THE CLONING AND CHARACTERISATION OF THE CHICKEN TYROSINASE-RELATED PROTEIN GENE FAMILY

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I, Craig Stuart April, hereby declare that the work on which this thesis is based is my own original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. I empower the university to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

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May 1998

To my Family

Audere est facere

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LIST OF PUBLICATIONS AND PRESENTATIONS

Publications

1. **April, C. S.**, Franz, T. and Kidson, S. H. (1996). The cloning and characterization of chick tyrosinase from a novel embryonic cDNA library. *Exp. Cell Res.* (224):372-378.

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3. **April, C. S.**, Jackson, I. J. and Kidson, S. H. (1998b). Molecular cloning and sequence analysis of a chicken cDNA encoding tyrosinase-related protein-2/DOPAchrome tautomerase. *Gene*. In press.

Conference Presentations

4. Ferguson, C. A., **April, C. S.** and Kidson, S. H. (1993). The onset and control of early melanogenesis in chick neural crest cells. Second International Workshop on the Genetic Control of Animal Development. Crete, Greece.

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CORRIGENDUM

Tyrosinase-related protein-2 (Tyrp2) has been renamed DOPAchrome tautomerase (Dct).

ABSTRACT

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The cloning and characterisation of the chicken Tyrosinase-related protein gene family.
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Very little is known about the molecular and genetic mechanisms controlling pigmentation within the bird kingdom. The aim therefore, of this study was to contribute towards the understanding of the genetic regulation of avian pigmentation by the cloning and characterisation of the chicken *Tyrosinase-related protein (TRP)* gene family. To accomplish this goal, neural crest cells from 500 black chick embryos were cultured under conditions supportive of melanocyte differentiation and proliferation. Using RNA extracted from these pigmented melanocyte cultures, a novel embryonic chick cDNA library was constructed. Screening of this library for chicken equivalents of the mammalian *TRP* gene family yielded more than 200 cDNA clones. After sequencing, three of these clones, B8.3, pcTRP-1.6 and pcTRP-2.10, were found to encode chicken Tyrosinase (Tyr), Tyrosinase-related protein-1 (Tyrp1) and Tyrosinase-related protein-2 (Tyrp2), respectively. In addition a chicken *Microphthalmia (Mi)* cDNA clone (M156) was isolated using a mouse *Mi* cDNA probe.

Comparative analyses revealed that chicken Tyr, Tyrp1 and Tyrp2 share approximately 68%, 72% and 70% amino acid sequence identity with their vertebrate orthologues. Northern blot hybridisation analysis demonstrated that the chicken *TRPs* are expressed in RNA from cultured retinal pigment epithelial (RPE) cells as well as in whole eye RNA. The major transcript sizes for the chicken *Tyr*, *Tyrp1* and *Tyrp2* genes are 2.5 kb, 2.3 kb and 3.5 kb, respectively. *In situ* hybridisation studies confirmed that both chicken *Tyr* and *Tyrp2* genes are expressed in a pigment cell-specific fashion with signals detected in both the skin and RPE of chick embryos.

Genomic Southern blot hybridisation analyses strongly suggested that all three chicken *TRP* genes contain several introns that are likely to be conserved within the vertebrate *TRP* gene family. Furthermore, the chick *Tyr*, *Tyrp1* and *Tyrp2* genes were found to span approximately 5-19 kb, 5-11 kb and 15-30 kb, respectively of the chicken genome. Comparisons between a black and white chick breed at the *Tyr* and *Tyrp1* loci revealed no gross rearrangements at either of these loci. However, 1-2 kb alterations were observed between the same breeds at the *Tyrp2* locus. The nature and significance of this alteration is not known.

The cloning of the chicken *Tyr*, *Tyrp1* and *Tyrp2* cDNAs constitutes the first molecular cloning and characterisation of any avian *TRP* gene family. Taken together therefore, this study contributes towards the further understanding of the molecular mechanisms regulating pigmentation as well as the evolution of gene families.

CHAPTER 1: INTRODUCTION AND AIMS

1.1 Introduction

Birds are one of the most strikingly colourful of all the vertebrates. These displays are important for social communication, concealment from predators and in radiative heat exchange (McFarland *et al.*, 1985). The colours of feathers may be classified into two main groups: structural and pigmentary (Smyth, 1990). Structural colours are produced by small particles or air pockets in the feathers, reflecting particular wavelengths of light. According to Stevens (1991) structural colours may also arise from thin films having different refractive qualities. These films produce the iridescent blues, greens and purples, that change in appearance depending on the angle of vision. The white appearance of some feathers may even be caused by the reflectivity of some of these thin film surfaces.

Most of the feather colours in the domestic fowl however, result from the presence of pigments. Avian pigments include two general types, the carotenoids and the melanins. The brightest pigments (yellows, oranges and reds) are carotenoids, consisting predominantly of the xanthophylls (Brush, 1990), which are obtained through plant diets. These colours are thought to be important in communication and identification (Brush, 1990). In the domestic fowl the carotenoids contribute to a limited extent to the yellow and orange colour of some feathers, skin, fat and egg yolk. The melanins are responsible for the principal black and red pigments in feathers, although both carotenoids and melanins may interact with each other to produce a variety of shank and eye colours as well as structural sheens in the plumage (Smyth, 1990). As Smyth (1990) notes, while many poultry fanciers still breed for the beauty and technical excellence of colouration, pigmentation is also of economic importance to the commercial poultry industry. For example, white feathers are economically desirable to the commercial poultry meat industry, because they leave no residual melanin in the follicle after feather removal. Plumage colour has also been used as a useful genetic marker for sex determination of day old chickens. Apart from studies on the classical genetics of domestic fowl pigmentation, very little is known about the molecular genetics of avian plumage and eye colour. The cloning and analysis of genes involved in this complex process will further our understanding of the molecular mechanisms regulating avian pigmentation.

Although there are several comprehensive texts and reviews available on mammalian melanogenesis (Silvers, 1979; Hearing and Tsukamoto, 1991; Jackson, 1994b), comparatively little is known about avian melanogenesis. For example, in the mouse it has

been suggested that nearly 100 genes have an effect on coat colour (Barsh, 1996). Seventeen of these genes have been cloned, and in humans, 14 homologous genes have been mapped to loci known to be responsible for human pigmentary disorders (Jackson, 1997). In stark contrast, although there are at least 40 loci known to play a role in chicken pigmentation (Somes, 1980), not a single chicken gene has been genetically mapped to any of these known pigmentary loci. Therefore, the broad aims of this thesis are:

i) to contribute towards the understanding of the molecular mechanisms regulating the development of chicken melanogenesis by providing the molecular tools with which such investigations may be conducted and

ii) to further our understanding of the genetics of avian pigmentation as well as the evolution of gene families.

This review is intended to provide a general overview of the current understanding of the genetics and biochemistry of chicken melanogenesis. Particular emphasis has been placed on areas, where currently very little is known, and where this thesis aims to contribute. In addition there is a brief overview of the discovery and molecular genetics of the *Tyrosinase-related protein (TRP)* gene family. In accordance with the guidelines as set out by the Poultry Species Committee of the National Animal Genome Research Program, 1993, and the Online Mendelian Inheritance in Man (OMIM) database, abbreviations of the *TRP* genes and loci, in this study, are as follows: *Tyrosinase (Tyr)*, *Tyrosinase-related protein-1 (Tyrp1)* and *Tyrosinase-related protein-2 (Tyrp2)*. Furthermore, all gene symbols, including genes, loci, alleles, mRNAs, cDNAs and primers in this thesis are *italicised*, with their protein products non-italicised. Only human gene and protein symbols are CAPITALISED, with all other animal and plant gene and protein products indicated by an initial Capital letter, followed by lower case letters.

1.2 Molecular markers for avian melanocytes

Ever since starlike cells carrying melanin granules were first observed in the integuments of vertebrates, several theories regarding the origin of these cells were put forward (Ris, 1941). It was not until Du Shane (1934) and Dorris (1936) demonstrated that in the axolotl, *Ambystoma*, and chicken respectively, that melanophores were derived from neither the epidermis nor the mesenchyme, but the neural crest (NC), that the debate was resolved. Once it was established that melanophores/melanocytes arose from the NC and migrated to the epidermis, the precise routes and timing of migration of avian melanocyte precursors were investigated. A large body of evidence that has accumulated over several decades from many laboratories has contributed to our current understanding of this process. More

comprehensive texts and reviews on the developmental biology of avian NC cells (NCCs) may be found elsewhere (Weston, 1970; Le Douarin, 1982; Weston, 1991; Le Douarin *et al.*, 1993).

Very little is known about the molecular mechanisms, and in particular the genetic regulation of avian melanocyte differentiation. As Anderson (1997) notes, the problems of NC development at the molecular genetic level can be stated rather simply: which genes are important for NC (and melanocyte) development, and what do their products do? When the current project was initiated in 1991, only a single chicken pigment cell-specific gene, MMP115, had been reported (Mochii *et al.*, 1991), along with a few antisera detecting antigens expressed by chicken pigmented cells or their progenitors. In almost all of these cases, the identity and function of the pigment cell-specific gene product/antigen is not known. Moreover, many of these markers are not expressed in a pigment cell-specific fashion. It is clear therefore, that in order to define transient, but distinct stages of avian melanocyte development, additional probes are required. To address the scarcity of chicken melanocyte-specific gene markers, a key aim of the current investigation was to clone cDNAs encoding *Tyr*, *Tyrp1* and *Tyrp2*, that together constitute the chicken *TRP* gene family.

While the value of genetic markers in tracing the course of avian melanocyte development is evident, the nature, function and control of expression of these molecules is just as important. In order to shed further light on the hierarchy of molecular control of avian melanogenesis it is necessary to investigate the regulation of avian melanogenesis at the genetic level.

1.3 The genetics of chicken pigmentation

In mammals there are at least 80 distinct loci known to be involved in the regulation of mouse pigmentation (Silvers, 1979; Jackson, 1997). Of these, at least 25 are thought to act on melanocyte development (Bennett, 1993), 11 others are thought to be involved in melanocyte function (Bennett, 1993; Jackson, 1997) and 16 thought to act systemically on the general metabolism (Bennett, 1993). Of the remaining loci, too little is know to allow classification into any grouping. It is unclear how many loci and alleles are involved in chicken melanogenesis. Somes (1980) lists 39 chicken loci that have been reported as being involved in either feather, eye or skin colouration. More recently, Smyth (1990) included *grey* and *erminette* as two additional loci involved in chicken pigmentation. Thus, at least 40 loci are known to be involved in chicken pigmentation. Similarly, Ito and Tsudzuki

(1994) reported that approximately 30 kinds of plumage mutations have been described for the Japanese quail. However, there are many similarities between mammalian and avian melanogenesis (Carver and Brumbaugh, 1974; Zimmerman *et al.*, 1982; Bowers, 1988), and it is likely that a similar number of loci (at least 80) are involved in the regulation of both mammalian and avian pigmentation. Although a number of chicken genes have been sequenced, quite remarkably, not a single one of these genes has been genetically mapped to a pigmentary chicken locus (it is thought and sometimes assumed, but not yet proven, that the chicken *Melanocortin 1 receptor* (*Mc1r*) and *Tyr* genes map to the E^+ and C^+ loci respectively (see sections 1.3.1 and 1.3.3).

The following section is restricted to a discussion of four key loci known to influence the pigmentary phenotype of the breeds of fowl relevant to the current study. These loci are the *Extended black (E)*, *Dominant white (I)*, *albino (c)* and *pink eye (pk)*.

1.3.1 The *Extended black (E)* locus

Dunn (1922) first reported a dominant autosomal gene which is primarily responsible for the completely black plumage of many breeds of fowl, including Black Orpingtons and one of its derivatives, the Black Australorp (Gericke, 1934). This gene he called "extension of melanic pigment", assigning the symbol E^m. For several decades E^m was erroneously considered a dominant allele of the *Columbian (Co)* restriction locus (then called *e*). *Co* restricts black pigment to the hackle, wing tip, foot and tail feathers. It is now known however, that *Extended black (E)* and *Co* are not allelic. The *E* locus, which according to Stevens (1991b) is the most studied of all the chicken pigmentary loci, is multi-allelic with eight alleles (Morejohn, 1955; Smyth, 1990) having a dominance hierarchy of *E (Extended black)* > E^R *(Birchen)* > e^{Wh} *(dominant wheaten)* > e^+ (wild type) > e^b *(brown)* > e^s *(speckled)* > e^{bc} *(buttercup)* > e^y *(recessive wheaten)*. Four additional alleles (e^m, e^n, e^{nl} and e^q) have been proposed but their relationship to the *E* locus remains to be established (Smyth, 1990). Because the phenotypes of the *E* locus alleles range from black eumelanin *(Extended black)* to red phaeomelanin *(recessive wheaten)* production, it is believed that the *E* locus is primarily concerned with black-red melanin distribution in the chicken feathers.

In attempting to attribute a specific cellular function to the *E* locus, Carver and Brumbaugh (1974) measured the oxygen consumption in developing *E/E* (black) and e^y/e^y (red) feather papillae. They suggested that the *E* locus regulates the expression of eumelanin or phaeomelanin by the synthesis of particular enzymes (dopa oxidase) at certain times during development. Zimmerman *et al.* (1982) however, in their ultrastructural analysis of

eumelanosomes, concluded that the *E* locus is a structural gene which controls basic eumelanosome matrix structure.

More recently the *E* locus has been investigated at the molecular level. Carefoot (1993) has mapped the *E* locus to chicken chromosome 1. Also, skin graft experiments revealed that the *E* locus acts in a cell-autonomous manner within the melanocyte (Brumbaugh, 1967). Because the chicken *E* locus alleles exhibit phenotypes similar to those reported for the mouse *extension (e)* locus (Robbins *et al.*, 1993), which encodes the melanocortin 1 receptor (Mc1r), Takeuchi *et al.* (1996a) cloned the chicken *Mc1r* gene in order to determine whether this gene is responsible for chicken *E* locus function. The *Mc1r* gene has been cloned in several animals (see Jackson, 1997) and encodes the α-MSH receptor. Recent work by Takeuchi *et al.* (1996b) has revealed that chicken *E* phenotypes possess *Mc1r* mutations that appear to correspond to specific dominant and recessive mouse *Mc1r* alleles responsible for mutant mouse coat colours. It is likely therefore, though not yet proven, that the chicken *Mc1r* gene is encoded by the chicken E^{+} locus.

Evidence accumulated over many years from several laboratories strongly suggests that in mammals, α-MSH and the α-MSH receptor are involved in enhancing eumelanogenesis by stimulating the enzymatic activity of TYR. Several different mechanisms have been proposed to explain how this enhanced enzyme activity may come about. For example, while there is evidence that α-MSH upregulates *Tyr* gene expression (Kuzumaki *et al.*, 1993) as well as Tyr protein translation (Aroca *et al.*, 1993), there is also good evidence that pre-existing Tyr enzyme is activated by MSH (Halaban *et al.*, 1983). Furthermore, it appears that α-MSH elicits different *TRP* transcriptional responses in different mouse melanoma cell lines. Whereas Kuzumaki *et al.* (1993), using B16-F1 cells, reported that α-MSH upregulated both *Tyr* and *Tyrp1* gene expression, Aroca *et al.* (1993), using JB/MS cells, found that α-MSH only upregulated *Tyr*, but had no effect on *Tyrp1* and *Tyrp2* transcription. More recent work seems to suggest that TYR activity is required for both eu- and phaeomelanin synthesis, whereas TYRP1 and TYRP2 are only required for eumelanin synthesis (Tsukamoto *et al.*, 1992; del Marmol *et al.*, 1993; Kobayashi *et al.*, 1994a; Sakai *et al.*, 1997).

In avians, the physiological role of α-MSH is unclear, although the ostrich (Li *et al.*, 1978), turkey (Chang *et al.*, 1980) and chicken (Hayashi *et al.*, 1991) α-MSH primary peptide sequences have been reported. Alpha-MSH presence has also been detected in the duck

pituitary (Iturriza *et al.*, 1980). There is also experimental evidence to suggest that α-MSH does indeed play a role in avian melanogenesis. Satoh and Ide (1987) report that α-MSH not only accelerates melanogenic differentiation in quails, but also affects the state of commitment of NCCs to melanogenic differentiation *in vitro*. Further evidence for a role for α-MSH and its receptor in regulating chicken melanogenesis stems from *in vitro* experimentation, where Bibosa and Bowers (1992), Marco (1994) and Bowers *et al.* (1997) have demonstrated that cultured chicken melanocytes respond to exogenous α-MSH or cAMP agonists, by dramatically enhancing eumelanogenesis.

1.3.2 The *Dominant white (I)* locus

Gericke (1934) writes that, "Darwin (1875) was the first investigator to draw our attention to the inheritance of white plumage in the domestic fowl... he showed that the white colour of the game (hen) was possibly a dominant factor." According to Smyth (1990), *Dominant white* was one of the first animal traits to be investigated following the rediscovery of Mendel's classical work. *Dominant white* was shown to be a dominant mutation by Bateson (1903) and later assigned the gene symbol *I* (Inhibitor of black pigment). *Dominant white* is responsible for the completely white plumage of many commercial breeds of fowl, including White Leghorns and Pile Games (Smyth, 1990). Currently it is believed that the *I* locus, like the *E* locus is multi-allelic. Three *I* locus alleles have been described, $I > I^{D}$ *(dun)* $> i^{+}$ (wild type). *Erminette* has been proposed to be allelic with *I* (Smyth, 1990), although this suggestion remains unproven.

Earlier in this century, there was much speculation regarding the cause and nature of *Dominant* and *recessive white* colouration in animals. For example, it was reported by Spiegler in 1904 (Gortner, 1910) that the hair and wool of white horses and sheep and even the white feathers of birds (Spillman, 1910) contained "white melanin". It was believed then that "oxidation was not carried far enough to produce color." This melanin subsequently became known as Spiegler's "white melanin". Gortner (1910) then demonstrated that Mendelian *Dominant* and *recessive whites* had no relation to the presence of Spiegler's "white melanin". Gortner (1910) suggested that Spiegler's "white melanin" was a decomposition product of keratin and that the *Dominant white* phenotype was due to the presence of an "anti-oxydase" which prevented pigment formation.

Grafting experiments by Willier and Rawles (1940) suggested that White Leghorn (WL) (carrying the *I* gene) melanophores were quantitatively fewer or had less ability to migrate

than in other breeds. They further suggested that WL melanophores had a limited capacity to deposit pigment and that these deficiencies were intrinsic properties of WL melanophores. A similar proposal, put forward by Hamilton (1940) suggested that the WL possessed white feathers because of the small number and low viability of differentiated melanophores, so that they did not thrive for long in the feather germ. The apparent decrease in the number of differentiated melanophores was suggested to be due to the fact that many of the melanophores died in the melanoblast stage before they could elaborate pigment granules.

Brumbaugh (1971), using a combination of DOPA histochemistry and ultrastructural analyses on single I/i^+ mutant stocks, reported that the *I* locus was responsible for a reduction in the synthesis of premelanosomes. This result prompted him to make the following observation regarding the "viability and early death" hypothesis forwarded by Hamilton (1940), "Dominant white melanocytes appear to die sooner than standard melanocytes because they have transferred and dispersed their few, poorly assembled granules into the epidermal cells and are therefore no longer dark enough to be identified." It remains unresolved however, whether melanocytes die early, or whether unmelanised melanocytes are indeed present in breeds carrying the *I* gene.

Several recent studies support the finding of Brumbaugh (1971), that the *I* locus causes a reduction in premelanosomal synthesis. First, ultrastructural studies by Jimbow *et al.* (1974) suggested that the *I* locus is responsible for a melanocyte-intrinsic defect that results in the formation of reduced numbers of irregularly assembled melanosomes. They also reported reduced cytochemical dopa oxidase activity in WL *(I/I)* melanocytes, implying that the *I* gene somehow affects the level of Tyr activity. However, as pointed out by Brumbaugh and Lee (1975), much of the earlier work on the *I* locus involved using the WL breed. This breed is now known to carry both the *Barring (B)* and *Blue (Bl)* mutations. Because both these dominant mutations produce hypomelanosis as single mutants, the effect of *I* alone on the WL pigmentation cannot be ascertained unambiguously.

The second line of evidence suggesting that the *I* locus plays a role in premelanosomal synthesis comes from Brumbaugh and Lee (1975). These authors measured the dopa oxidase (Tyr) activity of skin melanocytes from a single mutant (I/i^+) stock, and reported no reduction in cytochemical dopa oxidase activity in this breed, thus highlighting the importance of using single mutant stocks. However, they did report that there were fewer melanogenic organelles in *I/I* melanocytes by comparison with i^+/i^+ melanocytes. This

therefore suggested that the *I* locus either prevents the assembly of melanosome components into recognizable structures, reduces their synthesis, or allows premature degradation of these organelles. The authors concluded that the *I* locus contains a regulatory mutation which partially suppresses the production of premelanosomal components in NC-derived melanocytes. Subsequent studies by Zimmerman *et al.* (1982), supported this finding that *Dominant white* did not affect melanosome ultrastructure and that the *I* locus harboured a regulatory mutation instead.

Further evidence that the *I* locus is involved in premelanosome synthesis was provided by ultrastructural studies by Marco (1994), who reported that the White Plymouth Rock X Pile Game breed (which carries the *I* gene), is hypomelanotic because of a defect which impairs the rate of melanosome synthesis. In this study it was reported that both 9 and 10 day old skin melanocytes contained only a small number of premelanosomes relative to other organelles.

Wilkins *et al.* (1982), then further investigated the *I* locus, by analysing complementation in heterokaryons. In these experiments the authors found that I/i^+ Bl/bl^+ + I/i^+ Bl/bl^+ and I/i^+ C^+/c + I/i^+ C^+/c double heterozygotes failed to complement (produce pigment in culture) as somatic homokaryons, whereas I/I bl^+/bl^+ + i^+/i^+ Bl/Bl and I/I C^+/C^+ + i^+/i^+ c/c somatic heterokaryons produced copious amounts of pigment in culture. The authors suggested therefore that this complementation difference between heterokaryons and heterozygotes was because *I* has an inhibitory effect on some aspect of pigment synthesis and that this action is restricted to the nucleus.

While *I* bleaches eumelanin, it has little or no effect on phaeomelanin (Brumbaugh, 1971; Brumbaugh and Lee, 1975; Malone and Smyth, 1979). The *I* locus is also epistatic to both *E* and *c* loci and while inhibiting only NC-derived eumelanin, has no effect on eumelanin production in retinal pigment epithelial (RPE) cells (Brumbaugh, 1971; Brumbaugh and Lee, 1975). Although the preliminary mapping of the *I* locus has been reported (Ruyter-Spira *et al.*, 1997), the gene responsible for *I* locus function has not been identitifed and as yet no suitable candidate genes have been proposed for this locus. Whereas *Dominant white* is prevalent amongst birds, there appear to be no dominant mammalian coat colour equivalents of the chicken *I* allele. This therefore raises the intriguing possibility that the i^+ locus may encode a novel gene involved in the regulation of vertebrate pigmentation.

1.3.3 The *recessive white (c)* locus

According to Smyth (1990) *recessive white (c)*, like *Dominant white (I)* was another of the earliest animal traits to be studied during the first investigations of Mendel's principles. Brumbaugh *et al.* (1983) and Smyth (1990) report that *recessive white* is the most common form of white plumage that is inherited as a recessive mutation. *Recessive white* accounts for the white plumage of many breeds, including White Plymouth Rocks. The symbol *c* was used to denote the absence of chromagen material. As in mammals, the C^+ locus in chickens is multi-allelic, with 4 alleles described with a dominance order of C^+ (wild type) > *c (recessive white)* > c^{re} *(red-eyed)* > c^a *(autosomal albino)* (Brumbaugh *et al.*, 1983; Smyth *et al.*, 1986). Whereas *c* affects only the plumage, c^a affects the plumage as well as eye pigmentation. Brumbaugh *et al.* (1983) reported that c^a/c^a eyes contained very few melanin granules. *Red-eyed* chickens resemble *recessive whites*, except that the former has red eyes whereas the latter has dark pigmented eyes (Smyth *et al.*, 1986). More recently, Bitgood and Smyth (1991) reported a spontaneous C^+ locus mutation in White Leghorns. It is possible that this mutation might represent a fifth C^+ locus allele or it may be identical to the previously described c^a mutation. The *c* locus is thought to be epistatic to *E* but hypostatic to the *I* locus.

There are conflicting reports regarding the enzymatic activity of C^+ locus mutants. Carver and Brumbaugh (1974) reported that *c/c* chickens possessed very low levels of dopa oxidase (Tyr) activity. Similarly, Brumbaugh *et al.* (1983) found that both *c* and c^a feather and RPE cells were cytochemically Tyr-negative and contained numerous incompletely formed unpigmented granules (melanosomes).

In contrast, Oetting *et al.* (1985a), on examining both *c* and c^a chickens, reported that both loci produce normal premelanosomes and Tyr-like molecules (having cross-reactivity with a polyclonal anti-chicken Tyr antibody). These Tyr-like molecules however lacked Tyr activity and this prompted the authors to suggest that the C^+ locus is the structural locus for Tyr. This result directly conflicted with the study of Boissy *et al.* (1987) in which cultured c^a melanocytes were reported to lack Tyr protein as well as any identifiable premelanosomes. Boissy *et al.* (1987) state that there are no simple explanations for these discrepancies, although in the latter study, the authors used a polyclonal anti-hamster Tyr antibody. It is possible therefore that differences in the two anti-Tyr antisera used, might explain the differing immunoprecipitation results obtained.

1.3.4 The *pink eye (pk)* locus

Warren (1940) first described the *pink eye (pk)* mutation in White Plymouth Rocks. The autosomal recessive *pk* mutation, when homozygous dilutes black eumelanin to a blue-grey colour, resulting in grey plumage and pink eyes. Because *pk* eyes are very similar in colour to c^a ones, the two albinism conditions were originally considered identical, until a breeding test proved them to be non-allelic. Ultrastructural (Brumbaugh, 1968) and autoradiographic (Brumbaugh and Lee, 1975) analyses revealed the *pk* mutation to be a structural mutation retarding eumelanin binding to the premelanosomal matrix of both RPE cells and melanocytes. Because the *pk* mutation retards melanin deposition, the matrix ultrastructure of the *pk* premelanosomes is readily visible. It was this characteristic that assisted Brumbaugh (1968) in providing the first detailed description of avian premelanosomal structure.

In contrast to the study by Brumbaugh and Lee (1975), heterokaryon complementation analysis by Antoniou and Brumbaugh (1982), Wilkins *et al.* (1982) and Brumbaugh and Oetting (1986) suggested that the pk^+ locus is a nuclearly restricted regulator controlling the expression of the E^+ locus. These experiments were based on somatic cell hybridisations using two different genotypes (e^y and *pk*) having different deficiencies in melanin production. Heterokaryons, with pk/e^y nuclei, failed to complement (produce wild type pigment), whereas melanocytes from crossed pk/e^y birds produced copious amounts of pigment in culture. To reconcile these two differing results, the authors proposed that in the heterozygote cells (latter experiment), the wild type pk^+ and E^+ genes at each of the two loci are in the same nucleus, whereas in the heterokaryons (former experiment), the wild type genes at each locus are in separate nuclei. This suggested therefore that the nuclear membrane prevented pk^+ from being able to complement E^+ (previous experimentation with *pk* and other mutant loci had implicated *pk* as being responsible for non-complementation). Wilkins *et al.* (1982) also suggested that *pk* is epistatic to *I*.

Although the gene for *pk* locus function has not been identified yet, potentially homologous mutations have been reported in the mouse and human. Mutations at the human *P* locus have been implicated in type II oculocutaneous albinism (OCA) (OMIM #203200) (Rinchik *et al.*, 1993). In the mouse, mutations of the *pink-eyed dilution (p)* gene are associated with mutant phenotypes very similar to that of human (OCA) type II (Gardner *et al.*, 1992). As yet the functions of both the human *P* and mouse *p* loci are unknown, although the mouse p protein has been suggested to bind melanin to the melanosomal matrix (Donatien and Orlow, 1995), which is similar to the chicken *pk* locus function suggested by Brumbaugh

and Lee (1975). Both the mouse p and human P polypeptides include a total of 12 transmembrane domains suggesting that these proteins may be integral components of the melanosomal membrane. In the chicken, *pk* appears to have very little or no effect on phaeomelanin and in the mouse, *p* mRNA is only expressed in melanocytes that specifically synthesise eumelanin. Phaeomelanin-synthesising melanocytes do not express the *p* gene. This finding provides additional support for the suggestion made here, that the chicken *pk* gene is the avian homologue of the mouse *p* and human *P* genes.

Because there is evidence that mammalian TRPs form a high molecular weight (> 200 kDa) melanosomal complex with other melanogenic proteins, including the α-MSH receptor (Pawelek, 1991), lysosome-associated membrane protein-1 (Lamp-1) (Pawelek, 1991; Luo *et al.*, 1994; Jimbow *et al.*, 1994), Lamp-2 (Jimbow *et al.*, 1997), possibly Lamp-3 (Jimbow *et al.*, 1997), Pmel 17 (silver) (Lee *et al.*, 1996), and the p protein (Rosemblat *et al.*, 1994; Lamoreux *et al.*, 1995), and because mutations at the *p* gene have been associated with reduced levels of Tyr (Nakatsu *et al.*, 1993), Tyrp1 and Tyrp2 protein (Chiu *et al.*, 1993), it will be of interest to determine whether chicken *TRP* gene expression is altered in *pk* melanocytes. While there is histochemical evidence that *pk* melanocytes contain DOPA oxidase (Tyr) activity (Brumbaugh and Lee, 1975), this assay is not quantitative. Also, although there is evidence that mouse *p* gene mutations reduce TRP enzyme activity through a post-translational mechanism (Chiu *et al.*, 1993), there have been no reports on the effects of the *p* and *pk* mutations on *TRP* gene expression.

1.3.5 A model of pigment gene interaction for chicken melanogenesis

Using somatic cell hybridisation and heterokaryon complementation analyses, Antoniou and Brumbaugh (1982), Wilkins *et al.* (1982) and Brumbaugh and Oetting (1986) attempted to combine classical chicken pigmentary genetics with the ultrastructural and cytochemical studies of chicken pigmentation in order to dissect the biochemical steps involved in chicken melanogenesis. In their investigations, these authors used six pigmentary loci (*c^a*, *pk*, *e^y*, *I*, *sex-linked imperfect albinism* (*s^{al}*) and *Blue (Bl)*) and were able to construct a theoretical hierarchy of gene/biochemical control for melanosome synthesis. Two basic complementation groups were found, one group being involved in the formation of the enzyme Tyr, with the second group being involved in the formation of the melanosome. However, although this scheme provided a basic framework for future investigations, further progress in the understanding of the biochemical and genetic regulation of avian melanogenesis has been hindered by the absence of suitable melanoma and melanocyte cell lines. Much of our current understanding of the biochemistry of the melanin biosynthetic

pathway in avians has therefore been limited to the few ultrastructural (predominantly describing melanosomal biogenesis) and cytochemical studies conducted on the domestic fowl.

Thus far, the only enzymatic activity that has been demonstrated to be associated with chicken melanin biosynthesis is that of Tyr. Several laboratories have independently purified and characterised chicken Tyr (Doezema, 1973a; Yamamoto and Brumbaugh, 1984; Boissy *et al.*, 1987; Wang *et al.*, 1995). The biochemical characteristics of chicken Tyr appear very similar to that of mammalian TYR, with both tyrosine hydroxylase and DOPA oxidase activities present. Furthermore, ultrastructural evidence from several laboratories (Carver and Brumbaugh, 1974; Bagnara *et al.*, 1979; Zimmerman *et al.*, 1982; Oetting *et al.*, 1985b; Bowers, 1988) provides support that the regulation of chicken melanogenesis is at least as complex as that described for mammals.

1.4 The biochemistry of melanogenesis

Just over a century ago in 1895, Bourquelot and Bertrand first reported that a substance present in the mushroom, *Russula nigricans*, was turned black by an enzyme present in the same fungus. In 1896, Bertrand identified the chromagen (substrate) as the amino acid (aa) tyrosine and Bourquelot subsequently named the enzyme "tyrosinase". Ever since this discovery, considerable attention has been focused on elucidating the biochemical steps responsible for the biosynthesis of melanin from L-tyrosine. One of the first descriptions of the metabolic pathway responsible for the biosynthesis of melanin was reported by Raper (1928) and Mason (1948). In the Raper-Mason model of melanin biosynthesis, TYR catalyses the hydroxylation of L-tyrosine to DOPA, and the oxidation of DOPA to DOPAquinone, with the remaining steps in the cyclisation of DOPAquinone to melanin proceeding non-enzymatically. It was not until Logan and Weatherhead (1978) provided the first indirect evidence for post-Tyr inhibition of melanogenesis in the hair follicles of Siberian hamsters, that additional regulatory sites were included in the melanin biosynthetic pathway. Subsequent work from several laboratories (Körner and Pawelek, 1980; Hearing and Jiménez, 1987; Hearing and Tsukamoto, 1991; Kwon, 1993) revealed the mammalian melanin biosynthetic pathway to be a complex multi-enzymatic one. This complexity is highlighted by the discovery of:

i) at least three enzymes involved in the biosynthesis of melanin. These include the three members of the TRP gene family, TYR (EC 1.14.18.1), DHICA oxidase (TYRP1) and L-DOPAchrome tautomerase (EC 5.3.2.3) (TYRP2). A more detailed discussion of the TRP gene family follows in section 1.5.

ii) the Pmel 17 family (Kwon, 1993), whose members were originally suggested to consist of the mouse and human silver (Kwon *et al.*, 1991; Kwon *et al.*, 1994), the chicken MMP115 (Mochii *et al.*, 1988a) and the bovine RPE1 (Kim and Wistow, 1992) proteins. It is possible that at least two additional closely related proteins, the human nmb (Weterman *et al.*, 1995) and quail QNR-71 proteins (Turque *et al.*, 1996) may belong to this family.

iii) the pink-eyed dilution (p) protein which has been suggested to link the melanin matrix to the melanosomal membrane (Donatien and Orlow, 1995). The *p* gene was initially thought to encode a membrane-bound L-tyrosine transporter (Jara *et al.*, 1990), but this theory has since been disproved (Gahl *et al.*, 1995);

iv) an as yet partially characterised MART-1/Melan-A protein, which has been suggested to play a role in pigment synthesis and melanosome formation (Coulie *et al.*, 1994; Kawakami *et al.*, 1994; de Vries *et al.*, 1997).

v) a human and mouse visual pigment-like G-protein coupled receptor, termed peropsin (Sun *et al.*, 1997). Peropsin was found to be uniquely expressed in the microvilli of RPE cells and most likely plays a role in phototransduction rather than in eye melanogenesis. Recently another opsin-like molecule, melanopsin (Provencio *et al.*, 1998), was reported in frog dermal melanophores. Although melanopsin is also expressed by other cell types, it is thought that it may mediate photo-induced melanosome migration in the skin.

vi) several endogenous melanogenic inhibitors. Although some of these inhibitors have been purified and partially characterised (Hatta *et al.*, 1988; Chakraborty *et al.*, 1989; Kameyama *et al.*, 1989), their exact mechanisms of inhibition are unknown;

vii) catalases (Halaban and Moellmann, 1990) and peroxidases (d' Ischia *et al.*, 1991; Gesualdo *et al.*, 1997) that may also play roles within the melanin biosynthetic pathway and

viii) at least two genes involved in the switch from eu- to phaeomelanin biosynthesis. These genes encode the MC1R (Mountjoy *et al.*, 1992; Chhajlani and Wikberg, 1992) and Agouti protein (Bultman *et al.*, 1992; Wilson *et al.*, 1995). The roles of α-MSH and MC1R in melanogenesis have been discussed elsewhere (see sections 1.3.1 and 4.5). Recently a gene distantly related to *Agouti*, termed *Agouti-related transcript (protein)* (Shutter *et al.*, 1997; Ollman *et al.*, 1997), was cloned. Thus far, it appears that this gene has no effect on mouse pigmentation, but has been implicated in weight regulation (Ollmann *et al.*, 1997).

A scheme outlining the current understanding of the enzymatic steps involved in mammalian melanin biosynthesis is presented in Fig. 1.1.

1.5 The discovery of the TRP gene family

Since the discovery of Tyr in the last century, it has been accepted that this enzyme plays a pivotal role in regulating the biosynthesis of melanin. As noted earlier, for several decades it was assumed that Tyr was the only enzyme involved in the biosynthesis of melanin. It therefore came as a surprise when in 1988, Jackson found that a previously reported mouse *Tyr* cDNA (Shibahara *et al.*, 1986) did not in fact encode Tyr, but a closely related molecule. The subsequent discovery of a second Tyr-related protein (Jackson *et al.*, 1992) gave rise to the *Tyr-related protein (TRP)* gene family. Because the TRPs have contributed to the understanding of the developmental biology, genetics, biochemistry and more recently the evolution of vertebrate melanogenesis, and because the bulk of this thesis details the first reported cloning and characterisation of the chicken *TRP* gene family, it is necessary to review the molecular biology of this gene family. Recent comprehensive reviews on the *TRP* gene family have been published (Jackson, 1994b; Jackson *et al.*, 1994; Del Marmol and Beermann, 1996).

The first *Tyr* gene to be cloned was isolated from the bacterium, *Streptomyces antibioticus*, using interspecific insertional inactivation of an antibiotic (neomycin) resistance marker gene (Katz *et al.*, 1983; Bernan *et al.*, 1985). Soon afterwards, *Tyr* genes from *Streptomyces glaucescens* (Hintermann *et al.*, 1985), and plants (van Gelder *et al.*, 1997) were also cloned. Although many of these genes were isolated before several of their animal counterparts, not much is known regarding their genomic organisation and regulation. In plants most *Tyrs* are encoded by multigene families (in the tomato, there are more than 7) which produce 60-65 kDa proteins which are proteolytically processed to 40-45 kDa. In plants and fungi, although the physiological function/s of Tyr (polyphenol oxidase) is not fully understood, it is thought that Tyr may have a ripening function (Joy *et al.*, 1995) and play a protective role. For example, melanin scab formation occurs after attack from insects or microorganisms or browning occurs after bruising or damage to fruit or vegetables (van Gelder, 1997). As yet there have been no reports of a bacterial or plant TRP. A recent more comprehensive review of plant and fungal Tyrs has been published (van Gelder *et al.*, 1997).

In 1986, Shibahara *et al.* reported the first cloning and sequencing of a mammalian *Tyr* gene. Using differential hybridisation, their clone was isolated from a B16 mouse melanoma

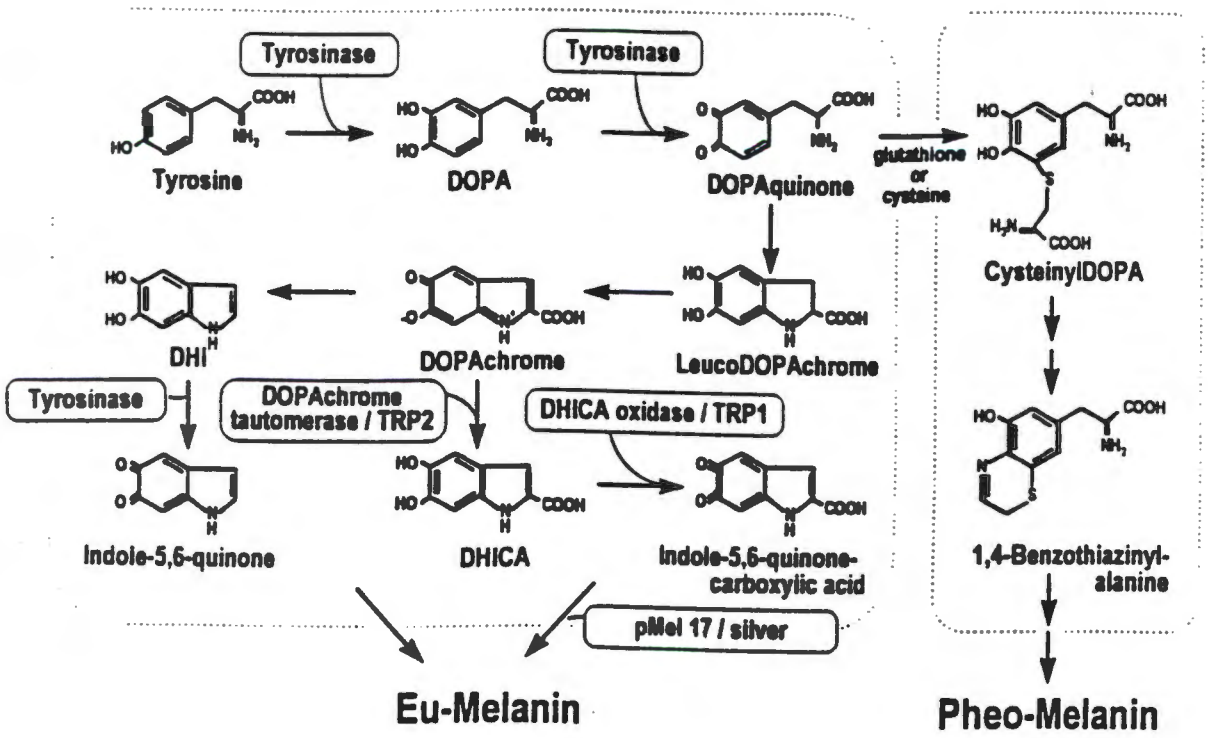

Fig. 1.1. Schematic diagram of the mammalian melanin biosynthetic pathway (adapted from Furumura *et al.*, 1996).

cDNA library using mouse neuroblastoma cDNA and black mouse (C57BL/6) skin cDNA as negative and positive probes respectively. Evidence to suggest that the two isolated clones (pMT4 and pMT5), encoded Tyr included:

i) northern blot analysis, showing specific hybridisation of pMT5 to a 2.3 kb transcript in B16 melanoma cell and C57BL/6 black mouse skin RNA, but not to neuroblastoma or K1735 amelanotic melanoma cell RNA;
ii) immunocytochemistry, showing that transient transfection of pMT4 into the amelanotic melanoma cell line, K1735, resulted in immunofluorescent-positive cells when cross-reacted with two anti-mouse T_4 Tyr monoclonal antibodies;
iii) sequence homology, showing that the deduced pMT4 aa sequence showed 26% sequence identity with the fungal, *Neurospora crassa*, Tyr protein sequence.

In 1988, Jackson, in attempting to clone a mouse *Tyr* gene, screened a B16 mouse melanoma λgt11 expression library with two anti-mouse Tyr antisera. A mouse melanoma cDNA clone (5A) was isolated, which was found to cross-hybridise to pMT4 (Shibahara *et al.*, 1986). Southern blot analysis on recombinant inbred mouse genomic DNA, demonstrated that pMT4 mapped to the mouse *brown (b)* locus and not the *albino (c)* locus, which had previously been shown to possess Tyr activity (Gluecksohn-Waelsch, 1979). Thus Jackson (1988) suggested that pMT4 did not encode authentic Tyr, but a related molecule, which he termed Tyr-related protein (TRP).

Meanwhile, in 1987, two additional mammalian *TYR* cDNAs were reported. The first of these by Yamamoto *et al.* (1987), provided the nucleotide (nt) sequence of a mouse *Tyr* cDNA, which was later shown to be an alternatively spliced variant of the full-length mouse *Tyr* primary transcript (Ruppert *et al.*, 1988). The second report detailed the cloning and characterisation of a human *TYR* cDNA (Kwon *et al.*, 1987). Genomic Southern blot hybridisation analysis revealed that this clone, Pmel 34, mapped very near to, or at the mouse *albino* locus. This clone, isolated from a normal human melanocyte cDNA library, using an anti-human TYR antiserum, was subsequently shown to be truncated at its 5' end, missing the initiator Met codon (Bouchard *et al.*, 1989).

With the partial human and mouse *TYR* cDNA sequences available, the isolation of the full-length mouse (Müller *et al.*, 1988) and human (Bouchard *et al.*, 1989) *TYR* cDNAs soon followed. Functional enzymatic assays of transfected *TYR* cDNA constructs confirmed the identity of these clones. Several cDNA clones for human *TYRP1* (Cohen *et al.*, 1990; Vijayasaradhi *et al.*, 1990; Urquhart, 1991) were also reported.

In 1992, clone 5A, originally reported by Jackson (1988) to be closely related to the *b* locus clone, pMT4 (*Tyrp1*), was sequenced and characterised and was identified as a novel TRP, termed Tyrosinase-related protein-2, thus heralding the existence of a three-member TRP gene family. Since the identification of the mammalian TRP gene family, several reports identifying TRPs in vertebrates, other than in mammals, have appeared, including TRPs from the fish (Inagaki *et al.*, 1994; Peng *et al.*, 1994), frog (Takase *et al.*, 1992; Miura *et al.*, 1995) and axolotl (Mason and Mason, 1995; Mason *et al.*, 1996). There is evidence to suggest that the TRP gene family diversification predated the radiation of the early vertebrates (Jackson *et al.*, 1994; Jackson, 1994a; Morrison *et al.*, 1994). With the cloning of the *Halocynthia roretzi* (Sato *et al.*, 1997) and *Ciona intestinalis* (Caracciolo *et al.*, 1997) *Tyr* genes, there has been great interest in determining whether there are other TRP representatives in invertebrates.

The following section provides a brief summary of the molecular genetics of the *TRP* gene family. A more detailed analysis of the molecular biology of cloned vertebrate *TRP* cDNAs as well as the evolution of the *TRP* gene family is presented in Chapter 4.

1.5.1 The molecular genetics of the *TRP* gene family

Much of the molecular biology of the *TRP* gene family has come from studies on mammals, largely because until recently, only mammalian *TRP* genes had been cloned. Biochemical and genetic evidence (Gluecksohn-Waelsch, 1979; Müller *et al.*, 1988) have mapped the mouse *Tyrosinase* gene to the *Tyr* (previously called the *albino (c)*) locus. The *TYR* gene spans 60-100 kb of the mammalian genome and consists of 5 exons and 4 introns (Ruppert *et al.*, 1988; Giebel *et al.*, 1991). Northern blot analysis indicates that the processed *TYR* transcripts are between 2 and 2.5 kb, with alternative splicing generating several smaller-sized transcripts (Porter and Mintz, 1991; Kelsall *et al.*, 1997).

The mammalian (Ruppert *et al.*, 1988; Klüppel *et al.*, 1991; Ponnazhagan *et al.*, 1994) and avian (Yamamoto *et al.*, 1992; Ferguson and Kidson, 1996) *TYR* promoters have been characterised with several potential cis-acting elements present, including an 11 bp M box thought to bind the Microphthalmia-associated transcription factor (MITF) (Bentley *et al.*, 1994; Ganss *et al.*, 1994; Bertolotto *et al.*, 1996; Yasumoto *et al.*, 1997). MITF is required for melanocyte differentiation (Opdecamp *et al.*, 1997), function (Moore, 1995) and survival (Steingrímsson *et al.*, 1996). *TYR* locus-control regions (Montoliu *et al.*, 1996) and enhancers (Porter and Meyer, 1994) 12-15 kb upstream of the coding region have also been reported. Mammalian TYR possesses tyrosine hydroxylase, DOPA oxidase and DHI

oxidase catalytic activites (Körner and Pawelek, 1982; Tripathi *et al.*, 1992). In animals, including the chick, mutations at the *Tyr* locus are thought to result in albinism (Online Mendelian Inheritance in Animals, OMIA #000202) and in humans are associated with type I OCA (OMIM #203100) (Spritz, 1994b).

The *Tyrosinase-related protein-1* gene maps to the *Tyrp1* (previously called the *brown (b)*) locus and spans approximately 15-18 kb of the mammalian genome (Shibahara *et al.*, 1991; Jackson *et al.*, 1991; Sturm *et al.*, 1995; Box *et al.*, 1998). The *TYRP1* gene consists of 8 exons (including the first non-coding exon) and 7 introns. The primary transcript is between 2.1 and 3.3 kb (Shibahara *et al.*, 1986; Boissy *et al.*, 1996). In the mouse, there is evidence for at least one alternatively spliced *Tyrp1* transcript (Shibahara *et al.*, 1991). This alternative transcript yields a truncated Tyrp1 molecule, lacking the carboxy (C)-terminus transmembrane domain. This mouse Tyrp1 isoform appears to be distinct from a post-translationally truncated mouse and human TYRP1 that is secreted by melanomas (Xu *et al.*, 1997). It is unclear whether these truncated TYRP1s perform any physiological functions. There has also been a report of the use of an alternative TYRP1 translational frame to generate a novel human melanoma antigen (see section 4.2 and Wang *et al.*, 1996).

The *TYRP1* promoter, like the *TYR* promoter, contains an M-box and has also been shown to be transactivated by MITF (Yavuzer *et al.*, 1995; Bertolotto *et al.*, 1998). The enzymatic function of TYRP1 has been a subject of much controversy (Halaban and Moellmann, 1990; Winder *et al.*, 1993; Zhao *et al.*, 1994; Winder *et al.*, 1994), although it has now been shown that mouse Tyrp1 possesses DHICA oxidase activity (Jiménez-Cervantes *et al.*, 1994; Kobayashi *et al.*, 1994a, 1994b). In the mouse, mutations at the *Tyrp1* locus may result in a brown, rather than a black coat colour (Jackson *et al.*, 1990), while in humans *TYRP1* mutations have been associated with type 3 OCA (OMIM #203290) (Spritz, 1994b; Boissy *et al.*, 1996; Manga *et al.*, 1997).

The *Tyrosinase-related protein-2* gene maps to the *Tyrp2* (previously called the *slaty (slt)*) locus and spans approximately 45-55 kb of the mammalian genome (Budd and Jackson, 1995; Sturm *et al.*, 1995). The *TYRP2* gene consists of 8 exons and 7 introns. Mammalian transcript sizes vary from 2.3 to 4.5 kb (Bouchard *et al.*, 1994; Yokoyama *et al.*, 1994a). Although Yasumoto *et al.* (1997) reported that the M-box-containing human *TYRP2* promoter was not transactivated by human MITF, recent work by Bertolotto *et al.* (1998) seems to indicate that the mouse Microphthalmia (Mi) protein is indeed able to transactivate

the human *TYRP2* promoter. In mammals, TYRP2 possesses L-DOPAchrome tautomerase activity (Tsukamoto *et al.*, 1992; Yokoyama *et al.*, 1994a; Kroumpouzos *et al.*, 1994). L-DOPAchrome tautomerase is a distinct enzyme from the recently identified rat liver D-DOPAchrome tautomerase, that specifically binds the substrate, D-DOPAchrome, a stereoisomer of L-DOPAchrome (Odh *et al.*, 1993; Zhang *et al.*, 1995). Mutations at the mouse *Tyrp2* locus may result in a grey (slaty) rather than black coat colour (Budd and Jackson, 1995). Currently there is no known human disorder associated with the human *TYRP2* locus.

Apart from sharing homology at the nt and aa level the TRPs also share several key conserved motifs that are thought to be important for TRP functioning, including an epidermal growth factor (EGF)-like domain, two metal ion-binding domains and a C-terminal consensus sequence (CTCS).

The EGF-like domain consists of 12 aa residues containing three conserved cysteine residues [CXCX(5)GX(2)C]. Because of its cysteine-rich nature, Jackson *et al.* (1992) first proposed that the EGF-like repeat may, through the formation of secondary disulphide bridges, facilitate the formation of a multi-enzymatic TRP complex on the lumenal (inner) face of the melanosome. Pawelek (1991), Pawelek *et al.* (1991) and Orlow *et al.* (1994) have all biochemically demonstrated the presence of a 200 to > 700 kDa high molecular weight complex, consisting of multimers of at least Tyr, Tyrp1 and Tyrp2, in mouse melanocytes. It is thought that this complex may be required for efficient eumelanin synthesis (Chiu *et al.*, 1993).

The mouse *albino* (Shibahara *et al.*, 1990; Jackson and Bennett, 1990; Yokoyama *et al.*, 1990) and *brown* (Zdarsky *et al.*, 1990) phenotypes are as a result of point mutations in the Tyr and Tyrp1 EGF-like repeats, respectively. Spritz (1994b) and Spritz and Hearing (1994), in their list of about 60 published human *TYR* mutations, include two point mutations which occur in the human TYR EGF-like repeat. Both these alterations, when homozygous, cause a form of OCA. Although this is not direct evidence that the EGF-like repeat mediates the formation of a multi-enzymatic TRP complex, it does emphasise a key role for this element in regulating TRP enzymatic activity.

Two metal ion-binding domains termed "CuA" and "CuB" (Lerch, 1988), are present in all TRPs cloned to date. Homologous Cu^{2+}-binding regions have also been found in bacterial, fungal and plant Tyrs (van Gelder *et al.*, 1997) as well as in arthropod and mollusc

haemocyanins (Morrison *et al.*, 1994). The CuA and CuB domains each contain 3 conserved histidine residues which have now been demonstrated to bind Cu^{2+} in human (Spritz *et al.*, 1997) and mouse (Furumura *et al.*, 1998) TYRs. Murine Tyrp2 has been shown to bind Zn^{2+} and small amounts of Fe^{2+} (Solano *et al.*, 1996; Martinez-Esparza *et al.*, 1997; Furumura *et al.*, 1998). Although Martinez-Esparza *et al.* (1997) were able to show that mouse Tyrp1 contained mostly Fe^{2+} and Cu^{2+}, they were unable to reconstitute Tyrp1 enzymatic activity from the apoprotein using these latter two ions. Recently however, Furumura *et al.* (1998) reported that mouse Tyrp1 did not bind Cu^{2+}, Zn^{2+} or Fe^{2+}. Whichever metal ions are bound by TYRP1, it is likely that each TRP molecule chelates two metal atoms and in this way binds a single molecule of O_2 in keeping with the respective oxidising functions of the TRPs (Oetting and King, 1994; Solano *et al.*, 1996).

Oetting and King (1994) and Spritz and Hearing (1994) list 9 and 8 point mutations which occur in the human TYR CuA and CuB regions respectively. Two of the CuB mutations occur at different histidine residues. Again, these lesions, when homozygous, are responsible for a form of OCA (OMIM #203100) by diminishing or abolishing TYR activity. Although there have been no reported mutations at either the mouse Tyr or Tyrp1 CuA and CuB regions yet, a point mutation at the mouse Tyrp2 CuA region is responsible for the *slaty (slt)* phenotype (Jackson *et al.*, 1992). This mutation reduces DOPAchrome tautomerase activity by 10-30% (Jackson, 1994b). It is clear therefore that the two conserved TRP metal-binding regions are crucial for the respective catalytic functions of the TRP family members.

Apart from the conserved EGF-like and metal ion-binding domains, both vertebrate Tyr and Tyrp1 also contain a C-terminal consensus sequence (CTCS). This consensus sequence (EXXQPLL) is present at the C-termini of all vertebrate TYRs and TYRP1s, but is absent from TYRP2s and ascidian Tyr (Sato *et al.*, 1997; Caracciolo *et al.*, 1997). Orlow *et al.* (1993) first suggested that because both TYR and TYRP1 are type-1 membrane proteins, with short cytoplasmic C-terminal tails and long intralumenal glycosylated portions, that the intracellular sorting of these proteins may be dictated by cytoplasmic C-terminal sequences. Winder *et al.* (1993) then, on comparing the C-termini of mouse and human TYR and TYRP1, reported on the conservation of a di-leucine motif. These authors suggested that this repeat might play a role as a Golgi sorting signal. Peng *et al.* (1994) extended this di-leucine repeat to include adjacent aas, yielding the consensus sequence EXXQPLL (EXQPLL for chicken Tyr). This sequence was suggested to participate in directing Tyr and Tyrp1 to the melanosome. Further support for this latter suggestion comes from

Vijayasaradhi *et al.* (1995) who, using a combination of chimaeric proteins and C-terminal deletion mutants, were able to identify the CTCS as a sorting signal for both melanosomal as well as lysosomal proteins. These melanosomal molecules included mammalian TYR, TYRP1, Pmel 17 and p proteins. Moreover, Jimbow *et al.* (1997) reported a role for the C-terminal cytoplasmic sequences as a targeting signal for transporting TYRP1 from the trans Golgi network to stage I melanosomes. It appears that this CTCS is also present at the C-terminus of the human nmb protein (Weterman *et al.*, 1995). This latter protein, as reported earlier (see section 1.4), is thought to belong to a second family of proteins involved in the polymerisation of melanin.

Genetic evidence for an intracellular sorting signalling function for the C-terminal of Tyr comes from work by Beermann *et al.* (1995). In their analysis of the *platinum* (c^p) allele, these authors reported that a point mutation resulted in a truncated Tyr molecule, lacking part of the C-terminus, including the CTCS. These mutated Tyr molecules were then shown to be misrouted to the cell periphery and extracellular surface, rather than to melanosomes. This aberrant trafficking was suggested to be the basis for the pigment-diluting effects of the *platinum* allele. Taken together then, there is strong support for an intracellular trafficking signalling function for the CTCS in TYR and TYRP1.

1.6 General and specific aims of study

Breeders of birds have provided a wealth of information on the genetic loci known to influence the pigmentation of several varieties of the domestic fowl (Bowers, 1988; Smyth, 1990; Stevens, 1991b). However, very few of these genes have been cloned and characterised. When the current study was initiated in 1991, only a single chicken pigment cell-specific gene, termed MMP115, had been reported (Mochii *et al.*, 1988a). In contrast, there has been substantial progress in identifying and cloning genes involved in the regulation of mammalian pigmentation (Spritz and Hearing, 1994; Barsh, 1996; Jackson, 1997). Probably the main reason for the lack of progress in cloning avian pigment cell-specific genes is the lack of suitable cell lines. Most mammalian genes have been obtained from cDNA libraries generated from melanoma cells or immortalised melanocytes, and as far as is known, there are no equivalent avian melanoma lines. Therefore in order to search for avian pigment cell-specific genes, bulk cultures of pigmented cells must be generated. Initial attempts to produce bulk, long-term cultures of pigmented chicken melanocytes were thwarted by poor proliferation and short survival times, along with contamination by undesirable cells. It is mainly for these reasons, that there have been so few reports on the

successful generation of bulk chicken melanocyte cultures (Giss *et al.*, 1982; Bowers and Gatlin, 1985; Boissy and Halaban, 1985).

Because the primary interest of this study is the genes involved in melanocyte differentiation, and more specifically in the avian homologues of the mammalian *TRP* gene family, an experimental approach was adopted to generate a cDNA library that would be representative of genes expressed within NC-derived melanocytes. The cloning of the chicken *TRP* gene family will contribute towards the understanding of the developmental biology, genetics, biochemistry and evolution of vertebrate melanogenesis by:

i) providing additional gene-specific markers of chicken melanocyte differentiation;
ii) allowing the genetic mapping of chicken pigmentary mutant loci;
iii) allowing the detailed dissection of the enzymatic control of chicken melanin biosynthesis; and
iv) shedding light on the possible mechanisms involved in the molecular evolution of gene families.

Therefore the **specific aims** of this study were:

i) to establish culture conditions supportive of chicken neural crest cell survival;
ii) to induce the differentiation of neural crest cells into large-scale proliferative, pigmented chicken melanocytes;
iii) to construct an embryonic chicken melanocyte cDNA library using mRNA purified from the pigmented chicken melanocyte cultures in ii);
iv) to screen the library constructed in iii) for full-length cDNAs encoding chicken *TRPs*;
v) to compare the nucleotide and deduced amino acid sequences of the chicken *TRP* gene family to other reported *TRP* gene family members; and
vi) to determine the expression patterns and genome complexities of the chicken *TRP* gene family.

CHAPTER 2: MATERIALS AND METHODS

2.1 Chicken neural crest cell and melanocyte cultures

In order to generate large numbers of neural crest-derived pigmented melanocytes in culture, two different approaches were attempted. In both approaches, fertile eggs of the Black Australorp X New Hampshire Red domestic fowl *(Gallus gallus)* breed were incubated at 37°C in a humidified (50-60%), rotating incubator.

The three breeds used in the present study have the following pigmentary genotypes (see section 1.3):

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

ii) Black Australorp X New Hampshire Red: *E/*e^{Wh} i^+/i^+ C^+/C^+ pk^+/pk^+. This cross breed has a predominantly black plumage, pigmented eyes and a small white throat patch.

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

2.1.1 Chicken melanocyte cultures using Buffalo Rat liver cell-conditioned medium

In the first approach, a method described by Giss *et al.* (1982) was adopted with slight modifications. This method used conditioned medium from Buffalo Rat liver (BRL-3A) cells in order to stimulate the proliferation of embryonic chicken melanocytes. Eggs were incubated for 72 hr, after which embryos were staged according to Hamburger and Hamilton (1951). The trunk regions (caudal to the heart), of stage 14-19 embryos were dissected to yield the neural tube (NT), notochord and accompanying somite pairs. These truncal segments were then cut into 2-3 mm^3 pieces, rinsed in Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate buffered saline (pH 7.4, DPBS) and incubated in 0.02% EDTA (pH 8.0) in DPBS for 20 min at room temperature. The EDTA solution was aspirated and the trunk pieces were further incubated in 0.25% trypsin in DPBS for 6 min at room temperature, with gentle agitation after 5 min. The trypsin solution was aspirated and replaced with Ham's F-12 medium containing 10% heat-inactivated foetal calf serum (FCS) (basic medium). Single cell suspensions were obtained by gentle trituration. Cells were counted using a haemocytometer and seeded in basic medium.

BRL-3A cells were obtained from ATCC (CRL 1442) and maintained in basic medium as described above. One day before chicken embryonic cell cultures were initiated, BRL-3A cells were plated at a density of 3.84×10^5 cells per 10 cm culture dish. BRL-3A-

conditioned medium was prepared and embryonic chicken neural crest cells (NCCs) were cultured according to the eight-day schedule outlined in Giss *et al.* (1982) (see section 3.1.1).

2.1.2 Chicken melanocyte cultures from neural tube explants

In the second approach, the method for culture of NCCs, described by Boissy and Halaban (1985), was adopted with slight modifications. This method used the phorbol ester, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) in order to stimulate the proliferation of embryonic chicken melanocytes in culture. Embryos corresponding to stages 14-15 (19-22 somite pairs) were dissected to yield the NT, notochord and somite pairs. Truncal segments were incubated in 0.05% collagenase in DPBS for 13 min at room temperature. Digested segments were then dissected free of the notochord, surrounding somites and mesenchyme using dissecting pins. Neural tubes were plated in basic medium as described previously, to allow NCC migration onto the dish surfaces, whereafter the NTs were removed using tungsten needles. Cells were maintained in a 37^{o}C incubator (95.5% air/5.5% CO_2, 65% humidity) and given fresh basic medium every third day. NCC cultures were treated with various growth factors as described in section 3.1.2.

2.2 RNA extraction and poly (A)$^+$ purification from cultured chicken melanocytes

To obtain the starting material for construction of a chicken melanocyte cDNA library, total RNA was extracted from five 75 cm^2 culture flasks containing three-month old primary chicken melanocyte cultures, according to the guanidine/caesium chloride method (Sambrook *et al.*, 1989). Poly (A)$^+$-enriched RNA was purified from total RNA using oligo dT-cellulose affinity chromatography (Aviv and Leder, 1972; Sambrook *et al.*, 1989).

2.3 Chicken melanocyte cDNA library construction

A cDNA library was constructed in Lambda Uni-ZAP XR (Stratagene) (Fig. 2.1) according to the manufacturer's instructions using 10 µg of poly (A)$^+$ RNA purified from chicken melanocytes. The initial stages of the library construction were carried out with the kind assistance of Dr Nicci Illing, Department of Chemical Pathology, University of Cape Town, South Africa. Briefly, the first cDNA strand was synthesised using M-MuLV reverse transcriptase and an oligo-dT linker/primer (5' AGAGAGAGAGAGAGAGAGAACTAGTCTC GAGTTTTTTTTTTTTTTTTT 3') containing an internal *Xho*I restriction enzyme site. The first strand reaction mix included 5-methyl-dCTP, thus protecting hemi-methylated cDNA from subsequent restriction enzyme (*Xho*I) digestion. Second cDNA strand synthesis was generated using *RNAase* H and *E. coli* DNA Polymerase I.

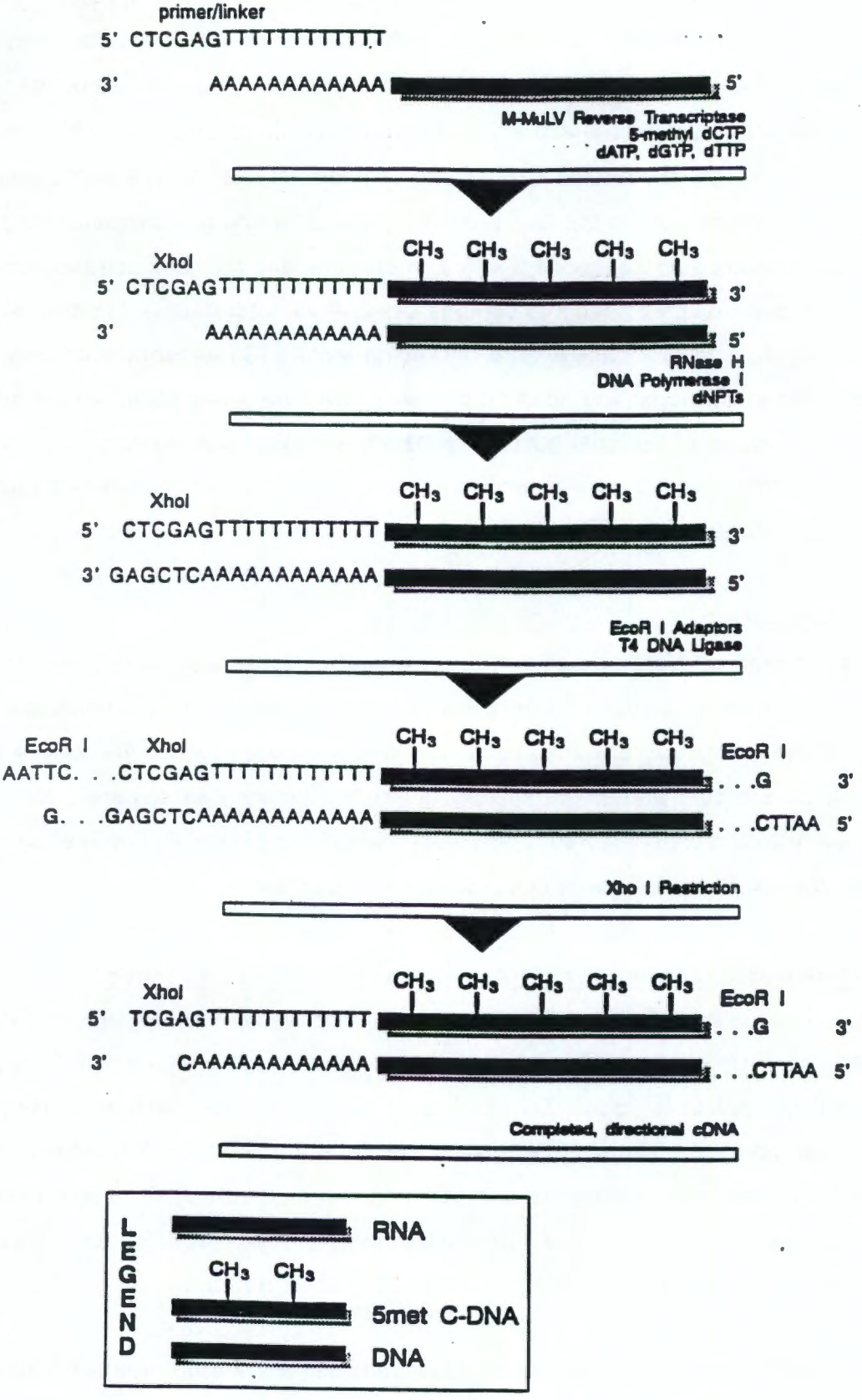

Fig. 2.1. Flow diagram showing the main steps involved in Lambda ZAP XR cDNA library construction (from Stratagene).

Complementary DNA termini were blunt-ended using T4 DNA polymerase and then ligated to *Eco*RI adaptors (kinased 9-mer: 3' GCCGTGCTC 5' and dephosphorylated 13-mer: 5' AATTCGGCACGAG 3') using T4 DNA ligase. After phosphorylating the *Eco*RI ends using T4 polynucleotide kinase, tailored cDNAs were digested using *Xho*I, thus generating cDNAs containing an *Eco*RI site at the 5' end and a *Xho*I site at the 3' (poly $(A)^+$) end. This allowed the completed cDNA to be inserted into Lambda Uni-ZAP XR in a sense orientation (*Eco*RI→*Xho*I) with respect to the *lacZ* promoter (Figs. 2.2a and b). Complementary DNAs were size-fractionated on Sephacryl S-400 spin columns and fractions corresponding to > 500 bp were directionally cloned into Lambda Uni-ZAP XR vector arms. Ligation reactions were packaged using Gigapack II Gold packaging extract (Stratagene) according to the manufacturer's instructions. The resulting primary cDNA library was plated and titred using the SURE (Stratagene) bacterial host. To generate a large stable quantity of a high titre library stock, the primary library was amplified (according to the manufacturer's instructions) and aliquots were stored at 4°C and -80°C.

2.4 Chicken melanocyte cDNA library screening

To isolate chicken cDNA equivalents of the mammalian *Tyrosinase-related protein (TRP)* gene family, the library constructed here, was screened in two independent rounds. In the first round, the library was screened for a chicken *Tyrosinase (Tyr)* cDNA using a mouse *Tyr* cDNA as a probe, and in the second round, the library was screened for chicken *Tyrosinase-related protein-1 (Tyrp1)* and *Tyrosinase-related protein-2 (Tyrp2)* cDNAs using a generic chicken *TRP*-PCR-generated product (details below).

2.4.1 Screening for chicken *Tyr* cDNAs using a mouse *Tyr* cDNA probe

The first screening round was performed in the Department of Anatomy and Cell Biology, Medical School, University of Cape Town, South Africa. Approximately $8x10^5$ pfus were transferred onto Nylon N^+ discs (Du Pont) according to the manufacturer's instructions. Prewashings, prehybridisations (10% dextran sulphate; 4 x SSC, pH 7.0; 20mM Tris, pH 7.4; 1 x Denhardts; 40% formamide and 100 µg/ml denatured salmon sperm DNA) and hybridisations were performed in a hybridisation oven (Hybaid) at 42°C for 3 and 16 hr respectively, according to standard procedures (Davis *et al.*, 1986).

The hybridisation solution included a nick-translated (Sambrook *et al.*, 1989) 1.9 kb mouse *Tyr* cDNA probe. The mouse *Tyr* cDNA (pmcTyr1) was a kind gift from Dr Günther Schütz, German Cancer Research Centre, Heidelberg, Germany. The 1.9 kb fragment was released from pmcTyr1 using *Eco*RI and corresponded to the full-length mouse *Tyr* cDNA

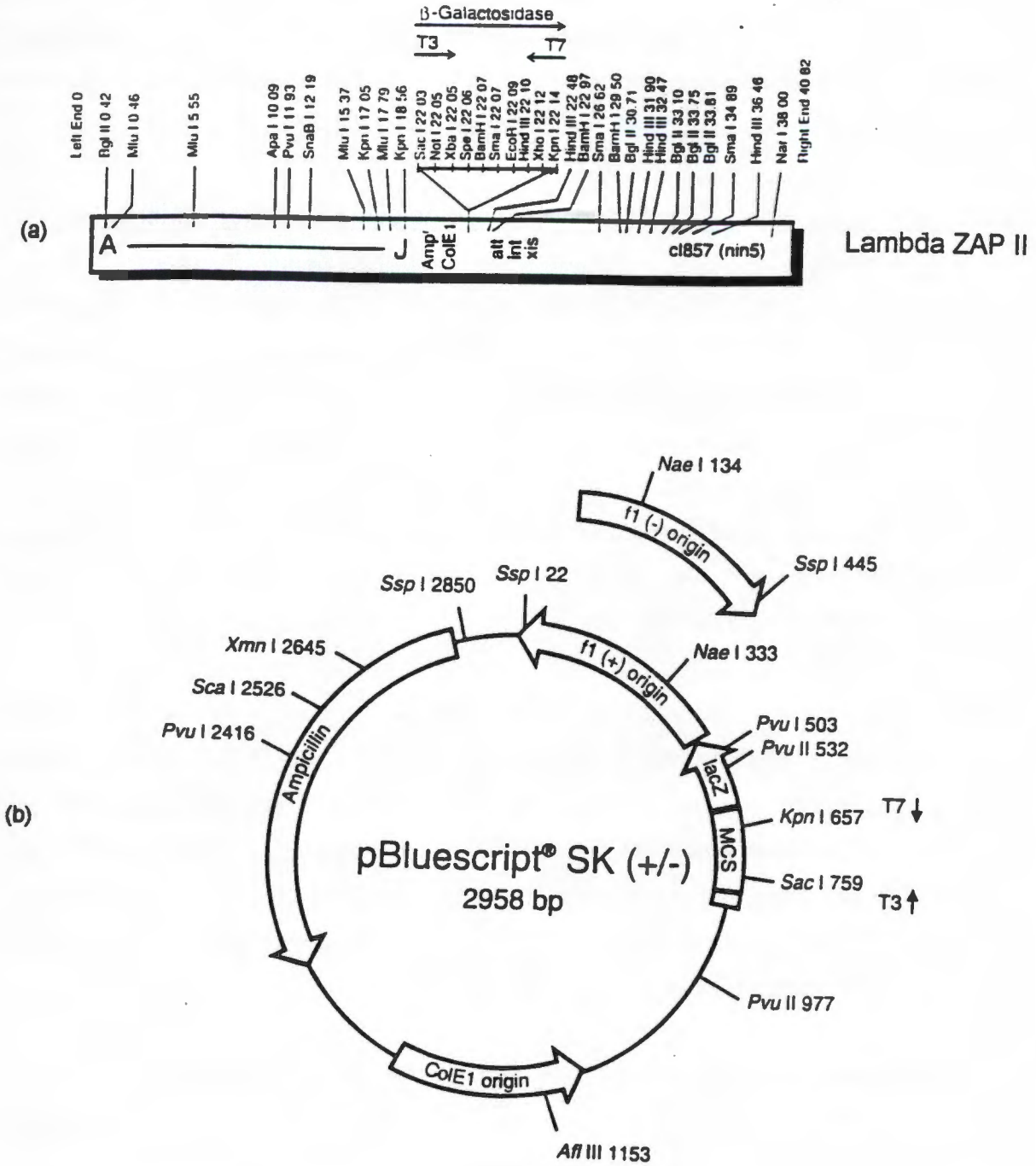

Fig. 2.2. Restriction maps of the Lambda Uni-ZAP XR phage (a) and excised pBluescript SK⁻ phagemid (b) vectors used in the construction of a chicken melanocyte cDNA library (from Stratagene).

(Müller *et al.*, 1988). The filters were first washed under low stringency (2 x SSC; 0.1% SDS; 4 x 5 min at room temperature) and then under moderate stringency conditions (1 x SSC; 0.1% SDS; 2 x 30 min at 55°C). Autoradiography was performed using x-ray film at -80°C for two days. Secondary and tertiary screenings were performed as for the primary screening, except phage were plated more sparsely to yield single isolates. Single positive isolates were cored and stored at 4°C until *in vivo* excision (section 2.5).

2.4.2 Screening for chicken *Tyrp1* and *Tyrp2* cDNAs using a chicken generic *TRP* PCR product

The second screening round was performed in the laboratory of Dr Ian Jackson at the MRC Human Genetics Unit, Western General Hospital, Edinburgh, United Kingdom. Approximately 1.2 x 10^6 pfus were transferred onto duplicate Nylon N^+ (Amersham) sheets (20 x 20 cm) according to the manufacturer's instructions. Prewashings, prehybridisations (10% dextran sulphate; 6 x SSC, pH 7.0; 5 x Denhardts; 0.1% sodium pyrophosphate; 0.5% SDS and 100 µg/ml denatured salmon sperm DNA) and hybridisations were performed in a hybridisation oven (Hybaid) at 65°C for 2 and 16 hr respectively, according to standard procedures (Davis *et al.*, 1986).

The hybridisation solution included a random primed 720 bp PCR-generated chicken *TRP* product (see below for a description of this probe). The 720 bp *TRP* PCR product was random-primed labelled in low-melting point agarose to high specific activity with [α-^{32}P]dCTP. Duplicate filters were first washed under low stringency (2 x SSC; 0.1% SDS; 3 x 20 min at 50°C) and then under moderate stringency conditions (1 x SSC; 0.1% SDS; 2 x 20 min at 55°C). Autoradiography, subsequent screenings and plaque isolations were performed as described earlier (see section 2.4.1).

The PCR-generated chicken generic *TRP* probe used for this round of cDNA library screening was prepared as follows: The polymerase chain reaction was used to generate partial chicken *TRP* cDNA fragments. Degenerate primers corresponding to homologous regions of all three members of the vertebrate *TRP* gene family were the kind gift of Dr Ian Jackson, MRC Human Genetics Unit, Edinburgh, United Kingdom. Because the spacing of these homologous regions within the individual *TRP* members of the vertebrate gene family is highly conserved (Jackson *et al.*, 1994; Mason and Mason, 1995), simultaneous amplification of *TRP* mRNAs/cDNAs will result in a "generic" PCR product. Although the design of these primers was initially based on mammalian *TRP* cDNA nucleotide (nt)

sequence information (Jackson *et al.*, 1994), the primers have been used successfully to amplify *TRP* members from other vertebrates (Mason and Mason, 1995).

The four degenerate primers used were 79M, 80M, 81M and 82M (Jackson *et al.*, 1994) (Table 2.1). Forward primer 79M corresponds to amino acids (aas) DDREXWP, forward 81M and reverse 82M primers correspond to aas FVWXHYY, with reverse primer 80M corresponding to aas APIGHNR of the vertebrate *TRP* gene family (Jackson *et al.*, 1994; Mason and Mason, 1995). PCR amplifications were performed in a Hybaid thermocycler using the Perkin Elmer Gene Amp Kit. Standard reaction conditions included 25 pmols each of the *TRP* primers and 5 µl chicken melanocyte cDNA library titre in a 50 µl reaction with PCR cycles of 94^{o}C for 3 min; 94^{o}C for 30 sec; 48^{o}C for 1 min; 72^{o}C for 2 min for 30 cycles.

2.4.3 Screening for chicken *Mi* cDNAs using a mouse *Mi* cDNA probe

In order to isolate a chicken *Microphthalmia (Mi)* cDNA, a mouse *Mi* cDNA probe was used to screen the chicken melanocyte cDNA library described here. A mouse *Mi* cDNA was a kind gift from Dr Colin Goding, Marie Curie Research Institute, Oxted, United Kingdom. To generate a probe for library screening, the mouse *Mi* cDNA (1.3 kb) insert was released from pBluescript SK^{-} using *Eco*RI. This cDNA fragment corresponds to the entire coding region of the mouse *Mi* gene (Yavuzer *et al.*, 1995). After random prime labelling with [α-^{32}P]dCTP, the mouse *Mi* probe was included in a moderate screening experiment (see section 2.4.2 for library screening conditions). Positively hybridising isolates were cored and stored at 4^{o}C until *in vivo* excision.

2.4.4 Screening for chicken *Mc1r* cDNAs using a human *MC1R* PCR probe

In an attempt to obtain a chicken orthologue of the mammalian *Melanocortin 1 Receptor (MC1R)* cDNA, a human *MC1R* PCR probe was used to screen the chicken melanocyte cDNA library constructed here. Human *MC1R* primers (amino-terminal 5' CAGCACCATGAAAGCAGGACACCTGG 3' and carboxy-terminal 5' TGCCCAGCACACTT AAAGCGCGTGCA 3') as well as a human *MC1R* PCR product (918) were the kind gifts of Dr Paloma Valverde, University of Newcastle upon Tyne, United Kingdom). The *MC1R* primers amplify the region corresponding to nts 420-1447 of the human *MC1R* gene (Mountjoy *et al.*, 1992). Using these *MC1R* primers, and an aliquot of the 918 PCR product as a DNA template, a 1007 bp *MC1R* product was generated. The PCR reactions were performed as described by Valverde *et al.* (1995). This 1 kb human *MC1R* product was random-primed labelled in low-melting point agarose to high specific activity with

Table 2.1. Primers used for chicken *TRP* cDNA sequencing

Primer	mer	Sequence
M13F	17	5' GTTTTCCCAGTCACGAC 3'
M13R	19	5' GGAAACAGCTATGACCATG 3'
TRP 79M	27	5' GCACTCGGAT/CGAT/CC/AGIGAA/GIINTGGCC 3'
TRP 80M	27	5' TAGGAGCCT/GG/ATTG/ATGICCIATNGGNGC3'
TRP 81M	28	5' GCTAGATCTTT/CGTITGGIIICAT/CTAT/CTA 3'
TRP 82M	28	5' TGGAGATCTAA/GTAA/GTGIIICCANACA/GAA 3'
TRP-1F1	22	5' GTGTGTGCAGGTGACAGTAGAC 3'
TRP-1F2	22	5' TGTGAATGCTCTTCACCAAGCC 3'
TRP-1F3	22	5' AATGGAACAGGAGGGCAAACAC 3'
TRP-2F1	23	5' GCTGAACCTCCATAATTTGGCTC 3'
TRP-2F3	22	5' CTGCTTGTACTTTTCCAGCACC 3'
TRP-2F4	23	5' GACTACCATCCACCCAGACTATG 3'
TRP-2R1	22	5' GCACCAACAGCTCTGGTTGAAG 3'
TRP-2R2	22	5' AATAGGGATGGACAAAGCCACC 3'
TRP-2R3	22	5' GTGATAGGATAAGGGAGAATGC 3'
N = all four bases; I = inosine		

[α-^{32}P]dCTP and used to screen the library described here (see section 2.4.2 for library screening conditions).

2.4.5 Screening for members of the chicken *Pmel 17* gene family using degenerate *Pmel 17* primers

In an attempt to generate a "generic" *Pmel 17* PCR probe for library screening, a strategy similar to that described in section 2.4.2 was employed here. By comparing the mouse (Kwon *et al.*, 1994) and human (Kwon *et al.*, 1991) *Pmel 17* as well as the chicken *MMP115* (Mochii *et al.*, 1991) cDNA nt sequences, degenerate primers were designed to simultaneously amplify all members of the *Pmel 17* gene family (Dr Ian Jackson, MRC Human Genetics Unit, Edinburgh, United Kingdom). As for the vertebrate *TRP* gene family, the spacing of homologous regions within the individual members of the mammalian *Pmel 17* gene family is highly conserved (Kwon *et al.*, 1994). Simultaneous PCR amplification of *Pmel 17* mRNAs/cDNAs will result in a "generic" *Pmel 17* PCR product. The degenerate *Pmel 17* primers used were MMP-1 (5' GAC/TTGC/TTGGC/AGNGGNGGATCCAGA 3'), MMP-2 (5' TTC/TGTNTAC/TGTNTGGATCCAAGCT 3'), MMP-3 (5' CCANACA/GTANAC G/AAAGCTTAGATC 3') and MMP-4 (5' A/GTCNCCG/AAAA/GTCCCTCTAGAGATC 3'). Forward primer MMP-1 corresponds to aas DCWRGG, forward MMP-2 and reverse MMP-3 primers correspond to aas FVYVW, with reverse primer MMP-4 corresponding to aas WDFGDS of the mammalian *Pmel 17* gene family (Mochii *et al.*, 1991; Kwon *et al.*, 1994). Degenerate PCR reactions were performed as described earlier (see section 2.4.2).

2.5 *In vivo* excision

To facilitate easier manipulation, cDNA inserts from Lambda ZAP II were "subcloned" into pBluescript SK$^-$ by *in vivo* excision, using ExAssist helper phage (Stratagene) and XL1-Blue cells, according to the manufacturer's instructions (Figs. 2.3a and b). SOLR colonies containing double-stranded pBluescript SK$^-$ phagemids were rescued after selection on bacterial plates containing ampicillin (50 µg/ml).

2.6 Plasmid Southern blot hybridisation analysis

To further analyse potential chicken *TRP* and *Mi* cDNAs, excised phagemid DNAs were probed with mouse *TRP* and *Mi* cDNAs. Phagemid DNA was prepared (Sambrook *et al.*, 1989) and digested with *Eco*RI and *Xho*I to yield cloned cDNA inserts. The DNA was separated on 1% agarose gels, capillary transferred in 20 x SSC onto Nylon N$^+$ (Amersham) membranes and hybridised to radiolabelled cDNA probes. Separate hybridisation experiments using the following cDNA probes were carried out:

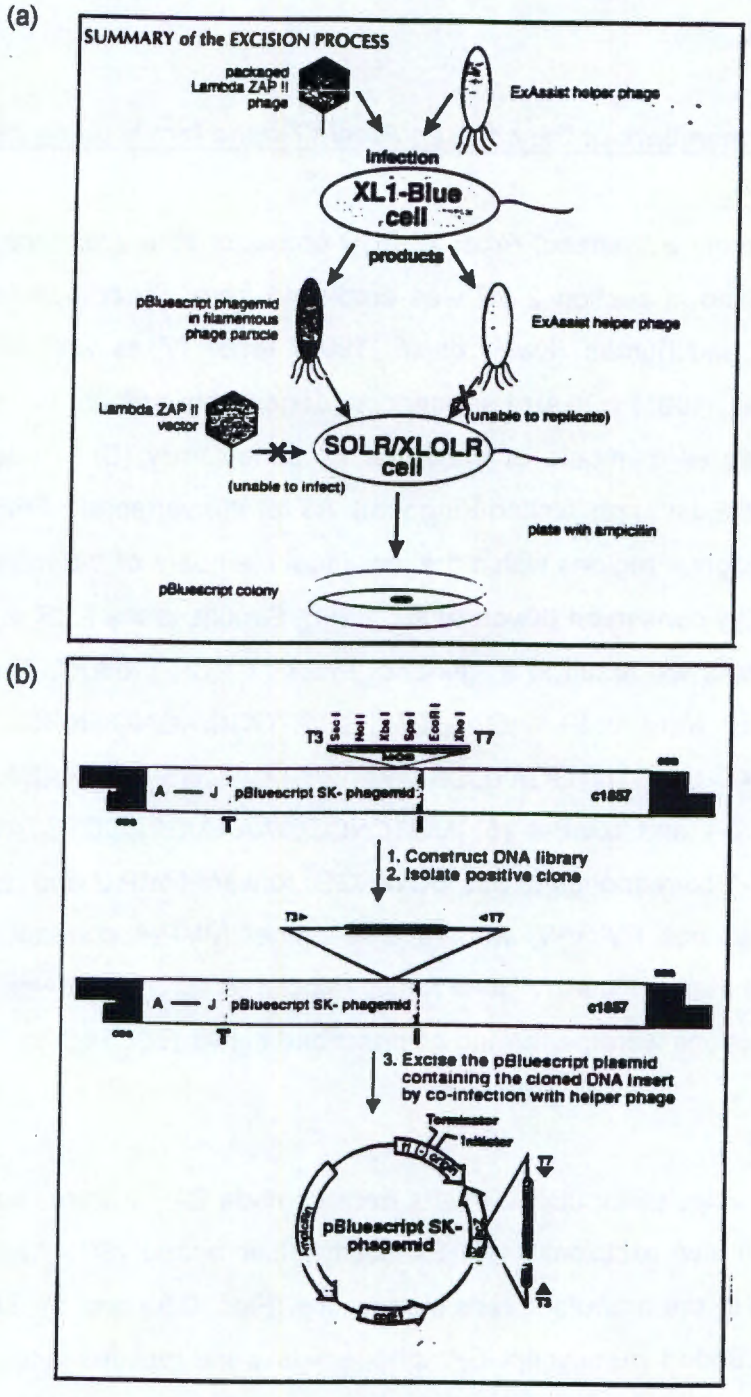

Fig. 2.3. Outline of the *in vivo* excision of pBluescript SK⁻ from Lambda ZAP II. (a) After screening, positively hybridising lambda pfus are isolated and allowed to infect XL1-Blue cells, which are co-infected with ExAssist filamentous helper phage. (b) Inside the cell, trans-acting proteins from the helper phage recognise two separate domains (Initiator and Terminator) positioned within the Lambda ZAP II vector arms. Both of these signals are recognised by the helper phage gene II protein and a new DNA strand is synthesised, displacing the existing strand. The displaced strand is circularised and packaged as a filamentous phage by the helper phage proteins and then secreted from the cell. pBluescript SK⁻ phagemids are recovered by infecting an F' strain (SOLR) and by plating on ampicillin plates, giving colonies (from Stratagene).

i) A mouse *Tyr* cDNA probe (pmcTyr1) has been described earlier (see section 2.4.1).

ii) A mouse *Tyrp1* cDNA (pMT4) was a kind gift from Dr Shigeki Shibahara, Tohoku University School of Medicine, Miyagi, Japan. A 1.6 kb *Hind*III cDNA fragment from pMT4 was prepared for use as a probe. This cDNA fragment corresponds to nts 619-2186 of the mouse *Tyrp1* cDNA sequence (Shibahara *et al.*, 1986).

iii) A mouse *Tyrp2* cDNA (pTRP2A) was a kind gift from Dr Ian Jackson, MRC Human Genetics Unit, Edinburgh, United Kingdom. A 1.75 kb *Eco*RI cDNA fragment from pTRP2A was prepared for use as a probe. This cDNA fragment corresponds to nts 452-2182 of the mouse *Tyrp2* cDNA sequence (Jackson *et al.*, 1992).

iv) A mouse *Mi* cDNA probe has been described earlier (see section 2.4.3).

These cDNA probes were all random-primed labelled in low-melting point agarose to high specific activity with [α-^{32}P]dCTP. Prehybridisations, hybridisations, stringency washes and autoradiography were performed as for library screening (see section 2.4.1).

2.7 Sequencing strategies for the chicken *TRP* gene family

In order to sequence the isolated cDNAs, two different strategies were adopted. In the first, the chicken *Tyr* cDNA clone was restriction mapped and four fragments subcloned. The subclones were then sequenced using universal vector primers. In contrast, the chicken *Tyrp1* and *Tyrp2* cDNAs were directly sequenced using a combination of vector, degenerate *TRP* and custom-designed internal primers. Sequencing strategies and primers are described in Fig. 2.4 and Table 2.1.

2.7.1 Sequencing of the chicken *Tyr* cDNA

Clone B8.3, containing the 1.9 kb chicken *Tyr* cDNA, was restriction mapped in order to generate fragments for subcloning and subsequent sequencing. Four restriction fragments, *Eco*RI-*Xho*I (600 bp), *Xho*I-*Bam*HI (430 bp), *Bam*HI-*Pvu*II (450 bp) and *Pvu*II-*Xho*I (450 bp), spanning the entire 1.9 kb chicken *Tyr* cDNA (Fig. 2.4a), were blunt-end subcloned (Sambrook *et al.*, 1989) into pUC19. These subclones were sequenced at least twice in both directions, using the forward (F) and reverse (R) M13 universal primers (Table 2.1).

2.7.2 Sequencing of the chicken *Tyrp1* and *Tyrp2* cDNAs

For both pcTRP-1.6 (containing a 1.7 kb chicken *Tyrp1* cDNA) and pcTRP-2.10 (containing a 2.9 kb chicken *Tyrp2* cDNA), F and R universal primers were used on the pBluescript SK$^-$-containing inserts, in order to generate DNA sequences from both the 5' and 3' termini of the chicken *Tyrp1* and chicken *Tyrp2* cDNAs. Next, four degenerate *TRP* primers (see

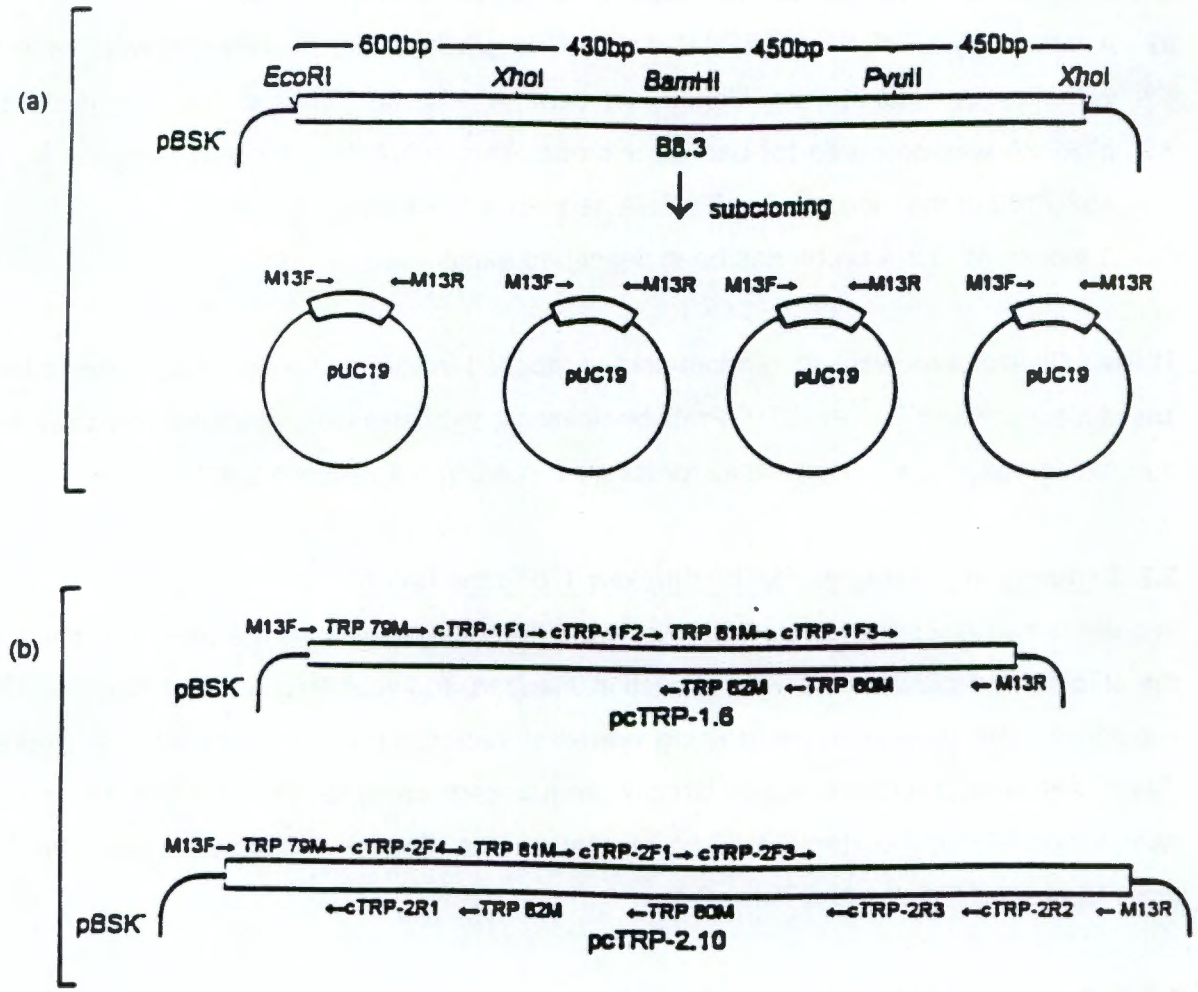

Fig. 2.4. Strategies adopted for sequencing of chicken *TRP* cDNAs. (a) The 1.9 kb cDNA insert carried by clone B8.3 was restriction mapped and subcloned in pUC19. Four subclones (400-600 bp) were generated and were sequenced using universal vector primers. (b) The 1.7 kb and 2.9 kb cDNA inserts carried by clones pcTRP-1.6 and pcTRP-2.10 respectively, were sequenced directly in pBluescript SK⁻ (pBSK) using a combination of universal vector, degenerate *TRP* and custom-designed internal primers. Arrows indicate the direction of primed sequencing.

Table 2.1, section 2.4.2 and Jackson *et al.*, 1994) were used to prime internal chicken *Tyrp1* and chicken *Tyrp2* sites. Finally, the gaps remaining after 5', 3' and internal sequencing, were "filled in" using custom-designed chicken *Tyrp1* and *Tyrp2* primers. These primers were designed using the prime function of Genetics Computer Group (GCG) program, version 9.0 (GCG sequence analysis software package, GCG, Inc., Madison, WI, USA) and were synthesised on a Beckman oligo 1000 DNA synthesizer by Prof Iqbal Parker, Department of Medical Biochemistry, University of Cape Town, South Africa. Each sequencing reaction was performed at least twice.

2.8 DNA sequencing and deduced protein analysis

All cDNAs were sequenced directly using the dideoxy chain termination method (Sanger *et al.*, 1977) using a Sequenase Kit (USB). Plasmid/phagemid DNA (5 µg) was extracted using alkaline lysis (Davis *et al.*, 1986), then alkali-denatured and radio-labelled using [^{35}S]dATP. Sequenced products were resolved on 6% polyacrylamide/8M urea gels electrophoresed on a Hoeffer Poker Face II sequencing apparatus. Gels were vacuum-dried at 80^{o}C for 1-1.5hrs and autoradiography was performed using x-ray film at room temperature for 2-3 days. DNA and deduced amino acid sequence analyses were performed using the Genetics Computer Group (GCG) program, version 9.0 (GCG sequence analysis software package, GCG, Inc., Madison, WI, USA), GenBank and EMBL databases. The Swiss-Prot and Prosite databases were used to identify protein patterns and motifs in the deduced protein sequences.

2.9 Northern blot hybridisation analysis

All tissues and cell cultures were from Black Australorp chicken embryos. The retinal pigment epithelium (RPE) was dissociated from seven day old chicken embryos and RPE cells were maintained in culture for 10-30 days. These RPE cultures were kindly generated by Mrs Toni Wiggins, Department of Anatomy and Cell Biology, University of Cape Town, South Africa, according to a protocol from Eguchi and Okada (1973) and Adler *et al.* (1982).

Poly (A)$^{+}$ RNA was isolated (Sambrook *et al.*, 1989) from these pigmented cultures and total RNAs were extracted (Chomczynski and Sacchi, 1987; Sambrook *et al.*, 1989) from the dissected eyes, skins, brains and hearts of ten day old chicken embryos. These RNAs were separated on denaturing 1.1% agarose/2.2 M formaldehyde agarose gels, capillary-transferred in 20 x SSC onto nitrocellulose membranes (Amersham) and hybridised to the following [α-^{32}P]dCTP random-primed labelled chicken *TRP* cDNA probes:

i) A 1.5 kb *Eco*RI-*Pst*I fragment from B8.3. This cDNA fragment corresponds to nts 1-1542 (Fig. 3.10) and therefore contains most of the chicken *Tyr* coding region.

ii) A 1.7 kb *Ava*I fragment from pcTRP-1.6. This cDNA fragment corresponds to the entire chicken *Tyrp1* coding region, including the 5' and 3' *UTRs* (Fig. 3.12).

iii) A 1.3 kb *Bam*HI fragment from pcTRP-2.10. This cDNA fragment corresponds to nts 330-1608 (Fig. 3.14) and therefore contains most of the chicken *Tyrp2* coding region.

Prehybridisations and hybridisations (10% dextran sulphate; 4 x SSC, pH 7.0; 20mM Tris, pH 7.4; 5 x Denhardt's reagent; 40% formamide and 100 µg/ml denatured salmon sperm DNA) were performed in a hybridisation oven (Hybaid) at 42^{o}C for 16 and 24 hr respectively. Membranes were first washed under low stringency (2 x SSC; 0.1% SDS; 2 x 20 min at 42^{o}C) and then under high stringency conditions (0.1 x SSC; 0.1% SDS; 2 x 20 min at 65^{o}C). Autoradiography was performed using x-ray film at -80^{o}C for 10 days.

2.10 Genomic Southern blot hybridisation analysis

As a preliminary analysis of the chicken *Tyr*, *Tyrp1* and *Tyrp2* loci, genomic Southern blot hybridisation analyses were performed. Genomic DNA was prepared (Strauss, 1987) from Black Australorp and White Plymouth Rock X Pile Game embryos. The DNAs were digested with various enzymes as described in section 3.6 and separated on 0.7% agarose gels. The chicken *TRP* cDNA probes used have been described earlier in section 2.9. Blotting, prehybridisations, hybridisations, stringency washes and autoradiography were performed as for northern blot hybridisation analysis.

CHAPTER 3: RESULTS

In order to address the specific aims of this study as set out in section 1.6, the major thrust of this investigation proceeded through three main stages. The first stage required the establishment of culture conditions conducive to the generation of large numbers of pigmented chicken melanocytes. The second stage involved the construction and screening of a chicken melanocyte cDNA library in order to isolate cDNAs uniquely expressed in pigment cells. The final stage of this project focused on the comprehensive characterisation of the three members of the chicken *Tyrosinase-related protein (TRP)* gene family.

3.1 Chicken neural crest cell and melanocyte cultures

In order to construct a cDNA library that would allow the cloning of chicken pigment cell-specific cDNAs, the first step of this study required the successful cultivation of large numbers of pigmented chicken melanocytes. Two different approaches were attempted to accomplish this first goal.

In the first approach, single cell suspensions from 72 hr old embryonic chicken trunk regions, containing mixed populations of cells from the neural tube, notochord and accompanying somite pairs, were cultured in conditioned medium prepared from Buffalo Rat liver cells (BRL-3A) (Giss *et al.*, 1982). BRL-3A cells produce mast cell growth factor (Zsebo *et al.*, 1990), which has been shown to promote the survival of neural crest cells (NCCs) as well as promote the rate of melanogenic differentiation in the chicken (Lahav *et al.*, 1994) and mouse (Morrison-Graham and Weston, 1993). At the same time, contaminating, non-NCCs die off.

In the second approach, neural tubes from 48 hr old chicken embryos were explanted in culture dishes, allowing the migration of NCCs. These cells were grown in the presence of the tumour promoter, 12-*O*-tetradecanoyl-13-phorbol acetate (TPA), and basic fibroblast growth factor (bFGF). TPA and bFGF have been used successfully as mitogens for avian (Boissy and Halaban, 1985; Stocker *et al.*, 1991), mouse (Sviderskaya *et al.*, 1995; Hirobe, 1992) and human (Eisinger and Marko, 1982; Halaban *et al.*, 1987) melanocytes.

3.1.1 Buffalo Rat liver cell-conditioned medium fails to stimulate the bulk culture of chicken melanocytes

To establish whether the conditions for growth in BRL-3A cell-conditioned medium (BRL-3ACM), as previously described by Giss *et al.* (1982) and Oetting *et al.* (1985a), could be

further optimised, the effects of different BRL-3ACM concentrations on the behaviour of cultured chicken melanocytes were assessed in the current study. Following the protocol of Giss *et al.* (1982), BRL-3ACM was diluted in Ham's F-12 medium containing 20% heat-inactivated foetal calf serum (FCS) (basic medium) as follows:

i) basic medium only;
ii) one part BRL-3ACM + three parts basic medium = 25% BRL-3ACM;
iii) two parts BRL-3ACM + three parts basic medium = 40% BRL-3ACM;
iv) three parts BRL-3ACM + one part basic medium = 75% BRL-3ACM

Cell cultures were fed with fresh BRL-3ACM every second day and the effects of different BRL-3ACM concentrations on four parameters (onset of melanogenesis, percentage melanised cells, melanocyte proliferation and survival) were investigated. The results are presented in Table 3.1.

Cultures grown in basic medium alone initially contained cells that were small and stellate (Fig. 3.1a), but within three to four days melanised cells were also seen (Fig. 3.1b). These cells represented less than a quarter of the total number of NCCs (Fig. 3.1c), eventually became larger and more flattened and persisted for approximately 10 days in culture before dying.

When embryonic trunk single cell suspensions were cultured in 25% BRL-3ACM, stellate, pigmented melanocytes were visible after three to four days in culture. After about eight to ten days in culture, pigmented clumps of melanocytes were observed. These clusters represented approximately half to two thirds of the total number of cells present, with large, single, flattened, unpigmented cells also present. Both the pigmented clusters and unpigmented cells however, failed to survive for longer than 19 days in culture (Table 3.1).

Cultures grown in more concentrated conditioned medium (40% BRL-3ACM) yielded visibly pigmented clumps of melanocytes after six days, thus delaying the onset of melanogenesis by two to three days by comparison with control and 25% BRL-3ACM cultures (Table 3.1). These pigmented clusters also contributed approximately 50-60% of the total cell population, with unpigmented cells present. These clusters survived for a similar period (18 days) to that of the 25% BRL-3ACM-treated cultures.

Cultures grown in 75% BRL-3ACM also exhibited delayed onset of melanogenesis when compared to control and 25% BRL-3ACM cultures (Table 3.1). The pigmented clusters

Table 3.1. Effect of BRL-3A conditioned medium on chicken melanocyte survival and proliferation

Medium	Onset of melanogenesis (days)	Percentage melanised cells (%)	Melanocyte proliferation	Survival time (days)	*n*
Ham's F-12 + 20% FCS	3-4	5-10	-	10-14	9
25% BRL-3A + 75% Ham's F-12 + 20% FCS	3-4	50-60	+	19	10
40% BRL-3A + 60% Ham's F-12 + 20% FCS	6	50-60	+	18	5
75% BRL-3A + 25% Ham's F-12 + 20 % FCS	6	70-80	+	25	22
+ visible increase; - no increase; *n* number of culture dishes					

Table 3.2. Effect of TPA and bFGF on chicken melanocyte differentiation and proliferation

Medium	NCC proliferation	Onset of melanogenesis (days)	Percentage melanised cells (%)	Melanocyte proliferation	Survival time (days)	*n*
Ham's F-12 + 20% FCS	+	3-4	30	-	10	26
Ham's F-12 + 20% FCS + 32 nM TPA	+++	7-8	25	+++	16	14
Ham's F-12 + 20% FCS + 32 nM TPA + 10 ng/ml bFGF	+++	7-8	70	+++	>100	6
+ visible increase; - no increase; *n* number of culture dishes						

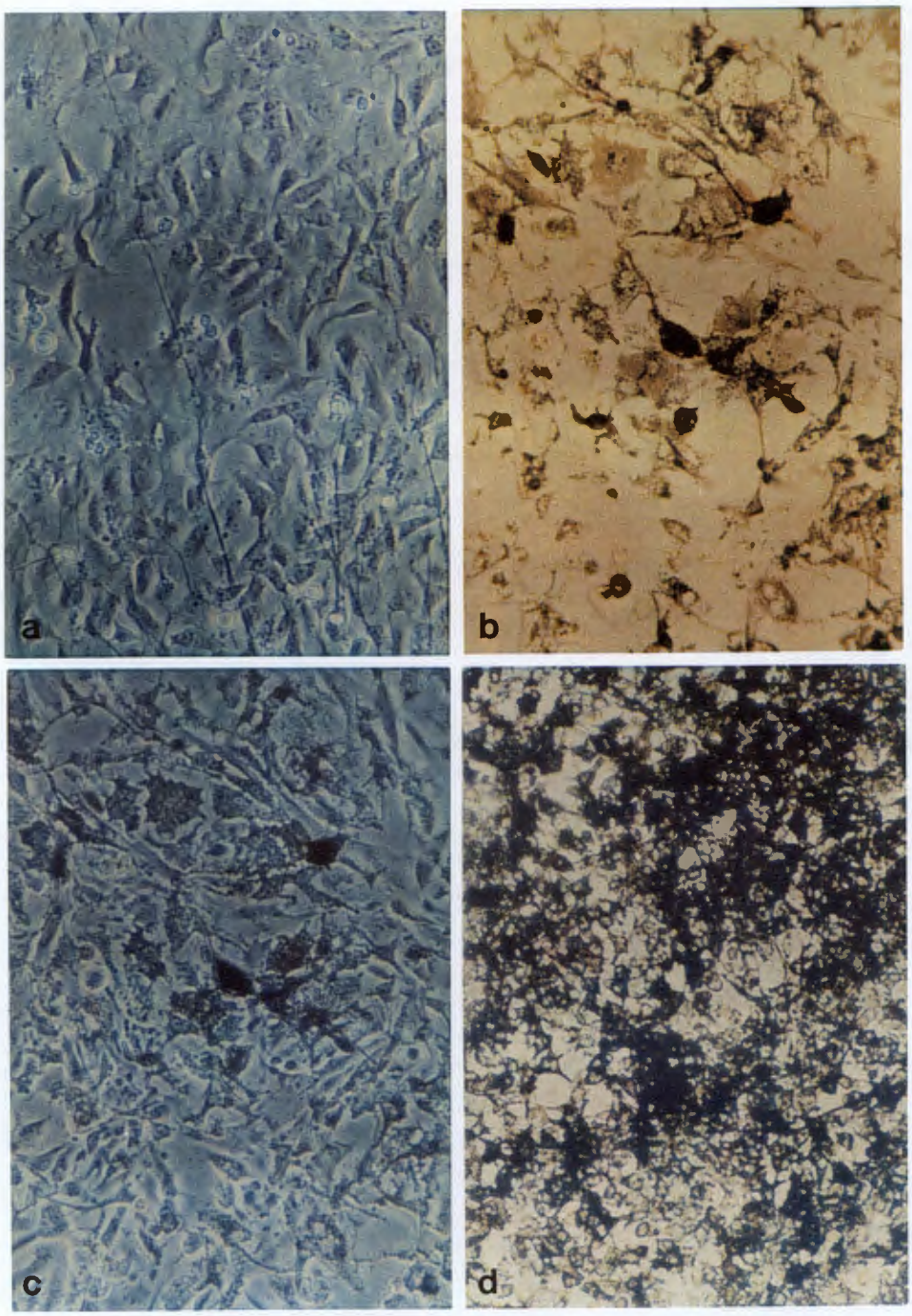

Fig 3.1. Chick neural crest cells cultured in Ham's F-12 medium with 20% FCS. (a) Small, stellate and unpigmented neural crest cells after 2 days of culture (phase contrast, x172). (b) Neural crest cells become pigmented after 3-4 days of culture (bright field, x172). (c) Same field as (b) showing both pigmented and unpigmented cells (phase contrast, x172). (d) Pigmented melanocytes cultured in Ham's F-12 medium, 20% FCS, 32 nM TPA and 10 ng/ml bFGF for more than 100 days (bright field, x172).

observed here, however contributed to 70-80% of the total cell number, with very few unpigmented cells present. Furthermore, these pigmented cells appeared more heavily melanised than those pigmented cultures grown in either basic medium alone or in less concentrated BRL-3A conditioned medium. These heavily pigmented clusters grew for more than three weeks in culture, but did not survive beyond 25 days of culture. Although, at best, the single cell suspension experiments and culture conditions described here yielded 70-80% pure cultures of heavily pigmented melanocytes, the total number of pigmented cells obtained never exceeded a few thousands (estimated from 1-2% confluency of a 60 mm culture dish). Therefore, an alternative approach that involved a combination of the approaches used by Boissy and Halaban (1985) and Stocker *et al.* (1991) was adopted in a further attempt to generate bulk cultures of pigmented chicken melanocytes.

3.1.2 TPA and bFGF enables the large scale culture of pigmented chicken melanocytes

In this procedure TPA and bFGF were evaluated for their ability to stimulate mitosis and differentiation of primary NCCs into pigmented melanocytes. Following the protocol of Stocker *et al.* (1991), the combined effects of 32 nM TPA and 10 ng/ml bFGF were evaluated for their ability to stimulate the survival and proliferation of NCCs and melanocytes. Cell cultures were fed every second day with fresh medium containing growth factors. The results of these experiments are presented in Table 3.2.

The cultures grown in basic medium alone behaved in a similar fashion to those control cultures described in section 3.1.1 (Fig. 3.1a).

Because different concentrations of TPA have been shown to function as a NCC (Payette *et al.*, 1980; Sieber-Blum and Sieber, 1981) and melanocyte (Boissy and Halaban, 1985) mitogen, a range of different TPA concentrations was evaluated for an optimal mitogenic effect on undifferentiated chicken NCCs. Dose-dependency experiments revealed that concentrations of 100 nM and 200 nM TPA were toxic to NCCs. 32 nM TPA appeared to stimulate NCC proliferation and 10 nM TPA had little or no effect on NCC proliferation.

When NCCs were grown in basic medium supplemented with 32 nM TPA, visible melanisation occurred only after 7-8 days in culture, thus delaying the onset of melanogenesis. These pigmented cells proliferated faster, were more dendritic and survived longer (16 days) than those grown in the absence of TPA (Table 3.2). Thus, the mitogenic

effect of 32 nM TPA enabled the expansion of potential chicken melanocytes as undifferentiated NCCs.

Because there is evidence that bFGF acts synergistically with TPA in inducing avian melanogenesis (Stocker *et al.*, 1991), as well as acting as a mitogen for human melanocytes (Halaban *et al.*, 1987, 1988a), chicken NCC cultures grown in 32 nM TPA were further supplemented with 10 ng/ml bFGF (Stocker *et al.*, 1991). Mitosis was stimulated (visual observation) and the onset of melanogenesis was delayed by four days (Table 3.2). Significantly, the combined effects of TPA and bFGF sustained the continued proliferation and survival of pigmented melanocytes beyond three months.

Having established the culture conditions for stimulating the proliferation and melanogenic differentiation of chicken NCCs, NCCs from approximately 500 embryos, dissected over several months, were grown in culture dishes containing basic medium supplemented with 32 nM TPA and 10 ng/ml bFGF. These cells remained small and stellate, initially with little pigmentation visible, although dividing cells were observed. Melanocytes were subcultured and pooled into 75 cm^2 flasks once dishes were 70-80% confluent.

Two days prior to RNA extraction, 100 ng/ml α-melanocyte stimulating hormone (α-MSH) was added to the cultures resulting in a dramatic visible increase in melanisation. Alpha-MSH has been shown to accelerate quail melanocyte differentiation *in vitro* (Satoh and Ide, 1987), as well as upregulate mouse *Tyr* gene transcription (Kuzumaki *et al.*, 1993; Aroca *et al.*, 1993). After three months in culture, approximately 10^8 cells were harvested, 70% of which were pigmented (Fig. 3.1d). It is possible that the remaining unpigmented cells (30%) represent lightly pigmented melanocytes that are indiscernable by light microscopy (Boissy *et al.*, 1986, 1989).

In summary then, whereas BRL-3A conditioned medium failed to sustain the bulk culture of chicken melanocytes, a combination of TPA, bFGF and α-MSH successfully permitted the differentiation and long term proliferation of pigmented chicken melanocytes. With an enriched source of melanised chicken cells, the next step of this study was to construct an embryonic chicken melanocyte cDNA library.

3.2 Construction of a novel embryonic chicken melanocyte cDNA library

RNA was extracted from approximately 10^8 cells (5 x 75 cm^2 flasks), yielding 195 µg of total RNA. An aliquot (10 µg) of this RNA was assessed by electrophoresis on denaturing agarose/formaldehyde gels (Fig. 3.2), revealing intact and undegraded total RNA. 10 µg of poly $(A)^+$-enriched RNA was obtained from the remaining 185 µg of total RNA and used to

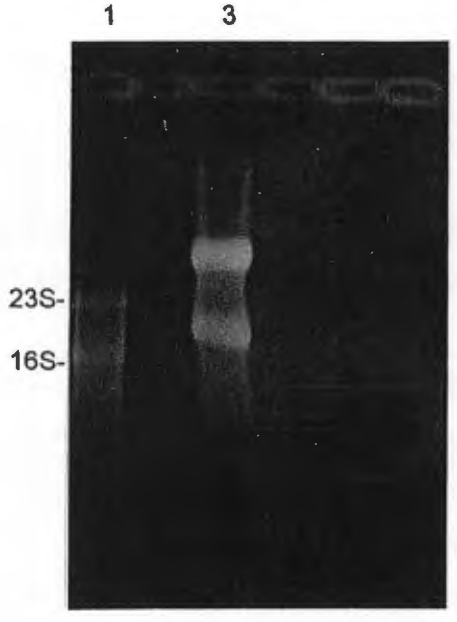

Fig. 3.2. Gel electrophoresis of 10 µg of total RNA extracted from pigmented chicken melanocyte cultures. Discrete ethidium bromide-stained 18S and 28S rRNA bands are visible (lane 3). *E. Coli* rRNA markers are indicated (lane 1).

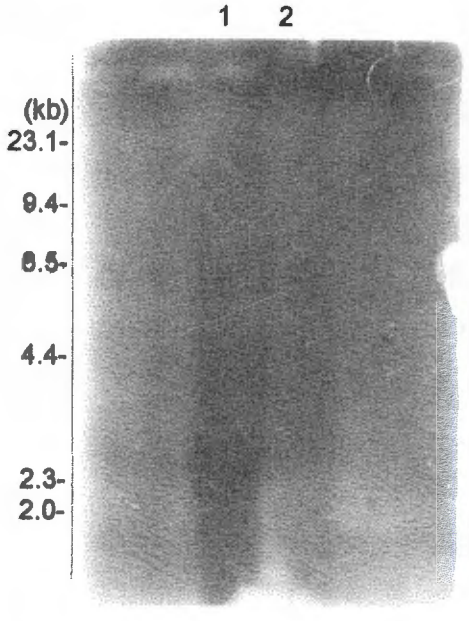

Fig. 3.3. Autoradiograph of alkaline agarose gel containing [α-^{32}P]dATP-labelled first (lane 1) and second (lane 2) strand chicken melanocyte-specific cDNAs. The molecular weight (kb) marker is λ/*Hind*III.

construct a cDNA library in Lambda Uni-ZAP XR (Stratagene), according to the manufacturer's instructions. Both the first and second synthesised strands ranged from 500 bp to 10 kb according to alkaline agarose gel electrophoresis (Fig. 3.3), confirming the presence of full-length, undegraded mRNA transcripts. After blunt-ending, *Eco*RI adaptors were ligated to the cDNA termini. These termini were then phosphorylated and digested with *Xho*I, yielding cDNAs with a 5' *Eco*RI end and a 3' *Xho*I (poly $(A)^+$) end. This step allowed the uni-directional cloning of cDNAs into Lambda ZAP XR vector arms.

In order to optimise the cloning of full-length cDNAs, tailored cDNAs were size-fractionated on Sephacryl S-400 spin columns according to the manufacturer's instructions (Stratagene). The first four fractions (> 500 bp - 10 kb) yielded approximately 50 ng cDNA each (by comparison with Lambda DNA standards). These fractions were pooled and ligated to Lambda Uni-ZAP XR arms. After packaging and titering (according to Stratagene's instructions), the estimated primary library size yielded approximately 7×10^5 recombinants. The amplified high titre cDNA library stock yielded approximately 4.5×10^9 pfus/ml.

3.3 Screening of a chicken melanocyte cDNA library for markers of pigment cell differentiation

As outlined in section 1.6, when this current project was initiated, the chicken *Tyr* cDNA had not been cloned. Therefore, the library constructed here, was screened for cDNAs likely to be specifically expressed in chicken melanoblasts, their precursors and differentiated melanocytes.

3.3.1 cDNA library screening strategies for the isolation of chicken *TRP* cDNAs

A chief goal of this study was to obtain chicken equivalents of the mammalian *TRP* gene family. This was achieved in two separate rounds of screening. In the first round, the library was screened for a chicken *Tyr* cDNA, using a mouse *Tyr* cDNA probe (Müller *et al.*, 1988). In the second screening, a chicken PCR-generated *TRP* probe was used to isolate cDNAs encoding chicken *Tyrp1* and *Tyrp2*.

3.3.1.1 Isolation of candidate chicken *Tyr* cDNAs

The chicken melanocyte library was screened (8×10^5 pfus) with a 1.9 kb *Eco*RI mouse *Tyr* cDNA (Müller *et al.*, 1988) probe. After primary and secondary screenings, 19 positive plaques were obtained (Fig. 3.4a). The inserts carried by the 19 Lambda ZAP II phages were then "subcloned" into pBluescript SK^- ($pBSK^-$) by *in vivo* excision. In order to further analyse the inserts carried by the 19 $pBSK^-$ clones, restriction enzyme digests, using *Eco*RI

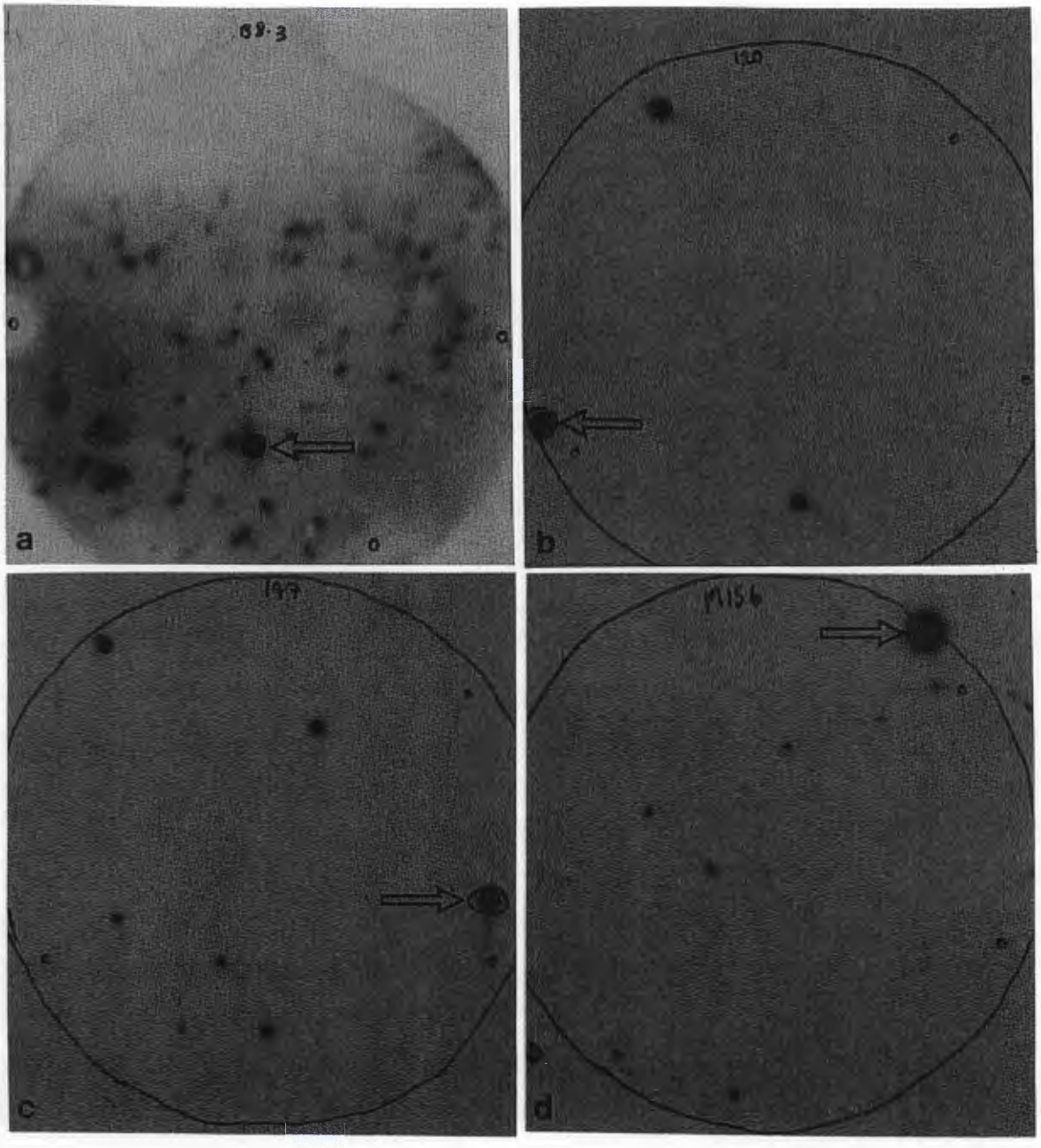

Fig. 3.4. Autoradiographs of Nylon N^+ membranes containing positively-hybridising pfus (arrows) isolated from an embryonic chicken melanocyte cDNA library. (a) Clone B8.3, probed with a 1.9 kb *Eco*RI mouse *Tyr* cDNA. (b) Clone pcTRP-1.6, probed with a 720 bp PCR-generated chicken *TRP* product. (c) Clone pcTRP-2.10, probed with a 720 bp PCR-generated chicken *TRP* product. (d) Clone M156, probed with a 1.3 kb *Eco*RI mouse *Mi* cDNA.

and *Xho*I, were performed on the phagemid DNAs. DNA gel electrophoresis revealed that the cloned inserts ranged from 1.0-2.0 kb in size (Fig. 3.5a).

To further characterise candidate *Tyr* cDNAs, the 19 pBSK$^-$-containing inserts were analysed by Southern blot hybridisation using a mouse *Tyr* cDNA probe. Only seven of these 19 chicken inserts cross-hybridised to the mouse *Tyr* cDNA probe, under moderate stringency conditions (Fig. 3.5b). These seven inserts were 1.2-2.0 kb in size. Partial restriction mapping and end-sequencing of these seven chicken clones revealed that six appeared to share a common restriction map with a chicken *Tyr* cDNA, that had just been published by another group (Mochii *et al.*, 1992). The seventh cDNA clone revealed a distinct unrelated restriction map. Of the six clones sharing a common chicken *Tyr* cDNA restriction map, the longest clone (B8.3) was selected for further analysis.

3.3.1.2 Isolation of candidate chicken *Tyrp1* and *Tyrp2* cDNAs

In order to isolate chicken *Tyrp1* and *Tyrp2* cDNAs the library constructed here was screened with a polymerase chain reaction (PCR)-generated chicken "generic" *TRP* cDNA probe. This *TRP* product was generated using aliquots of the cDNA library phage stock as DNA template as well as degenerate primers that were designed to amplify simultaneously all three known members of the *TRP* gene family (Jackson *et al.*, 1994). Details of the degenerate primer sequences, as well as the corresponding regions of the *TRP* gene family to which they anneal, have been described earlier (see section 2.4.2 and Table 2.1). Five µl aliquots of the amplified cDNA library phage stock were used as DNA templates to drive the degenerate PCR reactions. Of the three primer combinations used (79M and 80M; 80M and 81M; 79M and 82M), only one pair (80M and 81M) consistently yielded the expected product size of 720 bp (Fig. 3.6). This PCR product corresponds to the region spanning the middle to 3' (carboxy (C))-terminus of the *TRP* family. Based on these results, the chicken melanocyte cDNA library was screened using this 720 bp "generic" chicken *TRP* PCR product.

After the primary and secondary library screenings, 223 positive plaques were obtained. Twelve of the strongest 223 hybridising plaques were selected for further analysis (Figs 3.4b and 3.4c). These 12 positive clones were converted to pBSK$^-$-containing inserts by *in vivo* excision. Restriction enzyme analyses on phagemid DNA, using *Eco*RI and *Xho*I, yielded cloned insert sizes ranging from 1.0-3.0 kb (Fig. 3.7).

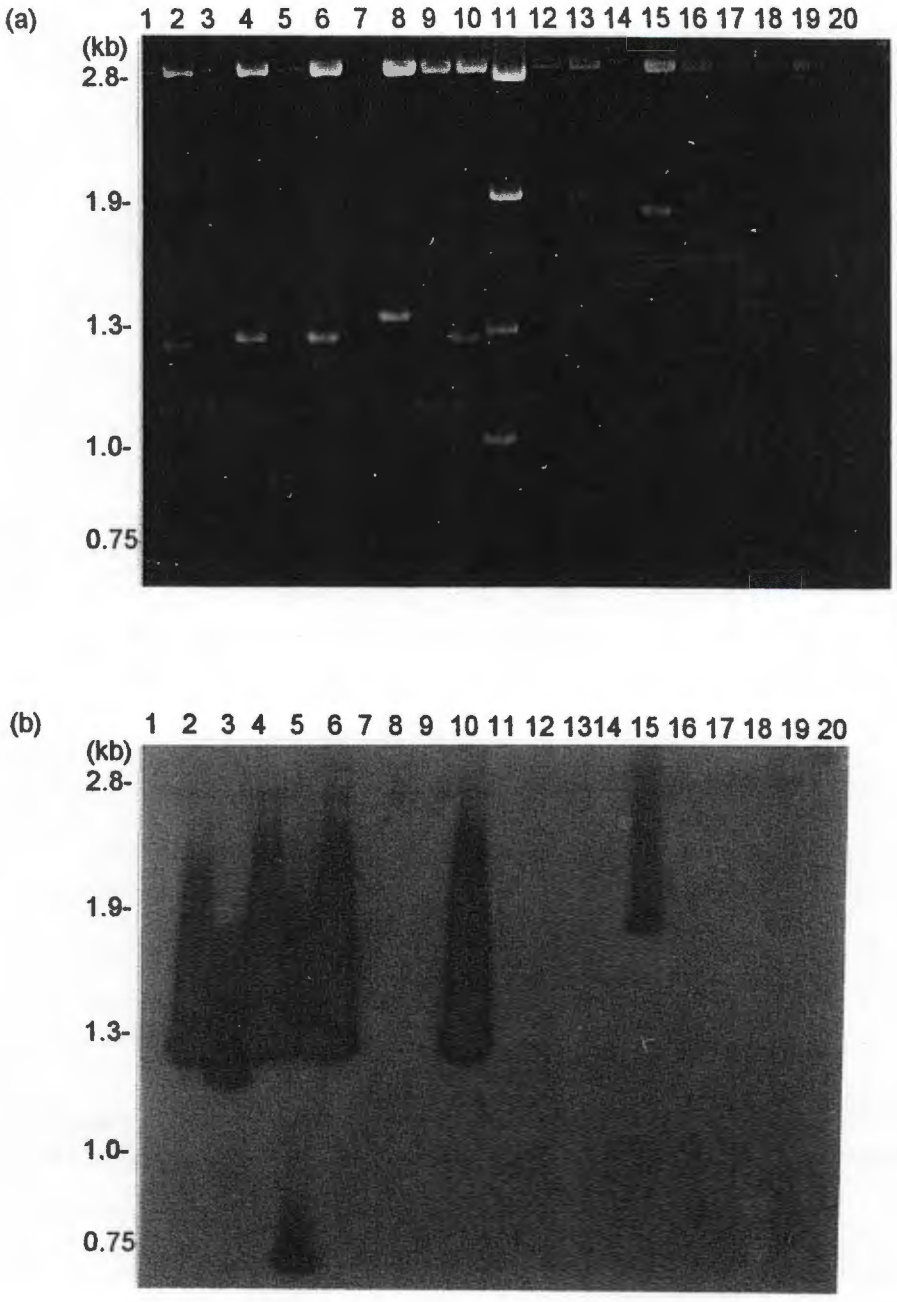

Fig. 3.5. (a) DNA gel electrophoresis of 19 positively-hybridising chicken clones (lanes 1-10, 12-20) isolated from an embryonic melanocyte cDNA library using a mouse *Tyr* cDNA probe. Phagemid DNA was digested with *Eco*RI and *Xho*I to release cloned cDNA inserts. The molecular weight (kb) marker is Pox/*Eco*RI (lane 11). (b) Autoradiograph of Southern blot of gel in (a). Seven (lanes 2, 3, 4, 5, 6, 10 and 15) out of 19 chicken cDNA inserts cross-hybridise to a radiolabelled 1.9 kb *Eco*RI mouse *Tyr* cDNA probe.

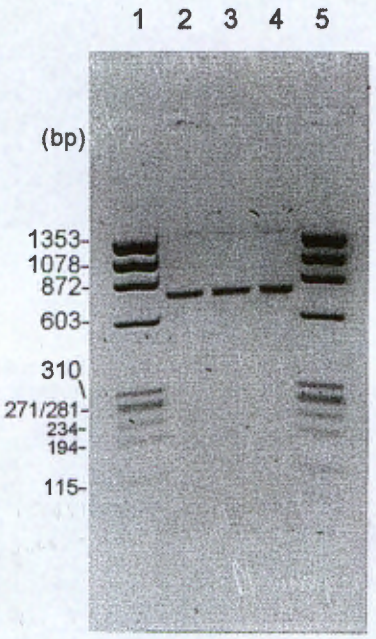

Fig. 3.6. DNA gel electrophoresis of a "generic" 720 bp chicken *TRP* product (lanes 2-4). This fragment was generated by PCR using degenerate *TRP* primers and aliquots of a chicken melanocyte cDNA library as template. The molecular weight marker (bp) is øX174/*Hae*III (lanes 1 and 5).

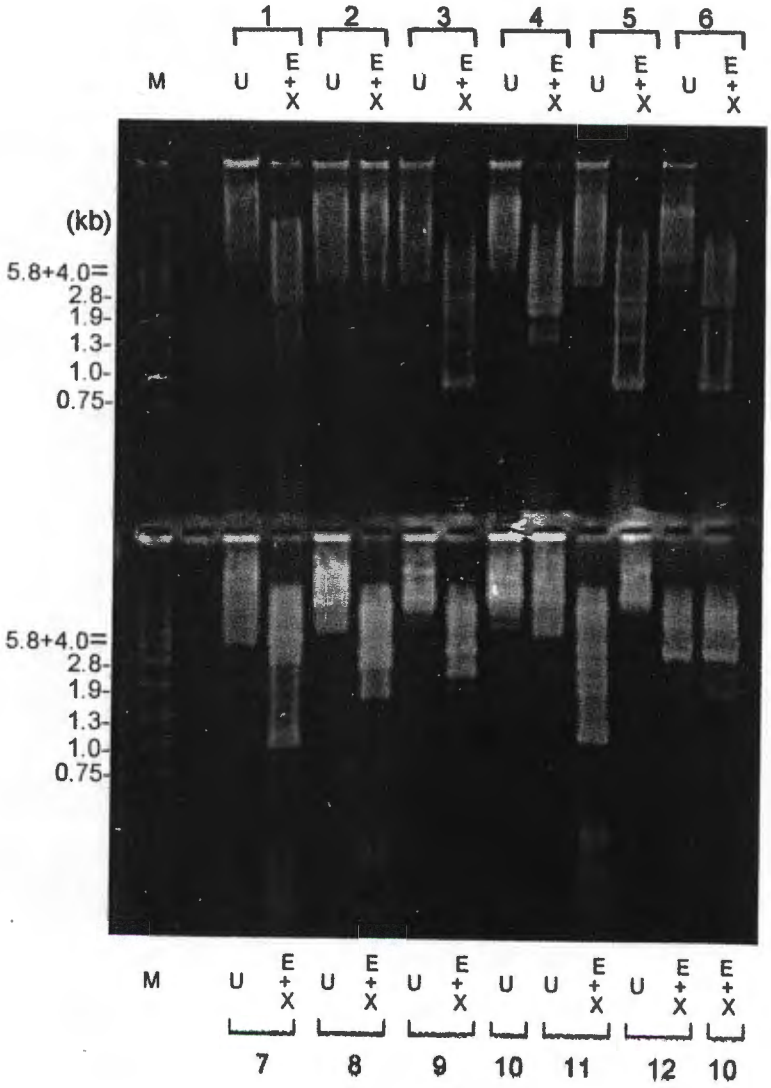

Fig. 3.7. DNA gel electrophoresis of 12 positively-hybridising chicken clones isolated from an embryonic chicken melanocyte cDNA library using a 720 bp PCR-generated chicken *TRP* product. Phagemid DNA was either undigested (U) or digested with *Eco*RI and *Xho*I (E+X) to release cloned cDNA inserts. The molecular weight marker (M) is Pox/*Eco*RI.

Because of the degenerate *TRP* screening procedure, it was not known which of these 12 clones corresponded to cDNAs encoding either chicken *Tyr*, *Tyrp1* or *Tyrp2*. In order to differentiate between these possibilities, these clones were subjected to Southern blot hybridisation analyses, using mouse *Tyrp1* (Shibahara *et al.*, 1986) and *Tyrp2* (Jackson *et al.*, 1992) cDNA probes. Out of these twelve clones, five cross-hybridised to a 1.6 kb *Hind*III mouse *Tyrp1* cDNA probe, with insert sizes ranging from 1.0-1.8 kb (Fig. 3.8a). The remaining seven clones all cross-hybridised to a 1.75 kb *Eco*RI mouse *Tyrp2* cDNA probe, with insert sizes of 1.8-3.0 kb (Fig. 3.8b). Because the partial restriction maps of the five *Tyrp1* cross-hybridising inserts were similar, the longest (1.7 kb) of these clones, pcTRP-1.6, was selected for further analysis. Similarly, pcTRP-2.10 (2.9 kb) was selected for further analysis, as it best represented an intact full-length cDNA candidate for chicken *Tyrp2*. The absence of any chicken *Tyr* cDNAs may reflect the relative abundance of the *TRP* cDNAs within the screened library. It is also possible that a sample size larger than twelve may have yielded *Tyr* cDNAs.

In summary then, from two separate screenings of an embryonic chicken melanocyte cDNA library, more than 200 putative chicken *TRP* cDNA clones were isolated. Out of these clones, 6 potential *Tyr*, 5 *Tyrp1* and 7 *Tyrp2* cDNAs were analysed further. The longest clone from each of these groups (B8.3, pcTRP-1.6 and pcTRP-2.10) was then selected for a more extensive characterisation (see section 3.4 below). Additionally, all three of these clones consistently yielded the expected product sizes after degenerate PCR, using all three *TRP* primer combinations, confirming the full-length and intact nature of these cDNAs.

While the library screening was underway, the opportunity was taken to screen for other genes that might be expressed in chicken melanocytes. The results of these screenings are briefly described below.

3.3.2 Isolation of chicken *Mi* cDNAs

The library was screened under moderate stringency conditions with a 1.3 kb cDNA, which contained the entire coding region of the mouse *Microphthalmia (Mi)* gene (Yavuzer *et al.*, 1995) (see section 2.4.3), yielding 11 candidate chicken *Mi* cDNA clones (Fig. 3.4d). Of these 11 clones, only a single clone (M156) yielded an insert after *in vivo* excision. PCR reactions, using M156 phagemid DNA as template and universal vector primers, yielded an insert >2.0 kb. Subsequent restriction enzyme analysis of the rescued phagemid, revealed that M156 carries a 2.5 kb insert. This insert consistently cross-hybridised to a mouse *Mi* cDNA probe on Southern blot hybridisation analysis. Further restriction enzyme mapping, end-sequencing and comparison to the mouse (Hodgkinson *et al.*, 1993) and human (Tachibana *et al.*, 1994) *Mi* cDNAs, by Karen Pinder and Toni Wiggins, Department of

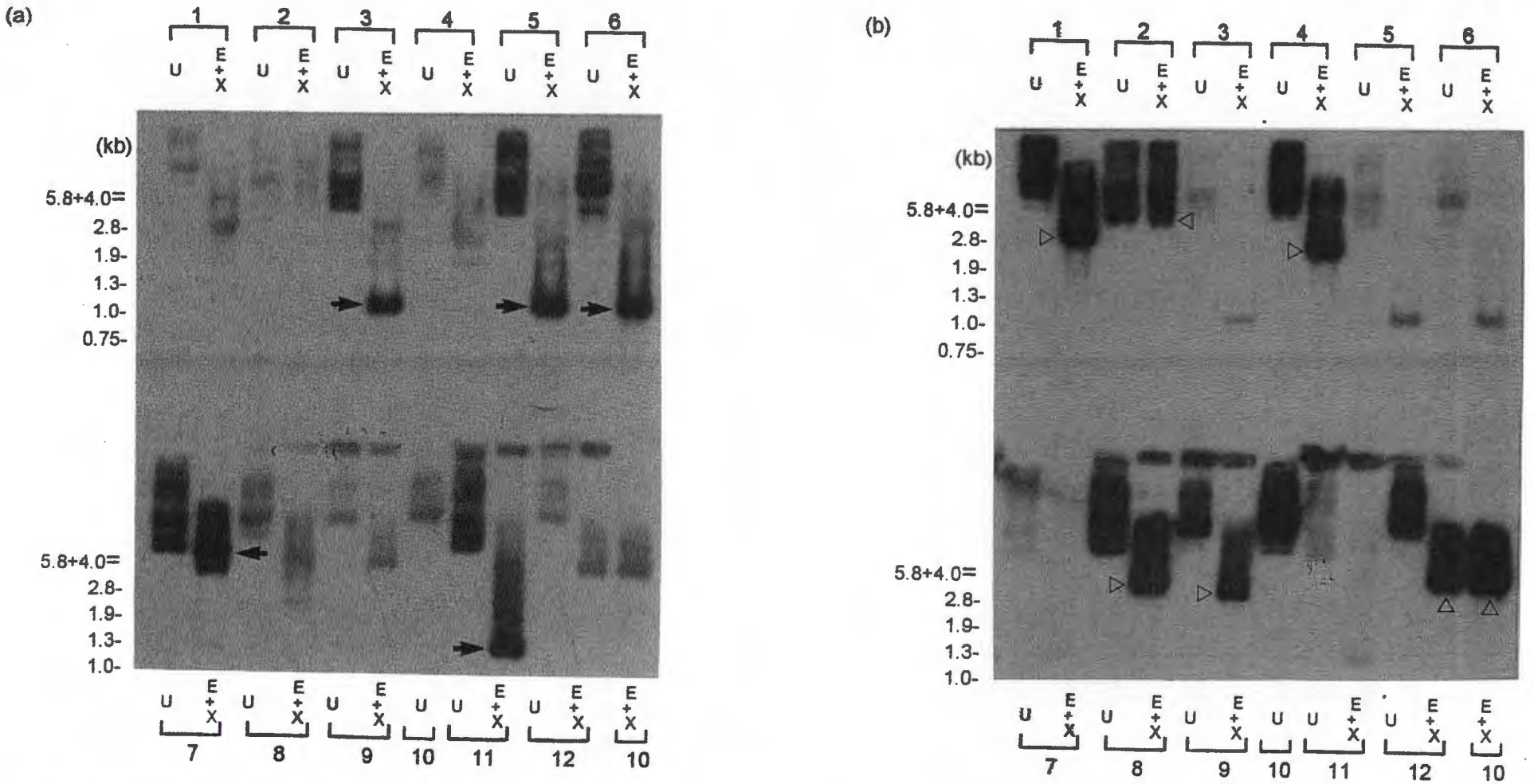

Fig. 3.8. Autoradiographs of Southern blot of gel shown in Fig. 3.7. Southern blots of undigested (U) and *Eco*RI + *Xho*I (E+X) digested phagemid DNA was subjected to (a) probing with a radiolabelled 1.6 kb *Hind*III mouse *Tyrp1* cDNA fragment, stripped and then (b) reprobed with a radiolabelled 1.75 kb *Eco*RI mouse *Tyrp2* cDNA fragment. Five (arrows) out of twelve cDNA inserts strongly cross-hybridised to the mouse *Tyrp1* cDNA probe (a), with the remaining seven inserts (arrow-heads) cross-hybridising to the mouse *Tyrp2* cDNA probe (b). The molecular weight marker (kb) is Pox/*Eco*RI.

Anatomy and Cell Biology, Medical School, University of Cape Town, South Africa, revealed that clone M156 harboured the entire coding region of the chicken *Mi* gene. During further characterisation of M156, another group (Mochii *et al.*, 1998) reported the cloning of a chicken *Mi* cDNA, cmi9. Preliminary comparisons appear to indicate that clone M156 is virtually identical to cmi9, thus verifying its authenticity as an avian equivalent of the mammalian *Mi* gene.

3.3.3 Attempted isolation of chicken *Mc1r* cDNAs

In an attempt to isolate chicken homologues of the mammalian *Melanocortin 1 Receptor (MC1R)* cDNAs, the library was also screened with a random-primed 1 kb human *MC1R* genomic DNA probe. This 1 kb human *MC1R* DNA (Fig. 3.9) was generated by nested PCR, using human *MC1R* primers (see section 2.4.4 and Valverde *et al.*, 1995). No positively hybridising chicken clones were obtained. Positive controls indicated that the lack of hybridising signals was not due to technical problems.

Why the *MC1R* screening experiment failed to yield potential chicken *Mc1r* equivalents is not known. One possibility is that the homology between the human and chicken *Mc1r* DNA is not sufficient for cross-hybridisation (even under moderate stringency conditions). However, shortly after these experiments had been carried out, a chicken genomic clone, containing the entire *Mc1r* coding region, was successfully cloned by another group (Takeuchi *et al.*, 1996a), using a mouse *Mc1r* cDNA probe. A comparison of the coding regions of the chicken and mouse *MC1R* DNAs revealed 72% nucleotide (nt) sequence homology, suggesting that the homology between the chicken and human *MC1R* genes may indeed be sufficient for cross-hybridisation.

An alternative possibility for why the screening experiment in the current study failed to yield cross-hybridising signals, may be due to low *Mc1r* cDNA abundance in the Black Australorp X New Hampshire Red library. In addition, this breed is heterozygous at the E^{+} locus (E/e^{Wh}), which is now thought to encode the *Mc1r* gene in chickens (Takeuchi *et al.*, 1996b). Because the molecular nature of the *E* and e^{Wh} alleles is not known, it may be possible that these lesions may have affected the cross-hybridisation of the human *MC1R* DNA probe to the chicken *E* and e^{Wh} (*Mc1r*) cDNAs.

3.3.4 Attempted PCR amplification of the chicken *Pmel 17* gene family

As a first step towards isolating cDNAs encoding members of the *Pmel 17* gene family, attempts were made to obtain a "generic" *Pmel 17* chicken cDNA with which to probe the

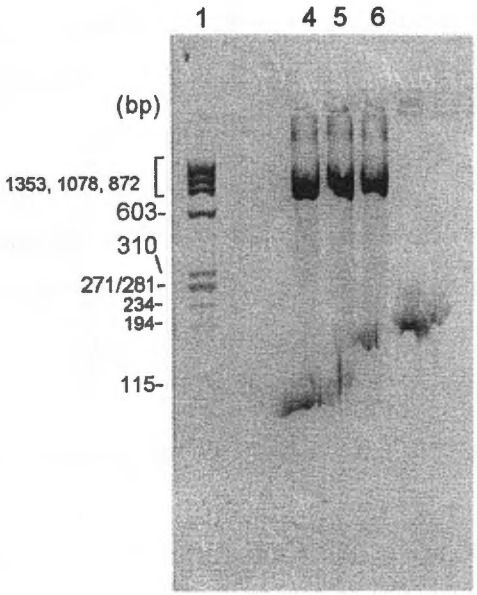

Fig. 3.9. DNA gel electrophoresis of a human 1 kb *MC1R* genomic product (lanes 4-6). This fragment was generated by nested PCR using human *MC1R* primers. The molecular weight marker (kb) is øX174/*Hae*III (lane 1).

chicken melanocyte cDNA library. Employing a similar strategy to that used in isolating chicken *Tyrp1* and *Tyrp2* cDNAs, degenerate PCR was performed using aliquots of the amplified cDNA library phage stock as DNA templates. Three different primer combinations were used (MMP-1 and MMP-4; MMP-1 and MMP-3; MMP-2 and MMP-4; see section 2.4.5). Although different aliquots of library template, as well as different annealing temperatures were used, these degenerate PCR reactions failed to yield products of the expected sizes. It is unclear why these PCR reactions were unsuccessful, although it is possible that the degenerate primers were not sufficiently homologous to the chicken cDNA templates, or that the chicken *Pmel 17 family* cDNAs are present at very low levels within the cDNA library.

To summarise, from an embryonic chicken melanocyte at least four candidate pigment cell-specific cDNAs were isolated. These include *Tyr*, *Tyrp1*, *Tyrp2* and *Mi*. Attempts to clone the chicken equivalents of the mammalian *MC1R* and *Pmel 17* gene family were unsuccessful. The characterisation of the chicken *Mi* cDNA falls beyond the scope of this project and is currently been addressed by a fellow student (Karen Pinder). The remaining sections of this chapter have therefore been devoted to the further characterisation of the chicken *Tyr*, *Tyrp1* and *Tyrp2* cDNAs.

3.4 Strategies for the DNA sequencing of the cDNAs encoding the chicken *TRP* gene family

Two different strategies were employed to sequence the three clones encoding chicken *Tyr* (B8.3), *Tyrp1* (pcTRP-1.6) and *Tyrp2* (pcTRP-2.10). In the first strategy, clone B8.3 was restriction mapped and four subclones (400-600 bp), corresponding to the entire 1.9 kb insert carried by B8.3, were sequenced manually using univeral vector primers. For the second strategy, clones pcTRP-1.6 and pcTRP-2.10 were sequenced using a combination of vector, degenerate *TRP* and custom-designed internal primers. Each sequencing reaction was performed at least twice (see sections 2.7.1 and 2.7.2, Fig. 2.4 and Table 2.1 for more sequencing details).

The results generated by the sequencing of clones B8.3, pcTRP-1.6 and pcTRP-2.10 have been described in two sections. The first section analyses the nt sequences of all three clones individually and compares the deduced amino acid (aa) sequences of the individual chicken clones to orthologous TRPs in other species. The second part of this sequence analysis compares the chicken TRP gene family to that of the mouse and human families. The salient features of the clones encoding the chicken *TRP* gene family have been

summarised under Table 3.3. A comparison of the chicken TRPs with all cloned full-length members of the vertebrate TRP family is presented as an identity matrix in Table 3.4.

3.4.1 Nucleotide and amino acid sequence analysis of cDNAs encoding the chicken *TRP* gene family

3.4.1.1 Clone B8.3 encodes a chicken *Tyr* cDNA

The entire nt and predicted aa sequence of B8.3 is presented in Fig. 3.10. Full-length B8.3 consists of 1909 bp, with 44 bp of 5' untranslated sequence *(UTR)* followed by an open reading frame (ORF) of 1587 nts. This ORF begins with an ATG codon (nts 45-47), which is homologous to the vertebrate translation initiation consensus sequence A/GNCAUG (Cavener and Ray, 1991) and ends with a TAA translation termination codon (nts 1632-1634). Following the ORF is 250 bp of 3' *UTR* and a poly $(A)^+$ stretch of 28 nts. A CAYUG recognition element (nts 1767-1771) (Berget, 1984) is also present. This element is thought to play a role in poly $(A)^+$ cleavage site selection. A putative polyadenylation signal (ATTAAA) (Wickens and Stephenson, 1984) (nts 1863-1868) is also present.

Clone B8.3 shows 99.7% nt sequence identity with a previously cloned chicken *Tyr* cDNA, Cty18 (Mochii *et al.*, 1992), with only four nt differences. Three of these substitutions occur within the predicted coding region of B8.3; two of these three substitutions are silent and therefore do not result in a corresponding aa change (nt 608: CTC→CTT = Leu; nt 1289: AAC→AAT = Asn) whilst the third substitution (nt 1351) results in a GGA = Gly→GAA = Gln change. The fourth substitution (nt 1712: A→G) occurs within the 3' *UTR* of the cDNA.

FASTA (GCG) homology searches revealed that clone B8.3 shares the highest nt sequence identities with orthologous *TYR* cDNAs from other species. Thus B8.3 shares 59.4%, 61.5%, 64.5% and 66.8% nt sequence identity with the fish (Inagaki *et al.*, 1994), frog (Takase *et al.*, 1992), mouse (Müller *et al.*, 1988) and human (Bouchard *et al.*, 1989) *TYR* cDNAs respectively (Table 3.4). The ascidian *Tyr* cDNA sequences (Sato *et al.*, 1997; Caracciolo *et al.*, 1997) were not available from the GenBank database at the time of writing this thesis. B8.3 also has 73.6% nt sequence identity (over 244 nts) with the partial cat (van der Linde-Sipman *et al.*, 1997) and 71.8% identity (over 871 nts) with the partial dog (Tang *et al.*, 1996, unpublished) *Tyr* cDNA sequences, respectively. At the aa level, the sequence identities between B8.3 and the partial cat and dog Tyrs are 75.0% (over 80 aas) and 75.6% (over 276 aas), respectively.

Table 3.3. Summary of key features of the chicken *TRP* cDNAs

Feature	*Tyr*		*Tyrp1*		*Tyrp2*	
cDNA length (bp)	1909		1771		2938	
5' UTR (bp)	44	nts 1-44	65	nts 1-65	185	nts 1-185
ORF (bp)	1587	nts 45-1631 aas 1-529	1605	nts 66-1670 aas 1-535	1563	nts 186-1748 aas 1-521
3' UTR (bp)	250	nts 1632-1909	101	nts 1671-1771	1186	nts 1749-2938
poly $(A)^+$ signal (number)	1	nts 1863-1868	1	nts 1752-1757	2	nts 2068-2073 nts 2898-2903
poly $(A)^+$ tail (nt)	28	nts 1882-1909	?	?	18	nts 2920-2938
mature protein length (aa)	512	nts 99-1631 aas 19-529	512	nts 135-1670 aas 24-535	498	nts 255-1748 aas 24-521
signal peptide (aa)	18	nts 45-98 aas 1-18	23	nts 66-134 aas 1-23	23	nts 186-254 aas 1-23
molecular weight (kDa)	58		58		56	
N-glycosylation sites (number)	9		6		9	
transmembrane domain (aa)	36	nts 1431-1538 aas 463-488	27	nts 1485-1565 aas 474-500	18	nts 1617-1670 aas 478-495

Table 3.4. Nucleotide and deduced amino acid sequence comparisons of full-length members of the TRP gene family

	fish Tyr	frog Tyr	chicken Tyr	mouse Tyr	human TYR	fish Tyrp1	chicken Tyrp1	mouse Tyrp1	human TYRP1	chicken Tyrp2	mouse Tyrp2
frog Tyr	60.3 59.4 67.3	% nucleotide identity % amino acid identity % amino acid similarity									
chicken Tyr	59.4 60.3 68.6	61.5 67.3 75.3									
mouse Tyr	58.9 60.6 68.3	60.5 65.5 73.5	64.5 71.8 77.9								
human TYR	60.0 58.8 67.1	61.2 70.0 75.0	66.8 73.1 77.7	78.8 85.8 89.4							
fish Tyrp1	46.8 37.3 44.3	50.0 38.0 46.4	47.9 39.6 47.3	47.0 37.9 46.1	48.3 38.4 46.0						
chicken Tyrp1	46.5 38.8 46.3	48.4 39.0 47.7	48.9 40.0 50.0	49.5 41.5 51.5	49.8 38.1 47.7	60.8 66.2 71.0					
mouse Tyrp1	46.7 39.1 46.9	47.0 38.7 48.9	45.8 40.5 49.2	47.4 39.2 47.6	48.0 39.0 47.4	55.7 64.2 69.5	68.6 72.9 79.1				
human TYRP1	47.8 40.5 48.6	45.8 38.7 49.0	48.1 40.2 50.0	47.5 39.5 48.9	49.0 39.9 49.1	58.6 64.8 70.3	70.1 75.9 81.9	77.2 85.3 88.1			
chicken Tyrp2	47.7 40.1 49.1	47.5 40.0 51.1	50.0 38.9 49.8	50.0 40.1 47.5	48.6 38.9 47.7	49.5 44.0 52.4	53.3 49.8 58.1	50.2 48.0 57.3	48.8 48.5 57.0		
mouse Tyrp2	48.0 37.9 47.5	49.8 40.1 50.6	48.5 39.4 50.1	48.9 39.5 49.6	49.6 39.2 48.9	50.7 44.8 54.8	53.0 47.2 56.1	51.4 48.3 56.2	50.9 49.5 57.9	63.4 69.2 75.4	
human TYRP2	47.2 38.2 46.9	50.8 39.8 51.1	48.3 38.5 48.7	48.5 39.6 48.5	52.3 39.1 48.8	49.8 44.7 53.5	54.3 47.0 55.9	49.7 48.9 57.1	51.5 48.3 56.6	65.5 69.9 75.3	78.6 83.4 85.9

DNA sequences were translated using the "Translate" programme and were then aligned and compared in a pairwise fashion using the "Gap" programme (Genetics Computer Group (GCG) program (GCG sequence analysis software package, version 9.0, GCG, Inc., Madison, WI, USA)). The three values for each comparison are percentage nucleotide sequence identity (top), amino acid sequence identity (middle) and amino acid sequence similarity (bottom), respectively. Fish Tyr (Inagaki *et al.*, 1994, D29687), frog Tyr (Takase *et al.*, 1992, D12514), chick Tyr (this study, S81675), mouse Tyr (Müller *et al.*, 1988, X12782), human TYR (Bouchard *et al.*, 1989, Y00819), fish Tyrp1 (Peng *et al.*, 1994, S71755), chicken Tyrp1 (this study, AF003631), mouse Tyrp1 (Shibahara *et al.*, 1986, X03687), human TYRP1 (Cohen *et al.*, 1990, X51420; Urquhart, 1991, X60955), chicken Tyrp2 (this study, AF023471), mouse Tyrp2 (Jackson *et al.*, 1992, X63349), human TYRP2 (Bouchard *et al.*, 1994, S69231).

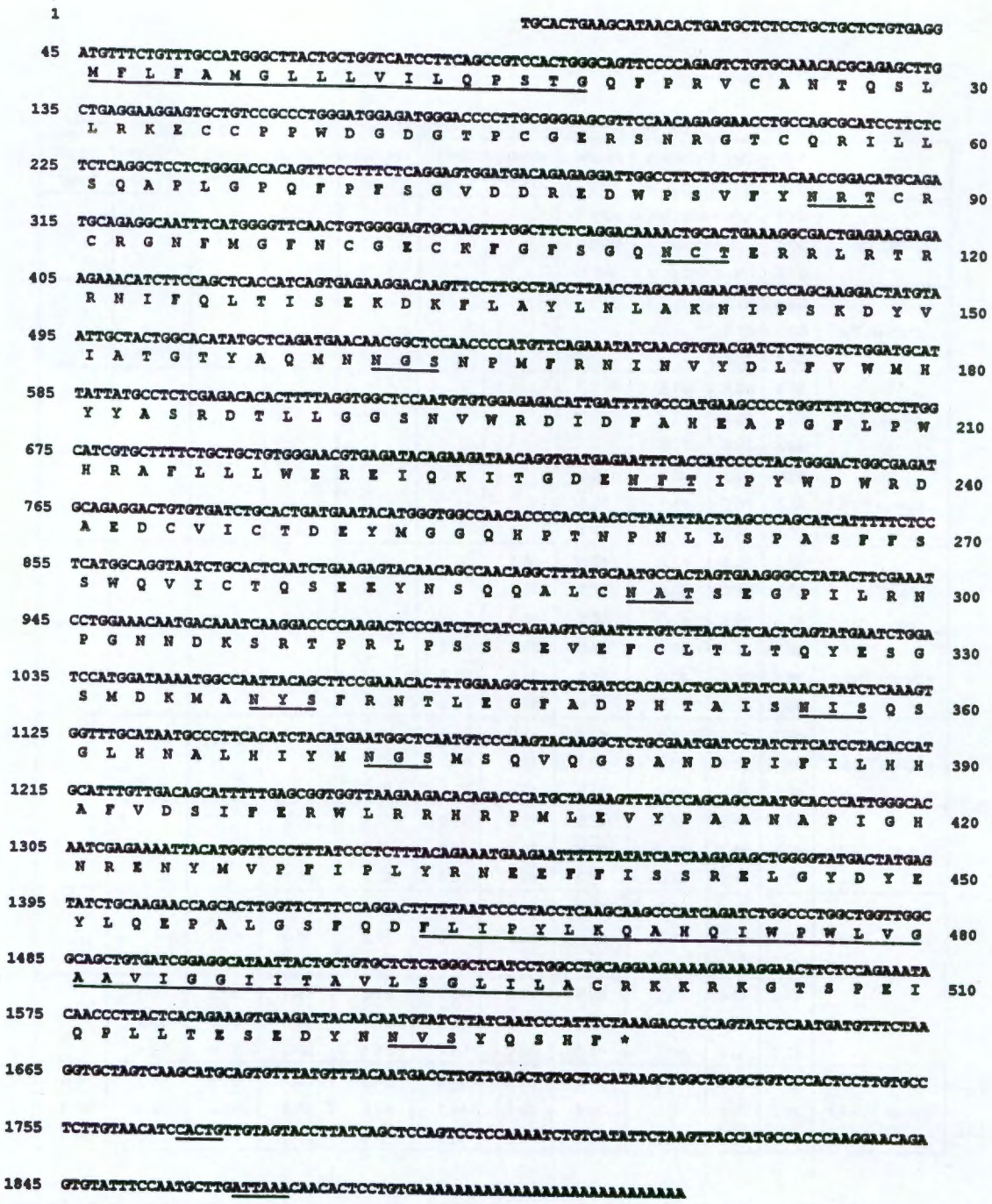

Fig. 3.10. Nucleotide and deduced amino acid sequence of the B8.3 cDNA insert, encoding chicken Tyr. A putative 18 amino acid leader sequence, nine *N*-linked glycosylation sites, a carboxy-terminal hydrophobic membrane-spanning domain, a CAYUG recognition element and a putative polyadenylation signal are all underlined. The stop codon is indicated with an asterisk. The nucleotide sequence shown here has been deposited in the GenBank database and assigned the accession number S81675.

The B8.3 ORF encodes a polypeptide of 529 aas with a predicted molecular weight of 60 kDa. The first 18 aa residues at the amino (N)-terminus are consistent with a signal sequence, containing a hydrophobic core and terminating with glycine (von Heijne, 1986) (Fig. 3.10). Thus the mature, processed B8.3 polypeptide consists of 511 aas with a predicted molecular weight of 58 kDa. The predicted protein sequence of B8.3 contains nine potential *N*-glycosylation sites (NXS/T) (Bause, 1983) and a potential 36 aa membrane-spanning domain near the C-terminus (Kyte and Doolittle, 1982).

The protein encoded by clone B8.3 shares respectively, 60.3%, 67.3%, 71.8% and 73.1% aa sequence identity with the fish (Inagaki *et al.*, 1994), frog (Takase *et al.*, 1992), mouse (Müller *et al.*, 1988) and human (Bouchard *et al.*, 1989) TYR proteins (Table 3.4). With conservative aa substitutions, B8.3 shares respectively, 68.6%, 75.3%, 77.9% and 77.7% aa sequence similarity with the fish, frog, mouse and human TYR proteins. An aa alignment of the predicted B8.3 protein with other published, full-length vertebrate TYR proteins was generated using the PILEUP GCG command (Fig. 3.11). Clone B8.3 encodes a protein of 529 aas, whereas the fish, frog, mouse and human TYR protein lengths are 540, 532, 533 and 531 aas respectively.

From the deduced aa sequences in Fig. 3.11, four conserved protein domains were identified by the Swiss-Prot and Prosite databases. The first of these domains (aas 93-104) is a cysteine-rich epidermal growth factor (EGF)-like domain [CXCX(5)GX(2)C], that has been found in several membrane-bound and secreted proteins. This domain is present in all members of the TRP gene family and it has been suggested that this domain may be involved in protein-protein interactions (Jackson *et al.*, 1992).

The conserved second (aas 185-217) [HX(4)F(L,I,V,M,T)XWHRX(2)(L,M)X(3)E] and third (aas 369-396) [DPXF(L,I,V,M,F,Y,W)X(2)HX(3)D] domains are restricted to the members of the TRP gene family and certain invertebrate haemocyanins (Lerch, 1988). These two histidine-containing motifs, termed "CuA" and "CuB" (Lerch, 1988), bind copper, thus facilitating mono-oxygenase activity (Martinez-Esparza *et al.*, 1997; Spritz *et al.*, 1997). The fourth domain (aas 523-528) (EXXPLL), termed a C-terminal consensus sequence (CTCS) (Peng *et al.*, 1994) contains a di-leucine sequence which has been suggested to play a role in the intracellular trafficking of melanogenic proteins from the Golgi to the melanosome (Winder *et al.*, 1993; Peng *et al.*, 1994; Vijayasaradhi *et al.*, 1995; Beermann *et al.*, 1995 and Jimbow *et al.*, 1997). This CTCS motif, although present in all vertebrate TYRs, is not present in the ascidian Tyr protein.

```
          1                                                                  60
cTyr      ~~~~MFLFAM GLLLVILQPS TGQFPRVCAN TQSLLRKECC PPWDGDGTPC GERSNRGTCQ
fiTyr     ~~~MKSLFLS AVLLQFFETC WSQFPRPCAN SEGLRTKECC PVWSGDGSPC GALSGRGFCA
frTyr     MESTTVLLAT STLLLVLHAS YGQFPRACST AQVLLSKECC PVWPGDNSSC GEVSGRGVCQ
mTyr      ~~~~MFLAVL YCLLWSFQIS DGHFPRACAS SKNLLAKECC PPWMGDGSPC GQLSGRGSCQ
hTYR      ~~~~MLLAVL YCLLWSFQTS AGHFPRACVS SKNLMEKECC PPWSGDRSPC GQLSGRGSCQ

          61                                  EGF-like domain                120
cTyr      RILLSQAPLG PQFPFSGVDD REDWPSVFYN RTCRCRGNFM GFNCGECKFG FSGQNCTERR
fiTyr     DVSVSDEPNG PQYPHSGIDD RERWPLAFFN RTCRCAGNYG GFNCGECRFG YWGSNCAEYR
frTyr     DVVPSNSPVG AQFPFSGIDD RENWPIVFYN RTCQCQGNFM GYNCGECRFG YTGPNCTVRR
mTyr      DILLSSAPSG PQFPFKGVDD RESWPSVFYN RTCQCSGNFM GFNCGNCKFG FGGPNCTEKR
hTYR      NILLSNAPLG PQFPFTGVDD RESWPSVFYN RTCQCSGNFM GFNCGNCKFG FWGPNCTERR

          121                                                                180
cTyr      LRTRRNIFQL TISEKDKFLA YLNLAKNIPS KDYVIATGTY AQM.NNGSNP MFRNINVYDL
fiTyr     ESVRRNIMSM STTEQQKFIS YLNLAKNTIN PDYVITTGTR AEMGENGESP MFSDINTYDL
frTyr     NMIRKDIFRM TTAEKDKLIA YLNLAKHTIS PDYVIATGTY EQM.NNGSNP MFADISAYDL
mTyr      VLIRRNIFDL SVSEKNKFFS YLTLAKHTIS SVYVIPTGTY GQM.NNGSTP MFNDINIYDL
hTYR      LLVRRNIFDL SAPEKDKFFA YLTLAKHTIS SDYVIPIGTY GQM.KNGSTP MFNDINIYDL

          181                Cu A domain                                     240
cTyr      FVWMHYYASR DTLLGG.SNV WRDIDFAHEA PGFLPWHRAF LLLWEREIQK ITGDENFTIP
fiTyr     FVWIHYYVSR DTFLGGPGNV WRDIDFAHES AAFLPWHRVY LLHWEQEIRK ITGDFNFTIP
frTyr     FVWIHYYASR DAFLED.GSV WADIDFAHEA PGFLPWHRFY LLLWEREIQK VTGDDNFTIP
mTyr      FVWMHYYVSR DTLLGG.SEI WRDIDFAHEA PGFLPWHRLF LLLWEQEIRE LTGDENFTVP
hTYR      FVWIHYYVSM DALLGG.YEI WRDIDFAHEA PAFLPWHRLF LLRWEQEIQK LTGDENFTIP

          241                                                                300
cTyr      YWDWRDAEDC VICTDEYMGG QHPTNPNLLS PASFFSSWQV ICTQSEEYNS QQALCNATSE
fiTyr     YWDWRDAQSC EVCTDNLMGG RNALNPNLIS PASVFSSWKV ICTQQEEYNN QEALCNATAE
frTyr     FWDWRDAQQC ELCTDEFFGG THPTSNNLLS PASFFSSWQI ICSRPEEYNS LRIICNGTNE
mTyr      YWDWRDAENC DICTDEYLGG RHPENPNLLI PASFFSSWQI ICSRSEEYNS HQVLCDGTPE
hTYR      YWDWRDAEKC DICTDEYMGG QHPTNPNLLS PASFFSSWQI VCSRLEEYNS HQSLCNGTPE

          301                                                                360
cTyr      GPILRNPGNN DKSRTPRLPS SSEVEFCLTL TQYESGSMDK MANYSFRNTL EGFADPHTAI
fiTyr     GPLLRNPGNH DPNRVPRIPT TADVEFTISL PEYETGSMDR FANNSFRNVL EGFASPETGM
frTyr     GPLLRSPGRH DRNRTPRLPT SADVEACLSL TDYETGAMDR SANFSFRNTL EGFAVPTSGI
mTyr      GPLLRNPGNH DKAKTPRLPS SADVEFCLSL TQYESGSMDR TANFSFRNTL EGFASPLTGI
hTYR      GPLRRNPGNH DKSRTPRLPS SADVEFCLSL TQYESGSMDK AANFSFRNTL EGFASPLTGI

          361                Cu B domain                                     420
cTyr      SNISQSGLHN ALHIYMNGSM SQVQGSANDP IFILHHAFVD SIFERWLRRH RPMLEVYPAA
fiTyr     AVQGQSTMHN ALHVFMNGSM SSVQGSANDP IFLLHHAFID SIFERWLRTH QPPRSIYPRT
frTyr     ANRSQSSMHN SLHVFLNGSM SSVQGSANDP IFVLHHAFVD SLFEQWLRRH QPSLDVYPEA
mTyr      ADPSQSSMHN ALHIFMNGTM SQVQGSANDP IFLLHHAFVD SIFEQWLRRH RPLLEVYPEA
hTYR      ADASQSSMHN ALHIYMNGTM SQVQGSANDP IFLLHHAFVD SIFEQWLRRH RPLQEVYPEA

          421                                                                480
cTyr      NAPIGHNREN YMVPFIPLYR NEEFFISSRE LGYDYEYLQE PALGSFQDFL IPYLKQAHQI
fiTyr     NAPIGHNDGY YMVPFLPLYR NGDYLLSNKA LGYEYAYLLD PGQRFVQEFL TPYLQQAQQI
frTyr     NAPVGHNREY NMVPFIPLFT NGEFFVQSRD LGYDYDYLAE S..GSIEDFL LPYLEQARQI
mTyr      NAPIGHNRDS YMVPFIPLYR NGDFFITSKD LGYDYSYLQE SDPGFYRNYI EPYLEQASRI
hTYR      NAPIGHNRES YMVPFIPLYR NGDFFISSKD LGYDYSYLQD SDPDSFQDYI KSYLEQASRI

          481                                                  CTCS          540
cTyr      WPWLVGAAVI GGIITAVLSG LI....LACR KKRKGTSP.. ..EIQPLITE SEDYNNVSYQ
fiTyr     WQWLLGAGIL GALIATIVAA VIVFARRKRR RNQKRKRAPS PGERQPLLQS SSEEGSSSYQ
frTyr     WQWLLGAAVL GGLITAVIAT IISLTC..RR KRKTKISE.. ..ETRPLIME AEDYQA.TYQ
mTyr      WPWLLGAALV GAVIAAALSG LSSRLCLQKK KKKKQPQE.. ..ERQPLIMD KDDYHSLLYQ
hTYR      WSWLLGAAMV GAVLTALLAG LVSLLC...R HKRKQLPE.. ..EKQPLIME KEDYHS.LYQ

          541
cTyr      SHF
fiTyr     TTL
frTyr     SNL
mTyr      SHL
hTYR      SHL
```

Fig. 3.11. Amino acid alignment of TYR proteins from the chicken (cTyr; this study), fish (fiTyr; Inagaki *et al.*, 1994), frog (frTyr; Takase *et al.*, 1992), mouse (mTyr; Müller *et al.*, 1988) and human (hTYR; Bouchard *et al.*, 1989) using the Pileup command (Genetics Computer Group (GCG) program (GCG sequence analysis software package, version 9.0, GCG, Inc., Madison, WI, USA)). The protein sequences are represented in one-letter code. Dots (..) have been introduced for optimal alignment. An amino-terminal EGF-like domain, two copper-binding domains, and a carboxy-terminal consensus sequence (CTCS) are all boxed.

3.4.1.2 Clone pcTRP-1.6 encodes a chicken *Tyrp1* cDNA

Clone pcTRP-1.6, encoding chicken *Tyrp1*, consists of 1771 bp, with 65 bp of 5' *UTR* (Fig. 3.12). The longest ORF of 1605 nts begins with the vertebrate translation initiation consensus sequence A/GNCAUG (Cavener and Ray, 1991) at nt 66 and ends with a TGA termination codon (nts 1671-1673). There is a 3' *UTR* of 101 bp, containing a putative polyadenylation signal (AATAAA) at nts 1752-1757 (Wickens and Stephenson, 1984).

Clone pcTRP-1.6 shares 60.8, 68.8% and 70.1% nt sequence identity with the goldfish (Peng *et al.*, 1994), mouse (Shibahara *et al.*, 1986) and human (Cohen *et al.*, 1990; Urquhart, 1991) *TYRP1* cDNA sequences respectively (Table 3.4). Clone pcTRP-1.6 also shares 70.3% (over 1057 nts) and 38.8% (over 954 nts) nt sequence identity with the partial axolotl (Mason and Mason, 1995) and bovine (Nonneman *et al.*, 1995, unpublished) *Tyrp1* cDNAs respectively. A full length axolotl *Tyrp1* cDNA has been reported (Mason *et al.*, 1996), but as yet this sequence is not available from the GenBank database.

Clone pcTRP-1.6 encodes a deduced polypeptide of 535 aas with a predicted molecular weight of 60.6 kDa. A signal sequence of 23 aa residues, consisting of a hydrophobic core and terminating in alanine (von Heijne, 1986), is present at the N-terminus (Fig. 3.12). Thus, the predicted mature protein is composed of 512 aas with a molecular weight of 58 kDa. There are six potential *N*-glycosylation sites (NXS/T) (Bause, 1983) and a possible 27 aa transmembrane domain near the C-terminus (Kyte and Doolittle, 1982).

The pcTRP-1.6 protein shares 67.6%, 72.8% and 76.2% aa sequence identity with the goldfish (Peng *et al.*, 1994), mouse (Shibahara *et al.*, 1986) and human (Cohen *et al.*, 1990; Urquhart, 1991) TYRP1 proteins respectively (Table 3.4). With conservative aa substitutions, pcTRP-1.6 shares 78.6%, 84.5% and 86.1% aa sequence similarity with the goldfish, mouse and human TYRP1 proteins respectively. The pcTRP-1.6 protein also has 78.1% aa (over 352 aas) sequence identity with the partial axolotl Tyrp1 protein.

Using the GCG command, PILEUP, an aa alignment of published, full-length TYRP1 proteins was generated (Fig. 3.13). The chicken, goldfish, mouse and human TYRP1 protein lengths are 535, 522, 537 and 527 aas respectively. The four conserved aa domains (EGF-like (aas 99-100), CuA (aas 192-224), CuB (aas 377-404) and CTCS (aas 509-415)) described for the vertebrate TYR proteins are all represented in the primary aa sequence of the four published TYRP1 proteins (Fig. 3.13). It is unclear whether the "CuA" and "CuB" sites in TYRP1, bind Cu^{2+} or Fe^{2+} ions (Martinez-Esparza, 1997). Furthermore,

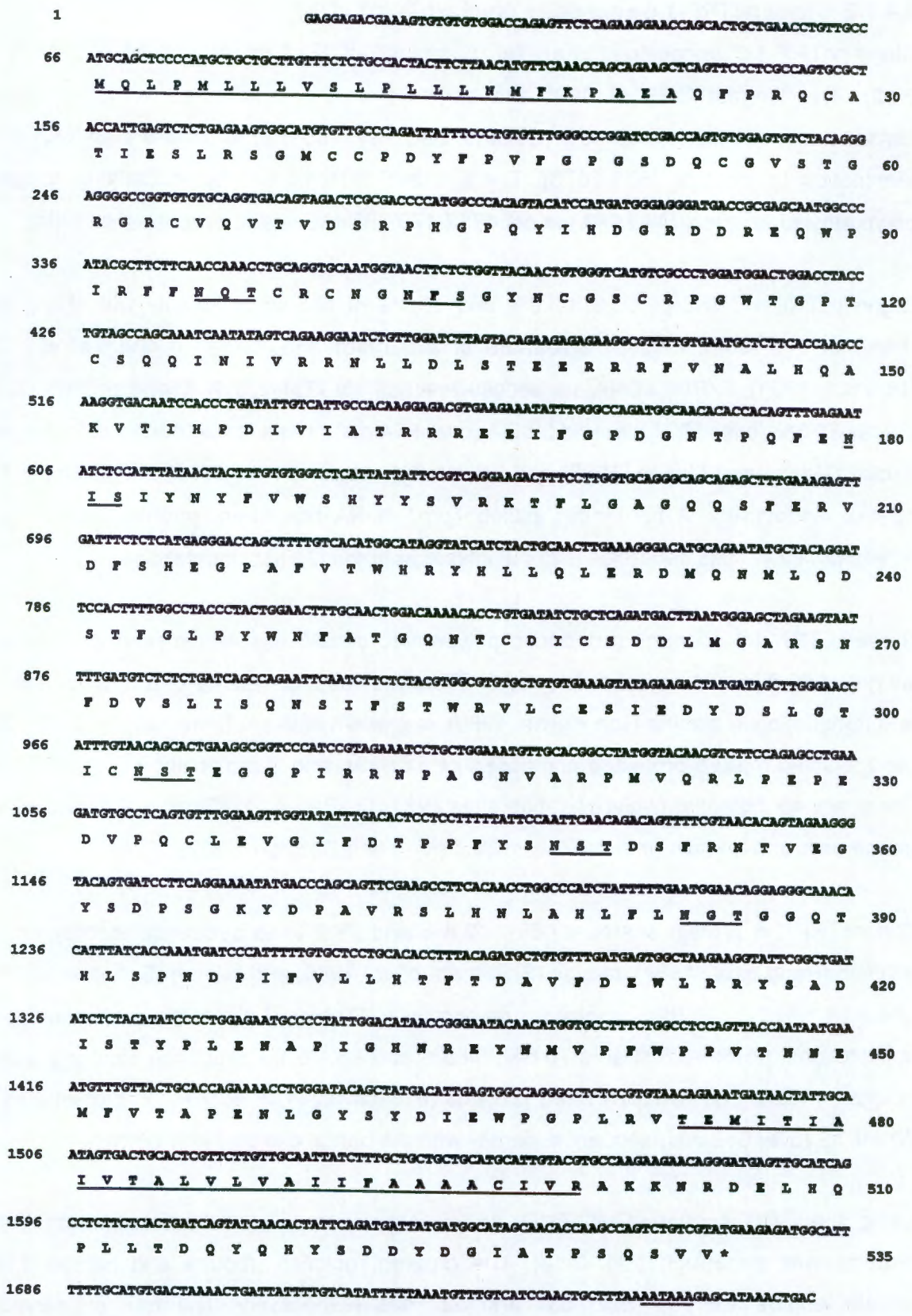

Fig. 3.12. Nucleotide and deduced amino acid sequence of the pcTRP-1.6 cDNA insert, encoding chicken Tyrp1. A putative 23 amino acid leader sequence, six *N*-linked glycosylation sites, a carboxy-terminal hydrophobic membrane-spanning domain and a putative polyadenylation signal are all underlined. The stop codon is indicated with an asterisk. The nucleotide sequence shown here has been deposited in the GenBank database and assigned the accession number AF003631.

Fig. 3.13. Amino acid alignment of TYRP1 proteins from the chicken (cTyrp1; this study), goldfish (gfTyrp1; Peng *et al.*, 1994), mouse (mTyrp1; Shibahara *et al.*, 1986) and human (hTYRP1; Cohen *et al.*, 1990 and Urquhart, 1991) using the Pileup command (Genetics Computer Group (GCG) program (GCG sequence analysis software package, version 9.0, GCG, Inc., Madison, WI, USA)). The protein sequences are represented in one-letter code. Dots (..) have been introduced for optimal alignment. An amino-terminal EGF-like domain, two metal-ion-binding domains, and a carboxy-terminal consensus sequence (CTCS) are all boxed.

the CTCS motif in the vertebrate TYRP1 proteins appears to be a heptapeptide (EXXQPLL) rather than the TYR (EXXPLL) hexapeptide.

3.4.1.3 Clone pcTRP-2.10 encodes a chicken *Tyrp2* cDNA

The nt sequence of the chicken *Tyrp2* cDNA carried by pcTRP-2.10 consists of 2938 bp, with 185 bp of 5' *UTR* (Fig. 3.14). The ORF of 1563 bp begins with GCAAUG at nt 186, a sequence that is similar to the vertebrate translation initiation consensus sequence of A/GNCAUG (Cavener and Ray, 1991) and ends with a TAG termination codon (nts 1749-1751). There is a 3' *UTR* of 1186 bp, including two putative polyadenylation signals (AATAAA) (Wickens and Stephenson, 1984) at nts 2068-2073 and 2898-2903 upstream of the 19 nt poly $(A)^+$ tail. A consensus YGTGTTYY "terminator" sequence (McLauchlan *et al.*, 1985) (nts 2662-2669) as well as four CAYUG recognition elements (nts 1863-1867; 1969-1973; 2129-2133; 2739-2743) (Berget, 1984) are also present. These two sequences are thought to be required for efficient mRNA 3' terminus formation and poly $(A)^+$ cleavage site selection respectively.

Quite unexpectedly, a stretch of 177 nts (nts 2266-2442) within the chicken *Tyrp2* 3' *UTR* (Fig. 3.14) showed 87% sequence identity with a recently sequenced region downstream of the chicken *T-cell receptor alpha chain constant region* and upstream of the *Defender against cell death 1* genes (Wang *et al.*, 1997). The potential significance of this homology is discussed further under section 4.1.

Clone pcTRP-2.10 has 63.4% and 65.5% nt sequence identity with the mouse (Jackson *et al.*, 1992) and human (Bouchard *et al.*, 1994) *TYRP2* cDNA sequences respectively (Table 3.4). Clone pcTRP-2.10 also shares 71.2% nt sequence identity (over 146 nts) with a partial bovine *Tyrp2* cDNA clone (Hawkins *et al.*, 1996).

The chicken *Tyrp2* ORF encoded by pcTRP-2.10 is 521 aas long, with a predicted molecular weight of 59.3 kDa. The first 23 aa residues at the N-terminus are consistent with a signal sequence, containing a hydrophobic core and terminating in alanine (von Heijne, 1986) (Fig. 3.14). Thus, the predicted processed chicken Tyrp2 polypeptide consists of 498 aas with a predicted molecular weight of 56.6 kDa. There are six potential *N*-glycosylation sites (NXS/T) (Bause, 1983) and a potential 18 aa residue transmembrane domain near the C-terminus (Kyte and Doolittle, 1982).

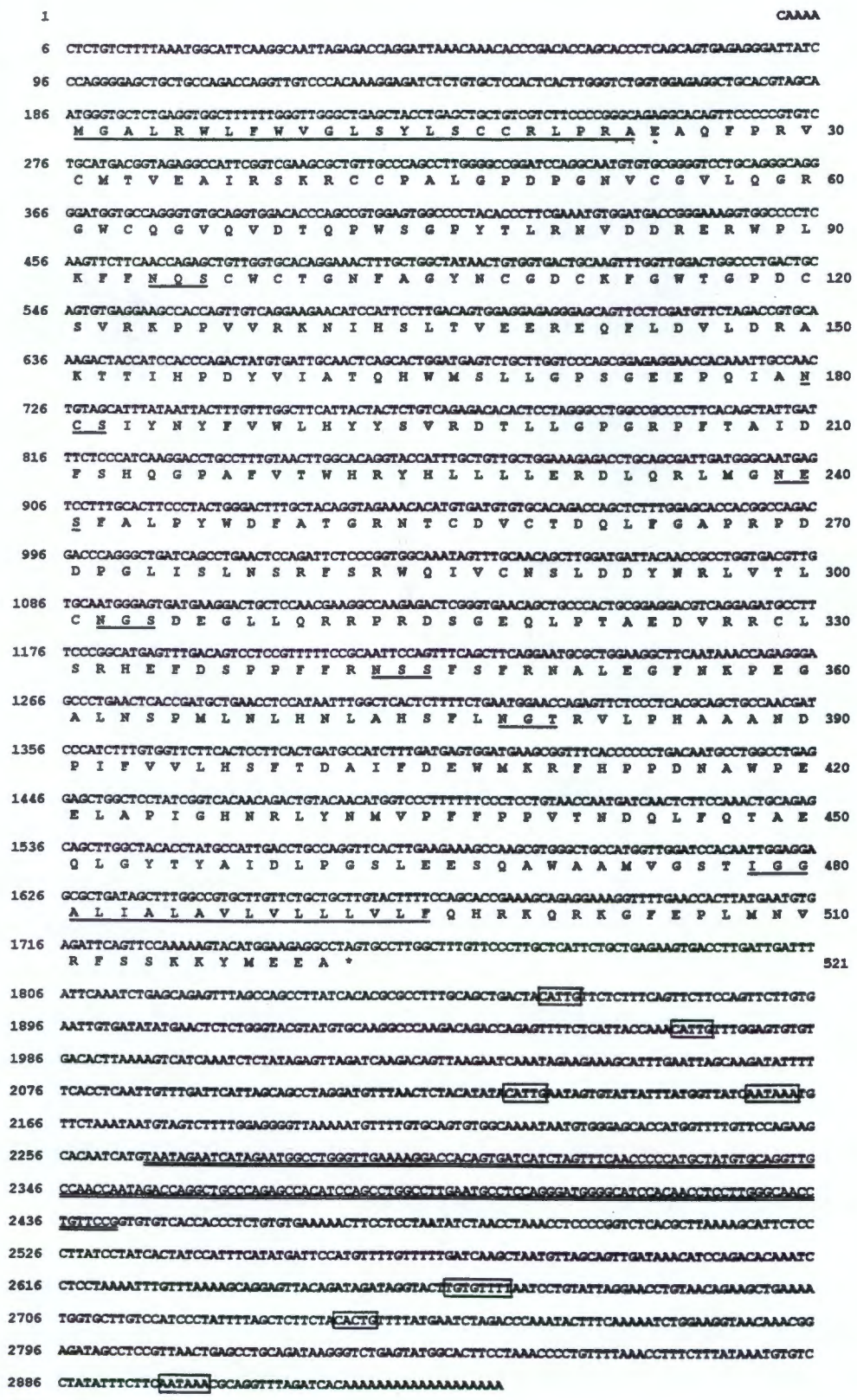

Fig. 3.14. Nucleotide and deduced amino acid sequence of the pcTRP-2.10 cDNA insert, encoding chicken Tyrp2. A putative 23 amino acid leader sequence, six *N*-linked glycosylation sites and a carboxy-terminal hydrophobic membrane-spanning domain are all underlined. The stop codon is indicated with an asterisk. Four CAYUG, a YTGTGTTYY and two putative polyadenylation signals are all boxed. The stretch of nucleotides within the 3' *UTR*, sharing 87% sequence identity with a region between the chicken *T-cell receptor alpha chain constant region* and the *Defender against cell death 1* genes (Wang *et al.*, 1997) is double underlined. The nucleotide sequence shown here has been deposited in the GenBank database and assigned the accession number AF023471.

The pcTRP-2.10 protein shares 69.1% and 69.8% aa sequence identity and 75.3% and 75.2% similarity with the mouse (Jackson *et al.*, 1992) and human (Bouchard *et al.*, 1994) proteins respectively (Table 3.4).

The chicken, mouse and human TYRP2 protein lengths are 521, 517 and 519 aas respectively. PILEUP (GCG) revealed that three (EGF-like (aas 97-108), CuA (aas 191-222), CuB (aas 370-398)) of the four previously described conserved motifs in TYR and TYRP1 proteins, were present in the avian and mammalian TYRP2 proteins (Fig. 3.15). The "CuA" and "CuB" sites have now been shown to chelate Zn^{2+} ions in mouse Tyrp2 (Solano *et al.*, 1996; Martinez-Esparza *et al.*, 1997). Also, the avian and mammalian TYRP2 proteins lack the CTCS present in their vertebrate TYR and TYRP1 paralogues.

From the primary aa sequences data it is clear that, although there are differences between the three vertebrate TRP members, there are also several similarities. This, therefore strongly suggests that the *TRP* gene family members are all homologues, implying a common evolutionary origin.

3.4.1.4 Comparison of the chicken and mammalian TRP gene families

Homologous genes may arise in two different ways. A lineage may split into two species generating orthologous genes (the same gene in different species), or a gene duplication event within a single genome may generate paralogous genes (homologous genes with different functions) (Jackson, 1994a; Kendrew, 1995). Sequence data have been used previously to infer orthologous and paralogous phylogenetic relationships within and between *TRP* gene family members (Morrison *et al.*, 1994; Jackson, 1994a; Budd and Jackson, 1995; Sturm *et al.*, 1995). In order to shed further light on the origin and phylogenetics of the *TRP* gene family, the aa sequences of the chicken TRPs were compared to the orthologous mouse and human TRP sequences.

The analyses presented here are restricted to TRPs from two vertebrate classes (Aves and Mammalia) and three species (chicken, mouse and human). The nine vertebrate TRP aa sequences have been aligned using PILEUP (GCG) and are shown in Fig. 3.16. Apart from the conservation of the EGF-like, CuA, CuB and CTCS motifs and five *N*-glycosylation sites, there are also a number of highly conserved aa residues within this family. These include 15 cysteine, 13 proline and 12 glycine residues. These clustered residues have been suggested to contribute towards the formation of secondary turns within the vertebrate TRP family (Peng *et al.*, 1994).

```
          1                                                          60
cTyrp2    MGALRWLFWV GLSYLSCCRL PRAEAQFPRV CMTVEAIRSK RCCPALGPDP GNVCGVLQGR
mTyrp2    MGLVGWGLL. .LGCLGCGIL LRARAQFPRV CMTLDGVLNK ECCPPLGPEA TNICGFLEGR
hTYRP2    MSPLWWGFL. .LSCLGCKIL PGAQGQFPRV CMTVDSLVNK ECCPRLGAES ANVCGSQQGR

          61                                  EGF-like domain        120
cTyrp2    GWCQGVQVDT QPWSGPYTLR NVDDRERWPL KFFNQSCWCT GNFAGYNCGD CKFGWTGPDC
mTyrp2    GQCAEVQTDT RPWSGPYILR NQDDREQWPR KFFNRTCKCT GNFAGYNCGG CKFGWTGPDC
hTYRP2    GQCTEVRADT RPWSGPYILR NQDDRELWPR KFFHRTCKCT GNFAGYNCGD CKFGWTGPNC

          121                                                        180
cTyrp2    SVRKPPVVRK NIHSLTVEER EQFLDVLDRA KTTIHPDYVI ATQHWMSLLG PSGEEPQIAN
mTyrp2    NRKKPAILRR NIHSLTAQER EQFLGALDLA KKSIHPDYVI TTQHWLGLLG PNGTQPQIAN
hTYRP2    ERKKPPVIRQ NIHSLSPQER EQFLGALDLA KKRVHPDYVI TTQHWLGLLG PNGTQPQFAN

          181                    Zn A domain                         240
cTyrp2    CSIYNYFVWL HYYSVRDTLL GPGRPFTAID FSHQGPAFVT WHRYHLLLLE RDLQRLMGNE
mTyrp2    CSVYDFFVWL HYYSVRDTLL GPGRPYKAID FSHQGPAFVT WHRYHLLWLE RELQRLTGNE
hTYRP2    CSVYDFFVWL HYYSVRDTLL GPGRPYRAID FSHQGPAFVT WHRYHLLCLE RDLQRLIGNE

          241                                                        300
cTyrp2    SFALPYWDFA TGRNTCDVCT DQLFGAPRPD DPGLISLNSR FSRWQIVCNS LDDYNRLVTL
mTyrp2    SFALPYWNFA TGKNECDVCT DDWLGAARQD DPTLISRNSR FSTWEIVCDS LDDYNRRVTL
hTYRP2    SFALPYWNFA TGRNECDVCT DQLFGAARPD DPTLISRNSR FSSWETVCDS LDDYNHLVTL

          301                                                        360
cTyrp2    CNGSDEGLLQ RRPRD.SGEQ LPTAEDVRRC LSRHEFDSPP FFRNSSFSFR NALEGFNKPE
mTyrp2    CNGTYEGLLR RNKVGRNNEK LPTLKNVQDC LSLQKFDSPP FFQNSTFSFR NALEGFDKAD
hTYRP2    CNGTYEGLLR RNQMGRNSMK LPTLKDIRDC LSLQKFDNPP FFQNSTFSFR NALEGFDKAD

          361                    Zn B domain                         420
cTyrp2    GALNSPMLNL HNLAHSFLNG TRVLPHAAAN DPIFVVLHSF TDAIFDEWMK RFHPPDNAWP
mTyrp2    GTLDSQVMNL HNLAHSFLNG TNALPHSAAN DPVFVVLHSF TDAIFDEWLK RNNPSTDAWP
hTYRP2    GTLDSQVMSL HNLVHSFLNG TNALPHSAAN DPIFVVLHSF TDAIFDEWMK RFNPPADAWP

          421                                                        480
cTyrp2    EELAPIGHNR LYNMVPFFPP VTNDQLFQTA EQLGYTYAID LPGSLEESQA WAAMVGSTIG
mTyrp2    QELAPIGHNR MYNMVPFFPP VTNEELFLTA EQLGYNYAVD L..SEEEAPV WSTTL.SVVI
hTYRP2    QELAPIGHNR MYNMVPFFPP VTNEELFLTS DQLGYSYAID LPVSVEETPG WPTTL.LVVM

          481                                                  522
cTyrp2    GALIALAVLV LLLVLFQHRK QRKGFEPLMN VRFSSKKYME EA
mTyrp2    GILGAFVLLL GLLAFLQYRR LRKGYAPLME TGLSSKRYTE EA
hTYRP2    GTLVALVGLF VLLAFLQYRR LRKGYTPLME THLSSKRYTE EA
```

Fig. 3.15. Amino acid alignment of TYRP2 proteins from the chicken (cTyrp2; this study), mouse (mTyrp2; Jackson *et al.*, 1992) and human (hTYRP2; Bouchard *et al.*, 1994) using the Pileup command (Genetics Computer Group (GCG) program (GCG sequence analysis software package, version 9.0, GCG, Inc., Madison, WI, USA)). The protein sequences are represented in one-letter code. Dots (..) have been introduced for optimal alignment. An amino-terminal EGF-like domain and two zinc-binding domains are all boxed.

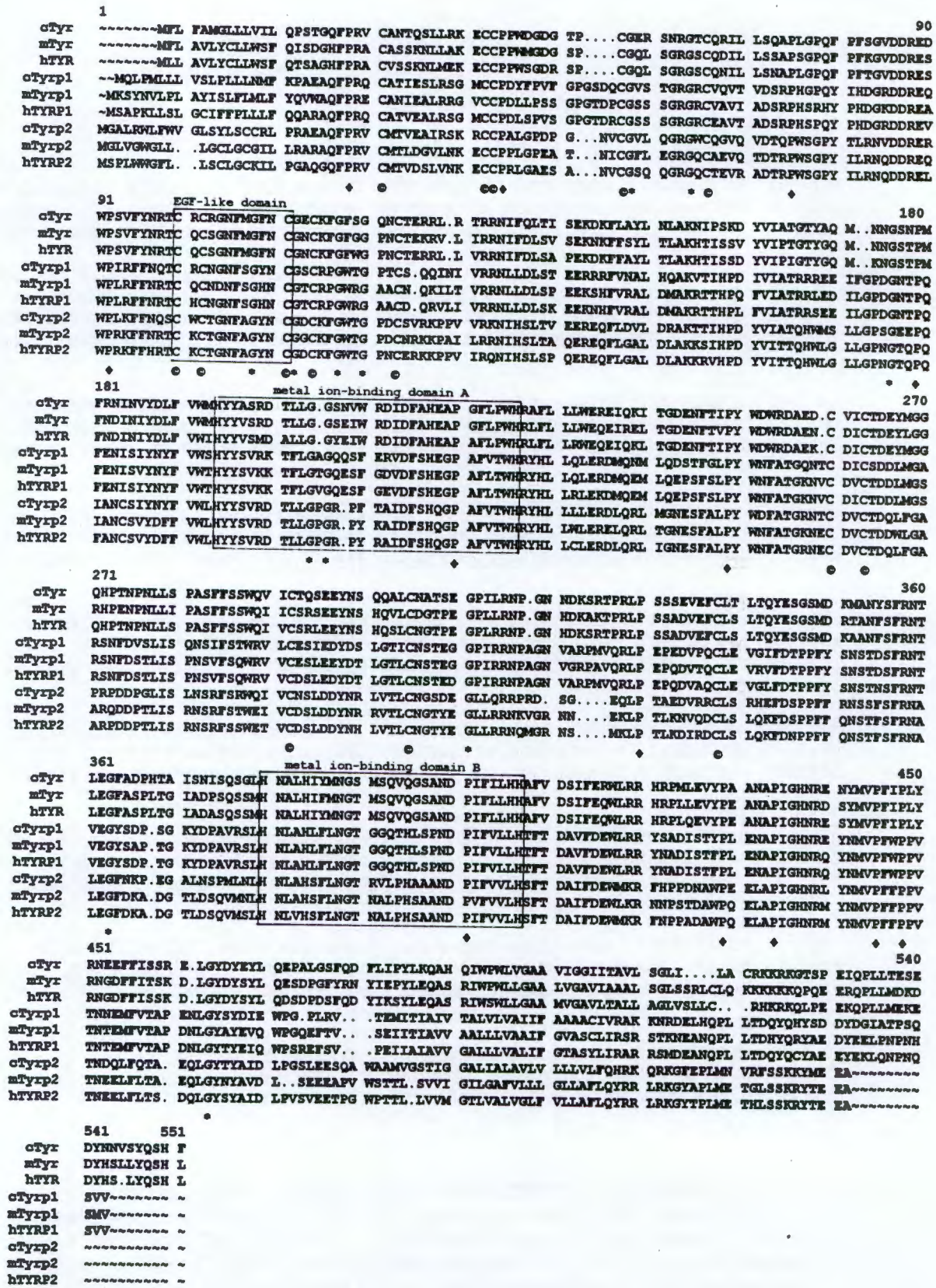

Fig. 3.16. Amino acid sequence alignment of the TRP family from chicken (cTyr = chicken Tyr, this study; cTyrp1 = chicken Tyrp1, this study; cTyrp2 = chicken Tyrp2, this study), mouse (mTyr = mouse Tyr, Müller *et al.*, 1988; mTyrp1 = mouse Tyrp1, Shibahara *et al.*, 1986; mTyrp2 = mouse Tyrp2, Jackson *et al.*, 1992) and human (hTYR = human TYR, Bouchard *et al.*, 1989; hTYRP1 = human TYRP1, Cohen *et al.*, 1990; Urquhart, 1991; hTYRP2 = human TYRP2, Bouchard *et al.*, 1994) using the Pileup command (Genetics Computer Group (GCG) program (GCG sequence analysis software package, version 9.0, GCG, Inc., Madison, WI, USA)). The protein sequences are represented in one-letter code. Dots (..) have been introduced for optimal alignment. An amino-terminal EGF-like domain and two histidine-rich metal-ion-binding domains are boxed. Conserved cysteine (©), proline (♦) and glycine (*) residues are also indicated.

A comparison of the individual chicken TRP members with each other demonstrated, on average, approximately 50% aa sequence identity (Table 3.4). Similar identities are obtained for the mouse and human TRPs. The orthologous chicken and human TRPs (TYR (73.1%), TYRP1 (75.9%) and TYRP2 (69.9) are more similar than the corresponding chicken and mouse Tyrp2 (Tyr (71.8%), Tyrp1 (72.9%) and Tyrp2 (69.2)) (Table 3.4). On comparing chicken TRP paralogues at the aa level, chicken Tyrp1 and Tyrp2 were found to share a higher sequence identity (49.8%) than either chicken Tyrp1 and Tyr (40.0%) or chicken Tyrp2 and Tyr (38.9%). Furthermore, chicken Tyrp1 and Tyr (40.5%) are more similiar than chicken Tyrp2 and Tyr (38.9%). Similarly, mammalian TYRP1s and TYRP2s are more identical than either mammalian TYRP1s and TYRs or TYRP2s and TYRs. Mammalian TYRP1s and TYRs are also more similar than TYRP2s and TYRs (Table 3.4).

In summary, clones B8.3, pcTRP-1.6 and pcTRP-2.10 each carries the entire coding sequence for chicken *Tyr*, *Tyrp1* and *Tyrp2*, respectively. These chicken cDNAs share significant nt and aa sequence homologies with their respective mammalian counterparts. Moreover, the chicken *TRP* gene family displays several highly conserved regions also present in the mouse and human *TRP* gene families. The following experimental sections provide evidence for the *in vivo* expression of the chicken *Tyr*, *Tyrp1* and *Tyrp2* clones isolated here.

3.5 Pigment cell-specific expression of the chicken *TRP* gene family

When this project was first initiated and before the appropriate species-specific *TRP* cDNA probes were available, attempts were made to determine whether pigmented chicken tissues expressed *Tyr* and *Tyrp1* transcripts. RNA was extracted from 10 day old Black Australorp skin and whole eyes and was subjected to northern blot hybridisation analysis using random-primed mouse *Tyr* (Müller *et al.*, 1988) and *Tyrp1* (Shibahara *et al.*, 1986) cDNA probes (see sections 2.4.1 and 2.6 for details of probes). Despite several attempts, which included both the analysis of total RNA and poly $(A)^+$-purified RNA from pigmented chicken tissues, under different stringency conditions, both mouse *Tyr* and *Tyrp1* probes failed to yield cross-hybridising signals. Positive control samples included total RNA from B16-F10 mouse melanoma cells and ethidium bromide staining of the RNA gels revealed that all RNA samples were of equal undegraded quantity and quality. Because it was likely that the hybridisation analysis employed here was not sufficiently sensitive to detect chicken *Tyr* and *Tyrp1* transcripts in both skin and whole eye samples, and because the level of homology between the mouse *Tyr* and *Tyrp1* cDNAs and the orthologous chicken cDNAs

was not known, further northern blot cross-hybridisation experiments were abandoned at that stage.

However, once the three members of the chicken *TRP* gene family had been isolated, northern blot hybridisation analyses were re-attempted in order to determine whether the chicken *TRP* gene family is expressed in a tissue-specific fashion. In addition to using chicken *TRP* cDNA probes, the experimental conditions were further optimised by using total and poly $(A)^+$ RNA from pure chicken retinal pigment epithelial (RPE) primary cell cultures.

In Fig. 3.17a, a single strongly hybridising 2.5 kb transcript for *Tyr* is visible in the lanes containing mRNA (lane 2) and total RNA (lane 3) from cultured chicken RPE cells. No hybridising signals were detected in RNAs from embryonic chicken heart, skin or eye (lanes 4, 5 and 6, respectively), although equivalent amounts of RNA were loaded in each lane (Fig. 3.17b, lanes 1-6).

In Fig. 3.17c, a weakly hybridising 2.3 kb transcript for *Tyrp1* is visible in the lane containing total RNA from 10 day old chicken eye (lane 1). No hybridising signals were detected in RNAs from embryonic chicken heart, skin or eye (lanes 4, 5 and 6, respectively). Ethidium bromide staining revealed that equivalent amounts of RNA were loaded in each lane (Fig. 3.17d, lanes 1-6).

In Fig. 3.17e, a transcript of 3.5 kb and a fainter signal at 5.0 kb are visible in lane containing total RNA from 10 day old chicken eye (lane 1). No *Tyrp2* hybridising signals were observed in RNAs from skin, brain, heart, liver or kidney (lanes 2, 3, 4, 5 and 6). Equivalent amounts of RNA were loaded in each lane as revealed by ethidium bromide staining (Fig. 3.17f, lanes 1-6).

The cloning of the chicken *Tyr*, *Tyrp1* and *Tyrp2* cDNAs in pBluescript SK^- allowed the further analysis of chicken *TRP* gene expression by *in situ* hybridisation. Concurrently with the study described here, *in situ* hybridisation experiments were carried out on embryonic chick tissue using *Tyr* and *Tyrp2* riboprobes. The chicken *Tyr* and *Tyrp2 in situ* hybridisation experiments were performed by Tamara Franz (Franz, 1996) (eye) and Dominique Koubovec (skin) in the Department of Anatomy and Cell Biology, Medical School, University of Cape Town, South Africa. Using digoxigenin-labelled antisense chicken *Tyr* and *Tyrp2* riboprobes, *Tyr* gene expression in the eye was found to be restricted to the RPE layer only.

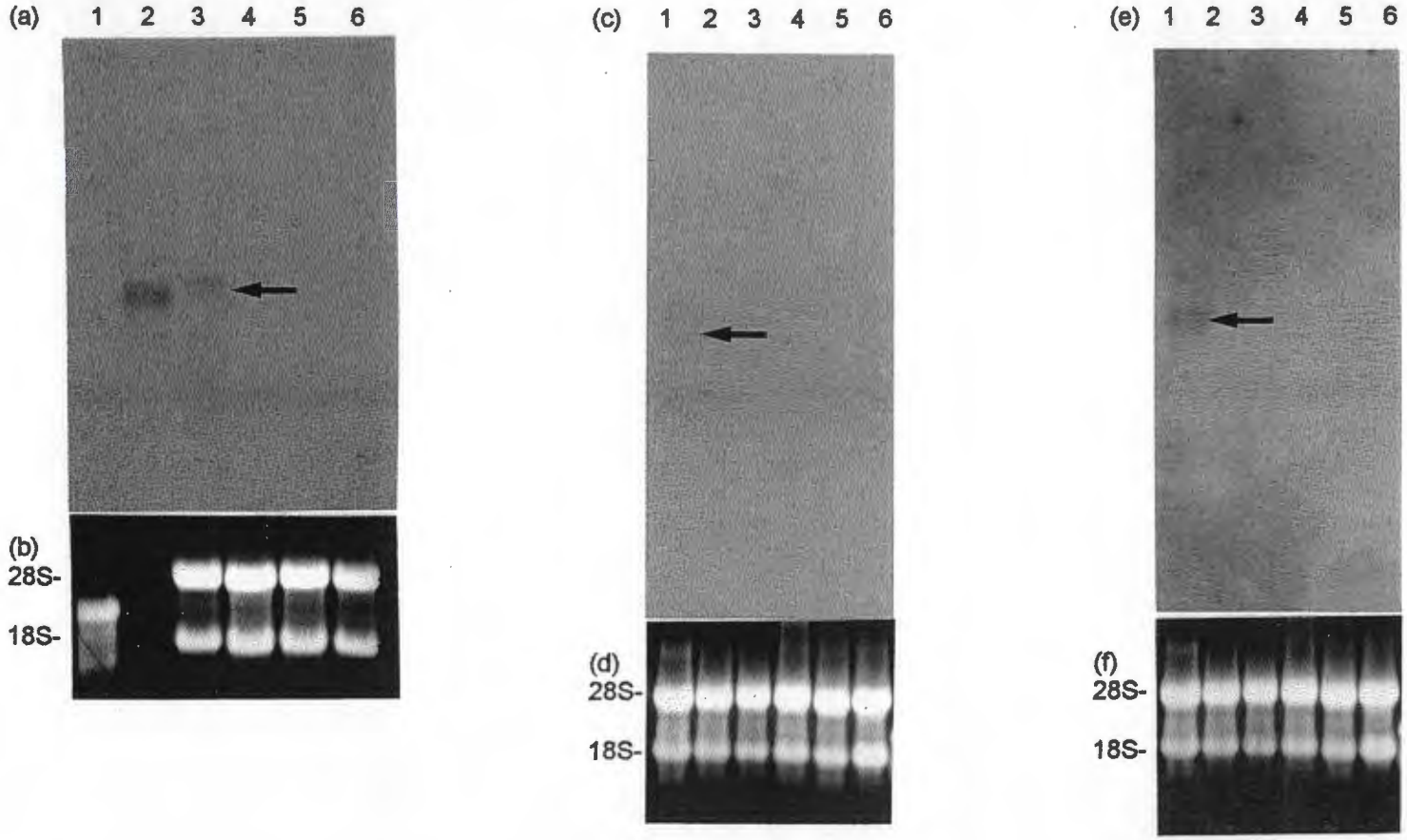

Fig. 3.17. Pigment cell-specific expression of the chicken *TRP* gene family. Northern blot hybridisation analysis was performed on pigmented and non-pigmented tissues from 10 day old Black Australorp embryos. (a) Ten micrograms of *E. coli* rRNA (lane 1), 3 µg poly A^+ RNA from RPE cultures (lane 2), 10 µg total RNA from each of RPE cultures (lane 3), skin (lane 4), eye (lane 5) and heart (lane 6) were probed with a 1.5 kb *Eco*RI-*Pst*I chicken *Tyr* cDNA probe. A single *Tyr* transcript of 2.5 kb (arrow) is visible in lanes 2 and 3 only. (c) Twenty micrograms total RNA from each of eye (lane 1), skin (lane 2), brain (lane 3), heart (lane 4), liver (lane 5) and kidney (lane 6) were probed with a 1.7 kb *Ava*I chicken *Tyrp1* cDNA probe. A single *Tyrp1* transcript of 2.3 kb (arrow) is visible in lane 1 only. (e) Twenty micrograms total RNA from each of eye (lane 1), skin (lane 2), brain (lane 3), heart (lane 4), liver (lane 5) and kidney (lane 6) were probed with a 1.3 kb *Bam*HI chicken *Tyrp2* cDNA probe. A major *Tyrp2* transcript of 3.5 kb (arrow) and a fainter signal of 5 kb is visible in lane 1 only. (b), (d) and (f) are the corresponding regions of the RNA gels showing the quality and quantity of the RNAs loaded as assessed by ethidium bromide staining of the 28S (4.6 kb) and 18S (1.6 kb) rRNAs.

with pigment granules concentrated apically, and *Tyr* transcripts localised more basally (Figs. 3.18a and b). Similar patterns of RPE-specific expression were obtained with the *Tyrp2* riboprobe, with *Tyrp2* expression preceeding that of *Tyr* expression. Furthermore, in 10 day old chicken skin, *Tyrp2* gene expression is restricted to pigment-containing melanocytes concentrated around the base of feather follicles (Figs. 3.18c and d). A few unpigmented cells were also labelled by the *Tyrp2* riboprobe. A similar pattern of melanocyte-specific expression, within the skin, is obtained for *Tyr* gene expression.

Taken together therefore, both northern blot and *in situ* hybridisation experiments clearly indicate that the chicken *TRP* gene family is indeed preferentially transcribed in a pigment cell-specific manner. To confirm that the chicken *TRP* cDNAs isolated here were encoded by separate loci, the next part of this study examined the genomic structure of the chicken *TRP* gene family.

3.6 The chicken and mammalian *TRP* genes share a common genomic structural organisation

As a preliminary step towards analysing the genomic structure of the chicken *TRP* gene family, as well as contributing towards the understanding of the molecular mechanisms of the evolution of the vertebrate *TRP* gene family, the chicken *Tyr*, *Tyrp1* and *Tyrp2* loci were investigated, using the chicken *TRP* cDNA probes described here.

Unlike Black Australorp (B) chickens, which have a black plumage and pigmented eyes, White Plymouth Rock X Pile Game (W) chickens exhibit a white plumage, with pigmented eyes. In addition, the W breed carries both the *Dominant white (I)* and *recessive white (c)* mutations (Smyth, 1991). In order to determine whether there are any large genomic differences between the B and W breeds at either the *Tyr*, *Tyrp1* or *Tyrp2* loci, Southern blot hybridisation analysis of genomic DNA from both B and W fowl breeds was carried out.

3.6.1 The chicken *Tyr* locus

Genomic DNA from B and W chickens was digested with restriction enzymes that cut the 1.9 kb chicken *Tyr* cDNA (Fig. 3.10) zero times (*Eco*RI and *Kpn*I) or once (*Bam*HI, *Hin*cII, *Pvu*II and *Xho*I). Several strongly hybridising genomic bands were observed after hybridisation with a chicken *Tyr* cDNA probe (Fig. 3.19). The average sum of labelled fragment sizes for the seven enzymes used in this analysis is 9 kb, with sizes ranging from 5 to 19 kb. These results suggest that the chicken *Tyr* gene contains several introns. No gross differences were observed between the B and W chicken *Tyr* loci.

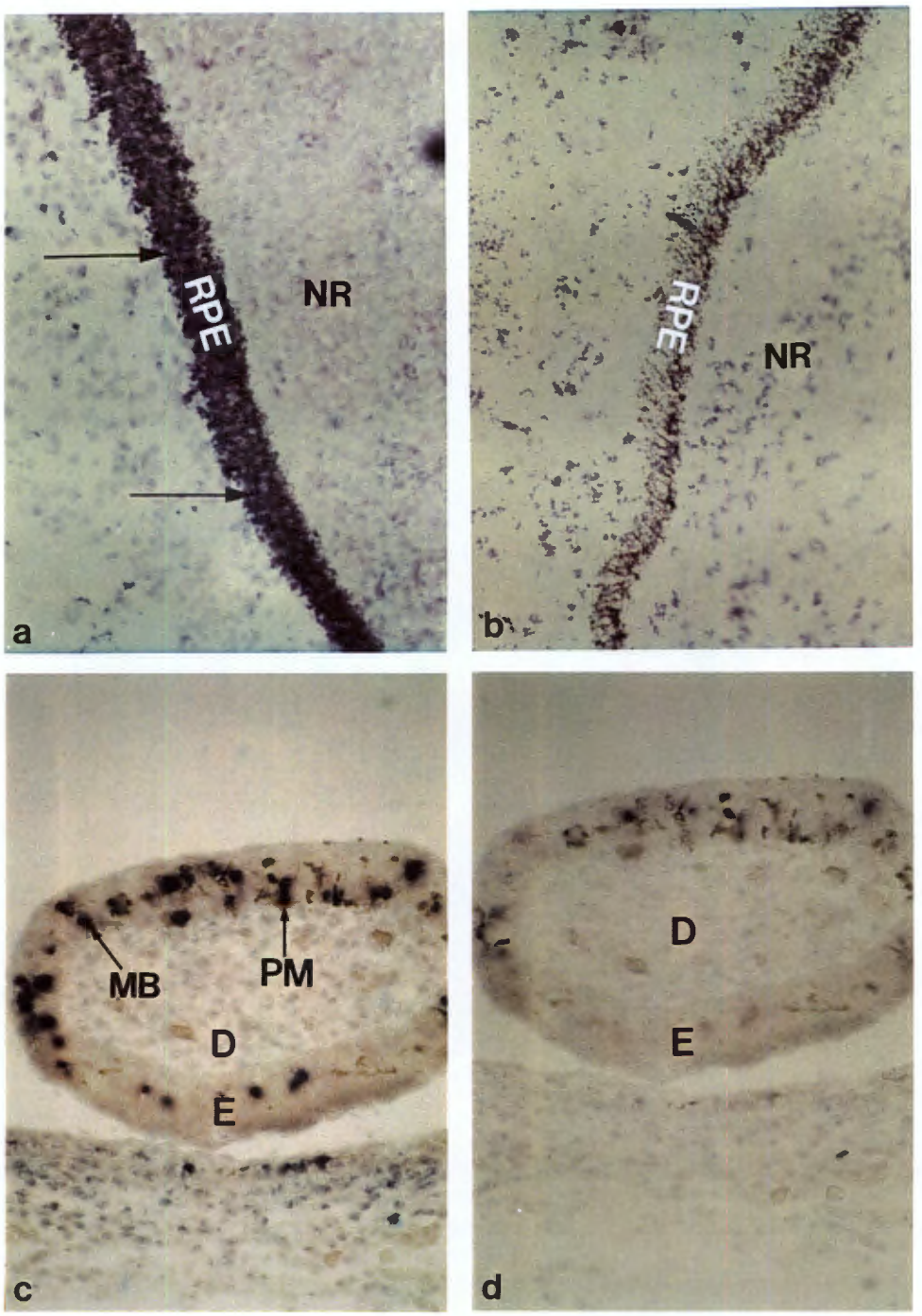

Fig. 3.18. Pigment cell-specific expression of the chicken *Tyr* and *Tyrp2* genes using *in situ* hybridisation. (a) Transverse section through a 6 day old White Plymouth Rock X Pile Game chick eye. *Tyr* transcripts (blue staining, arrows) are localised within the retinal pigment epithelium (RPE) only (bright field, x155) and not in cells of the neuroretina (NR). (b) Similar section as (a), but without *Tyr* riboprobe (bright field, x155). (c) Transverse section through the skin of a 10 day old Black Australorp embryo. *Tyrp2* transcripts (blue staining, arrows) are localised within pigmented melanocytes (PM) as well as possible unpigmented melanoblasts (MB) of the epidermis (E) and dermis (D) (bright field, x130). (d) Similar section as (c), but without *Tyrp2* riboprobe (bright field, x130). Micrographs courtesy of T. Franz (eye) and D. Koubovec (skin).

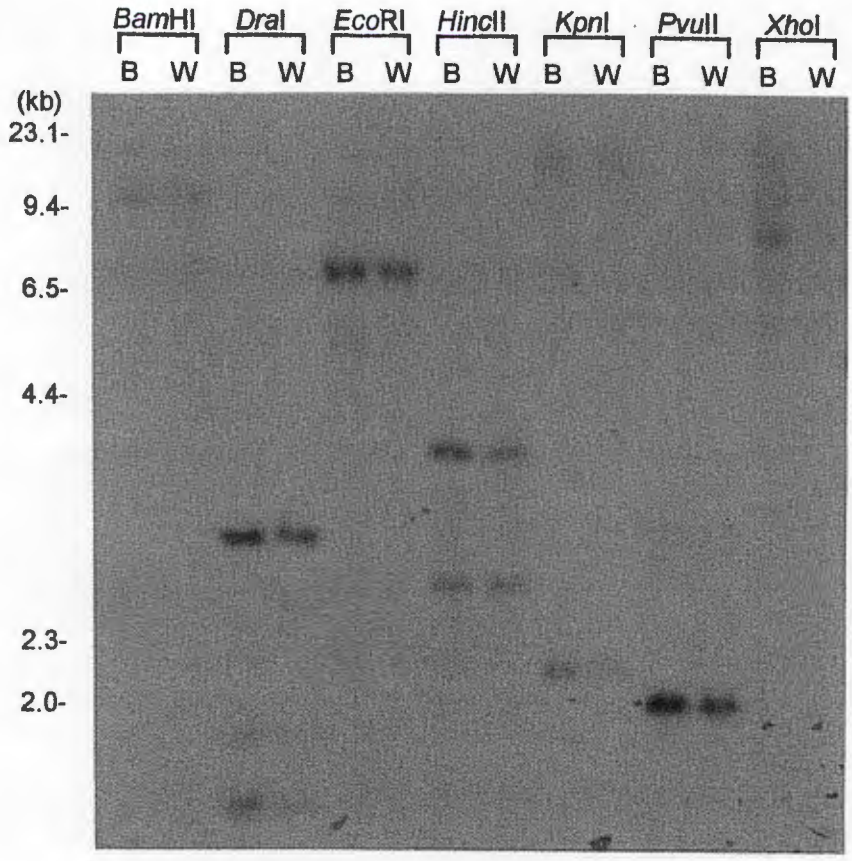

Fig. 3.19. Genomic Southern blot hybridisation analysis of the *Tyr* locus in Black Australorp (B) and White Plymouth Rock X Pile Game (W) chickens. Genomic DNA (20 µg) prepared from 10 day old B and W embryos was digested with *Bam*HI, *Dra*I, *Eco*RI, *Hinc*II, *Kpn*I, *Pvu*II and *Xho*I and subjected to Southern blot hybridisation using a radiolabelled *Eco*RI-*Kpn*I 1.9 kb chicken *Tyr* cDNA probe. The molecular weight (kb) marker is λ/*Hind*III.

3.6.2 The chicken *Tyrp1* locus

For analysis of the *Tyrp1* locus in chickens, genomic DNA was digested with *Dra*I, *Eco*RI, *Kpn*I, *Pvu*II and *Xho*I. This revealed respectively, three (2.5 kb, 2.1 kb and 1.2 kb), three (5 kb, 2.6 kb and 2.2 kb), two (6 kb and 5 kb), two (6 kb and 1.9 kb) and one (8 kb) strongly-hybridising bands (Fig. 3.20). Because there are no *Kpn*I, *Pvu*II and *Xho*I sites, one *Eco*RI and two *Dra*I sites in the chicken *Tyrp1* cDNA, these results suggest that the chicken *Tyrp1* gene has several introns and may span 5 to 11 kb. A comparison of the Southern blot hybridisation analysis of B and W genomic DNA revealed no gross differences (deletions or rearrangements) at the chicken *Tyrp1* locus.

3.6.3 The chicken *Tyrp2* locus

Genomic DNA from the B and W breeds was digested with several different restriction enzymes and subjected to Southern blot hybridisation analysis. A 2.7 kb *Sma*I-*Xho*I chicken *Tyrp2* cDNA fragment was used as a probe, since this fragment included almost the entire insert carried by pcTRP-2.10, except for 249 bp of the 5' end (Fig. 3.14). The results of this Southern blot hybridisation experiment are shown in Fig. 3.21. Despite repeated attempts, smeared signals were obtained in all lanes, with no clear bands visible.

To determine whether the non-specific hybridisation smears were due to partial digestion of the genomic DNA, the same stripped blot was reprobed with a 1.9 kb chicken *Tyr* cDNA probe. The results from this experiment were similar to that obtained for Fig. 3.19, indicating that the genomic DNA had indeed been digested sufficiently to yield an interpretable result. This result suggested that the non-specific hybridisation patterns obtained may have been peculiar to the chicken *Tyrp2* cDNA probe.

Because the chicken *Tyrp2* probe used in the above analyses carries an 18 nt poly $(A)^+$ tail, it is possible that this sequence may have contributed to the non-specific smeared signals obtained. To exclude this poly $(A)^+$-rich region from the probe, a 2.5 kb *Eco*RI-*Nde*I chicken *Tyrp2* cDNA fragment was used to reprobe the same stripped blot. This fragment contains the entire insert carried by pcTRP-2.10, apart from the last 389 nts of the 3' *UTR*. The results of this probing were not significantly different from the initial probing (Fig. 3.20), with smeared signals observed in all lanes (results not shown).

On further detailed analysis of the unusually long 1186 bp 3' *UTR* of clone pcTRP-2.10, it was discovered that 177 bp (nts 2262-2442) share a significantly high nt sequence identity (87%) with a region between the chicken *T-cell receptor alpha chain constant* and the

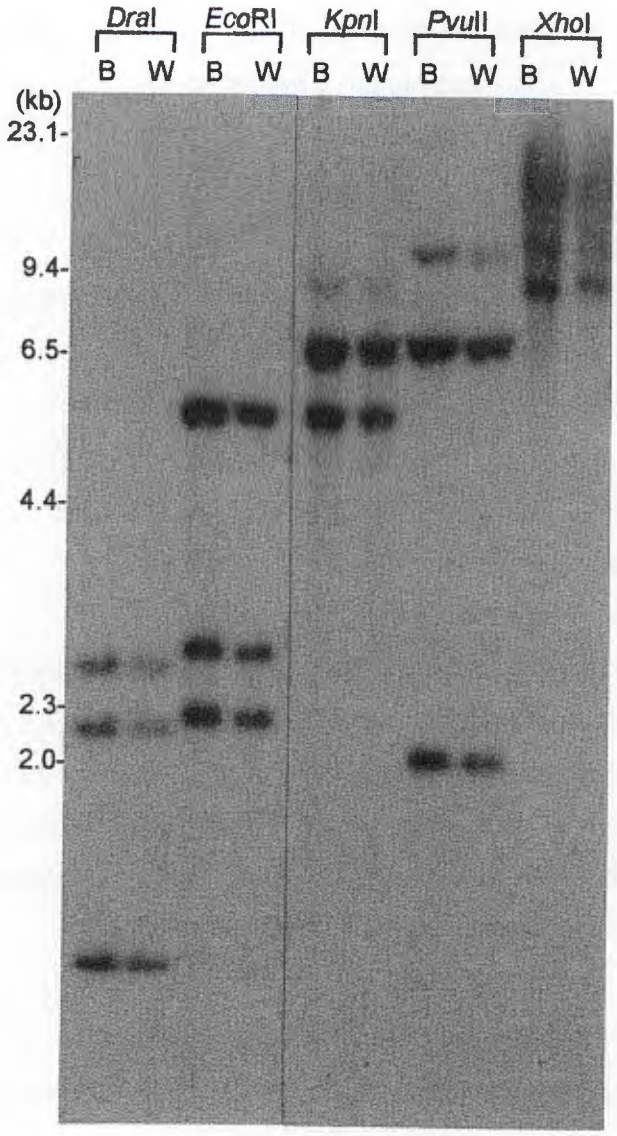

Fig. 3.20. Genomic Southern blot hybridisation analysis of the *Tyrp1* locus in Black Australorp (B) and White Plymouth Rock X Pile Game (W) chickens. Genomic DNA (20 µg) prepared from 10 day old B and W embryos was digested with *Dra*I, *Eco*RI, *Kpn*I, *Pvu*II and *Xho*I and subjected to Southern blot hybridisation using a radiolabelled *Ava*I 1.7 kb chicken *Tyrp1* cDNA probe. The molecular weight (kb) marker is λ/*Hind*III.

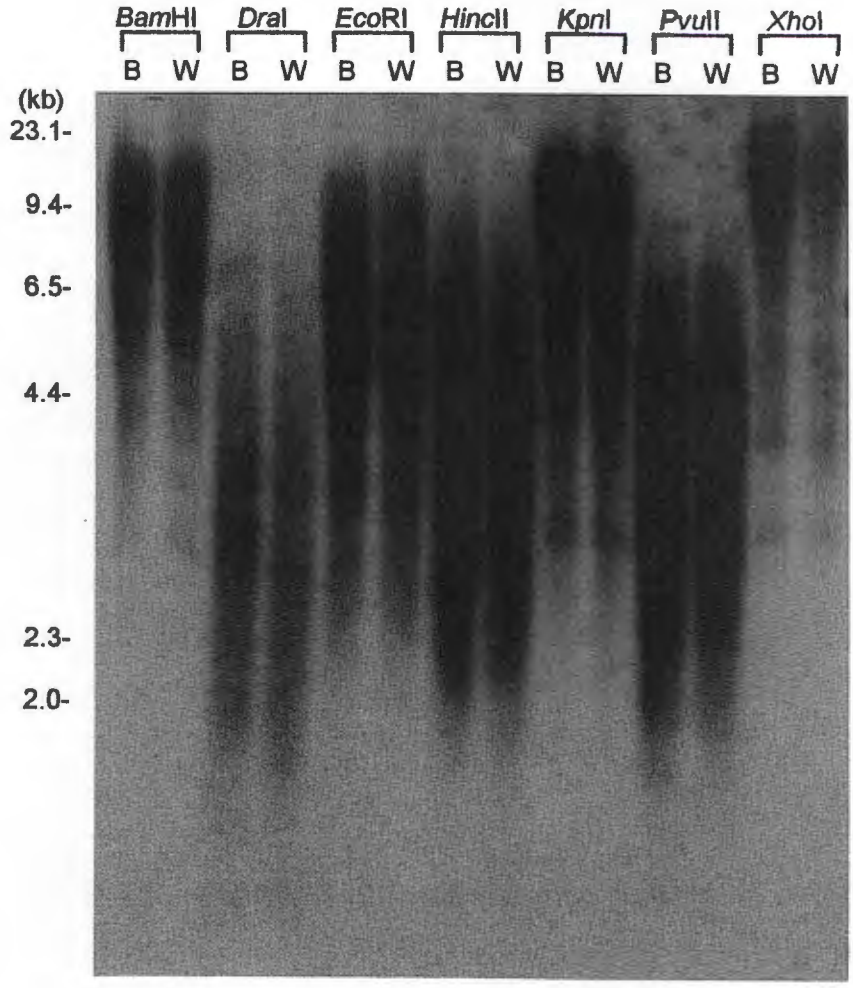

Fig. 3.21. Genomic Southern blot hybridisation analysis of the *Tyrp2* locus in Black Australorp (B) and White Plymouth Rock X Pile Game (W) chickens. Genomic DNA (20 µg) prepared from 10 day old B and W embryos was digested with *Bam*HI, *Dra*I, *Eco*RI, *Hinc*II, *Kpn*I, *Pvu*II and *Xho*I and subjected to Southern blot hybridisation using a radiolabelled 2.7 kb *Xho*I-*Sma*I chicken *Tyrp2* cDNA probe. The molecular weight (kb) marker is λ/*Hind*III. Smeared signals are visible in all lanes, suggesting non-specific hybridisation of the chicken *Tyrp2* cDNA probe.

defender of death protein-1 genes (Wang *et al.*, 1997) (Fig. 3.14). To investigate the possibility that both the poly $(A)^+$-rich tail as well as this 177 bp region of the chicken *Tyrp2* cDNA may be binding non-specifically to regions of the chicken genome, thus causing the smearing, a 1.3 kb *Bam*HI fragment, which contained most of the coding region of the chicken *Tyrp2* gene was prepared (Fig. 3.14 and section 2.9). The results of this re-hybridisation of the stripped blot with this probe are presented in Fig. 3.22. Several strongly-hybridising bands are clearly visible, indicating that the 177 bp 3' *UTR* and the poly $(A)^+$ tail were indeed responsible for the non-specific streaking obtained in the earlier experiments.

The average sum of labelled fragment sizes for the six restriction enzyme digests used in this analysis is 23 kb, with sizes ranging from 15 to 30 kb. Taken together, these results suggest the presence of a single copy *Tyrp2* gene, containing several introns, that spans approximately 15 to 30 kb of the chicken genome. In four out of six restriction enzyme digests larger *Tyrp2* hybridising bands were observed in the W lanes when compared to the adjacent B lanes. For example, whereas the *Eco*RI+*Hind*III digested DNA yields a 7.5 kb band (Fig. 3.22, arrow) in the W lane, the corresponding band is absent in the B lane. Although a 6.5 kb band is present in both the B and W *Eco*RI+*Hind*III digested samples, the W band is approximately half the intensity of the B band, suggesting a heterozygous difference between the B and W *Tyrp2* loci. Similar 1-2 kb differences were observed for the *Bam*HI, *Eco*RI+*Bam*HI and *Eco*RI+*Hinc*II digests. These results suggest that indeed there are differences between the B and W breeds at the *Tyrp2* locus

In summary, the following conclusions were drawn:

i) the chicken genome contains single copies of each of the *Tyr*, *Tyrp1* and *Tyrp2* genes;

ii) the chicken *TRP* genes each contains several exons and introns;

iii) the *Tyr*, *Tyrp1* and *Tyrp2* loci span approximately 5-19 kb, 5-11 kb and 15-30 kb of the chicken genome respectively.

iv) a comparison of B and W genomic DNA revealed no gross differences at the chicken *Tyr* or *Tyrp1* loci. However, differences between the B and W *Tyrp2* loci were evident. The molecular nature of these differences is as yet unknown.

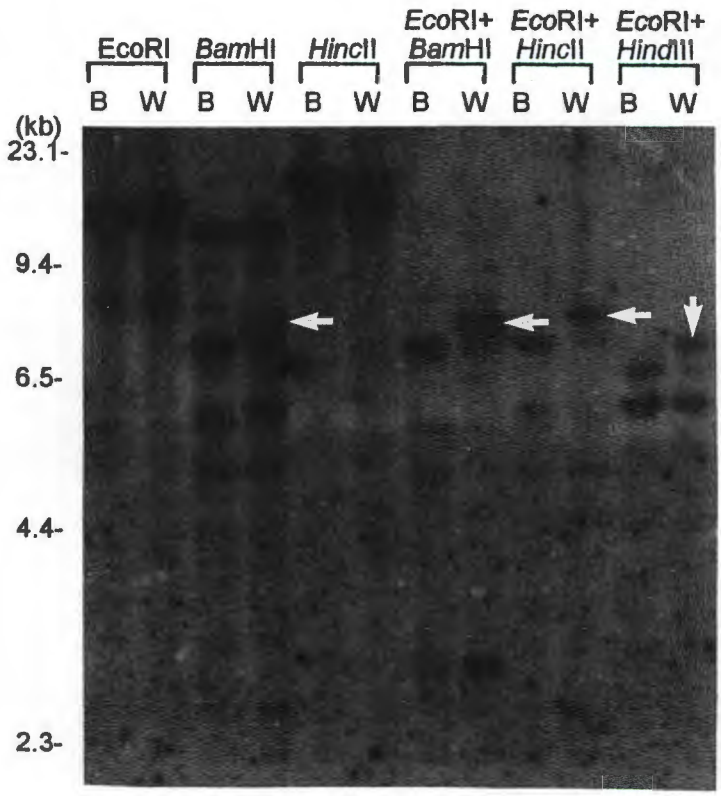

Fig. 3.22. Genomic Southern blot hybridisation analysis of the *Tyrp2* locus in Black Australorp (B) and White Plymouth Rock X Pile Game (W) chickens. Genomic DNA (20 µg) prepared from 10 day old B and W embryos was digested with *Eco*RI, *Bam*HI, *Hinc*II, *Eco*RI+*Bam*HI, *Eco*RI+*Hinc*II and *Eco*RI+*Hind*III and subjected to Southern blot hybridisation. A 1.3 kb *Bam*HI chicken *Tyrp2* cDNA fragment spanning most of the open reading frame was radiolabelled and used as a probe. This cDNA contains no *Bam*HI, *Eco*RI and *Hind*III and one *Hinc*II (position 2809) restriction sites. Arrows indicate differences between B and W *Tyrp2* hybridising bands. The molecular weight (kb) marker is λ/*Hind*III.

CHAPTER 4: DISCUSSION

The broad goal of this study was to further the understanding of the mechanisms responsible for the generation of colour diversity within the bird kingdom. The present study contributes to aspects of this issue by the cloning and characterisation of the chicken *Tyrosinase-related protein (TRP)* gene family and by exploring possible mechanisms whereby this gene family might give rise to diversity of avian colouration. This discussion deals with the evolutionary conservation and divergence of the members of the chicken *TRP* gene family as well as with the differential processing of chicken *TRP* transcripts. Genetic mutations and cellular signalling systems represent additional molecular mechanisms whereby diversity may be generated, and these latter two issues are explored in the context of the molecular genetics of chicken pigmentation, with particular emphasis being placed on candidate mutant pigmentary loci for the chicken *TRP* gene family. The final section of this discussion explores a few speculative issues stemming from this study and attempts to suggest areas of experimentation for future investigation.

4.1 The molecular biology of the chicken *TRP* gene family

This study is the first to describe the cloning and characterisation of all three members of the chicken *TRP* gene family. The chicken now represents only the third organism, after the mouse and human, in which the presence of all three members of the *TRP* gene family has been directly demonstrated. Although other vertebrate *TYR* and *TYRP1* cDNAs have been described, to date only mammalian *TYRP2* cDNAs have been reported (Jackson *et al.*, 1992; Bouchard *et al.*, 1994; Yokoyama *et al.*, 1994a; Cassady and Sturm, 1994; Hawkins *et al.*, 1996). The cell biology and biochemistry of avian melanogenesis has not been as well described as that for mammalian melanogenesis, and it is not known whether the chicken TRPs differ in structure or function from those in other organisms. It may be possible for example, that the diversity of avian colouration may be partially dependent on variations in the trafficking of avian TRPs and/or melanosomes, thus generating pigmentary patterns that are absent in other organisms. For example, the chicken *lavender* mutation which results in a dilution of melanin (Brumbaugh *et al.*, 1972) is thought to stem from an intermediate/microfilament defect in which melanosome translocation to the dendrites is impaired (Mayerson and Brumbaugh, 1981).

Having cloned all three members of the chicken *TRP* gene family, the next step of the present study was to compare the nucleotide (nt) and deduced amino acid (aa) sequences of the chicken TRPs to their orthologous chordate counterparts. This comparison was

carried out in order to learn more about the level of molecular conservation of the *TRP* gene family between avians and other organisms, as well as to determine whether the chicken TRPs possess any structural differences to previously reported TRPs.

The chicken TRP gene family members have significant nt and aa sequence identity with previously reported vertebrate TRPs. The chicken Tyr shares, on average, 65% aa sequence identity with the other vertebrate TYRs (Table 3.4). Similar average aa sequence identities are obtained when aligning either the chicken Tyrp1 or Tyrp2 aa sequences with their respective vertebrate counterparts. The deduced size of the chicken Tyr protein described here (58 kDa) is smaller than the sizes for chicken Tyr reported in biochemical studies (66-220 kDa) (Table 4.1). Given that these Tyrs most likely represent the post-translational modified forms of chicken Tyr, the protein size of 58 kDa described here, appears to be an accurate calculation of the unglycosylated chicken Tyr monomer. Similarly, taking post-translational modification into account, the deduced molecular weights of 58 kDa and 56.6 kDa described here correlate with the reported 65-80 kDa and 65-85 kDa sizes for chicken Tyrp1 and Tyrp2, respectively (Austin and Boissy, 1995). The differences in the molecular weights of reported chicken Tyr may also be attributed to intrinsic breed-specific differences. For example, the study by Doezema (1973b) was conducted on the retinal pigment epithelium (RPE) of White Leghorns, whereas Boissy *et al.* (1987) used cultured melanocytes from Light Brown Leghorns, and Wang *et al.* (1995) used skin melanocytes from White Silky fowls.

The present study demonstrates that the chicken TRPs also share common features with vertebrate TRPs, including an amino (N)-terminal signal sequence, several *N*-glycosylation sites and a carboxy (C)-terminal transmembrane domain. Although these features are not unique to the TRP gene family, the integrity of these three regions has been shown, in mammals, to be important for the subcellular trafficking, and therefore the functioning of the TRPs (Johnson and Jackson, 1992; Halaban *et al.*, 1988b; Kwon *et al.*, 1989; Beermann *et al.*, 1990; Budd and Jackson, 1995). It is likely therefore, that the homologous regions of the chicken TRPs play similar important roles in avian melanocytes.

The three chicken TRPs also contain domains unique to the vertebrate TRPs, including an epidermal growth factor (EGF)-like domain and two histidine-rich metal ion-binding sites. It is thought that the EGF-like domain may facilitate the formation of a melanosomal membrane TRP complex, which may be necessary for efficient melanin biosynthesis. On comparing the EGF-like domain in the chicken TRPs to the same domain in other chordate

Table 4.1. Molecular weights of chicken Tyrosinases

Tissue	Size (kDa)	Reference
RPE	72, 150, 220	Doezema (1973b)
feathers	66	Yamamoto and Brumbaugh (1984)
NCC	72	Oetting *et al.* (1985a, 1985b)
NCC	68, 82	Boissy *et al.* (1987)
NCC	82	Austin and Boissy (1995)
skin	66	Wang *et al.* (1995)
RPE = retinal pigment epithelium; NCC = neural crest cells		

TRPs, the degeneracy of the EGF-like consensus sequence may be further refined from [CXCX(5)GX(2)C] to [CXCXXN(F,Y)XGX(N,D)C]. This suggests that this motif plays a key role in chicken TRP functioning.

Two histidine-rich metal ion-binding domains in the TRP gene family, "CuA" and "CuB", have been shown to be important for the catalytic functions of TRPs (Tripathi *et al.*, 1992; Solano *et al.*, 1996; Martinez-Esparza *et al.*, 1997; Spritz *et al.*, 1997; Furumura *et al.*, 1998) and have been highly conserved in all cloned TRPs as well as in some invertebrate haemocyanins. The results of the present study demonstrate that, as expected, these domains have been conserved in all three chicken TRP orthologues. The first of the TYRP2 metal ion-binding domains, "ZnA", is highly conserved in the chicken with only one aa (Thr) difference out of 33. The same position is occupied by Lys and Arg residues in the mouse and human TYRP2 "ZnA" domains, respectively. Within the "CuB" domain, His389 is replaced by an invariant Leu in both chicken Tyrp1 and Tyrp2 as is the case in mammalian TYRP1 and TYRP2. Similarly, Ser192 and Ser375 are present in both chicken and mammalian TYRs, but are both absent from chicken and mammalian TYRP1s and TYRP2s. Furumura *et al.* (1998) suggest that the substitutions of His389, Ser192 and Ser375 in TYRP1 and TYRP2 may disrupt copper binding in these molecules. Thus far, the only enzymatic activities associated with the chicken melanin biosynthetic pathway have been tyrosine hydroxylase and DOPA oxidase (Yamamoto and Brumbaugh, 1984; Wang *et al.*, 1995). By expressing the cloned chicken *TRP* cDNAs, it will be possible to determine whether these TRPs fulfill similar catalytic functions to that described for the homologous mammalian enzymes.

The results from the present study also demonstrate that both chicken Tyr (EXQPLL) and Tyrp1 (EXXQPLL) contain a carboxy-terminal consensus sequence (CTCS), that is present in the C-termini of all vertebrate TYRs and TYRP1s. Recent evidence strongly suggests that this CTCS is involved in the trafficking of TYR and TYRP1 from the Golgi to the melanosomes (reviewed in section 1.5.1). This CTCS, however, is absent from chicken Tyrp2, which is in concordance with that reported for mammalian TYRP2s (Jackson *et al.*, 1992; Bouchard *et al.*, 1994; Yokoyama *et al.*, 1994a; Cassady and Sturm, 1994) and ascidian Tyr (Sato *et al.*, 1997; Caracciolo *et al.*, 1997). The absence of the CTCS in TYRP2 suggests that this enzyme is targeted to the melanosome by a mechanism different to that utilised by TYR and TYRP1. Winder *et al.* (1993) have suggested that a Gly-Tyr motif present in the mouse and human TYRP2 C-termini, but not present in any of the other TRPs, may be involved in the subcellular sorting of this molecule. However, this Gly-Tyr

motif is not present in the homologous region of the C-terminus of the chicken Tyrp2, and is substituted by Gly-Phe, weakening this hypothesis. On the other hand, a second Gly-Tyr motif (LGYXL), upstream of the CTCS and hydrophobic transmembrane domain, is present in all cloned TRPs, including chick Tyrp2 and ascidian Tyr. It will be of interest to determine whether this motif plays any role in the subcellular localisation of the TRPs.

In addition to the conserved EGF-like domain and the two metal ion-binding domains, the chicken TRPs also share several conserved aa residues with the mammalian TRPs. These clustered residues include 15 cysteines, 13 prolines and 12 glycines, and have been suggested to be "functionally conserved", contributing to the formation of secondary turns within the vertebrate TRP family (Peng *et al.*, 1994). At aa positions 62 and 370 (Fig. 3.16) an invariant Gly residue is found in all cloned vertebrate TRPs, except in chicken Tyr (this report and Mochii *et al.*, 1992), where instead, Asn and Ala residues are found, respectively. Similarly, Gly318 is conserved in all cloned TRPs, apart from chicken Tyrp2, where this position is occupied by an Asp residue. The functional significance, if any, of the substitutions of these Gly residues in chicken Tyr and Tyrp2 is not known.

The chicken *Tyrp2* cDNA sequence also differs from its mammalian counterparts in a second way. An unusually long chicken *Tyrp2* 3' *UTR* was found to contain a stretch of 177 nts sharing a significantly high (87%) sequence identity with a genomic clone spanning the chicken *T-cell receptor alpha chain constant region* and the *Defender against cell death 1* genes (Wang *et al.*, 1997). One explanation for the occurrence of this DNA segment is that it may represent a transposable element generated in the diversification of chicken T-cell receptors. *T-cell receptor* gene rearrangement during the development of mature functional T-cells in the thymus is a well documented immunological phenomenon (Kendrew, 1995). Furthermore, aberrant *T-cell receptor* gene rearrangements (particularly the α and δ genes), in chromosomal domains not encoding antigen receptor genes, have been reported before (Boehm and Rabbitts, 1989). Moreover, on closer inspection of the 177 nt region within the 3' *UTR* of the chicken *Tyrp2* cDNA, a conserved palindromic heptamer sequence (5'-CACAGTG-3') is identifiable. This sequence is known to serve as a recombination signal sequence for the variable and diversity segments of both *immunoglobulin* and *T-cell receptor* genes (Kendrew, 1995).

If this "foreign" DNA does indeed represent aberrant recombination of *T-cell receptor* genes, a number of interesting questions now arise. First, are there specific regions (hotspots) within the chicken *Tyrp2* gene that favour recombination? Secondly, do these regions occur

in other *TYRP2* genes (including other chicken breeds)? Thirdly, what effect, if any, does this foreign DNA region have on chicken *Tyrp2* gene expression and mRNA processing? Answers to questions like these might possibly contribute towards the understanding of mechanisms by which colour diversity is generated. For example, albino i^1/i^1 Medaka fish (*Oryzias latipes*) carry a transposable element, *Tol1*, that disrupts the *Tyr* gene, resulting in premature termination of the *Tyr* transcript (Koga *et al.*, 1995).

Whereas the genomic organisation of the mammalian *TRPs* has been well investigated (Ruppert *et al.*, 1988; Shibahara *et al.*, 1991; Jackson *et al.*, 1991; Ponnazhagan *et al.*, 1994; Sturm *et al.*, 1995; Budd and Jackson, 1995; Box *et al.*, 1998), very little is known about the genomic organisation and structure of the avian *TRP* genes. To date, only the partial quail (Yamamoto *et al.*, 1992) and chicken (Ferguson and Kidson, 1996) *Tyr* promoters have been analysed. In order to shed further light on this issue and to contribute towards the understanding of the evolution of the *TRP* gene family, the nt sequences of the chicken *Tyr*, *Tyrp1* and *Tyrp2* cDNAs were aligned with the orthologous mouse and human *TRP* genomic sequences.

Using the splice site consensus sequence, 5' A/CAGgtaagt....$(c/t)_n$nc/tagG 3' (Jackson, 1991), the sequence and relative positions of the published major 5' donor and 3' acceptor splice sites for the mammalian *TYR*, *TYRP1* and *TYRP2* genes were found to be highly conserved in the orthologous chicken *TRP* cDNAs (Tables 4.2-4.4). The conservation of the spacing of the splice site consensus sequence implies that the number and length of individual exons of the three chicken *TRP* genes are similar in number and size to the corresponding mammalian *TRP* exons (Tables 4.2-4.4). In keeping with this observation, genomic Southern blot hybridisation analysis in the present study, seemed to indicate the presence of several introns (and exons) at each of the chicken *Tyr*, *Tyrp1* and *Tyrp2* loci. Although in each case, the sizes of the chicken *Tyr*, *Tyrp1* and *Tyrp2* loci appear somewhat smaller than their mammalian counterparts (Table 4.5), these differences may not be due to different numbers of exons/introns at each locus, but to the sizes of each of the introns. For example the mouse *Tyr* intron I is 8.2 kb, whereas the homologous human *TYR* intron I is 22 kb (Table 4.2), even though the coding regions of these *TYR* genes span approximately 65 and 70 kb of the mouse (Ruppert *et al.*, 1988) and human (Giebel *et al.*, 1991; Ponnazhagan *et al.*, 1994) genomes respectively.

These structural similarities between the orthologous chicken and mammalian *TRP* genes suggest that the exon/intron splice boundaries for the chicken *TRP* gene family are found in

Table 4.2. Exon-intron boundaries of the chicken, mouse and human *TYR* genes

Gene	Exon no.	Exon size (bp)	5' donor.....3' acceptor	Intron no.	Intron size (kb)	Reference
chicken *Tyr*	1	?	CAG/?.....?/GTA Q V	1	?	this study
mouse *Tyr*	1	901	CAG/gtaa.....ccag/ATC	1	8.2	Ruppert *et al.* (1988)
human *TYR*	1	865	CAG/gtaa.....acag/ATT Q I	1	22	Giebel *et al.* (1991)
chicken *Tyr*	2	217	GAA G/?.....?/GC TTT	2	?	this study
mouse *Tyr*	2	217	GAA G/gtaa....acag/GA TTT	2	11.5	
human *TYR*	2	217	GAA G/gtaa....acag/GA TTT E S I	2	>26	Ruppert *et al.* (1988) Giebel *et al.* (1991)
chicken *Tyr*	3	148	GAC AG/?.....?/C ATT	3	?	this study
mouse *Tyr*	3	148	GAC AG/gttg.....gtag/T ATT	3	35.3	
human *TYR*	3	148	GAC AG/gttg....gcag/T ATT D S I	3	10	Ruppert *et al.* (1988) Giebel *et al.* (1991)
chicken *Tyr*	4	182	CCA G/?.....?/CA CTT P A L	4	?	this study
mouse *Tyr*	4	182	TCA G/gtaa....tcag/AT CCA	4	6.5	Ruppert *et al.* (1988)
human *TYR*	4	182	TCA G/gtaa....tcag/AC CCA S D P	4	9	Giebel *et al.* (1991)
chicken *Tyr*	5	?	-	-	-	Ruppert *et al.* (1988)
mouse *Tyr*	5	473	-	-	-	
human *TYR*	5	?	-	-	-	

Table 4.3. Exon-intron boundaries of the chicken, mouse and human *TYRP1* genes

Gene	Exon no.	Exon size (bp)	5' donor.....3' acceptor	Intron no.	Intron size (kb)	Reference
chicken *Tyrp1*	1	?	?	1	?	this study
	1	87-188	CATG/gtac.....tcag/CTGT	1A	0.591-0.7	Jackson *et al.* (1991); Shibahara *et al.* (1991)
mouse *Tyrp1*	1	93-194	ACTG/gtga.....tcag/CTGT	1B	0.585-0.7	Jackson *et al.* (1991)
	1	108-209	TGTG/gtgg.....tcag/CTGT	1C	0.57-0.7	Jackson *et al.* (1991)
human *TYRP1*	1	30	TGAG/gtaa.....tcag/CTGG	1	0.42	Sturm *et al.* (1995); Box *et al.* (1998)
chicken *Tyrp1*	2	?	ATA G/?.....?/TC AGA I V R	2	?	this study
mouse *Tyrp1*	2	470	ACA G/gtga....aaag/TC AGG T V R	2	2	Jackson *et al.* (1991); Shibahara *et al.* (1991)
human *TYRP1*	2	470	ATA G/gtaa. ..aaag/TC AGG I V R	2	1.133	Sturm *et al.* (1995); Box *et al.* (1998)
chicken *Tyrp1*	3	323	CAG/?.....?/AAT Q N	3	?	this study
mouse *Tyrp1*	3	323	CAG/gtat.....ctag/GAG	3	2.75/3	Jackson *et al.* (1991); Shibahara *et al.* (1991)
human *TYRP1*	3	323	CAG/gtat.....ctag/GAA Q E	3	2.614	Sturm *et al.* (1995); Box *et al.* (1998)
chicken *Tyrp1*	4	205	AAC A/?.....?/GC ACT	4	?	this study
mouse *Tyrp1*	4	205	AAC A/gtaa....ccag/GC ACT	4	1.6/4.5	Jackson *et al.* (1991); Shibahara *et al.* (1991)
human *TYRP1*	4	205	AAC A/gtaa....gcag/GC ACC N S T	4	3.619	Sturm *et al.* (1995); Box *et al.* (1998)
chicken *Tyrp1*	5	168	GAA G/?.....?/GG TAC	5	?	this study
mouse *Tyrp1*	5	168	GAA G/gcaa....gtag/GT TAC	5	1.7/1.8	Jackson *et al.* (1991); Shibahara *et al.* (1991)
human *TYRP1*	5	168	GAA G/gcaa....ctag/GT TAC E G Y	5	2.094	Sturm *et al.* (1995); Box *et al.* (1998)
chicken *Tyrp1*	6	180	GCT G/?.....?/AT ATC	6	?	this study
mouse *Tyrp1*	6	180	GCC G/gtga.....ttag/AT ATT	6	2.45-2.9	Jackson *et al.* (1991); Shibahara *et al.* (1991)
human *TYRP1*	6	180	GCT G/gtaa.....ttag/AT ATA A D I	6	3.291	Sturm *et al.* (1995); Box *et al.* (1998)
chicken *Tyrp1*	7	147	CCA G/?.....?/GG CCT P G P	7	?	this study
mouse *Tyrp1*	7	147	CCA G/gtcg....atag/GT CAG P G Q	7	0.7-0.8	Jackson *et al.* (1991); Shibahara *et al.* (1991)
human *TYRP1*	7	147	CCA A/gtga....atag/GT CGG P S R	7	0.834	Sturm *et al.* (1995); Box *et al.* (1998)
chicken *Tyrp1*	8	?	-	-	-	this study
mouse *Tyrp1*	8	1041	-	-	-	Jackson *et al.* (1991); Shibahara *et al.* (1991)
human *TYRP1*	8	1274	-	-	-	Sturm *et al.* (1995); Box *et al.* (1998)

Table 4.4. Exon-intron boundaries of the chicken, mouse and human *TYRP2* genes

Gene	Exon no.	Exon size (bp)	5' donor.....3' acceptor	Intron no.	Intron size (kb)	Reference
chicken *Tyrp2*	1	?	ACA G/?.....?/GA AAC	1	?	this study
mouse *Tyrp2*	1	758	ACA G/gtga.....tcag/GA AAC	1	8.8	Budd and Jackson (1995)
human *TYRP2*	1	722	ACA G/gtga.....ctag/GA AAC T G N	1	9.4	Sturm *et al.* (1995)
chicken *Tyrp2*	2	300	CTA G/?.....?/GG CCT	2	?	this study
mouse *Tyrp2*	2	300	TTA G/gtgg.....ttag/GT CCA	2	2.4	Budd and Jackson (1995)
human *TYRP2*	2	300	TTA G/gtgg.....tcag/GA CCA L G P	2	?	Sturm *et al.* (1995)
chicken *Tyrp2*	3	101	CAG/?.....?/CGA	3	?	this study
mouse *Tyrp2*	3	101	CAG/gttg.....ttag/AGA	3	0.75	Budd and Jackson (1995)
human *TYRP2*	3	101	CAG/gtag.....ttag/CGA Q R	3	?	Sturm *et al.* (1995)
chicken *Tyrp2*	4	167	AAC AG/?.....?/C TTG N S L	4	?	this study
mouse *Tyrp2*	4	167	GAC AG/gtga.....ttag/C TTG	4	3	Budd and Jackson (1995)
human *TYRP2*	4	167	GAT AG/gtaa.....ttag/C TTG D S L	4	?	Sturm *et al.* (1995)
chicken *Tyrp2*	5	177	TTC AG/?.....?/G AAT	5	?	this study
mouse *Tyrp2*	5	180	TTC AG/gtag.....tcag/G AAT	5	2	Budd and Jackson (1995)
human *TYRP2*	5	180	TTC AG/gtgg.....ctag/G AAT F R N	5	?	Sturm *et al.* (1995)
chicken *Tyrp2*	6	136	GTG/?.....?/GTT	6	?	this study
mouse *Tyrp2*	6	136	GTG/gtat.....gcag/GTC	6	?	Budd and Jackson (1995)
human *TYRP2*	6	136	GTG/gtat.....gcag/GTT V.....V	6	?	Sturm *et al.* (1995)
chicken *Tyrp2*	7	202	CCA G/?.....?/GT TCA P G S	7	?	this study
mouse *Tyrp2*	7	202	TCA G/gtga....gcag/AG GAA S S E	7	?	Budd and Jackson (1995)
human *TYRP2*	7	202	CCA G/gtaa.....ttag/TT TCA P V S	7	?	Sturm *et al.* (1995)
chicken *Tyrp2*	8	?	-	-	-	this study
mouse *Tyrp2*	8	>396	-	-	-	Budd and Jackson (1995)
human *TYRP2*	8	>179	-	-	-	Sturm *et al.* (1995)

Table 4.5. Genomic structure of the chicken, mouse and human *TRP* gene family

Locus name	Organism	Locus size (kb)	Exons (no)	Introns (no)	Reference
albino	chicken	5-19	5?	4?	this study
colour (c)	mouse	>100	5	4	Ruppert *et al.* (1988)
Tyr/TYR	human	>50	5	4	Giebel *et al.* (1991)
brown (b)	chicken	5-11	8?	7?	this study
Tyrp1/TYRP1	mouse	15-18	8	7	Jackson *et al.* (1991); Shibahara *et al.* (1991)
	human	24	8	7	Sturm *et al.* (1995); Box *et al.* (1998)
slaty (slt)	chicken	15-30	8?	7?	this study
Tyrp2/TYRP2	mouse	50-55	8	7	Budd and Jackson (1995)
	human	55	8	7	Sturm *et al.* (1995

virtually homologous positions to those reported for the corresponding mouse and human *TRP* genes. Although it is often difficult to predict with any certainty where exon/intron boundaries occur (Dibb, 1993), the predications made here may now be tested experimentally by primed sequencing of the exon/intron boundaries of the chicken *TRP* genes and by comparison to the corresponding mammalian counterparts. If the exon/intron boundaries of the mammalian *TRP* genes do indeed occur at homologous positions within the chicken *TRP* orthologues, this would imply that the chicken *TRP* gene family members possess a similar overall genomic structure to the orthologous mouse and human *TRP* genes.

In summary, chicken Tyr, Tyrp1 and Tyrp2 share a high degree of sequence homology with other cloned TRPs, as well as possibly having a similar genomic structure to mammalian *TRPs*, lending further support to the notion that the *TRP* gene family is highly conserved throughout the vertebrates. Although there are several structural similarities between the chick TRPs and their vertebrate orthologues, there are also some noteworthy differences, including a significantly different 3' *UTR* within the chicken *Tyrp2* transcript. It remains to be investigated whether these differences have any functional implications in the diversification of avian colouration.

4.2 Expression of the chicken *TRP* gene family

Gene duplication and divergence, yielding multigene families, constitutes one of the major evolutionary mechanisms whereby molecular diversity may be generated. A second mechanism whereby molecular diversity and thus functional and phenotypic diversity may be generated involves the differential production of gene transcripts, which may occur by alternative usage of promoters, alternative splicing or alternative usage of 3' polyadenylation sites (Kendrew, 1995). There is evidence to suggest that all of these mechanisms are found in the vertebrate *TRP* gene family. For example, heterogeneous *TYR* and *TYRP1* transcription start sites (Ruppert *et al.*, 1988; Jackson *et al.*, 1991; Ponnazhagan *et al.*, 1994; Sturm *et al.*, 1995; Ferguson and Kidson, 1996), alternative *TYR* and *TYRP1* splicing (Ruppert *et al.*, 1988; Bouchard *et al.*, 1989; Shibahara *et al.*, 1991; Inagaki *et al.*, 1994; Kelsall *et al.*, 1997; Sato *et al.*, 1997) and differential usage of 3' polyadenylation sites (Ruppert *et al.*, 1988; Takase *et al.*, 1992; Sato *et al.*, 1997) have all been reported. Furthermore, it also appears that patterns of alternative splicing of the *TYR* gene may differ, depending on the developmental origin of the pigment cell (Bouchard *et al.*, 1989; Inagaki *et al.*, 1994; Le Fur *et al.*, 1997; Kelsall *et al.*, 1997).

Interestingly, there has been a recent unprecedented report of the utilisation of an alternative open reading frame (ORF) of human *TYRP1*, resulting in the translation of a novel peptide (Wang *et al.*, 1996). The product encoded by this alternative ORF is a 24 aa peptide that is recognised by T cells in patients with metastatic melanoma and has therefore been suggested to function as a human tumour rejection antigen. Although the presence of this peptide was reported in normal human melanocytes (Wang *et al.*, 1996), the normal function of this peptide is unknown. This "alternative translation" represents the first example in eukaryotic cells, of two completely different peptides/proteins being translated from overlapping ORFs of a single gene. This recently discovered phenomenon may represent yet a further mechanism of generating functional diversity.

To determine whether chicken *Tyr*, *Tyrp1* and *Tyrp2* gene expression is restricted to pigment cells, and to investigate whether chicken *TRP* transcripts undergo differential processing, northern blot and *in situ* hybridisation analyses were performed on pigmented and non-pigmented chicken embryonic tissues. The results of the present study reveal that all three chicken *TRPs* are expressed in pigmented RPE cells. Strongly hybridising transcripts for chicken *Tyr* (2.5 kb), *Tyrp1* (2.3 kb) and *Tyrp2* (3.5 kb and 5.0 kb) were detected in RNA from pigmented RPE cultures and embryonic eyes, but not in other non-pigmented tissues. These results indicate that the chicken *TRPs*, like their vertebrate orthologues, are expressed exclusively in pigmented cells. In the current study northern blot hybridisation analysis failed to detect *TRP* transcripts in total RNA from the skin of 10 day old chicks. Unlike whole eye tissue or RPE cells, the paucity of melanocytes within the total skin cell population may require a more sensitive technique to allow the detection of *TRP* gene expression. A similar suggestion was put forward by Mochii *et al.* (1992) after they failed to detect *Tyr* transcripts in the skin of 9 day old White Leghorns.

A comparison of *TRP* transcript sizes is presented in Table 4.6. On comparing the chicken *TRP* transcript sizes with other chordate *TRP* transcripts, several observations were noted. First, the transcript sizes obtained in the current study correlate with previous studies where 2.5 kb (Mochii *et al.*, 1992) and 2.4 kb (Austin and Boissy) chicken *Tyr*, 2.1 kb *Tyrp1* (Austin and Boissy, 1995) and 3.4 kb *Tyrp2* (Austin and Boissy, 1995) mRNA sizes were reported. Secondly, the chicken *TYR* transcript is similar in size to the reported smaller transcripts (2.2 kb) for the ascidian (Sato *et al.*, 1997), fish (Inagaki *et al.*, 1994) and human *TYR* sizes (2.4 kb) (Kwon *et al.*, 1987; Naeyaert *et al.*, 1991). However, the chicken *Tyr* mRNA is larger than the reported mouse *Tyr* transcript sizes of 1.8-2.1 kb (Takeuchi *et al.*, 1988; Müller *et al.*, 1988; Shibahara *et al.*, 1988). The Japanese pond frog *Tyr* transcripts have

Table 4.6. Transcript sizes of the *TRP* gene family in chordates

	TYR		*TYRP1*		*TYRP2*	
Organism	Transcript size (kb)	Reference	Transcript size (kb)	Reference	Transcript size (kb)	Reference
chicken	2.5 2.4 2.5	Mochii *et al.* (1992) Austin and Boissy (1995) this study	2.1 2.3	Austin and Boissy (1995) this study	3.4 3.5	Austin and Boissy (1995) this study
ascidian	5.2 + 3.0 + 2.2 2.0	Sato *et al.* (1997) Caracciolo *et al.* (1997)	?		?	
fish	5.6 + 4.3 + 2.2	Inagaki *et al.* (1994)	?		?	
axolotl	?		2.0	Mason and Mason (1995)	?	
frog	?		?		?	
mouse	2.1 1.8-2.0[a] 2.0[a]	Takeuchi *et al.* (1988) Müller *et al.* (1988) Shibahara *et al.* (1988)	3.0 2.5 3.1	Shibahara *et al.* (1986) Jackson *et al.* (1990) Austin and Boissy (1995)	1.9 3.0[a] 2.3[a]	Jackson *et al.* (1990) Bouchard *et al.* (1994) Yokoyama *et al.* (1994a)
human	2.4 2.4 2.4 + 4.7[a]	Kwon *et al.* (1987) Naeyaert *et al.* (1991) Bouchard *et al.* (1989)	3.3	Boissy *et al.* (1996)	4.5[a] 2.3 + 4.3[a]	Bouchard *et al.* (1994) Yokoyama *et al.* (1994a)

[a]transcript sizes from melanoma cells

not been reported yet. Thirdly, the chicken *Tyrp1* transcript is similar in size to the axolotl (2.0 kb) *Tyrp1* transcript size (Mason and Mason, 1995), but is smaller than that reported for the mouse and human *TYRP1* transcripts. The goldfish *Tyrp1* cDNA has also been cloned (Peng *et al.*, 1994), but as yet there have been no reports of a fish *Tyrp1* transcript size. Fourthly, the chicken *Tyrp2* transcript is larger than that reported for the mouse (3.0 kb, Bouchard *et al.*, 1994), but smaller than the major human *TYRP2* (4.3 kb, Yokoyama *et al.*, 1994a) transcript.

Variation in *TRP* transcript sizes may be due to species-specific differences and differential processing of primary mRNAs. A further explanation for variation in *TRP* transcript sizes has been suggested by Le Fur *et al.* (1997) and Kelsall *et al.* (1997), who reported that melanoma cells may generate aberrantly processed *TYR* mRNAs. Eberle *et al.* (1995) also provide evidence that *TRP* gene transcription is regulated differently in human melanomas, by comparison with normal human melanocytes.

The chicken *Tyr* transcript length of 2.5 kb is also consistent with the reported positions for the initiation of chicken *Tyr* transcription (Ferguson and Kidson, 1996). Similarly, transcription start site analysis of the mouse (Budd and Jackson, 1995) and human (Yokoyama *et al.*, 1994b; Sturm *et al.*, 1995) *TYRP2* genes has revealed that approximately 480 and 420 bp respectively of the 5' end of the *TYRP2* primary transcript is not translated. A chicken *Tyrp2* mRNA transcript size of 3.5 kb is consistent with a 5' *UTR* of similar size.

In the current study, a second fainter 5 kb chicken eye *Tyrp2* transcript was also detected above the 3.5 kb major transcript. Although there is no experimental evidence for differential processing of either mouse or human *TYRP2* transcripts, Yokoyama *et al.* (1994a) do suggest that the human *TYRP2* gene may generate differently sized mRNAs by using different 3' polyadenylation signals. It may be possible therefore, that the 2.9 kb chicken *Tyrp2* cDNA clone described here represents the more abundant 3.5 kb major *Tyrp2* transcript. The minor 5 kb *Tyrp2* transcript may be generated by usage of a second downstream polyadenylation signal not present in the *Tyrp2* cDNA described here.

Although no additional *Tyr* and *Tyrp1* transcripts were detected in the current study, it may be possible that differentially processed chicken *Tyr* and *Tyrp1* transcripts may be present in the skin and/or in low abundance. In this light, it may be useful to further analyse the remaining uncharacterised *TRP* cDNA clones isolated from the melanocyte cDNA library described here. For example, it may be of interest to determine whether any of these clones

represent differentially processed chicken *TRP* transcripts and also whether any of these clones correspond to any of the 21 mouse *Tyr* alternatively spliced transcripts catalogued by Kelsall *et al.* (1997).

Concurrently with the study described here, *in situ* hybridisation experiments were performed on embryonic chick skin and eye sections. Riboprobes were generated using the chicken *Tyr* and *Tyrp2* cDNA clones isolated here. The results of these experiments confirmed that the chicken *Tyr* and *Tyrp2* genes are expressed in a pigment cell-specific fashion, with strongly hybridising signals present in both the skin and eye. Franz (1996) demonstrated that both *Tyr* and *Tyrp2* are expressed in the chick eye before visible pigmentation is observed and that *Tyrp2* expression preceeded that of *Tyr* expression. Furthermore, in 10 day old embryos *Tyrp2* expression was detected in a few unpigmented dermal cells. Because in the mouse *Tyrp2* expression has been used as a specific marker of melanoblasts (Steel *et al.*, 1992; Cable *et al.*, 1995; Opdecamp *et al.*, 1997; Mackenzie *et al.*, 1997; Nakayama *et al.*, 1998), it is feasible that in the chick these cells may also represent melanoblasts.

Taken together, the evidence presented here strongly suggests that the chicken *TRP* gene family is expressed in similar tissues and at similar times to that reported for mammalian *TRPs*. Moreover, it is likely that homologous molecular mechanisms regulating mammalian *TRP* gene family expression are involved in specifying the temporal and spatial expression patterns of the chicken *TRP* gene family. Support for this suggestion comes from Ferguson and Kidson (1996, 1997), who reported that the chicken *Tyr* promoter region contains an 11 bp M-box element. This E-box-containing motif binds mouse Microphthalmia (Mi) (Bertolotto *et al.*, 1996, 1998) and the human Microphthalmia-associated transcription factor (MITF) (Yasumoto *et al.*, 1997) and has been shown to be a key element facilitating transactivation of the mammalian *TRP* genes (Bentley *et al.*, 1994; Yasumoto *et al.*, 1994; Yavuzer *et al.*, 1995; Yasumoto *et al.*, 1997; Bertolotto *et al.*, 1996, 1998). The recent isolation of the chick *Mi* cDNA (this report and Mochii *et al.*, 1998) further strengthens the view that evolutionary conserved molecular mechanisms regulating melanogenesis are shared between avians and mammals. On the other hand, it remains possible that subtle genetic differences between avian and vertebrate TRPs may contribute towards the diversity of plumage colouration.

4.3 A tentative link between the molecular conservation and expression patterns of the vertebrate *TRP* gene family?

From the above comparative analyses it is clear that i) there is strong molecular conservation of the *TRP* gene family within vertebrates and ii) there is also conservation of the tissue specific expression pattern of all three *TRP* genes. This leads to the interesting question of whether there is any relationship between the molecular conservation of paralagous members (TYR, TYRP1 and TYRP2) within the vertebrate TRP gene family and the gene expression profiles of these paralogues.

Hastings (1996) proposed that within a gene family there is indeed a correlation between gene expression profile and protein molecular evolutionary rate. For his analysis, Hastings focused on the Troponin I (TnI) gene family. In tetrapods, this family consists of three TnI isoforms (paralogues), TnIcardiac, TnIfast and TnIslow, each encoded by a separate gene. Each TnI gene has a distinct developmental expression pattern, with TnIslow having the broadest and TnIcardiac having the narrowest expression patterns. On comparing the three TnI isoform aa sequences in quails and mammals (rat and human), Hastings found that quail TnIslow differed by only 10% when compared to mammalian TnIslow. Similar comparisons with quail and mammalian TnIfast and TnIcardiac yielded differences of 20% and 36%, respectively. From this analysis, Hastings proposed that broadly expressed isoforms show stronger sequence conservation than do narrowly expressed isoforms. He then extended his analysis to an additional 15 gene families and found that in 14 out of 15 cases the most strongly conserved orthologues were the most broadly expressed. He further suggested that broadly expressed isoforms were subjected to greater negative selection pressure, having had to function in a more diverse biochemical environment than narrowly expressed isoforms.

Although there is insufficient gene expression data for all three TRPs in avians and mammals, the aa sequences for all three TRP gene family members (TYR, TYRP1 and TYRP2) in three different organisms (chicken, mouse and human) (2 different Classes), permits the testing of part of Hastings's hypothesis. If this hypothesis holds true for the TRP gene family then the different levels of aa sequence difference among the three TRP paralogues should directly reflect differences in their respective rates of molecular evolution since the bird/mammal divergence at the Jurassic/Cretaceous period (Stevens, 1991a). By comparing the aa sequence differences for each of the three TRPs for chickens (avians) and mammals:

i) chicken Tyr shows approximately 24% aa sequence difference from mammalian TYRs;

ii) chicken Tyrp1 shows approximately 27% aa sequence difference from mammalian TYRP1s and

iii) chicken Tyrp2 shows approximately 31% aa sequence difference from mammalian TYRP2s;

Although these differences are not as marked as those reported for the Tnl gene family, the TRP paralogues may be ranked in order of decreasing sequence conservation (from strongest to weakest) as follows:

TYR > TYRP1 > TYRP2

This trend confirms the first part of Hastings's hypothesis suggesting that the "ancestoral" gene, TYR, shows the strongest aa sequence conservation, whereas the most recently evolved TRP paralogue, TYRP2, shows the weakest aa sequence conservation (fastest evolutionary rate) amongst vertebrates.

If as Hastings (1996) proposes, there is a correlation between molecular evolutionary rate within gene families and gene expression profiles, then a further prediction of this hypothesis would be that TYRP2 should be expressed more narrowly than TYRP1, with TYR exhibiting the broadest expression profile. From a preliminary analysis of the relevant literature the expression profiles of the TRPs were tabulated under Table 4.7.

While it may appear that the *TYR* gene is indeed expressed more broadly than either the *TYRP1* or *TYRP2* genes, two additional factors have to be considered. First, the *TRP* gene family expression studies were obtained using different detection methods (RT-PCR, *in situ*, western and northern blot hybridisation, immunocytochemistry, transgenic mice carrying *TRP* reporter constructs). Secondly, most studies on the expression of the *TRP* gene family have focused on mammalian *TYR*, with very little expression data on *TYRP1* or *TYRP2*. Given the different sensitivities of the techniques employed, the bias towards *TYR* expression data and the relative lack of information on complete expression profiles for the chicken *TRP* gene family, it currently is not possible to determine whether in fact there is a correlation between TRP gene family evolutionary rate and expression profile. However, with the cloning of the chicken *TRP* gene family, the outstanding expression data, once available, will allow a more accurate and rigorous testing of the possibility of evolutionary relationships between gene family conservation and expression.

Table 4.7. Expression profiles of the vertebrate TRP gene family

TYR		*TYRP1*		*TYRP2*	
Expression	Reference	Expression	Reference	Expression	Reference
Pigmented cells/tissues	Müller *et al.* (1988)	Pigmented cell/tissues	Shibahara *et al.* (1986) Raymond and Jackson (1995)	Pigmented cells/tissues	Steel *et al.* (1992) Cable *et al.* (1995)
Forebrain	Tief *et al.* (1996a, 1996b, 1997)	Forebrain	Tief *et al.* (1996b)	Forebrain	Steel *et al.* (1992) Pavan and Tighman (1994) Cable *et al.* (1995) Tief *et al.* (1996b)
Olfactory bulb	Tief *et al.* (1996b) Mackenzie *et al.* (1997)			Olfactory bulb	Tief *et al.* (1996b) Mackenzie *et al.* (1997)
Neural retina	Tief *et al.* (1996a)	Neural retina	Tief *et al.* (1996a)		
				Endolymphatic duct	Steel *et al.* (1992)
Testes	Müller *et al.* (1988)				
Schwann cells Satellite cells	Hanicec and Vachtenheim (1988)				
Central nervous system	Garai *et al.* (1992)				
Several organs	Battyani *et al.* (1993)				
Neural tube Neural ectoderm	Franz (1996)				
Pineal gland	Tief *et al.* (1996a, 1996b)				

4.4 Are there mutant *TRP* alleles in chickens?

Genetic mutations constitute a third common mechanism whereby phenotypic diversity may be generated. In the mouse (Gluecksohn-Waelsch, 1979; Halaban *et al.*, 1988b; Jackson *et al.*, 1990; Schmidt and Beermann, 1994; Jackson, 1994b; Budd and Jackson, 1995; Wu *et al.*, 1997) and human (Oetting and King, 1994; Spritz, 1994b; Boissy *et al.*, 1996; Manga *et al.*, 1997) there are more than 70 distinct mutations that occur at either the *TYR*, *TYRP1* or *TYRP2* loci. In stark contrast, only three (possibly four) mutant recessive (*Tyr*) *c* locus alleles (see section 1.3.3) have been described in the chicken. Attempts have been made to understand the biochemical nature of the *c* and c^a alleles (Brumbaugh *et al.*, 1983; Oetting *et al.*, 1985a; Boissy *et al.*, 1987), but as yet the genetic basis for the hypomelanosis associated with these mutant alleles (when homozygous) is unknown (see section 1.3.3). In an attempt to shed further light on the nature of the *recessive white (c)* allele, genomic DNA from two chicken breeds (C^+/C^+ and C^+/c) was probed with a chicken *Tyr* cDNA. Although the White Plymouth Rock X Pile Game (WPR X PG) breed used in this study is heterozygous (C^+/c) at the *c* locus, no gross differences at the chicken *Tyr* locus were observed between this and the Black Australorp X New Hampshire Red (BA X NHR) (C^+/C^+) breed. Although there are several possible explanations for this result, the most likely interpretation is that the mutation at the *c* allele may be too small to detect by Southern blot hybridisation analysis. Support for this possibility comes from Oetting *et al.* (1985a), who showed by immunoprecipitation experiments, the presence of enzymatically non-functional Tyr protein in both *c* and c^a homozygotes. A second, but less likely explanation is that the *Tyr* gene does not map to the chicken C^+ locus.

Although both the WPR X PG and BA chicken breeds do not exhibit a brown phenotype, genomic DNAs from both these breeds were probed with a chicken *Tyrp1* cDNA, in order to learn more about the chicken *Tyrp1* locus. No gross differences were observed at this locus between the black and white breeds examined. Several avian loci have been reported to be involved in the generation of brown plumage and/or eye colour (Somes, 1980; Ito and Tsudziki, 1993, 1994; Tsudzuki, 1995b, 1995c; Carefoot, 1996; Satoh *et al.*, 1997). However, it is not known whether any of these avian "browns" are homologous to either the mouse *brown* or human "brown" mutations. In commenting on brown phenotypes in the chicken, Smyth (1970) writes, "Apparently in the fowl it is not uncommon to have similar or identical phenotypes resulting from a number of different genetic combinations... Such situations are suggested as one of the major reasons for the surprisingly incomplete knowledge of plumage pattern inheritance in the domestic fowl."

Having found no large differences between the BA and WPR X PG breeds at either the *Tyr* or *Tyrp1* loci, the same breeds were then compared with regard to the chicken *Tyrp2* locus. Quite unexpectedly, genomic Southern blot hybridisation analysis revealed that indeed there is a difference between the BA and WPR X PG *Tyrp2* loci. The observed differences were evident in four out of six restriction digests and are consistent with a 1-2 kb heterozygous alteration at the WPR X PG *Tyrp2* locus. Because of the nature of the Southern blot hybridisation analysis, it is unclear whether this alteration represents a sequence polymorphism or indicates the presence of a mutant chicken *Tyrp2* allele. One approach in determining whether this alteration occurs within the coding region of the *Tyrp2* gene would be to PCR amplify RNA extracted from WPR X PG pigmented cells that are homozygous for this difference. DNA sequence comparisons between the BA and WPR X PG *Tyrp2* DNA would then distinguish between sequence polymorphisms or mutations occurring within the WPR X PG *Tyrp2* coding region.

A few loci have been reported to dilute wild type black/brown chicken plumage to a bluish-grey colour (Somes, 1980; Smyth, 1990; Tsudzuki, 1995a). Although, at this stage it is premature to suggest that any of these loci might encode a mutant chicken *Tyrp2* gene, one locus in particular, *Blue* (*Bl*), might be considered a potential candidate for chicken *Tyrp2*.

The *Bl* locus yields a modified eumelanin, resulting in a bluish-slate plumage (Smyth, 1990). In addition, *Bl* melanosomes are ultrastructurally different from bl^+/bl^+ melanosomes, being more spherical and sparsely distributed than wild type melanosomes (Zimmerman *et al.*, 1982; Smyth, 1990; Stevens, 1991b). In the mouse and human, the *Tyrp2* locus encodes the melanogenic enzyme, DOPAchrome tautomerase (Tsukamoto *et al.*, 1992; Yokoyama *et al.*, 1994a). Because this enzyme plays a key role in the composition and colour of melanin synthesised (Aroca *et al.*, 1992) as well as being a melanosomal membrane-bound protein (Hearing and Tsukamoto, 1991; Tsukamoto *et al.*, 1992), the bl^+ locus may be a possible candidate for the chicken *Tyrp2* gene. Smyth (1990) writes that several varieties of the Old English Game carry the *Bl* allele. Although the mixed white breed used in the current study partly derives from Pile Game, it is unclear whether the *Bl* allele is present in WPR X PG chickens. If the WPR X PG breed (I/I C^+/c) used in the present study does indeed possess a mutant *Tyrp2* allele, then the resulting mutant phenotype (if dominant) would have to be expressed on a wild-type i^+/i^+ background in order to avoid the epistatic effects of the *I* locus.

4.5 The possible roles of α-MSH, Mgf and the endothelins in regulating the behaviour of chick neural-crest derived melanocytes

Together with mutant genes, signalling systems operational within the pigment cell environment constitute a further mechanism whereby diverse colours and patterns may be generated. Apart from melanogenic enzymes and transcription factors, at least three key ligand-receptor interactions have been shown to play important roles in the early development of mammalian pigment cells. These systems include α-MSH, Mast cell growth factor (Mgf), the endothelins and their respective receptors. Several of these signalling components have been identified in avians and a few of the corresponding genes (cDNAs) have now been cloned in the chick and quail (Tables 4.8 and 4.9). The final section of this discussion explores the possible roles that these signalling systems may have had on the chick neural crest-derived melanocyte cultures in the current study and also speculates on the mechanisms whereby these systems may give rise to plumage colour and patterning diversity.

In a first attempt to generate large numbers of pigmented chick melanocytes, single cell suspensions derived from truncal neural tube segments were cultured in Buffalo rat liver (BRL-3A) conditioned medium (BRL-3ACM) as described by Giss *et al.* (1982). In the present study, unlike that of Giss *et al.* (1982), BRL-3ACM failed to stimulate the bulk production of cultured embryonic pigmented chick melanocytes. One difference between the present study at that of Giss *et al.* (1982), is the strain of fowl breed used. Two arguments are presented below that suggest that genetic differences between strains of fowl breeds may influence the behaviour of melanocytes *in vitro*.

The Black Australorp X New Hampshire Red (BA X NHR) breed used in the current study carries the genotype E/e^{wh} (Smyth, 1990). As discussed earlier, there is some evidence to suggest that the chick E^{+} locus may encode the *Mc1r* gene (Takeuchi et al., 1996b) that α-MSH/Mc1r has a direct role in the development of avian melanocytes. They reported that α-MSH seemed not only to accelerate quail melanogenic differentiation *in vitro*, but also to affect the state of commitment of neural crest cells (NCCs) to melanogenic differentiation *in vitro* via cAMP signalling. Because there is also evidence to suggest that α-MSH/Mc1r may play a role in the early development of melanoblasts (Barsh, 1996) and melanocyte growth (De Luca *et al.*, 1993), it is possible that the compound heterozygous (E/e^{wh}) melanocytes used in the current study may have responded differently to the BRL-3ACM than those melanocytes used by Giss *et al.* (1982).

Two additional genes known to influence melanocyte development are the c-kit tyrosine kinase receptor (Kit) (Geissler *et al.*, 1988) and its ligand Mgf (Huang *et al.*, 1990). Mutations at both the *KIT* and *MGF* loci result in spotting in mice and piebaldism in humans (Williams *et al.*, 1992; Spritz, 1994a). There is evidence now that suggests that Mgf/Kit interactions are also important for avian melanocyte development, including the survival (Lahav *et al.*, 1994; Lecoin *et al.*, 1995) and differentiation (Sheng Guo *et al.*, 1997) of melanocyte progenitors. Because there are significant levels of Mgf within BRL-3ACM (Zsebo *et al.*, 1990) it is conceivable that this Mgf might be partially responsible for the stimulatory activity of BRL-3ACM reported by Giss *et al.* (1982) in their chick melanocyte cultures. A possible explanation for the differences obtained in the present study when compared to that of Giss *et al.* (1982) is that the chick breed used in the current study, may carry a mutation at the *Kit* locus, thus diminishing the survival and proliferative signals supplied by Mgf-containing BRL-3ACM. This hypothesis however, is weakened by the observation that the BA X NHR breed does not exhibit any overt spotting, unlike the breeds described by Schaible (1968; 1972) and Somes (1980). Both the chicken *Mgf* (Zhou *et al.*, 1993) and *Kit* (Sasaki *et al.*, 1993) cDNAs have now been cloned, and it will be of interest to determine whether the BA X NHR and WPR X PG breeds carry wild type *Kit* genes.

In summary then, potential genetic differences at at least three loci (*Mc1r*, *Mgf* and *Kit*) may provide theoretical explanations for the behavioural differences observed when comparing the effect of BRL-3ACM on melanocyte cultures obtained in the present study with those obtained by Giss *et al.* (1982). Two further sets of genes, the endothelins and their receptors, have recently been implicated in the regulation of melanocyte development, and therefore are of potential relevance to the present study.

In a further attempt to generate large quantities of embryonic pigmented chick cultures in the present study, NCCs derived from neural tube explants were cultured in a combination of TPA (Boissy and Halaban, 1985) and bFGF (Stocker *et al.*, 1991). This approach allowed the successful generation of approximately 10^8 cells, 70% of which were pigmented. Whereas the effects of 12-O-tetradecanoyl-13-phorbol acetate (TPA), a potent tumour promoter (Abrahm and Rovera, 1980; Ashendel, 1985), on avian melanogenesis have been investigated (Payette *et al.*, 1980; Glimelius and Weston, 1981; Sieber-Blum and Sieber, 1981; Boissy and Halaban, 1985; Oetting *et al.*, 1985b), it is unclear which physiological molecule TPA is mimicking. Recent evidence suggests that the effects of endothelins (Edns) and their receptors (Ednrs) may partially explain some of the reported effects of TPA on pigmentation (Yada *et al.*, 1991; Hara *et al.*, 1995; Lahav *et al.*, 1996; Reid *et al.*, 1996;

Imokawa *et al.*, 1996; Nataf *et al.*, 1996). These effects, also observed in the current study, include potent stimulation of avian NCC proliferation, with a vast increase in pigmented melanocytes. Because both the quail and chick *Ednrb* (Nataf *et al.*, 1996) cDNAs, as well as a novel quail *Edn* receptor cDNA (Lecoin *et al.*, 1997) have been cloned, the role of TPA and the Edn signalling system in avian melanogenesis may now be investigated in more detail.

4.6 Concluding comments and future directions

This study has focused on several aspects regarding the genetics, biochemistry and evolution of avian pigmentation. Of particular interest here, has been the development of the appropriate tools with which to address several questions related to the disciplines stated above. From this investigation, several other issues have arisen, some of which have already been alluded to in this discussion. These additional areas are expanded upon here.

In the mouse and human, the *TYR* (Ruppert *et al.*, 1988; Ponnazhagan *et al.*, 1994), *TYRP1* (Jackson *et al.*, 1991; Shibahara *et al.*, 1991) and *TYRP2* (Budd and Jackson, 1995; Sturm *et al.*, 1995) promoter regions have been cloned and extensively characterised. In addition, the human *MITF* promoter has been isolated (Fuse *et al.*, 1996). Apart from the cloning of the chicken (Ferguson and Kidson, 1996) and partial quail (Yamamoto *et al.*, 1992) *Tyr* promoters, very little else is known regarding the regulation of avian pigment cell-specific gene transcription (Ferguson and Kidson, 1997).

Towards the final stages of the current study, as an initial step towards the eventual investigation of the transcriptional regulation of the chicken *Mi* and *TRP* gene family, a Lambda Charon4A White Leghorn genomic library (Dodgson *et al.*, 1979) was screened for the 5' regulatory regions of the chicken *Tyrp1*, *Tyrp2* and *Mi* genes. Using chicken *Tyrp1*, *Tyrp2* and *Mi* cDNA probes several strongly hybridising genomic clones were isolated. Although these clones have as yet not been analysed further, it is likely that some of these clones may yield information that will contribute towards the understanding of the genomic structure and transcriptional regulation of the chicken *Mi* gene and *TRP* gene family.

When the current study was initiated in 1991, only a single avian pigment cell specific gene (cDNA) had been cloned (Mochii *et al.*, 1988b). Since then, 18 antisera and 12 gene probes have been developed and used in expression studies of avian pigmentation (Tables 4.8 and 4.9). However, of these markers, only 10 out of 18 antisera and 6 (*MM2* (Mochii *et al.*, 1988b; *Cty18* (Mochii *et al.*, 1992; *QNR-71* (Turque *et al.*, 1996; *CMC1* (Takeuchi *et al.*,

Table 4.8. Cloned genes expressed in avian pigment cells and their precursors

Clone#	Organism	Gene product	Cell/tissue specificity	Reference
MM-2	chicken	melanosomal matrix protein	yes	Mochii *et al.* (1988b)
Cty18 B8.3	chicken chicken	Tyr Tyr	yes yes	Mochii *et al.* (1992) this study
-	chicken	Mast cell growth factor	no	Zhou *et al.* (1993)
clone 3	chicken	Kit receptor	no	Sasaki *et al.* (1993)
pP334	chicken	serpin?	yes	Agata *et al.* (1993)
QNR-71	quail	melanosomal protein	yes	Turque *et al.* (1996)
CMC1	chicken	Melanocortin 1 receptor	yes	Takeuchi *et al.* (1996a)
pqSCF17	quail	Mast cell growth factor	no	Petitte and Kulik (1996)
-	quail	Endothelin B receptor	no	Nataf *et al.* (1996)
pcTRP-1.6	chicken	Tyrp1	yes	this study
pcTRP-2.10	chicken	Tyrp2	yes	this study
cmi9 M156	chicken chicken	Microphthalmia Microphthalmia	no no	Mochii *et al.* (1998) this study

Table 4.9. Antigens expressed in avian pigment cell and their precursors

Antibody#	Organism	Antigen	Cell/tissue specificity	Reference
HNK-1	chicken	?	no	Tucker *et al.* (1984)
-	chicken	Tyr	yes	Oetting *et al.* (1985a)
MC/1 and MMP115	chicken	melanosomal matrix protein	yes	Mochii *et al.* (1988a)
3G11	chicken	RPE antigen	yes	Chu and Grunwald (1990a)
3C10	chicken	RPE antigen	yes	Chu and Grunwald (1990a)
1D5	chicken	RPE antigen	yes	Chu and Grunwald (1990a)
1H2	chicken	?	no	Chu and Grunwald (1990a)
2A10	chicken	cadheren?	no	Chu and Grunwald (1990b)
MEBL-1	chicken	?	yes	Kitamura *et al.* (1992)
MelEM	quail	glutathione-*S*-transferase subunit	no	Nataf *et al.* (1993)
Mel1	quail	premelanosomal associated protein	yes	Nataf *et al.* (1993)
Mel2	quail	premelanosomal associated protein	yes	Nataf *et al.* (1993)
SL	chicken	Tyrp1	yes	Austin and Boissy (1995)
71Cter	quail	melanosomal protein?	yes	Turque *et al.* (1996)
-	chicken	Mast cell growth factor	no	Sheng Guo *et al.* (1997)
-	chicken	Kit receptor	no	Sheng Guo *et al.* (1997)
-	chicken	Microphthalmia	no	Mochii *et al.* (1998)

1996a); *Tyrp1* and *Tyrp2* (this study) out of 12 gene probes are avian pigment cell-specific. Many of these molecules were first identified and cloned in mammals (particularly the mouse) and have since also been found to play key roles in the development and functioning of other vertebrate pigment cells (Jackson, 1997).

Because the cDNA library described here contains cDNAs derived from melanocytes as well as from undifferentiated NCCs, it should theoretically be possible to clone novel markers of the chicken neural crest as well as of the melanocyte lineage. Already, Kelsh *et al.* (1996) have identified 285 mutations (alleles) and 94 genes (complementation groups) affecting all aspects of *Danio rerio* (zebrafish) larval pigmentation. These include NCC specification and pigment cell fate (6 genes); pigment cell patterning (5 genes); pigment cell proliferation (2 genes); pigment cell survival (33 genes) and pigment cell differentiation (52 genes). Although these genes include those involved in iridophore and xanthophore development, it is clear that lower vertebrate pigment cell development is at least as genetically complex as mammalian pigmentation. For example, zebrafish candidates for *Tyr* (*Sandy*; Kelsh *et al.*, 1996) and *Mi* (*piegus*^{m286}, *punktata*^{m288} and *mizerny*^{m293}; Malicki *et al.*, 1996) have been suggested. Furthermore, it appears that the Medaka fish possesses *Tyrp1* and *Tyrp2* genes (Matsumoto, 1998), in addition to the *Tyr* gene (Inagaki *et al.*, 1994; Hyodo-Taguchi *et al.*, 1997). Sato *et al.* (1997) have also reported the identification of a *TRP*-like gene in the ascidian. As yet, it is not known whether this gene represents a tunicate *Tyrp1* or *Tyrp2*.

Because the primary goal of this study was to contribute towards the molecular biology of chicken pigmentation, only those mammalian pigmentary loci that are relevant to the current study have been referred to here. Recently several other mammalian pigmentary genes have been cloned, including the *agouti/ASP* (Bultman *et al.*, 1992; Wilson *et al.*, 1995), *beige/LYST/CHS* (Perou *et al.*, 1995; Nagle *et al.*, 1995), *HPS/pale ear* (Oh *et al.*, 1996; Feng *et al.*, 1997) and *mottled/ATP7A* (Cecchi *et al.*, 1997; Vulpe *et al.*, 1993) genes. Although there are no clear candidates for these genes in chickens, avian homologues for these genes will no doubt be identified eventually. Conversely, at least three avian pigmentary genes, *MMP115* (Mochii *et al.*, 1988b); *pP334* (Agata *et al.*, 1993) and *QNR-71* (Turque *et al.*, 1996) have been cloned, that as yet, have no reported mammalian equivalents. Genes like these, as well as the plethora of primary and secondary plumage patterns that abound in the avian kingdom, will continue to afford a unique and exciting model with which to explore the molecular and developmental genetics of pigmentation.

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