-310		M-box/Q-homologue
	-383	AGCCAACTGCT	-373		Q-homologue
	-1205	GGT**CATGTG**AT	-1195		TDE
	-1543	TCT**CATGTG**TA	-1533		
	-2044	CCT**CATGTG**CC	-2054	cs	

Note: The CATGTG motifs are in boldface type. Numbering is from the ATG translation start site. cs indicates that the element is present in the complementary strand. Q-homologue = the homologue of a p-MSE reported for the quail sequence. The positions of the seven p-MSE sequences identified in CTYR4.3 are shown in Fig. 3.2 (printed in boldface).

3.3.2. Transcription start site analysis

To determine whether the 2125 nt of the 5′ flanking untranslated sequence of chicken tyrosinase in clone CTYR4.3 does contain information necessary for transcription of tyrosinase, it was important to show that transcription start sites exist within this sequence. Therefore, primer extension analysis was used to map the 5′ ends of RNA transcripts of chicken tyrosinase to reveal the positions of the transcription start sites.

Pigmented tissues dissected from chick embryos including skin, whole eyes and retinal pigment epithelial cell layers, were used as a source of tyrosinase transcripts. As a negative control, either mRNA extracted from musculo-skeletal tissues i.e. the body of an embryo minus eyes, skin, brain, gut, liver, kidney, heart and limbs, or total RNA from embryonic brain was used. Unlabelled primer was annealed to the template RNA and extended using reverse transcriptase and a nucleotide mix in which cold dCTP was replaced with ^{32}P-dCTP. To determine the sizes of the primer extension products, they were run on a sequencing gel alongside a sequencing reaction in which the same primer was extended using CTYR4.3 DNA as a template. The number of nt by which the primer had been extended and thus the position of the 5′ end of each product could then be deduced.

Typical autoradiographs showing primer extension products obtained using different RNA templates are shown in Fig. 3.4. Each figure represents a portion of the sequencing gel showing the position of some of the products that could be obtained in repeat experiments, for instance the products with 5′ ends at nt positions -62 and -71. As primer extension was carried out using incorporated label as opposed to end-labelled primer, the procedure was prone to non-specific incorporation of the labelled nucleotide and thus high background on autoradiographs. This is because any short RNA or single-stranded DNA molecule present in the reaction mix could potentially serve as a random primer. Therefore, several primer extension runs were attempted under different conditions (such as varying the amount of primer or cold dCTP included in the reaction) in order to detect specific extended products above non-specific background on autoradiographs. The optimised conditions under which the 20-mer primer was annealed to the template RNA and extended, are outlined in the materials and methods section.

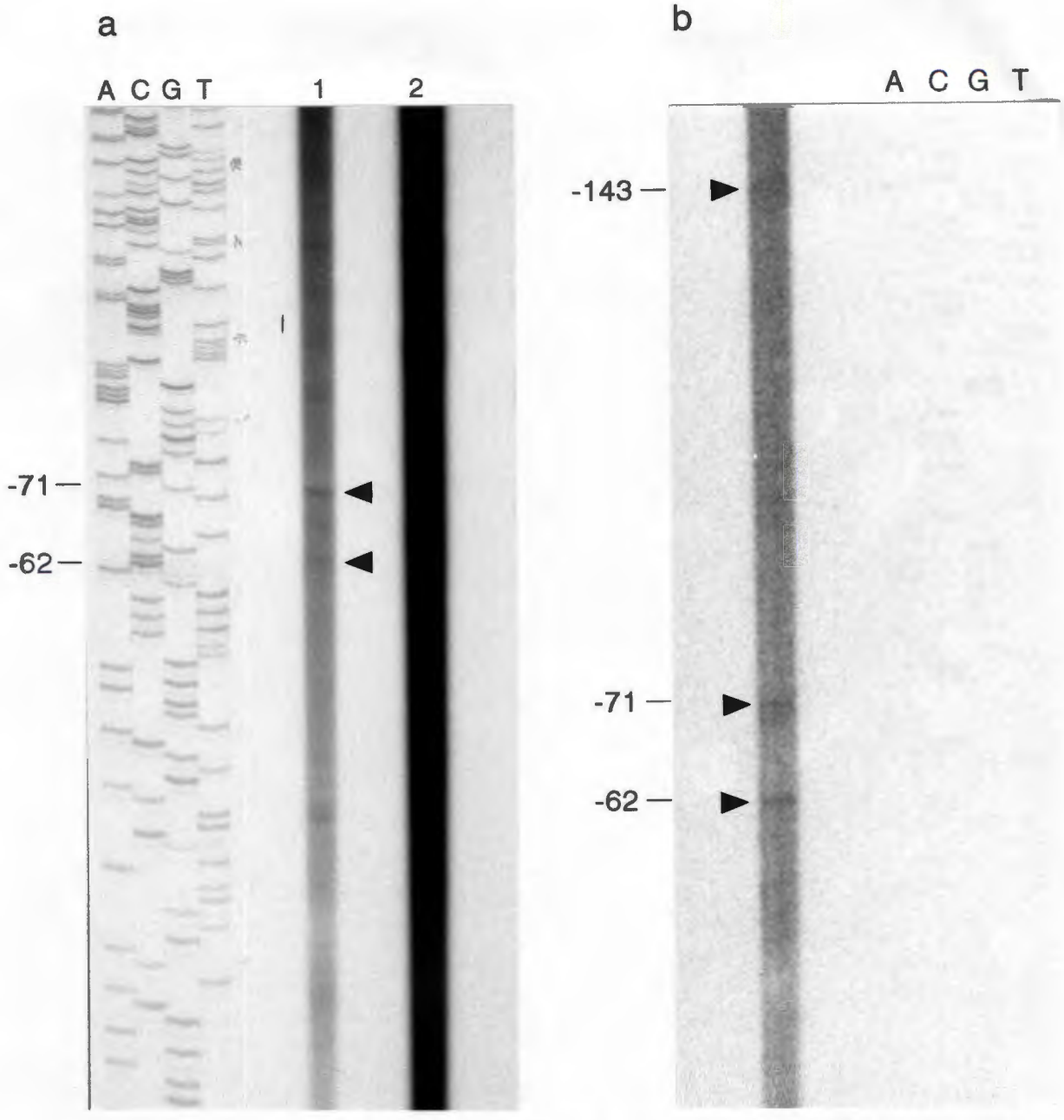


Fig. 3.4. Primer extension analysis to determine the transcription initiation site(s) of the chicken tyrosinase gene. **(a)** Primer extension analysis of poly(A^+) RNA isolated from pigmented chick embryo eye. **Lane 1**, Primer extension products obtained using the "optimised" experimental conditions described in the materials and methods. The products seen above the background smear that were repeatable are indicated by arrowheads. These correspond to transcription start sites at nt positions -62 and -71. The sequencing ladder of the complementary strand of CTYR4.3 obtained with the same primer, was used to locate the position of transcription start sites. **Lane 2**, a primer extension reaction performed under non-optimal conditions, which resulted in very high background. The experimental conditions employed were those typically used during first strand cDNA synthesis, which involve fewer manipulations but are less stringent. **(b)** Primer extension analysis of poly(A^+) RNA isolated from pigmented skin of chick embryos, using the "optimised" experimental conditions. The products corresponding to transcription start sites at nt positions -62, -71 and -143 are indicated by arrowheads. A very faint sequencing ladder of the complementary strand of CTYR4.3 obtained with the same primer, was used to locate the position of transcription start sites.

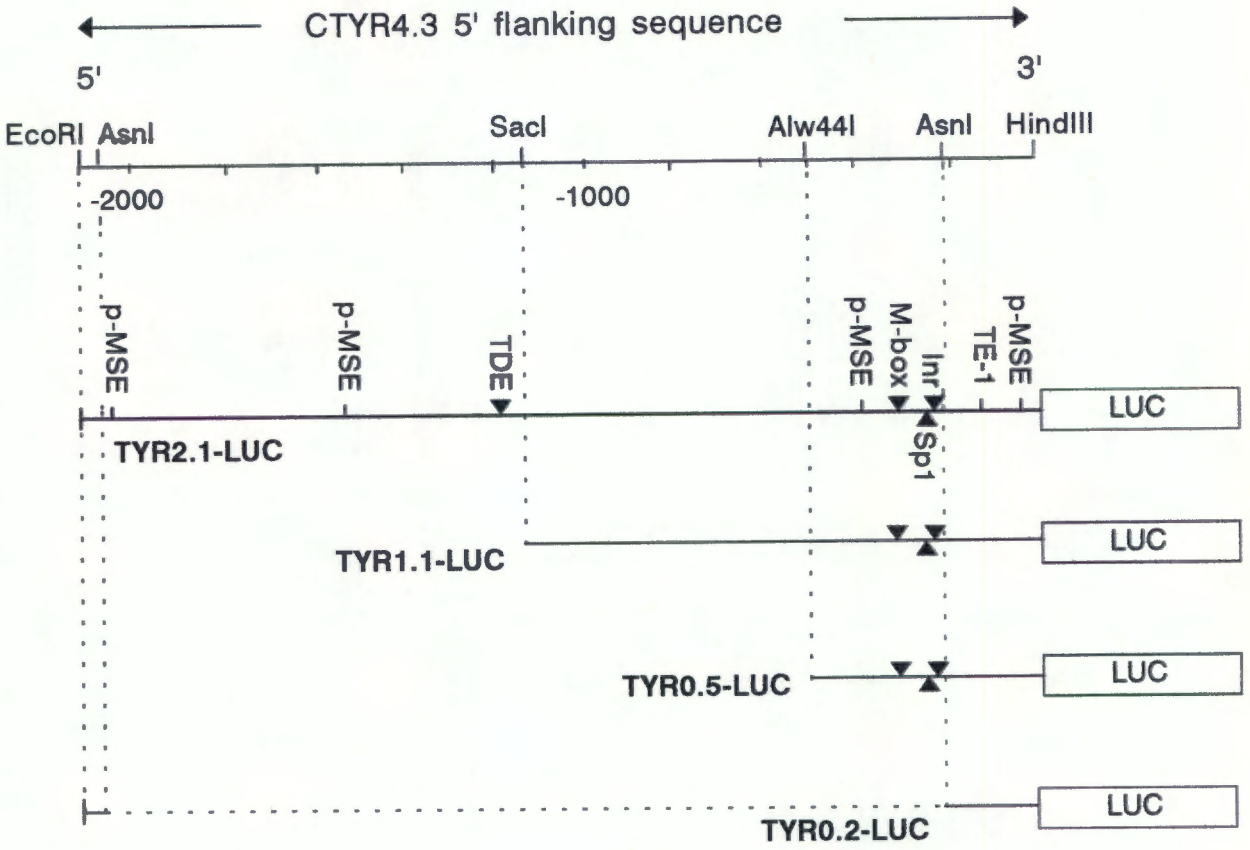


Fig. 3.5: Maps of the tyrosinase promoter-luciferase reporter gene constructs used for transient transfection. **TYR2.1-LUC** contains the entire 5′ flanking sequence cloned to date for the chicken tyrosinase gene. **TYR1.1-LUC** was constructed to specifically exclude the TDE-like element. **TYR0.5-LUC** containing the proximal 504 nt of 5′ flanking sequence, was constructed to compare promoter activity with that of the 519 nt quail promoter, which has been functionally analysed (Akiyama et al, 1994). **TYR0.2-LUC** was constructed to specifically exclude the putative M-box, SP1-binding site and Inr element. The positions of other potentially important sites within the constructs are indicated. Details of the construction may be found in section 3.2.3.

Data presented in Fig. 3.2 were derived from the combined results obtained from several different primer extension experiments. Five potential transcription start sites were identified (these are circled and indicated in bold) at nt positions -62, -71, -143, -200 and -248, with the product corresponding to the start site at nt position -62 being most consistently obtained. It should be noted that in some experiments additional transcription start sites were noticed, but these were not reproduced in repeat experiments and thus could not be considered to be authentic sites. However, one cannot rule out the possibility that minor species of tyrosinase transcripts may be initiated at these isolated sites. Interestingly, different potential start sites were obtained for eye- versus skin-derived tyrosinase transcripts. Using skin mRNA, primer extension products were obtained with 5′ termini at the three most proximal sites, whereas products that correlated with all five transcription start sites were obtained when using eye- and/or RPE-derived RNA. None of the signals produced by the five repeatable products was notably stronger than the others and thus a predominant start site was not apparent amongst the five sites indicated. These results suggest that the transcription start site for the chicken tyrosinase gene is heterogeneous.

3.3.3. Functional analysis of the 5′ flanking sequence of CTYR4.3 in transfected cells

The aim of the transfection experiments was twofold: firstly to confirm that the 5′ flanking region of CTYR4.3 can function as a promoter, and secondly, to begin to assess the significance of the conserved promoter elements (M-box, Inr, TDE) in the functioning of the chicken tyrosinase promoter.

To this end, reporter gene recombinants were constructed in which a series of putative chicken tyrosinase promoter sequences progressively deleted from the 5′ end, were cloned upstream of the luciferase reporter gene. The positions of the deletion endpoints relative to the putative regulatory elements are shown in Fig. 3.5.

Initially, attempts were made to generate the promoter deletions by Exonuclease III digestion. However, progressive deletions could only be generated from the 3′ end of CTYR4.3 and all attempts to generate progressive deletions from the 5′ end of CTYR4.3 were unsuccessful. Specific promoter

fragments were therefore obtained by restriction enzyme digestion, an approach that was greatly limited by the availability of unique restriction enzyme sites in CTYR4.3. As a result of cloning limitations, it should be noted that TYRO.2-LUC contains the 5′ flanking sequences between nt positions -205 and -3, as well as 45 bp from the extreme 5′ end (-2125/-2080) of CTYR4.3.

3.3.3.1. Characteristics of cell types used in transient transfection experiments

Functional analysis of the mammalian melanocyte-specific gene promoters has been facilitated by the availability of melanoma cell lines and immortalised melanocytes. There are, however, no equivalent avian cell lines available. The most concentrated source of pigmented cells in vertebrates is the RPE cell layer in the eye, and therefore primary cultures of RPE cells derived from chicken embryos were initiated for this study. These could be used as "tyrosinase-positive" cells for transient transfection experiments, as the cells are visibly pigmented (see Fig. 3.6.a) and continue to express the tyrosinase gene in culture as shown by Northern blot hybridisation analyses (see Fig. 3.7 and April et al, 1996).

A recently established quail neural crest-derived cell line, MQTNC (Fauquet et al. 1990), was also explored for its potential for use as a tyrosinase-positive cell line. These quail trunk neural crest cells were immortalised by infection with the avian *myc*-carrying MC29 retrovirus. Transformation of avian cells by MC29 either blocks or maintains differentiation, depending on the cell type. Fauquet et al (1990) have shown that immortalisation of the quail neural crest cells by MC29 does not suppress the appearance of catecholaminergic traits within the subpopulation originally destined to give rise to the catecholaminergic neurons. Fauquet et al (1990) observed differentiated melanocytes in some of their original immortalised cell cultures, indicating that a subpopulation of these heterogeneous cells have melanogenic potential. The MQTNC cultures used in this study were, however, visibly unpigmented (see Fig. 3.6.b and c) and therefore attempts were made to detect tyrosinase gene expression in these cells by Northern blot hybridisation using a radiolabelled chicken tyrosinase cDNA probe. As can be seen in Fig. 3.7, a single hybridisation signal was obtained in the 10 μg sample of total RNA extracted from cultured RPE cells (lane three), but no signals could be detected in

a

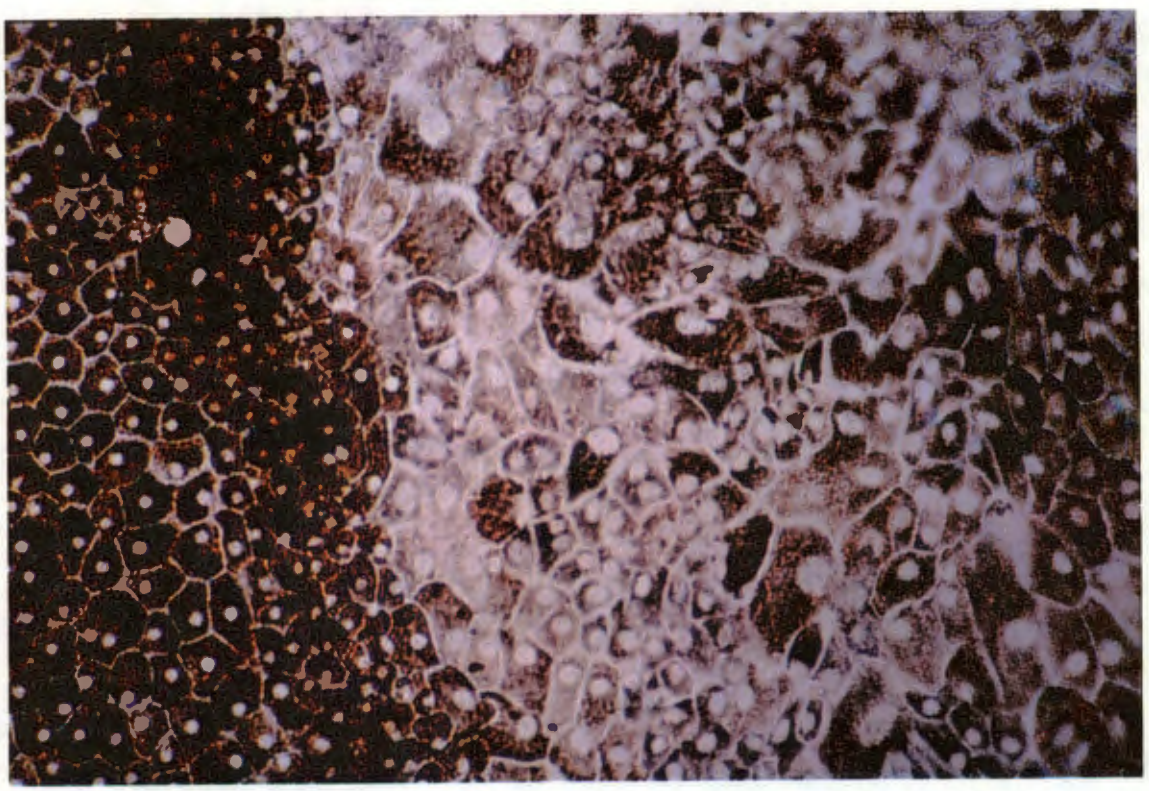


Fig. 3.6: (a) Primary cultures of chicken RPE cells. The characteristic "cobblestone" morphology of RPE cells is seen clearly on the left-hand side of the micrograph. All cells in this confluent culture are visibly pigmented. The cells were visualised with bright-field illumination. Magnification, X 275.

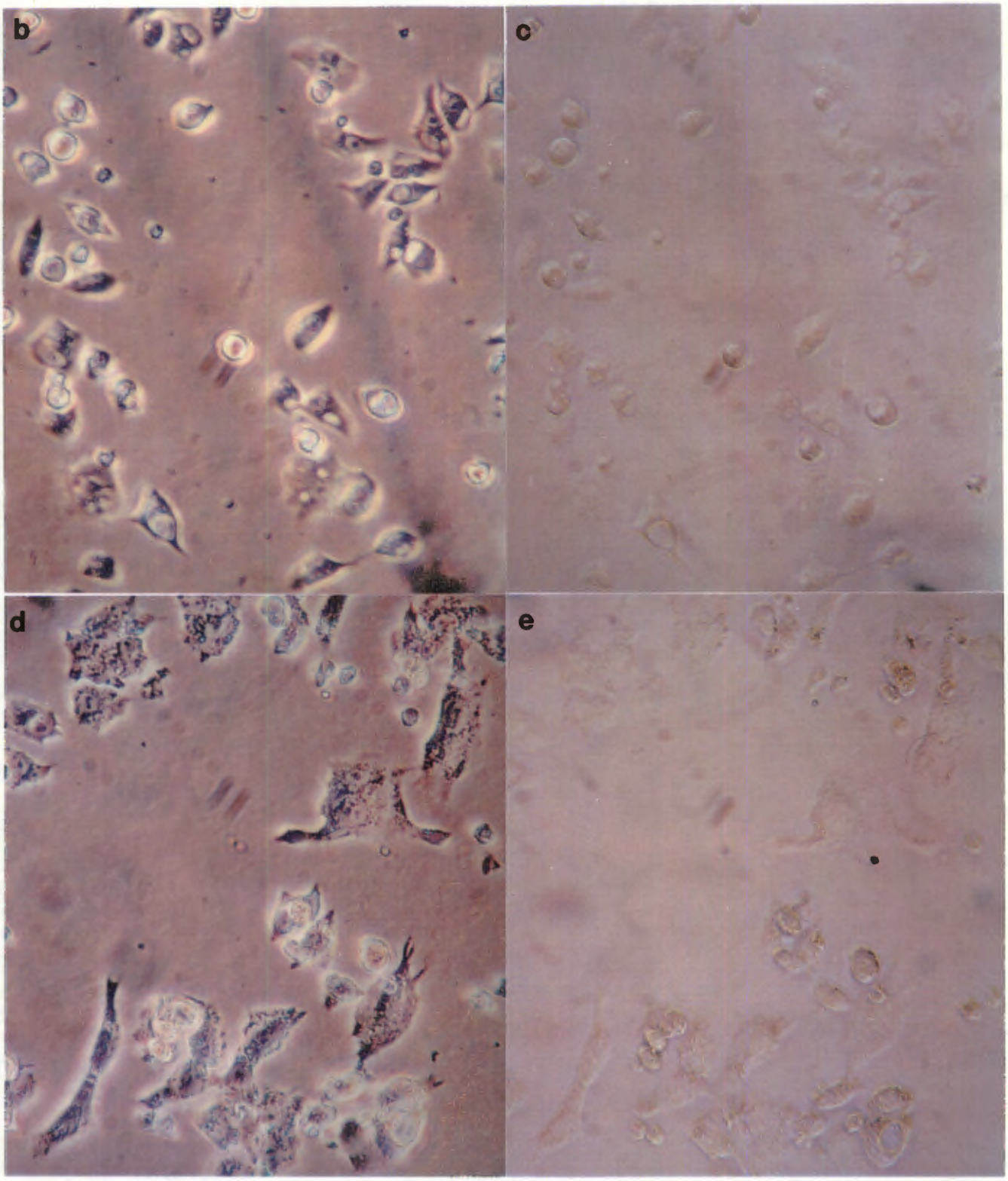


Fig. 3.6: (b-e) The phenotype of MQTNC and Hep G2 cells in culture. MQTNC cells (b and c) and Hep G2 cells (d and e) were visualised with both phase-contrast (b and d) and bright-field illumination (c and e). The cells are clearly unpigmented. Magnification, X 275.

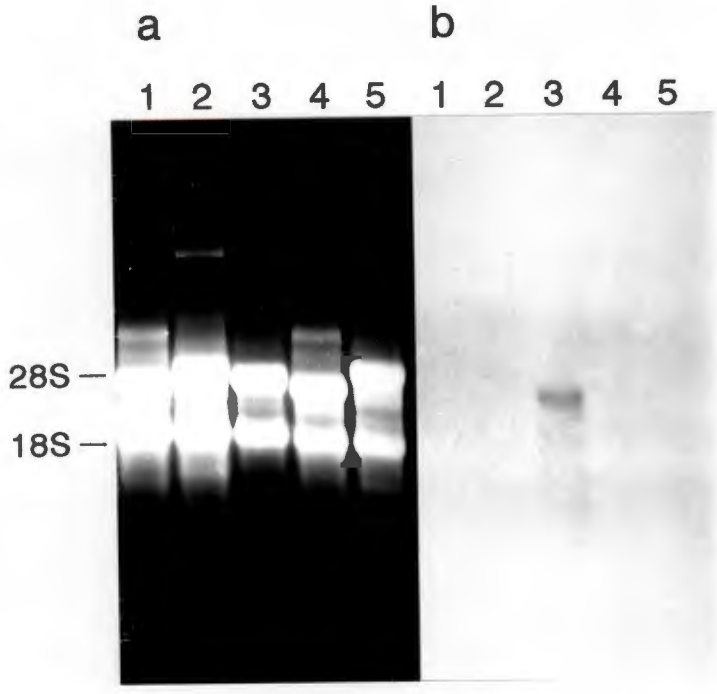


Fig. 3.7: Northern blot hybridisation analysis of tyrosinase gene expression in RPE, MQTNC and Hep G2 cells. **(a)** Electrophoretic analysis of RNA extracted from MQTNC cells (20 μg, lane 1; 10 μg, lane 4), Hep G2 cells (20 μg, lane 2; 10 μg, lane 5) and RPE cells (10 μg, lane 3). The 18S and 28S ribosomal RNA bands are clearly evident in all samples, indicating that the RNA is intact. The intensity of ethidium bromide staining confirms that approximately equal amounts of RNA were analysed in lanes 1 and 2, and in lanes 3,4 and 5. **(b)** Northern blot hybridisation of the RNA that was separated on the gel in (a). A 1.5 kb *Eco*RI/*Pst*I fragment of the chicken tyrosinase cDNA was radiolabelled and used as a probe. A hybridising RNA transcript of approximately 2.5 kb in size was detected only in the RPE RNA sample (lane 3), confirming that these cells are "tyrosinase-positive". It should be noted that these RPE cells had been in culture for 4 weeks and passaged twice.

either the 10 μg or 20 μg sample of total RNA extracted from MQTNC cells (lanes four and one, respectively). There is a possibility that the conditions used above may not have been sufficiently sensitive to detect a rare transcript within a subpopulation of the heterogeneous MQTNC cells, and thus, at this stage, it is not known for certain whether these cells are tyrosinase-negative or -positive. Attempts are currently being made to determine whether a subset of these cells are expressing the tyrosinase gene using *in situ* hybridisation (T. Franz and S. Kidson, personal communication).

Despite the uncertainty about the melanogenic potential of the MQTNC cells, it was deemed worthwhile to test tyrosinase promoter activity in these cells. This decision was made because of the interesting possibility that if the chicken tyrosinase promoter is active in MQTNC cells, its activity may be regulated differently in these neural crest-derived cells than in RPE cells which are derived from the optic cup. Furthermore, Fauquet and Boni (1993), who have focused on the catecholaminergic neurons that arise from neural crest cells, have shown that MQTNC cells are easily transfected using the calcium phosphate-mediated technique, and have successfully analysed the activity of tyrosine hydroxylase promoter-reporter gene constructs in MQTNC cells by transient transfection. This method of transfection also proved to be successful when tested on the primary RPE cell cultures initiated for this study, but required a few modifications because of the greater sensitivity of primary cultures to manipulation and their relatively slow growth rate.

Quail fibrosarcoma (QT6; ATCC no. 1708-CRL) cells were to be used as an avian "tyrosinase-negative" cell line. However, these cultures proved to be difficult to maintain in culture and to transfect using the calcium phosphate-mediated method. Hep G2 cells (human hepato-blastoma cells, ATCC no. HB8065; Fig. 3.6.d and e) were used as an alternative as they grow well in culture and are easily transfectable using the calcium phosphate-mediated method. Furthermore it has previously been shown by Northern blot hybridisation analysis that Hep G2 cells do not express tyrosinase (Kwon et al, 1987). This was confirmed by the lack of hybridisation signals in Hep G2 RNA samples probed with a radiolabelled chicken tyrosinase cDNA sequence (Fig. 3.7, lanes two and five).

To determine optimal conditions for calcium phosphate-mediated transfection of RPE, MQTNC and Hep G2 cells, a series of pilot experiments (transfection

experiments 1 to 11, results not shown) was conducted. Parameters that were varied included the seeding density of cells, the amount of DNA transfected, and the length of exposure of the cells to the calcium phosphate-DNA precipitates. The effects of using different methods of DNA purification (that is, Wizard Maxiprep DNA Purification [Promega Corporation] versus purification by centrifugation in cesium chloride-ethidium bromide gradients) and glycerol shocking were also assessed. The conditions that were finally used to test chicken tyrosinase promoter activity are specified in the materials and methods section 3.2.6.

3.3.3.2. Tyrosinase promoter activity in RPE, MQTNC and Hep G2 cells

Promoter activity of 5′ flanking sequences derived from CTYR4.3 was then assessed in transfection experiments 12 to 22. Results of individual experiments are shown in Fig. 3.8 (a-c). The results were not pooled because of the inter-experimental variation, but as can be seen from the graphs, similar results were obtained on each occasion for the different cell types.

The first aim of the transfection experiments was to show that the 5′ flanking sequences of CTYR4.3 are able to function as a promoter. Transfections with all constructs containing 5′ flanking sequences of CTYR4.3 resulted in luciferase activities significantly greater than those observed when using the promoterless luciferase construct (pGL2-Basic, indicated by a "0" on the x-axis of each experiment). These results confirm that the 5′ flanking sequences of CTYR4.3 possess promoter activity. The level of expression driven by the various promoter fragments, however, differed markedly in the different cell types. In the tyrosinase-negative Hep G2 cells, low levels of expression were observed with all constructs (Fig. 3.8.b). A similar result was reported by Hughes et al (1995) who observed promoter activity above background of a 529 bp human tyrosinase promoter in Hep G2 cells. Greatly increased luciferase activities were observed with some constructs in the RPE and MQTNC cells, but surprisingly, the highest levels of expression were driven by different constructs in these two cell types (as will be described below).

In RPE cells, in all three transfection experiments, the highest level of luciferase activity was obtained with TYR0.2-LUC, the construct containing the smallest (0.2 kb) promoter fragment (Fig. 3.8.a). Indeed, this was the only

promoter fragment with significantly higher activity in RPE cells than in Hep G2 cells, indicating that it must contain elements that can direct both basal transcription and RPE-specific expression of a downstream gene. Because deletion of promoter sequences between -0.5 and -0.2 kb resulted in this significant increase in promoter activity, it is possible that there is a strong negative regulatory element located between nt positions -0.5 and -0.2 kb.

Somewhat surprisingly, the 2.1 kb, 1.1 kb and 0.5 kb fragments had lower promoter activity than the 0.2 kb fragment. When comparing the activities of TYR2.1- and TYR1.1-LUC, it was noted that in all three experiments TYR2.1-LUC activity was higher (by 15-40%) than that of TYR1.1-LUC. This suggests the possibility of a positive regulatory element located between -1.1 and -2.1 kb from the ATG site.

Interestingly, the promoter fragments have very different activities in MQTNC cells. In all cases, the longest construct, TYR2.1-LUC, had the highest activity. Deletion of sequences upstream of -1.1 kb resulted in a dramatic reduction in promoter activity, especially in experiments 12 and 20, where activity dropped by 79% and 86%, respectively (See Fig. 3.8.c). This suggests the presence of a strong positively acting element located upstream of the -1.1 kb deletion endpoint, which appears to mediate a stronger positive effect in the MQTNC cells than in the RPE cells. The expression driven by the smaller promoter fragments containing 0.5 kb and 0.2 kb of chicken tyrosinase promoter sequences, remained at a relatively low level in MQTNC cells, comparable with the levels obtained in the Hep G2 cells. Moreover, the 0.2 kb fragment displayed the weakest promoter activity in these cells, in contrast to its high activity in RPE cells. Thus transcription driven by the chicken tyrosinase promoter appears to be regulated differently in RPE cells and MQTNC cells.

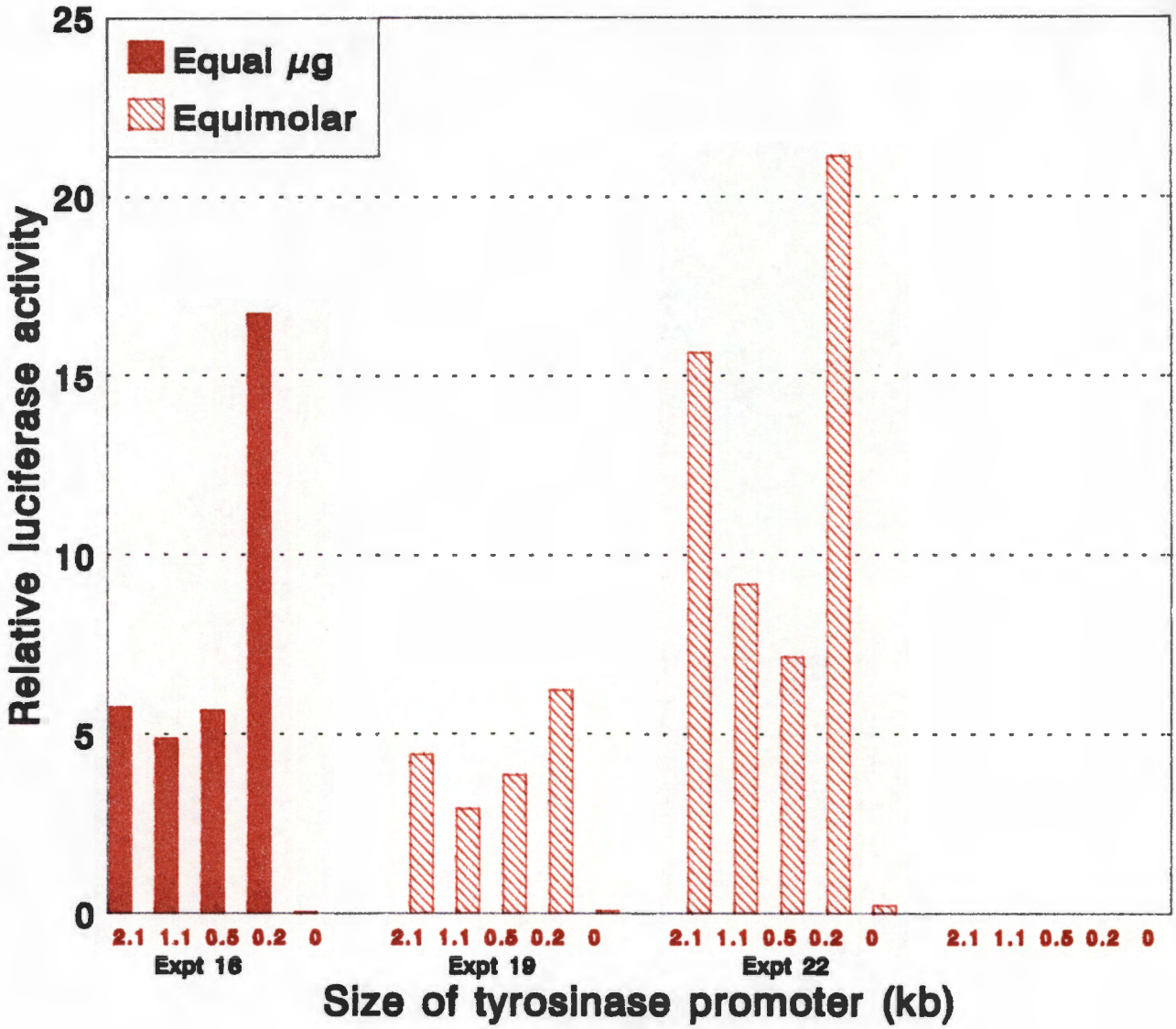


Fig. 3.8: (a) Activities of chicken tyrosinase gene promoter deletion mutants in RPE cells. Cells were transfected with either equimolar (hatched bars) or equal microgram amounts (solid bars) of each promoter-luciferase construct (The reason for this is discussed in the materials and methods section 3.2.6.2). Results were normalised with respect to β-gal activity in the same extract and are expressed as a percentage of the luciferase activity obtained with pGL2-Control, shown as units of relative luciferase activity. The figures 2.1, 1.1, 0.5, 0.2 and 0 on the x-axis represent the constructs TYR2.1-LUC, TYR1.1-LUC, TYR0.5-LUC, TYR0.2-LUC and pGL2-Basic, respectively.

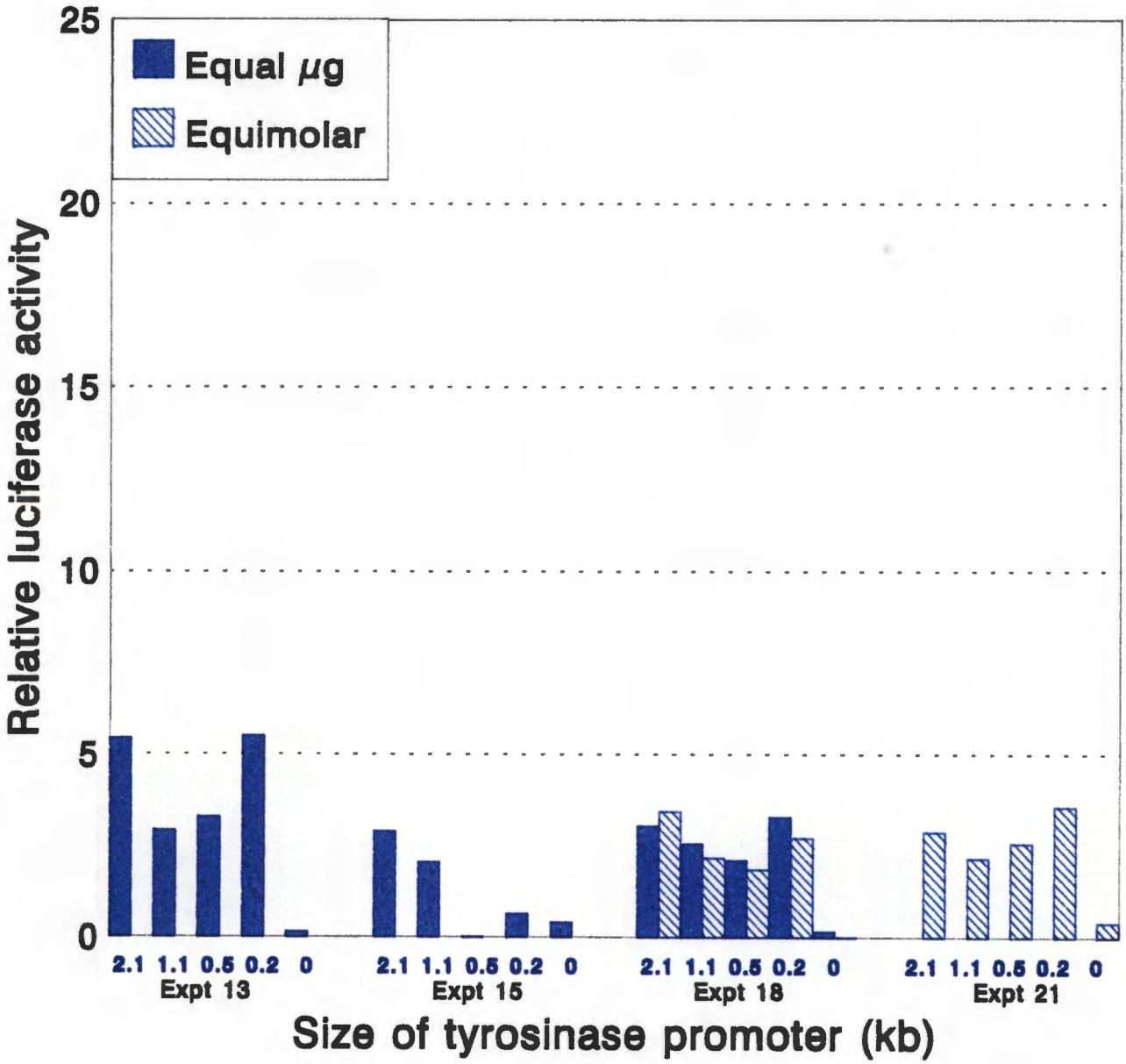


Fig. 3.8: (b) Activities of chicken tyrosinase gene promoter deletion mutants in **Hep G2** cells. Cells were transfected with either equimolar (hatched bars) or equal microgram amounts (solid bars) of each promoter-luciferase construct (The reason for this is discussed in the materials and methods section 3.2.6.2). Results were normalised with respect to β-gal activity in the same extract and are expressed as a percentage of the luciferase activity obtained with pGL2-Control, shown as units of relative luciferase activity. The figures 2.1, 1.1, 0.5, 0.2 and 0 on the x-axis represent the constructs TYR2.1-LUC, TYR1.1-LUC, TYR0.5-LUC, TYR0.2-LUC and pGL2-Basic, respectively.

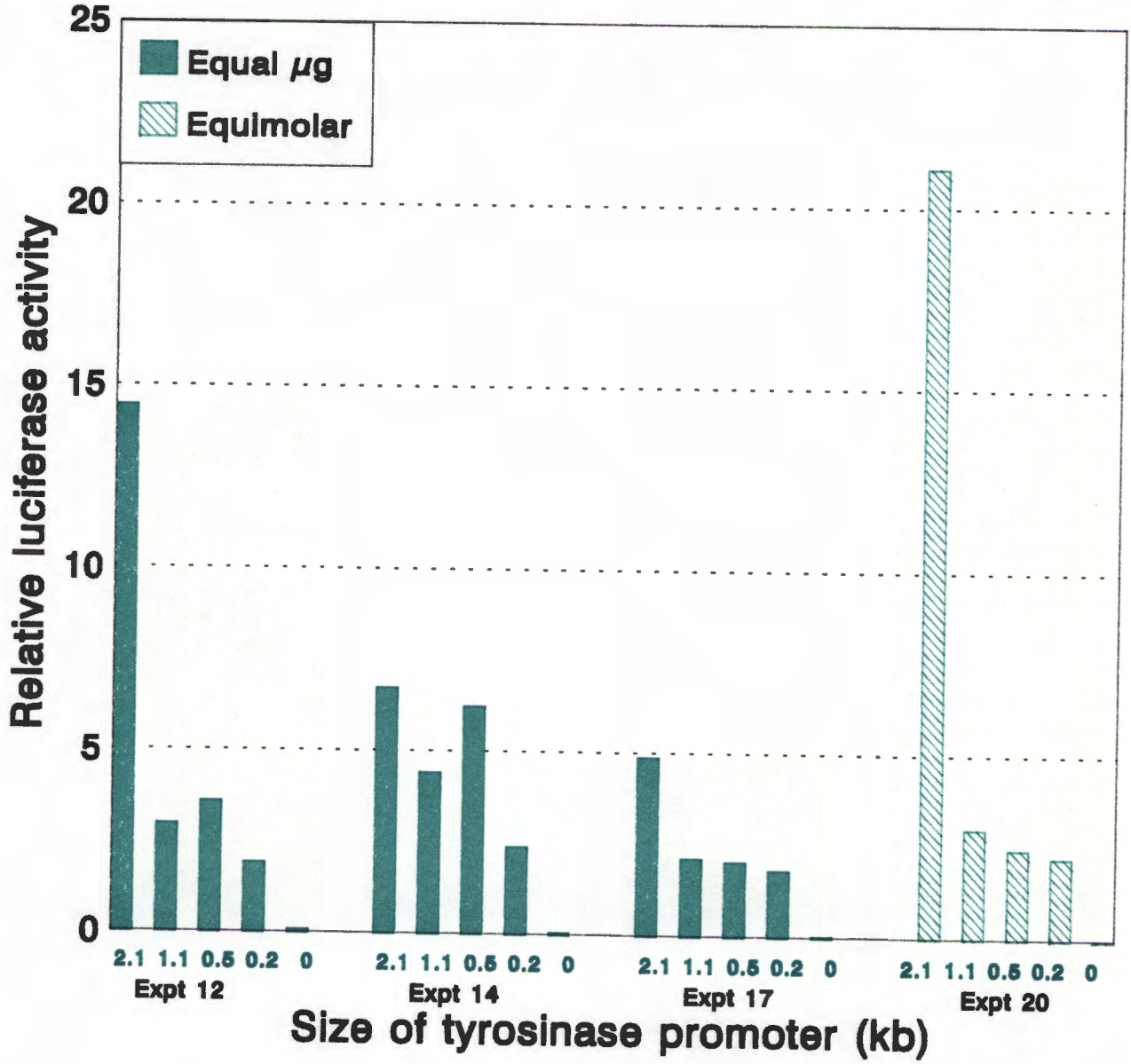


Fig. 3.8: (c) Activities of chicken tyrosinase gene promoter deletion mutants in **MQTNC** cells. Cells were transfected with either equimolar (hatched bars) or equal microgram amounts (solid bars) of each promoter-luciferase construct (The reason for this is discussed in the materials and methods section 3.2.6.2). Results were normalised with respect to ß-gal activity in the same extract and are expressed as a percentage of the luciferase activity obtained with pGL2-Control, shown as units of relative luciferase activity. The figures 2.1, 1.1, 0.5, 0.2 and 0 on the x-axis represent the constructs TYR2.1-LUC, TYR1.1-LUC, TYR0.5-LUC, TYR0.2-LUC and pGL2-Basic, respectively.

3.4. DISCUSSION

3.4.1. Comparison of vertebrate tyrosinase gene promoters

Sequence analyses, primer extension and transient transfection experiments were undertaken in this study to characterise the 5′ flanking sequence cloned in CTYR4.3 and to assess its promoter activity. The purpose of this section is to put forward an interpretation of the results from these experiments and to discuss their significance to tyrosinase gene regulation in chickens and lower vertebrates in general.

Sequence alignments were performed to assess the similarity of the chicken tyrosinase 5′ flanking sequence to the well characterised promoter regions of tyrosinase genes from other vertebrates. Results indicate that the chicken tyrosinase gene promoter is most closely related to those of the quail and turtle tyrosinase genes. Furthermore, the chicken promoter sequence appears to be more closely related to the mammalian tyrosinase sequence than to the frog and medaka fish tyrosinase 5′ flanking sequences which, surprisingly, could not be aligned with the tyrosinase gene promoter sequences of any other vertebrate. This finding is supported by the report of Morrison et al (1994), who constructed a phylogenetic tree based on cDNA sequence data to illustrate the evolutionary relationships between the members of the tyrosinase gene family. Their tree groups the chicken, mouse and human tyrosinase gene sequences together on a branch separate from that of the frog sequence (See Fig. 3.9).

Alignment of sequences from human, mouse, chicken, quail and turtle tyrosinase gene promoters in this study revealed two conserved regions, here named the proximal and distal conserved regions. The mammalian tyrosinase sequences present in these conserved regions have previously been analysed in other studies and shown to contain important regulatory elements (discussed in section 1.3). These regulatory elements include the Inr and SP1-binding site, which are required for basal transcription, and the M-box (p-MSE) and TDE, which are enhancers that mediate melanocyte-specific upregulation of tyrosinase gene expression. These sequence similarities raise the possibility that tyrosinase gene expression in chickens (and other avians), reptiles and mammals may be regulated by similar sets of

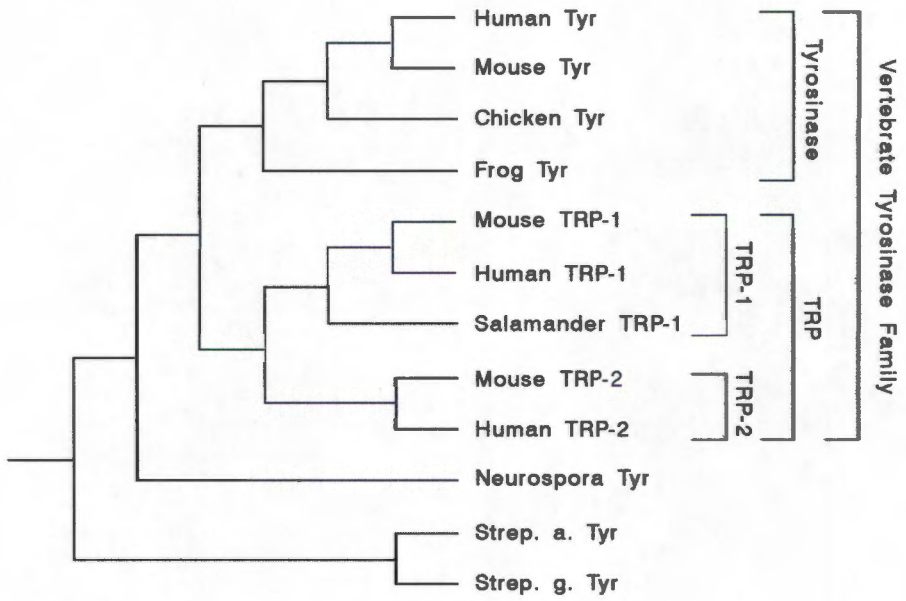


Fig. 3.9: Phylogeny of the vertebrate members of the tyrosinase gene family. Groups sharing common ancestry are bracketed. Tyr, tyrosinase; Strep. a., Streptomyces antibioticus; Strep. g., Streptomyces glaucescens. (After Morrison et al, 1994.)

cis- and trans-acting factors, and that these may well be distinct from those employed in the lower vertebrates, namely, amphibians and fish.

In support of this idea are the results of some "cross species" analyses of vertebrate tyrosinase promoters. For instance, Yamamoto and co-workers have shown that 5′ flanking sequences from quail and turtle tyrosinase genes can direct melanocyte-specific expression in the mouse system (cited in Jackson, 1994) and similarly that the mouse tyrosinase promoter can function melanocyte-specifically in cultured chicken cells (Akiyama et al, 1994). On the other hand, it has been reported that the mouse tyrosinase promoter is able to direct melanophore-specific expression of a downstream sequence in transgenic fish (Matsumoto et al, 1992). Thus, while there is very little sequence similarity between mammalian and fish tyrosinase promoters, functionally they appear to employ similar mechanisms to achieve melanocyte-specific expression.

3.4.2. Transcription of the chicken tyrosinase gene is initiated at heterogeneous start sites

Transcription start site analysis was performed in an attempt to determine the 3′ boundary of the chicken tyrosinase gene promoter. Using primer extension, several potential transcription start sites were mapped in the 5′ flanking sequence between nt positions -62 and -248 from the ATG site.

Several technical problems were encountered during transcription start site analysis. Tyrosinase mRNA transcripts, due to their rarity, are notoriously difficult to detect in whole tissue samples, especially skin samples (Mochii et al, 1992; April et al, 1996; Steel et al, 1992). The use of pigmented cell lines to obtain sufficient quantities of transcripts, has relieved this problem for *in vitro* studies of mammalian tyrosinase mRNA. However, as mentioned earlier, there are no such avian cell lines available. A further problem was the relatively high backround of autoradiographs from the primer extension analyses; this high background could have masked the extended products, and have been responsible for the inconsistency in results obtained from different experiments. It is unlikely that the heterogeneous results arose by reverse transcription of degraded RNA templates or that they represent products caused by premature halting of the reverse transcriptase

due to secondary structure formation by GC-rich templates because the chicken tyrosinase gene sequence is 52% AT-rich over the proximal 248 nt upstream from the ATG site. The authenticity of these primer extension results would, however, be strengthened if the results could be confirmed using techniques such as RNase or S1 nuclease protection.[1] Nevertheless, the identification of these heterogeneous transcription start sites for the chicken tyrosinase gene is supported by several observations.

Firstly, there is a lack of consensus in the literature regarding the 5′ terminus reported for different avian tyrosinase gene transcripts. A transcription start site was reported for the quail tyrosinase gene at the CACT sequence 81 nt upstream from the ATG site (Akiyama et al, 1994). In CTYR4.3 this site is exactly conserved in sequence and relative position from the ATG, and thus may also function as a transcription start site for chicken tyrosinase. However, since Akiyama et al (1994) do not indicate whether the location of this site was derived empirically or by extrapolation (see section 1.3.1), the authenticity of this site is questionable. Two groups of investigators have isolated chicken tyrosinase cDNA sequences from libraries generated using RNA from RPE cells (Mochii et al, 1992) or neural crest-derived melanocytes (April et al,1996). April et al (1996) found the 5′ terminus of their longest clone to be at -42 nt upstream of the ATG, whereas Mochii et al (1992) report that the 5′ terminus of two independent RPE tyrosinase cDNA clones is at nt position -108. Whilst the 5′ terminus of a cDNA molecule is not necessarily a good indication of the position of the transcription start site since this depends on the efficiency of the reverse transcription during cDNA synthesis, one can nevertheless deduce that the transcription start sites from which the tyrosinase RNA transcripts originate would lie upstream of the cDNA 5′ termini. Given the fact that these cDNA clones represent RNA transcripts of melanocytes from different tissues (viz RPE and neural crest-derived melanocytes), the different 5′ termini may reflect the use of alternative promoters in these tissues and not simply sites at which cDNA synthesis may have stopped prematurely. In summary, putative avian transcription start sites are located at nt position -81 and at a second site 5′ to nt position -108. Possibly a third site (distinct from the site at

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1. With RNase protection in mind, a *DraI/Hin*dIII fragment containing ±430 nt of 5′ flanking sequence and ±170 nt of exon 1, was excised from CTYR4.3 exonuclease deletion clone 10.10 (see Fig. 2.10). This fragment, cloned into a pGEM-3 vector (Promega Corporation), is being used to generate riboprobes for RNase protection.

-81) may be found 5′ to nt position -42. The proximity of the five transcription start sites identified in CTYR4.3 to the 5′ termini of the cDNA sequences is encouraging. The two proximal start sites are positioned at 18 nt and 27 nt upstream of the cDNA terminus at -41 nt, respectively and close to the CACT site at -81 nt, while the 3 distal start sites are positioned 35 nt, 92 nt and 140 nt upstream from the cDNA terminus at -108 nt.

The identification of heterogeneous start sites for chicken tyrosinase transcripts is consistent with the absence of a TATA box in the proximal 5′ flanking sequence of CTYR4.3. As mentioned in chapter 1 (section 1.3.1), the binding of a TATA element by transcription factor TFIID serves to direct accurate transcription initiation by RNA polymerase II at a single site approximately 30 nt downstream of the TATA element. Thus, for TATA-less promoters transcription has been found to occur from more heterogeneous sites (reviewed by Smale and Baltimore, 1989). Although several TATA motifs were identified in the 5′ flanking sequence of CTYR4.3 that match sites in the transcription factors database, the most proximal of these is at -552 nt from the ATG site, that is, more than 200 nt from the most distal transcription initiation site. These therefore cannot be functioning as TFIID binding sites. Similar conclusions can be drawn for TATA elements in the quail and turtle tyrosinase 5′ flanking sequences. A similar search of these sequences revealed that the most proximal TATA box is found at nt position -288 from the ATG site in the quail and at -289 in the turtle sequence. The TATA box is unlikely to play a role in transcription that is initiated at the quail start site (CACT) at -81 nt reported by Akiyama et al (1994), as it lies too far upstream of this site. Although TATA motifs have been reported for the mouse and human tyrosinase genes, these genes lack a canonical TATA box (Bentley et al, 1994) and there has been speculation that the TATA boxes might also not be functioning as true TFIID binding sites.

By what mechanism then would RNA polymerase II bind and initiate transcription at the TATA-less tyrosinase gene promoter? As has been discussed in chapter one (section 1.3.1), previous studies of mammalian tyrosinase gene regulation implicate an SP1-binding site and an initiator region containing a functionally significant E-box and octamer motif and an Inr element. These elements fall within the proximal conserved region which was also identified within the chicken, quail and turtle tyrosinase gene promoters. Interestingly, the position of the proximal conserved region relative to the

ATG site is not conserved between mammals and lower vertebrates - in the mouse and human tyrosinase promoters the Inr element (which lies at the 3′ boundary of the proximal conserved region) is found 80 nt upstream of the ATG site, whereas in CTYR4.3, the quail and turtle tyrosinase promoters, it is found approximately 200 nt upstream of the ATG site. Within the proximal conserved region, however, the positions of the putative regulatory elements are maintained relative to each other. The chicken Inr E-box and octamer motifs are identical to the human counterparts. In addition, the sequence at the 3′ terminus of the Inr region (5′TCAGATT) fits the Inr consensus motif exactly (as is the case for the homologous mouse, quail and turtle sequences) and lies 5 nt upstream of the putative transcription start site at nt position -200. Furthermore, the nonconsensus SP1-binding sequence (5′CGGGGGTGGGG) closely resembles the quail element (5′AGGGGGTGGGA) which was found by Bentley et al (1994) to be functional (see Fig. 3.3). Given the striking homology, it is tempting to speculate that in the absence of a TATA box, these elements may play a major role in transcription initiation at the chicken tyrosinase gene promoter. However, results from the promoter analyses in the tyrosinase-expressing RPE cells indicate that this might not necessarily be the case and furthermore raise the possibility that the additional 120 nt between the Inr and ATG site may be functionally significant.

3.4.3. Chicken tyrosinase promoter activity in transfected RPE cells

Results from all of the transfections of tyrosinase promoter-reporter gene constructs in RPE cells show that the progressive removal of promoter sequences from the 5′ terminus results in an initial decrease followed by little change in expression of the luciferase reporter gene. This trend is altered drastically by the marked increase in expression observed on transfection with the shortest construct, TYRO.2-LUC. This increase in expression was surprising given that the promoter sequences in TYRO.2-LUC exclude the putative Inr region, SP1-binding site and M-box motif. There are two possible explanations for this result. The first possibility is that deletion of sequences between -506 and -206 results in the removal of negative regulatory elements that are responsive to RPE-specific transacting factors. The second possibility is that the high levels of expression observed when using TYRO.2-LUC are due to the inclusion of an upstream (distal) 45 bp sequence (nucleotides -2125 to -2080) that was ligated to the 200 bp (-205 to -3) proximal sequence. (This was brought about by the cloning limitations

described in section 3.3.3. The promoter sequence of the TYRO.2-LUC construct is thus 247 bp in length.) Thus it is possible that the extra 45 bp sequence contains a strong positive regulatory element that is responsible for the high level of activity of the TYRO.2-LUC construct. Each of these hypotheses will be considered separately below.

Hypothesis one: there is a strong negative regulatory element between nt positions -506 and -206. The removal of such an element would result in increased expression driven by factors binding to regulatory elements within the proximal 200 bp of 5′ flanking sequence. This would imply that the most proximal 200 bp fragment of 5′ flanking sequence is sufficient to drive high levels of reporter gene expression in pigment-producing cells. As the Inr E-box and SP1-binding motifs are not included in TYRO.2-LUC, these highly conserved motifs appear not to be essential for transcription initiation at the chicken tyrosinase promoter in this case, in spite of their significant role in the mammalian tyrosinase promoters. What elements might then be responsible for directing transcription initiation at the four putative start sites found within the 200 bp fragment? Sequence analysis reveals no further potential TATA boxes or SP1-binding sites in this promoter fragment. A search for putative Inr elements using the PyPyAN$^{T}/_{A}$PyPy consensus, however, revealed a site at nt position -84/-78 (5′TCACACT) that overlaps the position of the quail transcription start site (CACT at -81) reported by Akiyama et al (1994). Although this site does not overlap any of the transcription start sites identified in CTYR4.3 by primer extension, it is found just 5′ of transcription start site 2 (at nt position -71) and may well prove to play a role in basal transcription of the chicken tyrosinase gene. The putative TE-1 element and the most proximal p-MSE are also found within this sequence (see Fig. 3.5). These two elements are direct homologues of the quail elements which were identified by Yamamoto et al (1992) and Akiyama et al (1994), but their functional significance in avian tyrosinase gene transcription regulation has not yet been assessed. Binding at either the TE-1, pMSE or other as yet unidentified elements within the proximal 200 bp of chicken promoter may prove to be necessary to direct transcription initiation from one or more of the four putative start sites or to confer tissue-specificity on the promoter activity.

The presence of a negative regulatory element positioned within TYRO.5-LUC (between nt -206 and -506) would be consistent with the identification of such

elements in the mammalian tyrosinase promoters. Using deletion analysis and mutagenesis, a negative regulatory element was identified in the human sequence at 115 nt from the Inr and in the mouse sequence at 125 nt from the Inr (Bentley et al, 1994; Ganss et al, 1994) (see section 1.3.2 and Fig. 1.7). More recently, Eisen et al (1995) showed that binding at the octamer element within the proximal conserved region by transcription factor Brn-2/N-Oct3, appears to mediate down-regulation of the tyrosinase gene promoter (see section 1.3.1). Since the degenerate octamer in the chicken sequence is identical to the human motif, binding by a chicken POU domain protein similar to Brn-2/N-Oct3 may also repress chicken tyrosinase promoter activity. Detection of N-Oct3 mRNA at elevated levels in melanoma cells as compared to levels in normal human melanocytes, suggests that Brn-2/N-Oct3 is associated with dedifferentiating melanogenic cells. This, together with other evidence provided by Eisen et al (1995), raises the possibility that Brn-2 may play an important role in melanocyte differentiation and transformation. Of relevance to this point is the observation that the cultures of RPE cells that were maintained for the transfection experiments became progressively less pigmented with time. Possibly, a protein such as Brn-2/N-Oct3 is upregulated in these depigmenting RPE cells in culture, which then binds to the tyrosinase gene promoter and progressively down-regulates its activity.

Hypothesis two: an alternative explanation for the high levels of expression obtained from TYRO.2-LUC, is that it is due to the presence of the 45 bp fragment included in the construct upstream of the 200 bp of proximal promoter sequence. In other words, it is possible that there is no negative regulatory element in TYRO.5-LUC, and that the deletion of ±300 nt between TYRO.5-LUC and TYRO.2-LUC may actually cause a decrease in expression which one might expect to accompany the removal of the conserved M-box, SP1-binding and Inr E-box motifs. The high levels of expression from TYRO.2-LUC would then simply be the result of the serendipitous placement of a potential regulatory sequence adjacent to the 200 bp fragment, which, due to its unnatural physical proximity, could stimulate transcription initiation from the 200 bp fragment. Given that the 200 bp promoter sequence has the potential to initiate basal levels of transcription (as discussed in the paragraph above), the 45 bp fragment may be supplying a tissue-specific enhancer element. Furthermore, the promoter elements responsible for RPE-specific gene expression have not yet been identified and thus one cannot exclude the possibility that the 45 bp fragment may contain such elements. Sequence analysis of the 45 bp fragment revealed the presence of several putative regulatory elements (see Table 3.3): firstly, the 3' end of the fragment contains a TATA-like sequence that might provide the core basal promoter; secondly, there exists a site recognised by the POU domain factor Pit-1. It is possible that RPE cells contain multiple POU domain proteins of unknown binding

specificity and function that may be able to recognise this sequence. Finally, an ATF site is present which may be functional in these cells.

The explanations above raise the possibility that there are two regulatory regions capable of directing basal transcription in the chicken tyrosinase 5′ flanking sequence: one that is undefined but responsible for the expression driven by the 0.2 kb promoter, and a second "mammalian-type" regulatory region positioned in the 0.5 kb promoter, 5′ to the 0.2 kb endpoint that is comprised of the conserved Inr and SP1-binding elements. The upstream regulatory region may be active under certain circumstances, for instance in the absence of an inhibitory factor such as Brn-2/N-Oct3, and would possibly direct transcription initiation at the more distal start sites identified in CTYR4.3. These "alternative promoters" may be used in different melanin-producing tissues. Alternative promoters have been proposed for the human and mouse tyrosinase genes (Ruppert et al, 1988; Yamamoto et al, 1989; Giebel et al, 1991; Ponnazhagan et al; 1994); transcription start site analyses of the mammalian tyrosinase genes have revealed a common site at -80 nt from the ATG (see section 1.3.1), which is overlapped by the Inr element at the 3′ terminus of the proximal conserved region. Additional transcription start sites have also been mapped in the mammalian sequences by some but not all investigators. Giebel et al (1991) suggest that the discrepancy in number and position of start sites reported may reflect differences between the human or mouse pigment cell lines used in each case. Thus several start sites are available in tyrosinase genes, but the site(s) used may depend on the cell type and trans-acting factors that are available.

To date there have been only two other reports on the functional analysis of promoter sequences from lower vertebrate tyrosinase genes (Akiyama et al, 1994; Miura et al, 1995). Miura et al (1995) simply showed that the proximal 748 bp of 5′ flanking sequence of the frog tyrosinase gene can drive transcription of a reporter gene in cultured frog melanophores. Akiyama et al (1994) assessed the promoter activity of quail tyrosinase 5′ flanking sequences in cultured chicken cells using two constructs, containing 519 nt and 369 nt of 5′ flanking sequence, respectively (detailed in section 1.3.2). They found that the proximal 369 bp of 5′ flanking sequence could drive low levels of expression of a reporter gene (mouse tyrosinase cDNA) in cultured albino melanocytes, fibroblasts and hepatocytes. Higher levels of expression

Table 3.3

Potential transcription factor binding sites in the 45 bp fragment of TYRO.2-LUC.

Binding site	Transcription factor	Significance	Reference
TGACGA -2122 TGACGA -2117	GCN4	Non-consensus binding site found in the yeast His3.1 gene 8520 (1986)	Proc. Natl Acod. Sci. U.S.A. Pnas 83: 8516-
TGACGAW -2116 TGACGAT -2122^{CS}	ATF	Binds at site found in adenovirus and cellular genes. Found to bind co-operatively in the presence of TFIID	Genetika 26: 804-816 (1990)
CATTCCT -2012 CATTCCT -2108^{CS}	MCBF	Novel factor found to play a role in basal transcription of cardiac muscle troponin T promoter.	Mol. Cell Biol. 10: 4271-4283 (1990)
ATGAAAA -2104 ATGAAAA -2098	Pit-1	Non-consensus binding site found in human Prolactin gene promoter.	Mol. Cell Biol. 10: 4271-4283 (1990)
YCATTC -2101 TCATTC -2106^{CS}	unknown	Regulatory site found in H2A histone protein gene promoter.	Nucleic Acids Res. 14 Supp: r119-149 (1986)

Note: This information appears in the GCG file, TFsites.
^{CS} indicates that the element is present in the complementary strand.

were obtained using the 519 bp promoter fragment, and this expression was found exclusively in the melanocytes. Thus, 519 bp of quail tyrosinase promoter is sufficient for appropriate tissue-specific expression in melanocytes of neural crest-derived origin. The lower level of expression driven by the 369 bp fragment in melanocytes suggests that a positive regulatory element is missing from this sequence but is present in the 519 bp fragment. The activation of reporter gene expression in the fibroblasts and hepatocytes using the truncated promoter sequence also suggests the loss of sequences necessary for repression of tyrosinase promoter activity in the non-melanin-producing cells.

Functional analysis by transient transfection of the proximal 500 nt of the chicken tyrosinase promoter in this study gives results that are not easily reconciled with the results of Akiyama et al (1994). Given the difference in length between TYRO.2-LUC and the 369 nt quail promoter fragment, and the fact that interpretation of the results of transfection experiments using TYRO.2-LUC is complicated by the presence of the extraneous 45 bp fragment in this construct, it would be premature to compare the activities of these two constructs. The chicken 5′ flanking sequence (506 nt) contained in TYRO.5-LUC, however, is roughly equivalent in length and nucleotide sequence to the 519 nt quail promoter sequence. Unlike the equivalent quail sequence, the activity of the chicken 506 nt promoter sequence was not tissue-specific, but was observed in both RPE and Hep G2 cells, although consistently at higher levels in the RPE cells. Although these promoter sequences are 92% homologous, the conflicting results may nevertheless be due to the slight differences in sequence. This has been demonstrated previously for the mouse and human tyrosinase promoters. Using transient transfections Shibata et al (1992) showed that proximal promoter sequences of human and mouse tyrosinase genes were not functionally equivalent, in spite of their sequence similarity. The discrepancy in results may also be due to the use of different systems to test promoter activity. Akiyama and coworkers made use of a retroviral vector, RCANBP, that includes the Rous sarcoma virus (RSV) long-terminal repeat (LTR i.e. viral promoter sequences) and *pol* sequences derived from the Bryan high-titre strain of RSV, but apparently requires an internal promoter to control the expression of the reporter gene. Although infections using vector alone (RCANBP) were included as negative controls, Akiyama et al do not indicate whether they tested for background activity of the retroviral vector linked to the reporter (RCANBP-mouse tyrosinase cDNA) in infected cultured chicken cells. Therefore, it is not known whether background reporter activity contributes to the activity

driven by the different quail promoter fragments. It has been reported that proviral sequences within the LTR and *pol* regions do influence the levels of expression of reporter genes driven

from an inserted promoter (Petropoulos and Hughes, 1991), but it is not clear whether these "enhancer" sequences affect the activities of the two quail promoter fragments in different ways. Another factor to consider is that the conflicting results may stem from the use of cultured chicken melanocytes for the retroviral infections, which were neural crest-derived, as opposed to RPE cells used for the chicken promoter analyses.

3.4.4. Chicken tyrosinase promoter activity in MQTNC cells

Prior to using the MQTNC cells for transfection, an attempt was made to test whether they have melanogenic potential. However, results from melanin formation assays (data not shown) and Northern blot analyses suggest that they do not have melanogenic potential. Transfection of such cells with the tyrosinase promoter-reporter constructs would possibly give results characteristic of a "tyrosinase-negative" cell line. In line with this expectation, similar levels of expression were seen in MQTNC cells and Hep G2 cells transfected with TYR1.1-LUC, TYR0.5-LUC and TYR0.2-LUC. However, an unexpectedly high level of transcriptional activity was obtained using TYR2.1-LUC. This suggests the presence of positive factors in the MQTNC cells that are able to transactivate the full-length tyrosinase promoter sequence, and therefore raises the possibility that these cells may be premelanogenic. In further support of this idea, there is a striking resemblance between the profile of the histogram representing the MQTNC transfection results and that obtained when melanoma cells were transfected with human tyrosinase promoter-reporter constructs (Shibata et al, 1992). The results of Shibata et al led to the identification of the TDE, a strong upstream enhancer element in which the E-box plays a central role. A TDE-like element is present in the chicken promoter within the distal conserved region at approximately -1200 nt upstream of the ATG site (see Fig. 3.3.b). While only the central nucleotides of the human TDE enhancer are conserved in CTYR4.3, these include the CATGTG motif which plays an essential role in the functioning of the enhancer. Given that this element is excluded from all of the constructs except the TYR2.1-LUC construct, the transfection results suggest that the chicken TDE-like element may well mediate the high level of MQTNC-specific transcriptional activity seen for the 2.1 kb promoter sequence.

Thus if the quail cells do have melanogenic potential, the different promoter activities in these cells and the RPE cells raises the possibility that tyrosinase gene expression in neural crest-derived melanocytes and RPE melanocytes is regulated differently *in vitro*. At a first glance, these deductions seem reasonable. However, another interesting explanation is also possible. The MQTNC cells were immortalised using a *myc*-carrying retrovirus. Myc proteins are bHLH-ZIP transcription factors that bind to E-box motifs (CANNTG) and activate transcription of linked genes (reviewed by Meichle et al, 1992). Myc proteins bind preferably to the sequence CACGTG, but have also been shown to bind at CATGTG sites with lower affinity. The raised levels of Myc proteins in the MC29-infected MQTNC cells (that is, the endogenous c-Myc plus v-Myc shown by immunostaining in Fig. 3 a and b of Fauquet et al, 1990) may facilitate binding of Myc to the E-box motif within the TDE-like element of TYR2.1-LUC. This may well be responsible for the anomalous activation of luciferase expression from TYR2.1-LUC specifically in these cells.

CHAPTER FOUR

CONCLUDING COMMENTS

In recent years, there have been significant advances in the understanding of the mechanisms involved in regulating expression of the mammalian tyrosinase gene. Not only have factors required for melanocyte-specific expression been identified, but most exciting is that the findings are beginning to reveal the link between the level of tyrosinase gene expression and the differentiated state of the melanocyte. For example, transcription factors such as Mi and Brn-2/N-Oct3 have been shown to be associated with melanocytes in different states of differentiation. The detection of Brn-2 mRNA in melanoblasts and at elevated levels in melanoma cells, suggests that Brn-2 is associated with immature or dedifferentiating melanogenic cells (Eisen et al, 1995). Mi, on the other hand, is associated with active pigment-producing melanocytes, and its expression is down-regulated in transformed melanocytes (Yavuzer et al, 1995). Both factors have been shown to regulate tyrosinase gene expression via elements found within the 5′ flanking region of the tyrosinase gene (Bentley et al, 1994; Ganss, 1994; Yasumoto et al, 1994; Yasumoto et al, 1995).

It is still unclear whether any of the mechanisms regulating tyrosinase gene transcription in mammals are at play in lower vertebrates. The present study on the chicken tyrosinase gene promoter sequence represents the most extensive analysis of a lower vertebrate tyrosinase gene promoter to date. The results begin to reveal whether there is conservation of regulatory mechanisms between higher and lower vertebrates.

4.1. FUNCTIONAL ANALYSIS OF THE CHICKEN TYROSINASE PROMOTER IN RPE CELLS

Perhaps the most controversial result from this study is that the putative regulatory elements in the chicken tyrosinase gene promoter which are highly conserved and almost entirely responsible for promoter functioning in mammals, are apparently not essential for high levels of tissue-specific expression in chicken RPE cells. Thus, deletion of promoter sequences that include the potential Inr, SP1-binding site and M-box resulted in unexpectedly high expression of the reporter gene in RPE cells. This is in direct contrast to

equivalent studies with the mammalian promoters which show that deletion or mutation of these sequences, especially the Inr, results in dramatic reduction in promoter activity (Bentley et al, 1994; Ganss et al, 1994; Yasumoto et al, 1994).

Two possible explanations for the unexpectedly high expression from the TYRO.2-LUC construct were put forward in the previous chapter (see section 3.4.3.). The first was that deletion to 0.2 kb of the promoter sequence may result in the removal of a negative regulatory element. A more detailed functional analysis of the sequences between nt position -506 and the ATG site would shed further light on this issue. The second suggestion was that the high level of expression may be due to the presence of an additional 45 bp fragment in the TYRO.2-LUC construct that has potential enhancer activity. To exclude anomalous effects that may have been caused by the presence of the distal 45 bp sequence in TYRO.2-LUC, it will be necessary to assess the promoter activity of a construct from which this 45 bp sequence is eliminated.

Assume for the moment that the 45 bp fragment plays no role in this issue and that the 200 bp of proximal promoter is indeed sufficient to drive tissue-specific transcription in RPE cells. This would suggest the existence of regulatory mechanisms that are unique to avians and possibly other lower vertebrates. In this regard it is interesting to note that while the tyrosinase gene promoter sequences of avians (chicken and quail) and turtle can be aligned, these cannot be aligned with the amphibian and fish sequences. This raises the possibility of an avian/reptile grouping distinct from a amphibian/fish grouping.

When comparing the results of avian and mammalian transfection experiments, it may be significant that the present studies were carried out in RPE cells whereas equivalent mammalian studies were carried out in epidermal melanocytes or melanoma cells. Therefore, perhaps the conflicting results arising from this study reflect the functional differences between RPE cells and epidermal melanocytes. Epidermal melanocytes are required to continue synthesizing and secreting melanin throughout their lifespan; therefore the tyrosinase gene must remain activated throughout this period. On the other hand, RPE cells cease synthesizing melanin early in development (Hu and Mah, 1979; Boissy et al, 1988) suggesting that the tyrosinase gene becomes down-regulated. Thus there must be regulatory factors present in RPE cells but not in epidermal

melanocytes (and vice-versa) that account for the different levels of tyrosinase gene expression. This hypothesis could be readily tested by gel shift analyses of nuclear extracts from RPE and epidermal melanocytes.

4.2. VALIDITY OF TRANSFECTION STUDIES IN THE IMMORTALISED QUAIL NEURAL CREST CELL LINE

The aim of transfecting the immortalized quail neural crest cells was to determine whether the chicken tyrosinase gene promoter would be active in these cells, and if so, whether the level of activity would reflect differences in regulation of melanocyte-specific genes in pigment cells of different embryonic origin. Results showed that in contrast to the activities observed in RPE cells, only the full length 2.1 kb 5′ flanking sequence had significant promoter activity in these cells. A simple interpretation of the results would be that transcription driven by the chicken tyrosinase gene promoter is regulated differently in RPE cells and neural crest-derived cells. However, this interpretation is questionable in the light of uncertainties regarding the validity of the use of the MQTNC cells. Firstly, their melanogenic potential is uncertain and secondly, the effect of high levels of *myc* expression in these cells is not known. As suggested earlier, it is possible that Myc may transactivate the 2.1 kb tyrosinase promoter via the E-box motif in the TDE-like element at nt position -1200. To pursue this question further, studies involving gel shift assays and site-directed mutagenesis of the TDE could be carried out. If the results from such studies suggest that Myc is not responsible for the high levels of expression of the reporter gene, the next step would be to investigate further the melanogenic potential of the immortalised neural crest cells.

The attempts so far to determine the melanogenic potential of the immortalised quail neural crest cells by Northern blot hybridisation and melanin formation assays suggest that they do not display any melanogenic characteristics. It may be possible, however, to select for and enhance the differentiation of the melanogenic subpopulation of the neural crest cells (if they are still represented) by addition of exogenous factors such as α-MSH, endothelin-1 or steel growth factor. (Steel factor is the cognate ligand for the c-kit tyrosine kinase receptor. Addition of chicken recombinant steel factor to

developing quail neural crest cells in culture, has been shown to enhance the differentiation rate of melanocyte precursors [Lahav et al, 1994].)

If attempts to induce melanogenesis in the immortalised quail cells is unsuccessful, a more viable option would be to repeat the transfection experiments in an established avian neural crest-derived melanocyte cell line. No such cell line is available, however, and attempts to date in our laboratory to generate bulk cultures of avian melanocytes, using the technique described by Oetting et al (1988), have been unsuccessful (S.Kidson, personal communication). (This technique involves the addition of Buffalo rat liver cell (BRL-3A)-conditioned medium (containing Steel factor) to neural crest cells dissociated from somites. Thus the success of this procedure depends on the differential survival of melanoblasts and the concomitant death of all the other somitic cells and it would seem that it is quite difficult to establish conditions ideal for this differential survival.)

A third option would be to carry out the transfection studies in melanocytes derived from another vertebrate. Attempts to transfect murine B16 melanoma cells and SV40-immortalised mouse melanocytes (S. Prince, Dept Anatomy and Cell Biology, UCT) using the calcium phosphate-mediated technique, were unsuccessful. Other transfection methods such as electroporation could be explored. It should be remembered, however, that although others have successfully carried out "cross species" analyses, the results from this study suggest that elements which are important for regulating mammalian tyrosinase gene expression may not be essential for expression of the avian gene (in RPE cells, at least). Therefore, results from any cross-species transfection studies would have to be interpreted with caution.

4.3. THE MICROPHTHALMIA PROTEIN - A CENTRAL FACTOR REGULATING TRANSCRIPTION OF THE TYROSINASE GENE IN MAMMALS AND LOWER VERTEBRATES?

As discussed in chapter one, the microphthalmia protein (Mi/MITF) has been identified as a promising candidate for a transcription factor that co-ordinately activates the melanocyte-specific tyrosinase gene family. Mi/MITF is able to transactivate the mammalian tyrosinase and TRP-1 gene promoters via binding to CATGTG E-box motifs within the TDE, Inr region and M-box elements (see section 1.3.2). These elements are highly conserved in the promoters of the chicken, quail and turtle tyrosinase genes which suggests that they may

also function as Mi/MITF binding sites in these vertebrates. However, the role or even the existence of a Mi/MITF-like protein in lower vertebrates remains to be demonstrated. The cDNA sequences encoding mouse Mi and human MITF have been cloned (Hodgkinson et al, 1993; Hughes et al, 1993; Tachibana et al, 1994), but to date no Mi/MITF gene sequences have been reported for a lower vertebrate. Moreover, the results of the present study leads one to further question whether Mi/MITF has a functional role in avian melanocyte-specific gene expression (because the very elements to which Mi/MITF is reputed to bind appear to be unnecessary for transcription of the tyrosinase gene in RPE cells).

Encouraging, however, is the report of the isolation of several clones from a chicken melanocyte cDNA library that cross-hybridise to a human MITF cDNA probe (C.S. April et al, personal communication). These clones are currently being sequenced. Successful cloning of a chicken Mi/MITF-like gene sequence would confirm that Mi/MITF is expressed in chicken melanocytes and provide the means to test directly whether it can transactivate the avian tyrosinase gene promoter.

If Mi/MITF proves to be one of the key factors co-ordinating the expression of melanocyte-specific genes, the next task would be to dissect out the upstream events that regulate Mi/MITF gene expression. This would ultimately lead us closer to solving the central developmental question of what determines the melanocyte lineage.

APPENDIX I - MODIFIED TECHNIQUES USED

1. Preparation of a radiolabelled probe by nick translation

Nick translation was carried out according to Rigby et al (1977) as follows: 0.8-1 μg of DNA was mixed with 2.5 μl 10 x nick translation buffer (0.5 M Tris.Cl, pH 7.5; 0.1 M $MgSO_4$; 1 mM dithiothreitol; 0.5 mg/ml BSA), 5 μl [α-^{32}P]-dCTP (3000 Ci/mMol, Ammersham) and 20 μM dATP, dGTP, dTTP in a total volume of 25 μl. Single stranded breaks (nicks) were introduced into the DNA by digestion with 0.02 μg/ml DNAse I for 10 min at RT. The nicks were then repaired using 1.5 units DNA polymerase, for 1 h at 15°C. Radio-labelled probe was separated from unincorporated [α-^{32}P]-dCTP by passing the reaction mixture over a Sephadex G50 column of 5 ml bed volume, equilibrated with 10 mM TrisCl, 0.1 mM EDTA pH 7.5. The reaction mixture separates into 2 peaks of radioactivity (monitored using a hand-held Geiger counter). The first peak containing the probe was collected in a volume of approximately 1 ml. The 2nd peak containing unincorporated nucleotides was discarded. An aliquot (10 μl) of probe was counted in 10 ml Instagel using a scintillation counter programmed for 1 min counts on a ^{32}P channel. Probes with specific activities > 1 x 10^7 cpm/ug DNA were generated in this way.

2. Large-scale preparation of bacteriophage DNA

An overnight culture of *E. coli* strain LE392 in LB broth containing 10 mM $MgSO_4$ was seeded from a liquid N_2 stock. The following day, this culture was diluted 1:500 in LB broth containing 0.4% maltose. After 6 h of shaking at 37°C, 1 ml was removed to an Eppendorf tube, 50 μl phage (from plate lysate stock) was added and allowed to adsorb to the bacteria for 15 min at 37°C. This was innoculated into two 500 ml Ehrlenmeyer flasks containing 200 ml of LB broth plus 10 mM $MgSO_4$. The cultures were shaken overnight at 37°C to allow lysis to occur. If lysis was not apparent the following morning (i.e. no floculent debris visible), the cultures were exposed to ultra-violet light for 10 min, then shaken at 37°C for a further 2-4 h. Lysed cultures were treated with 2.5 ml chloroform, left shaking for 10 min, decanted into centrifuge tubes (avoiding chloroform transfer) and centrifuged at 6000 g for 10 min at 4°C. To each supernatant (200 ml), 3.85g NaCl, 1 μg/ml pancreatic DNase I and 1 μg/ml RNase was added. Mixtures were incubated at 37°C for 30 min (to allow for digestion of nucleic acids released from the lysed bacteria)

then placed on ice for 5 - 10 min. Bacteriophage particles were precipitated by adding very slowly, 50 ml polyethylene glycol (50% PEG 6000 in 0.5 M NaCl) and stirring on a magnetic stirrer at 4°C overnight (or for a minimum of 2 h). Bacteriophage were recovered by centrifugation at 6000 g for 30 min at 4°C. Pellets were resuspended in 5 ml of 10 mM $MgSO_4$ using a pipette with a wide-bore tip. The PEG and bacterial debris were removed from the bacteriophage suspension by adding an equal volume of chloroform, vortexing the solution for 30 s and then centrifuging at 3000 g for 15 min at 4°C. The aqueous phase containing bacteriophage, was removed and centrifuged at 25 000 rpm for 1 h at 4°C in a Beckman vTi 65.2 rotor. The glassy pellet (which was spread down one side of the tube) was resuspended in 2 ml SM by gentle agitation on a rocking platform overnight at 4°C.

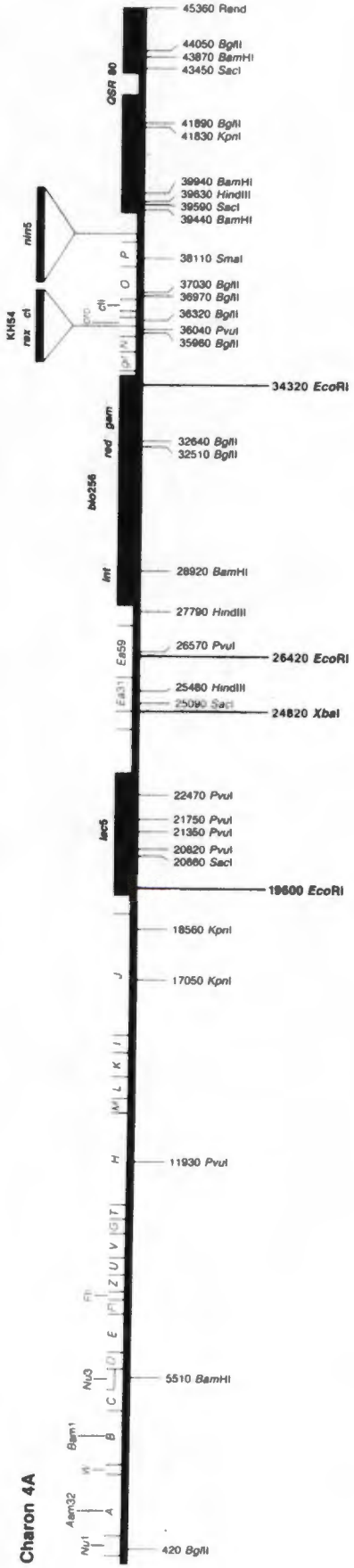
To extract the DNA from bacteriophage particles: 50 µg/ml Proteinase K and 0.5% SDS was added to the bacteriophage suspension in a 15 ml corex tube. The mixture was incubated in a waterbath at 37°C for 15 min, then at 65°C for 45 min. After cooling to RT, the mixture was extracted once using 1 volume of TE-buffered phenol, twice using 1 volume of phenol: chloroform: isoamyl alcohol:: 25: 24: 1 and finally, once using 1 volume of chloroform: isoamyl alcohol:: 24: 1. The aqueous phase was decanted and the nucleic acid precipitated by adding 1/10 volume 3 M sodium acetate (pH 7.0) and 1 volume isopropanol and storing the solution at -70°C for 10 min or at -20°C overnight. The nucleic acid was collected by centrifugation at 4000 g for 30 min at -20°C, the pellets were washed with 70% ethanol and recentrifuged for 10 min. Pellets were dried at RT and resuspended in 1 ml TE. The DNA was quantified and the purity determined spectrophotometrically. The DNA was characterised by restriction enzyme digestion followed by electrophoresis on 0.6% agarose gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). To get rid of contaminating RNA, the extracted DNA sample was digested with 55 µg/ml DNase-free RNase A for 30 min at 37°C. One tenth volume 3 M sodium acetate was added, after which the samples were re-extracted with phenol: chloroform: isoamyl alcohol, precipitated, resuspended and re-quantified.

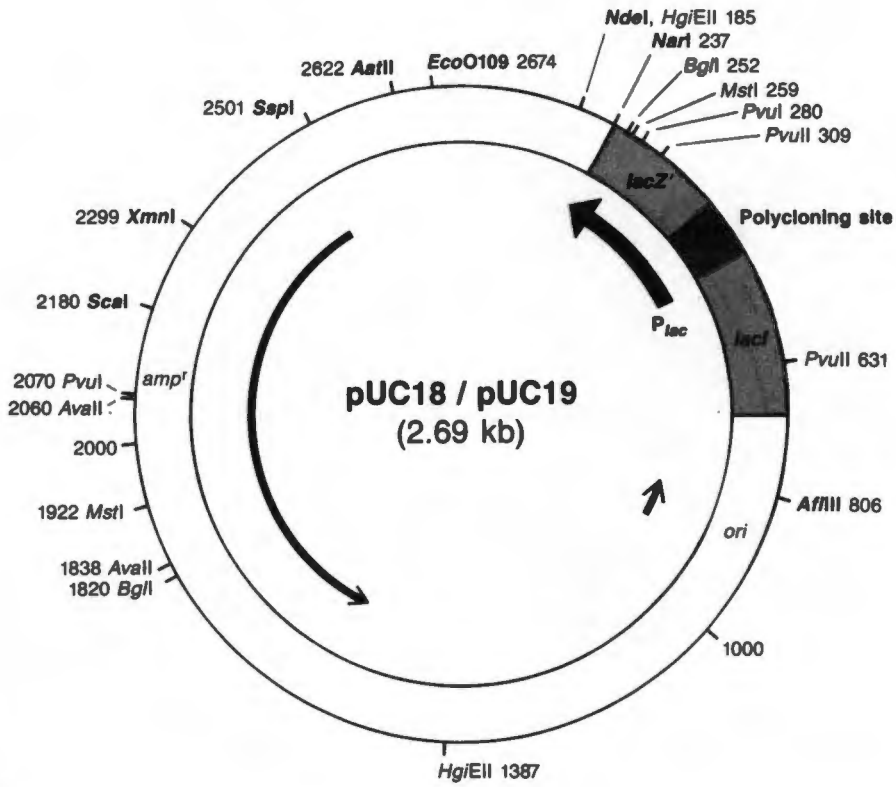
3. Plasmid mini-preparation for sequencing

The following procedure was adapted from a method supplied with the QIAGEN plasmid mini-preparation kit, and makes use of the buffer recipes recommended by the manufacturer (DIAGEN GmbH, Germany).

E. coli cultures containing the desired plasmid were grown overnight in 5 ml LB broth containing 100 μg/ml ampicillin. Bacteria were harvested by centrifugation at 3 000g for 10 min at RT, resuspended in 0.3 ml buffer P1 (100 μg/ml RNase A in 50 mM Tris HCL, 10 mM EDTA, pH 8.0, 4°C), and lysed by adding 0.3 ml buffer P2 (200 mM NaOH, 1% SDS, RT), mixed by inversion and incubated at RT for 5 min. Chromosomal DNA was precipitated by adding 0.3 ml buffer P3 (2.55 M Kac, pH 4.8, RT) and mixing by inversion. Bacterial debris and chromosomal DNA was removed by centrifugation at 10 000g for 15 min at 4°C. The supernatant fluid containing plasmid was transferred to a fresh tube and recentrifuged as before. The supernatant was divided into two aliquots (of approximately 0.4 ml each) to which 0.4 ml of buffered phenol (Tris HCL, pH 8.0) and 0.4 ml of chloroform:isoamyl alcohol (24:1) was added. The phases were gently mixed by inversion (not by vortexing) and then separated by centrifugation at 10 000g for 2 min at 4°C. The non-aqueous phase and denatured protein layer was removed and the aqueous phase re-extracted as before. The aqueous phase was extracted for a third time with chloroform:isoamyl alcohol before precipitating the plasmid DNA with 0.6 volumes of isopropanol for 30 min at RT. Following centrifugation at 15 000g for 30 min at RT, the DNA pellets were washed with 70% ethanol, dried (8 min in a Kartell vacuum drier) and dissolved in 30 μl ultra-pure water (BDH). The DNA was reprecipitated overnight at -20°C using 0.1 volume of 4 M LiCl and 2.5 volumes of absolute ethanol. Plasmid DNA was collected by centrifugation at 10 000g for 15 min at 4°C, washed, dried and resuspended in 10 μl ultrapure water. One microlitre of the DNA was subjected to diagnostic restriction enzyme digestion and analysed for quantity and quality by agarose gel electrophoresis. The remaining high purity plasmid DNA (approximately 4-5 μg in 20 μl ultrapure water) was used for sequencing with a Sequenase version 2.0 kit.

APPENDIX II - CLONING VECTORS USED





Polycloning Sites

pUC18

	1	2	3	4	5	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	7	8
	Thr	Met	Ile	Thr	Asn	Ser	Ser	Ser	Val	Pro	Gly	Asp	Pro	Leu	Glu	Ser	Thr	Cys	Arg	His	Ala	Ser	Leu	Ala	Leu	Ala
ATG	ACC	ATG	ATT	ACG	AAT	TCG	AGC	TCG	GTA	CCC	GGG	GAT	CCT	CTA	GAG	TCG	ACC	TGC	AGG	CAT	GCA	AGC	TTG	GCA	CTG	GCC

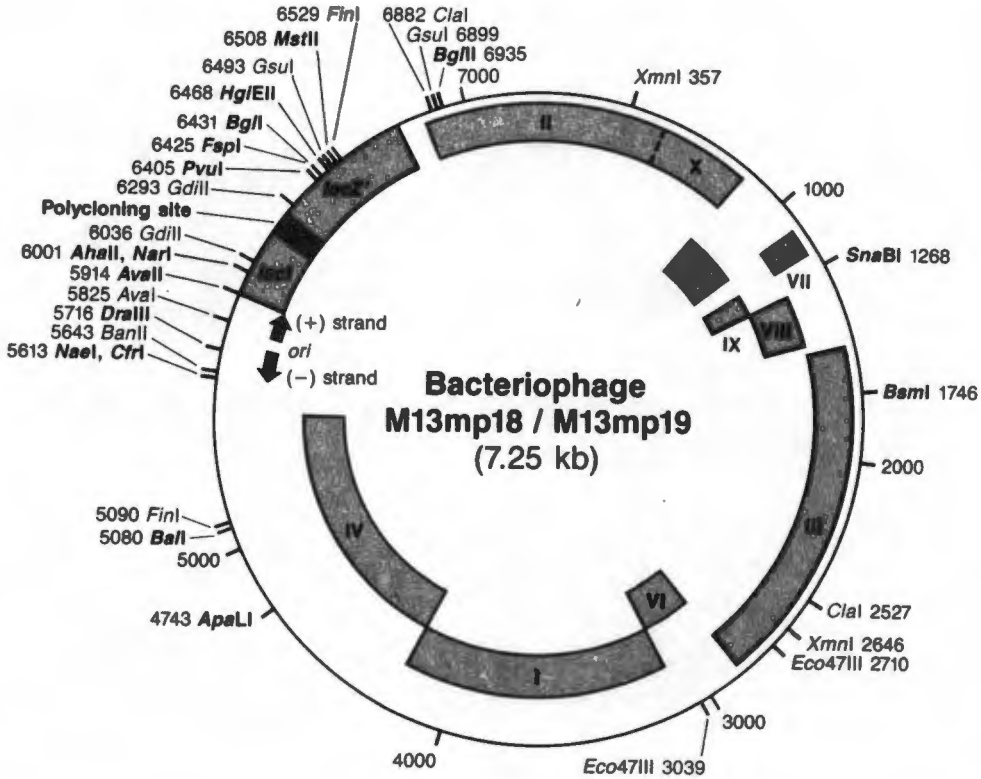
EcoRI *SacI* *KpnI* *SmaI* *XmaI* *BamHI* *XbaI* *SalI* *AccI* *HincII* *PstI* *SphI* *HindIII*

pUC19

	1	2	3	4	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	5	6	7	8
	Thr	Met	Ile	Thr	Pro	Ser	Leu	His	Ala	Cys	Arg	Ser	Thr	Leu	Glu	Asp	Pro	Arg	Val	Pro	Ser	Ser	Asn	Ser	Leu	Ala
ATG	ACC	ATG	ATT	ACG	CCA	AGC	TTG	CAT	GCC	TGC	AGG	TCG	ACT	CTA	GAG	GAT	CCC	CGG	GTA	CCG	AGC	TCG	AAT	TCA	CTG	GCC

HindIII *SphI* *PstI* *SalI* *AccI* *HincII* *XbaI* *BamHI* *SmaI* *XmaI* *KpnI* *SacI* *EcoRI*

In pUC18, the *Eco*RI site lies immediately downstream from P_{lac}.
In pUC19, the *Hind*III site lies immediately downstream from P_{lac}.



Polycloning Sites
M13mp18

	1	2	3	4	5	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	7`	8
	Thr	Met	Ile	Thr	Asn	Ser	Ser	Ser	Val	Pro	Gly	Asp	Pro	Leu	Glu	Ser	Thr	Cys	Arg	His	Ala	Ser	Leu	Ala	Leu	Ala
ATG	ACC	ATG	ATT	ACG	AAT	TCG	AGC	TCG	GTA	CCC	GGG	GAT	CCT	CTA	GAG	TCG	ACC	TGC	AGG	CAT	GCA	AGC	TTG	GCA	CTG	GCC

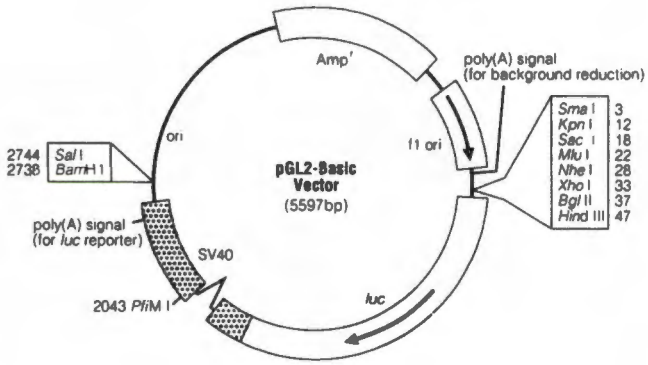
EcoRI *SacI* *KpnI* *SmaI* *XmaI* *BamHI* *XbaI* *SalI* *AccI* *HincII* *PstI* *SphI* *HindIII*

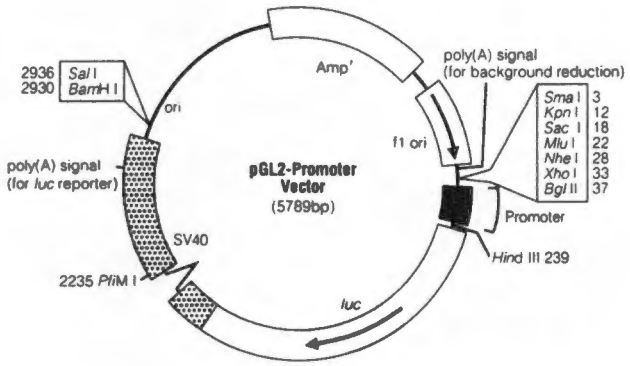
M13mp19

	1	2	3	4	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	5	6	7	8
	Thr	Met	Ile	Thr	Pro	Ser	Leu	His	Ala	Cys	Arg	Ser	Thr	Leu	Glu	Asp	Pro	Arg	Val	Pro	Ser	Ser	Asn	Ser	Leu	Ala
ATG	ACC	ATG	ATT	ACG	CCA	AGC	TTG	CAT	GCC	TGC	AGG	TCG	ACT	CTA	GAG	GAT	CCC	CGG	GTA	CCG	AGC	TCG	AAT	TCA	CTG	GCC

HindIII *SphI* *PstI* *SalI* *AccI* *HincII* *XbaI* *BamHI* *SmaI* *XmaI* *KpnI* *SacI* *EcoRI*

In M13mp18, the *Eco*RI site lies immediately downstream from P_{lac}.
In M13mp19, the *Hin*dIII site lies immediately downstream from P_{lac}.


Amp'
poly(A) signal
(for background reduction)
ori
f1 ori
2744
2738
Sal I
BamH I
pGL2-Basic
Vector
(5597bp)
Sma I 3
Kpn I 12
Sac I 18
Mlu I 22
Nhe I 28
Xho I 33
Bgl II 37
Hind III 47
poly(A) signal
(for luc reporter)
SV40
2043 PflM I
luc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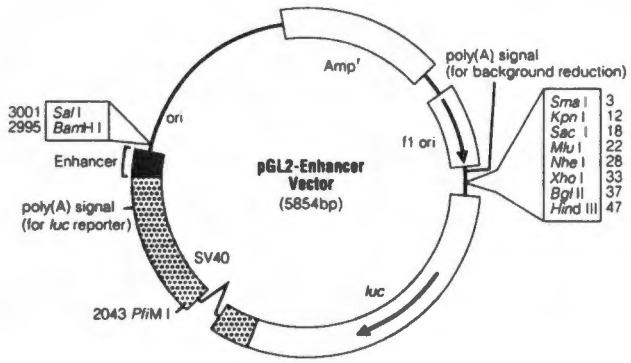
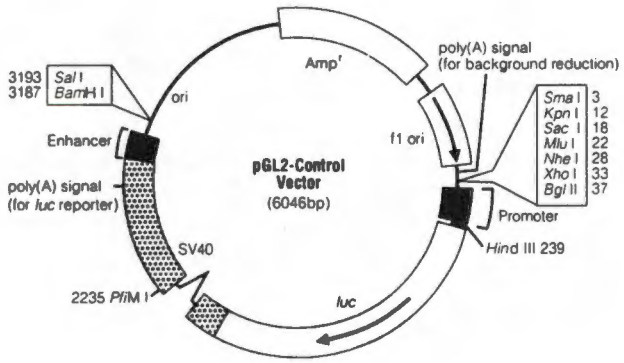
poly(A) signal
(for background reduction)
2936
2930
Sal I
BamH I
ori
f1 ori
poly(A) signal
(for luc reporter)
pGL2-Promoter
Vector
(5789bp)
Sma I 3
Kpn I 12
Sac I 18
Mlu I 22
Nhe I 28
Xho I 33
Bgl II 37
Promoter
Hind III 239
SV40
2235 PflM I
luc


Amp'
poly(A) signal
(for background reduction)
3001
2995
Sal I
BamH I
ori
f1 ori
Enhancer
pGL2-Enhancer
Vector
(5854bp)
Sma I 3
Kpn I 12
Sac I 18
Mlu I 22
Nhe I 28
Xho I 33
Bgl II 37
Hind III 47
poly(A) signal
(for luc reporter)
SV40
2043 PflM I
luc


Amp'
poly(A) signal
(for background reduction)
3193
3187
Sal I
BamH I
ori
f1 ori
Enhancer
poly(A) signal
(for luc reporter)
pGL2-Control
Vector
(6046bp)
Sma I 3
Kpn I 12
Sac I 18
Mlu I 22
Nhe I 28
Xho I 33
Bgl II 37
Promoter
Hind III 239
SV40
2235 PflM I
luc

APPENDIX III - TRANSCRIPTION FACTOR BINDING SITES IDENTIFIED IN CTYR4.3

Results of a search conducted using the FINDPATTERNS function of GCG and the transcription factor binding sites recorded in the GCG file, TFsites. TFsites is a composite from the following datasets: TFD (release 7.4) SITES dataset file, 1/95 and Transfac (release 2.2) SITES dataset selected entries, 12/94 (Nucleic Acids Res 21 (1993) 3117-8; J Biotechnol 35 (1994) 273-380; *In* Gene transcription: A practical approach (1993) 321-345).
Note: nt numbering of CTYR4.3 is from +1 to +3122 instead of -2125 to +997.

Transcription factor binding site	Consensus sequence and nt position in CTYR4.3
GCRE	TGACTC 104: TTTAA TGACTC TACAG
GCRE /Rev	GAGTCA 1,525: TTGTA GAGTCA CCTTG 1,999: TAGCA GAGTCA GTGGT
LVb_RS /Rev	TATCCTG 1,935: AAGTG TATCCTG TGTGT
NFI_CS1 /Rev	TTGGCNNNNNKCCA 1,888: GACAC TTGGCTTTCATCCA CAACA
NFI_CS3	TGGNNNNNNGCCA 1,345: CCTGA TGGGAGAAAGCCA GAAGT
NFI_CS3 /Rev	TGGCNNNNNNCCA 1,889: ACACT TGGCTTTCATCCA CAACA
PEBP2_RS /Rev	GCGGTC 2,061: TTGGA GCGGTC AGAGA
PRL_conserved_motif	CCTGAWWA 863: ATCTC CCTGAATA TCTGA
PRL_conserved_motif /Rev	TWWTCAGG 1,493: TAGAT TTATCAGG GGCTT
MRE_CS2 /Rev	GNGYGCA 1,450: GATTT GGGTGCA GGTTT 2,429: GTGGG GAGTGCA AGTTT
TATA-box.2	TATAAA 415: TTGAA TATAAA CATAA 1,573: TGAAA TATAAA AAAAG
TATA-box.2 /Rev	TTTATA 157: TGTTG TTTATA GATGG
FSE2.1	GAGAGGA 2,355: TGACA GAGAGGA TTGGC
BGP1_RS1 /Rev	CCGCCC 2,234: GCTGT CCGCCC TGGGA
C/EBP_CS1	TKNNGYAAK 1,300: TTGTC TGGAGTAAT GTTCT
CK-2 /Rev	TACCTGA 1,338: GGTGC TACCTGA TGGGA
CK-8-mer	AANCCAAA 1,587: AGACC AAGCCAAA TAATT
CK-8-mer /Rev	TTTGGNTT 2,438: GCAAG TTTGGCTT CTCAG
TATA-box.1 /Rev	ATTATA 708: CCGTG ATTATA TCTGT
his3-Tr-TATA	TATAAA 415: TTGAA TATAAA CATAA 1,573: TGAAA TATAAA AAAAG

```
his3-Tr-TATA /Rev
                                TTTATA
   157: TGTTG TTTATA GATGG

Sp1_CS2 /Rev
                                CCGCCC
 2,234: GCTGT CCGCCC TGGGA

engrailed_CS
                                HCWATHAAA
 1,475: AGTTA TCTATTAAA ATAGT

E-alpha_H_box /Rev
                                CAGGTCC
 1,377: GTTTC CAGGTCC TGCTA

GH-CSE1 /Rev
                                TAATTTA
 1,150: CGACA TAATTTA AGTGC
 1,595: CCAAA TAATTTA TCATG
 2,905: AACCC TAATTTA CTCAG

GH-CSE2
                                AATAAAT
   680: AATAA AATAAAT CAAAC
 1,839: TTTAC AATAAAT GCCTT

MLC1f/MLC3f-IR /Rev
                                TCCTGCAG
 1,125: TAAAC TCCTGCAG TTTTA

HC3
                                CCACCA
 2,895: ACACC CCACCA ACCCT

rPrl.A /Rev
                                TAATCAG
 1,912: TGTGA TAATCAG ATTAA

C/EBP_CS2
                                TCNTACTC
 1,774: TTTAA TCTTACTC CTTCT

C/EBP-TTRS3
                                TCTTACTC
 1,774: TTTAA TCTTACTC CTTCT

C/EBP-DEI.1 /Rev
                                GGGTAGGA
 1,958: ATCCA GGGTAGGA ATGGG

NFI.2 /Rev
                                GATTGGC
 2,360: GAGAG GATTGGC CTTCT

AP-2_CS4
                                YCSCCMNSSS
 2,234: GCTGT CCGCCCTGGG ATGGA

AP-2_CS4 /Rev
                                SSSNKGGSGR
 1,868: AGTCG GGGGTGGGGG AAAAA
 1,869: GTCGG GGGTGGGGGA AAAAG

PEA3_RS
                                AGGAAG
   215: ATTAA AGGAAG CCTTT
 2,219: TGCTG AGGAAG GAGTG

PEA3_RS /Rev
                                CTTCCT
 1,005: CCTGC CTTCCT GAGAT

XRE_CS1
                                CACGCW
 1,322: CAAAG CACGCA GAGTT
 2,203: GCAAA CACGCA GAGCT

TFIID-EIIa /Rev
                                TTTGTA
 1,519: TTCAC TTTGTA GAGTC

MTVGRE_NRS
                                AGGATGT
 2,123: CTGTG AGGATGT TTCTG

NF-E1_CS1
                                MYWATCWY
    87: GTGTG ACTATCTC ATGTT
   829: GAGTC CCTATCAC AGGCA
   855: CAGAG ATAATCTC CCTGA

NF-E1_CS1 /Rev
                                RWGATWRK
   705: TTTCC GTGATTAT ATCTG
   852: ACTCA GAGATAAT CTCCC
 1,812: ATCAT GTGATAGG CAGAA
 1,908: AACAT GTGATAAT CAGAT

NF-E1.3
                                CTATCA
   830: AGTCC CTATCA CAGGC

NF-E1.3 /Rev
                                TGATAG
 1,813: TCATG TGATAG GCAGA
```

```
NF-E1.4 /Rev
                                  GAGATAA
                852: ACTCA GAGATAA TCTCC

NF-E1.5
                                  TATCTT
              1,686: CTCTT TATCTT TTTTA

NF-E1.5 /Rev
                                  AAGATA
                424: AACAT AAGATA TTAAA
              2,795: TACAG AAGATA ACAGG

NF-E1.6
                                  TATCTC
                 89: GTGAC TATCTC ATGTT

NF-E1.6 /Rev
                                  GAGATA
                852: ACTCA GAGATA ATCTC
              2,786: AACGT GAGATA CAGAA

NF-E1.7 /Rev
                                  GTGATGA
                927: GTCAT GTGATGA CCTGC
              2,805: AACAG GTGATGA GAATT

NF-E1.8
                                  CCAATCT
              3,035: ACTAT CCAATCT GACTT

PUT2_UAS.1
                                  GAAGCCAA
                882: GCGTA GAAGCCAA CAGGA

PUT2_UAS.1 /Rev
                                  TTGGCTTC
              2,439: CAAGT TTGGCTTC TCAGG

STE6.1
                                  ATGTAATT
              2,571: GGACT ATGTAATT GCTAC

STE6.2 /Rev
                                  GTGTAAAT
              1,943: CCTGT GTGTAAAT GCATC

BAR1.1
                                  ATGTAATT
              2,571: GGACT ATGTAATT GCTAC

BAR1.2 /Rev
                                  ATGTAATT
              2,571: GGACT ATGTAATT GCTAC

MAT-alpha-2_CS1
                                  ATGTAATT
              2,571: GGACT ATGTAATT GCTAC

a-actin_US
                                  GTCGCC
              1,606: ATCAT GTCGCC ATCCT

a-actin_US /Rev
                                  GGCGAC
              2,469: TGAAA GGCGAC TGAGA

actin_5c_US
                                  TATAAAA
              1,573: TGAAA TATAAAA AAAGA

Ad2MLP_US.3
                                  TATAAA
                415: TTGAA TATAAA CATAA
              1,573: TGAAA TATAAA AAAAG

Ad2MLP_US.3 /Rev
                                  TTTATA
                157: TGTTG TTTATA GATGG

Ad2MLP_US.4
                                  TATAAAA
              1,573: TGAAA TATAAAA AAAGA

Ad2MLP_US.5
                                  TATAAAA
              1,573: TGAAA TATAAAA AAAGA

RC2-CYC1.2
                                  GATGACC
                929: CATGT GATGACC TGCTG

RC2-CYC1.2 /Rev
                                  GGTCATC
              2,155: CTGCT GGTCATC CTTCA

GAL1-TATA
                                  ATATAA
                414: ATTGA ATATAA ACATA
              1,572: TTGAA ATATAA AAAAA

GAL1-TATA /Rev
                                  TTATAT
                709: CGTGA TTATAT CTGTT

GCN4-HIS3.1
                                  TGACGA
                  4:   TAT TGACGA TAGGC
```

GCN4-HIS3.2

```
                GAGTCA
1,525: TTGTA GAGTCA CCTTG
1,999: TAGCA GAGTCA GTGGT
```

GCN4-HIS3.2 /Rev

```
                TGACTC
  104: TTTAA TGACTC TACAG
```

GCN4-HIS3.4

```
                TTACTC
1,028: AGTCA TTACTC TTGTA
1,776: TAATC TTACTC CTTCT
2,909: CTAAT TTACTC AGCCC
```

GCN4-HIS3.4 /Rev

```
                GAGTAA
1,302: GTCTG GAGTAA TGTTC
```

GCN4-HIS

```
                TGACTC
  104: TTTAA TGACTC TACAG
```

GCN4-HIS /Rev

```
                GAGTCA
1,525: TTGTA GAGTCA CCTTG
1,999: TAGCA GAGTCA GTGGT
```

GCN4-HIS3.5

```
                TGACTC
  104: TTTAA TGACTC TACAG
```

GCN4-HIS3.5 /Rev

```
                GAGTCA
1,525: TTGTA GAGTCA CCTTG
1,999: TAGCA GAGTCA GTGGT
```

GCN4-HIS4.1

```
                TGACTC
  104: TTTAA TGACTC TACAG
```

GCN4-HIS4.1 /Rev

```
                GAGTCA
1,525: TTGTA GAGTCA CCTTG
1,999: TAGCA GAGTCA GTGGT
```

GCN4-HIS4.2

```
                TGACTC
  104: TTTAA TGACTC TACAG
```

GCN4-HIS4.2 /Rev

```
                GAGTCA
1,525: TTGTA GAGTCA CCTTG
1,999: TAGCA GAGTCA GTGGT
```

GCN4-HIS4.3

```
                CAGTCA
  112: CTCTA CAGTCA TGCAG
  266: CTCAT CAGTCA CATTT
```

GCN4-HIS4.4

```
                TGACTA
   85: AGGTG TGACTA TCTCA
```

GCN4-HIS4.4 /Rev

```
                TAGTCA
1,022: CTCTG TAGTCA TTACT
1,630: AGTAA TAGTCA TTTTC
```

HiNF-Ahist

```
                AGAAATG
  339: GTTTC AGAAATG TGTCA
```

HiNF-Ahist /Rev

```
                CATTTCT
  272: AGTCA CATTTCT CATTC
```

CTF/CBP-hs

```
                GATTGG
2,360: GAGAG GATTGG CCTTC
```

CTF/CBP-hs /Rev

```
                CCAATC
3,035: ACTAT CCAATC TGACT
```

hsp70_US

```
                GAAGGGAAAA
3,005: CAGGG GAAGGGAAAA GCTGA
```

hsp70.5

```
                GATTGG
2,360: GAGAG GATTGG CCTTC
```

hsp70.5 /Rev

```
                CCAATC
3,035: ACTAT CCAATC TGACT
```

(TFIID/TBF)-RS

```
                TATAAA
  415: TTGAA TATAAA CATAA
1,573: TGAAA TATAAA AAAAG
```

(TFIID/TBF)-RS /Rev

```
                TTTATA
  157: TGTTG TTTATA GATGG
```

```
IE1.2                                  CTTTCC
                                 798: CACTC CTTTCC CATTG
                               3,092: AACTG CTTTCC AGATT

IE1.2 /Rev                             GGAAAG
                                 166: TAGAT GGAAAG GTTGA
                               1,551: GTCTG GGAAAG TCAGC

Sp1-IE-3.1                             CCGCCC
                               2,234: GCTGT CCGCCC TGGGA

Sp1-IE-3.2 /Rev                        CCGCCC
                               2,234: GCTGT CCGCCC TGGGA

Sp1-IE-3.4                             CCGCCC
                               2,234: GCTGT CCGCCC TGGGA

Sp1-IE-3.5                             CCGCCC
                               2,234: GCTGT CCGCCC TGGGA

Sp1-IE-4/5.2                           CCGCCC
                               2,234: GCTGT CCGCCC TGGGA

GCN4-ILV1.1                            GAGTCA
                               1,525: TTGTA GAGTCA CCTTG
                               1,999: TAGCA GAGTCA GTGGT

GCN4-ILV1.1 /Rev                       TGACTC
                                 104: TTTAA TGACTC TACAG

GCN4-ILV1.2 /Rev                       CACTCA
                                 846: ACTGG CACTCA GAGAT
                               2,045: TTTCA CACTCA GGTCA

GCN4-ILV1.3                            AAGTCA
                               1,359: GCCAG AAGTCA AGGTn
                               1,554: TGGGA AAGTCA GCTTA

GCN4-ILV1.3 /Rev                       TGACTT
                               3,041: CAATC TGACTT TAGGT

GCN4-ILV2 /Rev                         GAATCA
                                 194: TATCT GAATCA CTGAC
                                 736: GCATG GAATCA GACAA

GR-lysozym.1                           ATTCCTCTGT
                                 315: AGGAG ATTCCTCTGT TAACC

PR-lysozym                             ATTCCTCTGT
                                 315: AGGAG ATTCCTCTGT TAACC

GR-lysozym.3 /Rev                      ATTCCTCTGTT
                                 315: AGGAG ATTCCTCTGTT AACCA

lysozyme-TATA /Rev                     TTTTAAC
                                 384: ATTTT TTTTAAC TTTGA

MT-I.1                                 GCACTC
                                 845: CACTG GCACTC AGAGA

MT-I.1 /Rev                            GAGTGC
                               2,225: GGAAG GAGTGC TGTCC
                               2,429: GTGGG GAGTGC AAGTT

GR-MT-IIA                              TGTCCT
                               1,657: TTCAC TGTCCT TATGT

GR-MT-IIA /Rev                         AGGACA
                               1,973: GGGGA AGGACA TTGGA
                               2,449: TTCTC AGGACA AAACT
                               2,517: TGAGA AGGACA AGTTC
                               2,948: AGGTA AGGACA GGCAA

AP1-MT-II /Rev                         AGAAGTCA
                               1,357: AAGCC AGAAGTCA AGGTn

LVb-Mo-MuLV /Rev                       TATCCTG
                               1,935: AAGTG TATCCTG TGTGT

c-mos_DS3                              GTTTTAA
                                  97: CTCAT GTTTTAA TGACT

LSF-SV40                               CCGCCC
```

```
                                2,234: GCTGT CCGCCC TGGGA

Sp1-SV40.4 /Rev                                 CCGCCC
                                2,234: GCTGT CCGCCC TGGGA

SV40.6                                        TGGAAAG
                                  165: ATAGA TGGAAAG GTTGA

SV40.6 /Rev                                   CTTTCCA
                                3,092: AACTG CTTTCCA GATTT

SV40.16                                       TGGAAAG
                                  165: ATAGA TGGAAAG GTTGA

SV40.16 /Rev                                  CTTTCCA
                                3,092: AACTG CTTTCCA GATTT

SV40.13                                       TGGAAAG
                                  165: ATAGA TGGAAAG GTTGA

SV40.13 /Rev                                  CTTTCCA
                                3,092: AACTG CTTTCCA GATTT

GT-IIBa-SV40                                ACAGCTG
                                1,183: AACCA ACAGCTG TCCCn

GT-IIBa-SV40 /Rev                           CAGCTGT
                                  329: TTAAC CAGCTGT TTCAG
                                1,184: ACCAA CAGCTGT CCCnC

GT-IIBb-SV40                                ACAGCTG
                                1,183: AACCA ACAGCTG TCCCn

GT-IIBb-SV40 /Rev                           CAGCTGT
                                  329: TTAAC CAGCTGT TTCAG
                                1,184: ACCAA CAGCTGT CCCnC

(Sp1)-U2snR.1 /Rev                              CCGCCC
                                2,234: GCTGT CCGCCC TGGGA

(Sp1)-U2snR.3 /Rev                              CCGCCC
                                2,234: GCTGT CCGCCC TGGGA

GR-uteroglobin.1                            TGTTCT
                                  807: CCCAT TGTTCT TTCTG
                                1,308: AGTAA TGTTCT GAGCA

GR-uteroglobin.1 /Rev                       AGAACA
                                2,550: AGCAA AGAACA TCCCC

GR-uteroglobin.2                            TGTTCT
                                  807: CCCAT TGTTCT TTCTG
                                1,308: AGTAA TGTTCT GAGCA

GR-uteroglobin.2 /Rev                       AGAACA
                                2,550: AGCAA AGAACA TCCCC

PR-uterogl.3                                TGTTCACT
                                1,650: TTTTA TGTTCACT GTCCT

GR/PR-uteroglobin.1                         TGTTCACT
                                1,650: TTTTA TGTTCACT GTCCT

WAP_US5 /Rev                                ACTTGG
                                1,886: AAGAC ACTTGG CTTTC

WAP_US6                                     TTTAAA
                                1,693: TCTTT TTTAAA CAACA

SP1_CS3 /Rev                                    CCGCCC
                                2,234: GCTGT CCGCCC TGGGA

malT_CS                                     GGAKGA
                                  617: TCACA GGAGGA ACACT
                                2,026: AGTTG GGAGGA AAAGT
                                2,347: GGAGT GGATGA CAGAG
                                2,961: CAAAG GGATGA AGGTA

malT_CS /Rev                                TCMTCC
                                  564: TGTTA TCCTCC GAGAT
                                1,895: GGCTT TCATCC ACAAC
                                2,157: GCTGG TCATCC TTCAG
```

```
alpha-factor_stim_element /Rev
                                  TGTTTCA
                          333: CCAGC TGTTTCA GAAAT

malT-malPp
                                  TCCTCC
                          564: TGTTA TCCTCC GAGAT

malT-malPp /Rev
                                  GGAGGA
                          617: TCACA GGAGGA ACACT
                        2,026: AGTTG GGAGGA AAAGT

H2B-CCAAT /Rev
                                  TNATTGG
                        2,053: TCAGG TCATTGG AGCGG

H1_conserved_US /Rev
                                  TGTGTTT
                        1,420: AATTT TGTGTTT TCCCA

histone_H4_CS.2
                                  YCCTGA
                          862: AATCT CCCTGA ATATC
                        1,007: TGCCT TCCTGA GATTC

histone_H4_CS.2 /Rev
                                  TCAGGR
                        1,496: ATTTA TCAGGG GCTTG
                        2,339: CTTTC TCAGGA GTGGA
                        2,447: GCTTC TCAGGA CAAAA

H2A_conserved_US
                                  YCATTC
                          278: ATTTC TCATTC AGAGT
                          292: GTTTC TCATTC ATACA

H2A_conserved_US /Rev
                                  GAATGR
                           20: TGAAG GAATGA AAAGT
                        1,964: GGTAG GAATGG GGAAG

INF.1
                                  AAGTGA
                        3,056: TACTT AAGTGA AGGTG

INF.1 /Rev
                                  TCACTT
                          976: AGCTC TCACTT ATTTC
                        1,515: GTTTT TCACTT TGTAG

nitrogen_regulatory_CS
                                  TTTTGCA
                          598: AGCAG TTTTGCA CTAGC

GR-lysozyme-2
                                  ATTCCTCTGT
                          315: AGGAG ATTCCTCTGT TAACC

uteroglobin_HS-2.4_CS
                                  RYYWSGTG
                          902: AAAGC ACCTCGTG CTCCT

JCV_repeated_sequence
                                  GGGNGGRR
                        1,869: GTCGG GGGTGGGG GAAAA
                        2,025: CAGTT GGGAGGAA AAGTT

vaccinia-term-sequence
                                  CTATTC
                          479: AAGTT CTATTC TGGTT

vaccinia-term-sequence /Rev
                                  GAATAG
                        1,739: ATGCC GAATAG CCAAC

oli1_DS
                                  ATTCTTA
                          373: CTTGT ATTCTTA TTTTT

yeast.term.2 /Rev
                                  TATAAAAA
                        1,573: TGAAA TATAAAAA AAGAC

zeste-white /Rev
                                  CACTCA
                          846: ACTGG CACTCA GAGAT
                        2,045: TTTCA CACTCA GGTCA

AP-1_CS3
                                  TGANTMA
                          193: CTATC TGAATCA CTGAC

AP-1_CS3 /Rev
                                  TKANTCA
                          193: CTATC TGAATCA CTGAC
                        1,920: TCAGA TTAATCA CATAA
                        2,909: CTAAT TTACTCA GCCCA

TRE-GPEI.2 /Rev
                                  CTGAATCA
                          192: ACTAT CTGAATCA CTGAC

AP1-HPV16-fp4e1
                                  CTGAATCAC
                          192: ACTAT CTGAATCAC TGACA
```

```
Pu_box                                  GAGGAA
                                  618:  CACAG GAGGAA CACTA
                                2,027:  GTTGG GAGGAA AAGTT
                                2,218:  TTGCT GAGGAA GGAGT
                                2,280:  CAACA GAGGAA CCTGC

Pu_box /Rev                             TTCCTC
                                  316:  GGAGA TTCCTC TGTTA

TATA-box-CS                             TATAWAW
                                  465:  TTAAA TATATAT TTAAG
                                  552:  GAAAA TATATAT TGTTA
                                1,573:  TGAAA TATAAAA AAAGA

TATA-box-CS /Rev                        WTWTATA
                                  464:  CTTAA ATATATA TTTAA
                                  551:  AGAAA ATATATA TTGTT

AP-2_CS5                                GSSWGSCC
                                2,881:  TACAT GGGTGGCC AACAC

NRE_Box1_CS /Rev                        RGAGAGGNT
                                2,354:  ATGAC AGAGAGGAT TGGCC

bicoid_CS /Rev                          BRGATTAVV
                                1,091:  GAAAA GAGATTAAA GCAAC

junB-US2 /Rev                           ATTGGCC
                                2,361:  AGAGG ATTGGCC TTCTG

E1A-F_CS                                NGGAYGT
                                2,123:  CTGTG AGGATGT TTCTG

E1A-F_CS /Rev                           ACRTCCN
                                2,553:  AAAGA ACATCCC CAGCA

silencer_binding_site_2                 TATTAAAA
                                1,477:  TTATC TATTAAAA TAGTA

eve-stripe2-hb2                         TTATTTTTTT
                                  377:  TATTC TTATTTTTTT TAACT

TFII-I-HIV-1-Inr2                       AGCTCTCT
                                  450:  TGTAT AGCTCTCT ACTTA

MyoD-MCK-right_site /Rev                CAGGTG
                                2,802:  GATAA CAGGTG ATGAG

AP-1_CS4 /Rev                           TKASTCA
                                2,909:  CTAAT TTACTCA GCCCA

E2A_CS                                  RCAGNTG
                                1,183:  AACCA ACAGCTG TCCCn
                                2,391:  GACAT GCAGATG CAGAG
                                2,801:  AGATA ACAGGTG ATGAG

E2A_CS /Rev                             CANCTGY
                                  329:  TTAAC CAGCTGT TTCAG
                                1,101:  TAAAG CAACTGC AACAA
                                1,184:  ACCAA CAGCTGT CCCnC
                                1,746:  ATAGC CAACTGC TGCCC
                                2,419:  GGGTT CAACTGT GGGGA

Ets-1_CS                                SMGGAWGY
                                2,122:  TCTGT GAGGATGT TTCTG

F-ACT1_RS                               TGGCGA
                                2,837:  GGGAC TGGCGA GATGC

F-ACT1_RS /Rev                          TCGCCA
                                1,607:  TCATG TCGCCA TCCTA

H-APF-1_RS                              CTGGRAA
                                1,548:  CTGGT CTGGGAA AGTCA

H-APF-1_RS /Rev                         TTYCCAG
                                  961:  ATCGT TTCCCAG CAGAG
                                1,373:  TnTCG TTTCCAG GTCCT
                                2,016:  AAGCA TTCCCAG TTGGG
                                3,093:  ACTGC TTTCCAG ATTTT

HiNF-A_RS                               ATTTNNNNATTT
                                1,635:  TAGTC ATTTTCTCATTT TTATG
```

```
HiNF-A_RS /Rev                                AAATNNNNAAAT
                                    541: ACTGA AAATTAGAAAAT ATATA

HNF-5_CS                                      TRTTTGY
                                  2,133: GTTTC TGTTTGC CATGG

HNF-5_CS /Rev                                 RCAAAYA
                                    743: ATCAG ACAAACA ACAGT
                                  2,198: TCTGT GCAAACA CGCAG

MCBF_RS /Rev                                  AGGAATG
                                     18: GCTGA AGGAATG AAAAG
                                  1,962: AGGGT AGGAATG GGGAA

NFI_CS6 /Rev                                  TGGCNNNNNKCCR
                                  1,889: ACACT TGGCTTTCATCCA CAACA

NF-GMb_CS /Rev                                TAYCTGA
                                    189: AATAC TATCTGA ATCAC
                                    869: CTGAA TATCTGA GGCGT
                                  1,338: GGTGC TACCTGA TGGGA

PEA3_CS                                       AGGAAR
                                    215: ATTAA AGGAAG CCTTT
                                  1,038: CTTGT AGGAAA AAATA
                                  2,028: TTGGG AGGAAA AGTTT
                                  2,219: TGCTG AGGAAG GAGTG

PEA3_CS /Rev                                  YTTCCT
                                  1,005: CCTGC CTTCCT GAGAT

PuF_RS                                        GGGTGGG
                                  1,869: GTCGG GGGTGGG GGAAA

Sp1_CS4 /Rev                                  MYYMGCCYM
                                  2,912: ATTTA CTCAGCCCA GCATC

keratinocyte_CS                               AARCCAAA
                                  1,587: AGACC AAGCCAAA TAATT

keratinocyte_CS /Rev                          TTTGGYTT
                                  2,438: GCAAG TTTGGCTT CTCAG

P2I_CS                                        ACATRAS
                                     75: GCGGC ACATGAG GTGTG
                                    420: TATAA ACATAAG ATATT
                                  1,926: TAATC ACATAAG TGTAT

P2I_CS /Rev                                   STYATGT
                                     92: ACTAT CTCATGT TTTAA
                                    584: CTCTT CTCATGT GTAGC
                                    922: GCACG GTCATGT GATGA
                                  1,661: CTGTC CTTATGT AACTG

P2II_CS                                       AGGGKTA
                                  1,201: CTTCA AGGGGTA ATTTC

p53_CS                                        RRRCWWGYYY
                                  2,518: GAGAA GGACAAGTTC CTTGC

AntP_CS /Rev                                  TAATGNNNNT
                                    101: TGTTT TAATGACTCT ACAGT
                                  1,230: ACTTC TAATGGTTTT CATAT

ATF_CS /Rev                                   TGACGAW
                                      4:   TAT TGACGAT AGGCT

GT-2B_RS                                      CCAGCTG
                                    328: GTTAA CCAGCTG TTTCA

IBP-1_CS2                                     AAGTGA
                                  3,056: TACTT AAGTGA AGGTG

IBP-1_CS2 /Rev                                TCACTT
                                    976: AGCTC TCACTT ATTTC
                                  1,515: GTTTT TCACTT TGTAG

GATA-1_CS2                                    WGATAMS
                                  1,407: AAGTC TGATACC AAATT
                                  2,796: ACAGA AGATAAC AGGTG

GATA-1_CS2 /Rev                               SKTATCW
```

```
                                1,471: ATGGA GTTATCT ATTAA

NF-mu-E1_CS /Rev                                GCCANCTG
                                1,744: GAATA GCCAACTG CTGCC

TFIIIA_CS /Rev                                  YTCNRNCCNG
                                2,912: ATTTA CTCAGCCCAG CATCA

GAGA-E74A.1                                     CTCTCTT
                                  577: GATGG CTCTCTT CTCAT

CAP-box                                         CTTYTG
                                2,367: TTGGC CTTCTG TCTTT

CAP-box /Rev                                    CARAAG
                                  948: CCCAG CAAAAG GCATC
                                1,356: AAAGC CAGAAG TCAAG
                                1,820: ATAGG CAGAAG ATTAA
                                2,792: AGATA CAGAAG ATAAC

histone_CAP_box                                 YCATTCR
                                  278: ATTTC TCATTCA GAGTT
                                  292: GTTTC TCATTCA TACAG

URE_(1) /Rev                                    TGTTGTCA
                                1,395: AATAC TGTTGTCA AGTCT

CNBP-SRE /Rev                                   CRCSSCAS
                                2,891: GCCAA CACCCCAC CAACC

HNF-5_site                                      RCAAAYA
                                  743: ATCAG ACAAACA ACAGT
                                2,198: TCTGT GCAAACA CGCAG

HNF-5_site /Rev                                 TRTTTGY
                                2,133: GTTTC TGTTTGC CATGG

ZRE7                                            TTGCTCA
                                  224: AGCCT TTGCTCA TCAGT
                                1,165: GCAAT TTGCTCA CAAGT

ZRE7 /Rev                                       TGAGCAA
                                1,313: TGTTC TGAGCAA AGCAC

ZRE6                                            TGTGTCA
                                  344: AGAAA TGTGTCA TATGA

ZRE5                                            TGTGCAA
                                2,195: GAGTC TGTGCAA ACACG

ZRE5 /Rev                                       TTGCACA
                                  640: ATAGA TTGCACA GGTTT

ZRE4/TGTG2 /Rev                                 TGTGTCA
                                  344: AGAAA TGTGTCA TATGA

ZRE3/TGTG1 /Rev                                 TGTGTCA
                                  344: AGAAA TGTGTCA TATGA

ZRE2                                            TGAGCAA
                                1,313: TGTTC TGAGCAA AGCAC

ZRE2 /Rev                                       TTGCTCA
                                  224: AGCCT TTGCTCA TCAGT
                                1,165: GCAAT TTGCTCA CAAGT

ZRE1 /Rev                                       TGTGTAA
                                1,942: TCCTG TGTGTAA ATGCA

CCAAT_site_4                                    GATTGG
                                2,360: GAGAG GATTGG CCTTC

CCAAT_site_4 /Rev                               CCAATC
                                3,035: ACTAT CCAATC TGACT

undefined_site_2                                TTTCCA
                                1,373: TnTCG TTTCCA GGTCC
                                3,093: ACTGC TTTCCA GATTT

undefined_site_2 /Rev                           TGGAAA
                                  165: ATAGA TGGAAA GGTTG

betaP-F1 /Rev                                   TTTATC
```

	1,492: GTAGA TTTATC AGGGG
	1,598: AATAA TTTATC ATGTC
	1,684: TTCTC TTTATC TTTTT
betaE-F3	
	GGGTGGGG
	1,869: GTCGG GGGTGGGG GAAAA
site_d5(m)	
	AACAGGTG
	2,800: AAGAT AACAGGTG ATGAG
site_k	
	CAGGTC
	1,377: GTTTC CAGGTC CTGCT
	2,049: ACACT CAGGTC ATTGG
site_k /Rev	
	GACCTG
	932: GTGAT GACCTG CTGAT
CT-II	
	CTAATG
	1,229: AACTT CTAATG GTTTT
D6	
	ATGAATA
	2,871: CACTG ATGAATA CATGG
D4	
	CATTCAT
	293: TTTCT CATTCAT ACAGT
D3	
	GATGCAT
	2,659: GTCTG GATGCAT TATTA
D3 /Rev	
	ATGCATC
	1,949: TGTAA ATGCATC CAGGG
D1	
	ATGAAAA
	22: AAGGA ATGAAAA GTCTT
D1 /Rev	
	TTTTCAT
	1,236: AATGG TTTTCAT ATAGC
HB3	
	GAAAAAGAAAAA
	1,062: AAAAA GAAAAAGAAAAA AGAAA
CAAT_site(1) /Rev	
	ATTGGC
	2,361: AGAGG ATTGGC CTTCT
TATA_(3) /Rev	
	TCTTATA
	1,270: GGCTT TCTTATA AGGTG
RIPE3b	
	CTTCAGCC
	2,162: TCATC CTTCAGCC GTCCA
RIPE3b /Rev	
	GGCTGAAG
	12: CGATA GGCTGAAG GAATG
GR-intron-site-2 /Rev	
	GGCACA
	72: GATGC GGCACA TGAGG
	2,585: CTACT GGCACA TATGC
GR-intron-site-3	
	TGTCCC
	1,188: ACAGC TGTCCC nCCTT
GR-intron-site-4	
	GACACA
	2,681: CTCGA GACACA CTCTT
GR-intron-site-4 /Rev	
	TGTGTC
	344: AGAAA TGTGTC ATATG

Total finds:	360
Total length:	3,122
Total sequences:	1
CPU time:	10.81

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