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INDUCTION OF THE RETINAL PIGMENT EPITHELIUM OF THE CHICKEN EMBRYONIC EYE.

by

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ABSTRACT

INDUCTION OF THE RETINAL PIGMENT EPITHELIUM OF THE CHICKEN EMBRYONIC EYE.

During development of the eye, invagination of the optic cup gives rise to a double layered neuroepithelium, part of which differentiates into the retinal pigment epithelium (RPE). The molecular mechanisms which control differentiation of the RPE are not known. The present study was undertaken to determine 1) when induction of the RPE has occurred in chicken embryos and 2) to investigate whether contact with the presumptive neural retina (NR) is required for RPE differentiation.

In order to investigate when RPE induction has occurred, early expression of two genes involved in pigmentation were investigated. Digoxigenin-labeled tyrosinase and tyrosinase-related protein-2 (TRP-2) riboprobes were synthesised and used in ISH reactions on embryonic eye tissue. Tyrosinase transcripts were first detected at stage 19.5 (70-71 hours) and TRP-2 transcripts were detected a few hours earlier at stage 18.5 (67-69 hours) of embryonic development. These results indicate that induction has occurred by stage 18.5, approximately ten hours before distinct granules are visible in the RPE.

The tyrosinase and TRP-2 transcripts were always localised first in the optical axis of the eye in the region where pigment granules are first present. This indicates that differentiation of the RPE proceeds from the optical axis of the eye cup outwards towards the periphery and that induction of the RPE may also proceed in this direction.

To determine whether the presumptive NR is required for RPE induction, synthetic barriers were inserted into the uninvaginated optic vesicle of chicken embryos at stage 11 (40-45 hours) of development. The embryos were cultured *in vitro* until the optic vesicle had invaginated and sectioned to locate the barrier. Results suggest that contact with the presumptive NR may not be necessary for RPE induction.

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GLOSSARY

[1] Induction is the process by which one embryonic tissue interacts with a second to influence the second tissue's differentiation. A tissue is said to be induced once this interaction has occurred.

[2] Commitment. A committed cell will develop into a particular tissue type unless instructed (or induced) to change its pathway of instruction. That is committment is not irreversible.

[3] Determination is the process whereby an embryonic tissue is irreversibly committed to develop into a specific tissue irrespective of its environment.

"The lineage decision from a neuroepithelium to a pigment epithelial phenotype is a striking example of pattern formation in the early embryo and is the focus of this study." (Buse et al. 1993)

CHAPTER ONE

GENERAL INTRODUCTION

During development of the eye, spatially and temporally regulated interactions are responsible for the final differentiation and complex organisation required for a fully functional organ. Some aspects of these interactions or inductive events are fairly well understood (for example, induction of the lens by the optic cup), whereas others are poorly understood (for example, induction of the retinal pigment epithelium). This study investigates the induction and development of the retinal pigment epithelium in the chicken embryo.

1.1 Early eye development

The first evidence of eye development is the bilateral outpocketing of the wall of the diencephalon which forms the optic vesicles (Fig.1.1) (Balinsky, 1983). The optic vesicles enlarge and push outward until they reach the overlying ectoderm. The distal portion of the optic vesicle then flattens out and begins to invaginate inward, resulting in the formation of a double walled optic cup (Fig.1.1). As invagination progresses, the original lumen (optocoel) of the optic vesicle is reduced to a vestigial slit separating the inner and outer layers of the newly formed optic cup. The outer layer thins and eventually becomes pigmented, giving rise to the retinal pigment epithelium (RPE) of the retina. The inner layer of the optic cup thickens markedly (from the pre-invagination stage) and becomes the multi-layered sensory portion of the retina, the neural retina (NR). These two layers come into close association as development proceeds.

The lens, which is derived from ectoderm, initiates development when the optic vesicle is in very close contact with the overlying surface ectoderm. The ectodermal layer thickens to form a lens placode. During later stages of development, the indented lens placode (lens vesicle) detaches from the ectoderm and the developing lens settles into the newly formed optic cup. The remaining structures of the eye, the iris, ciliary body, choroid, sclera and cornea are of mesodermal origin and begin developing shortly after the lens settles into the optic cup. The development of the associated glands, eyelids and conjunctiva occur later.

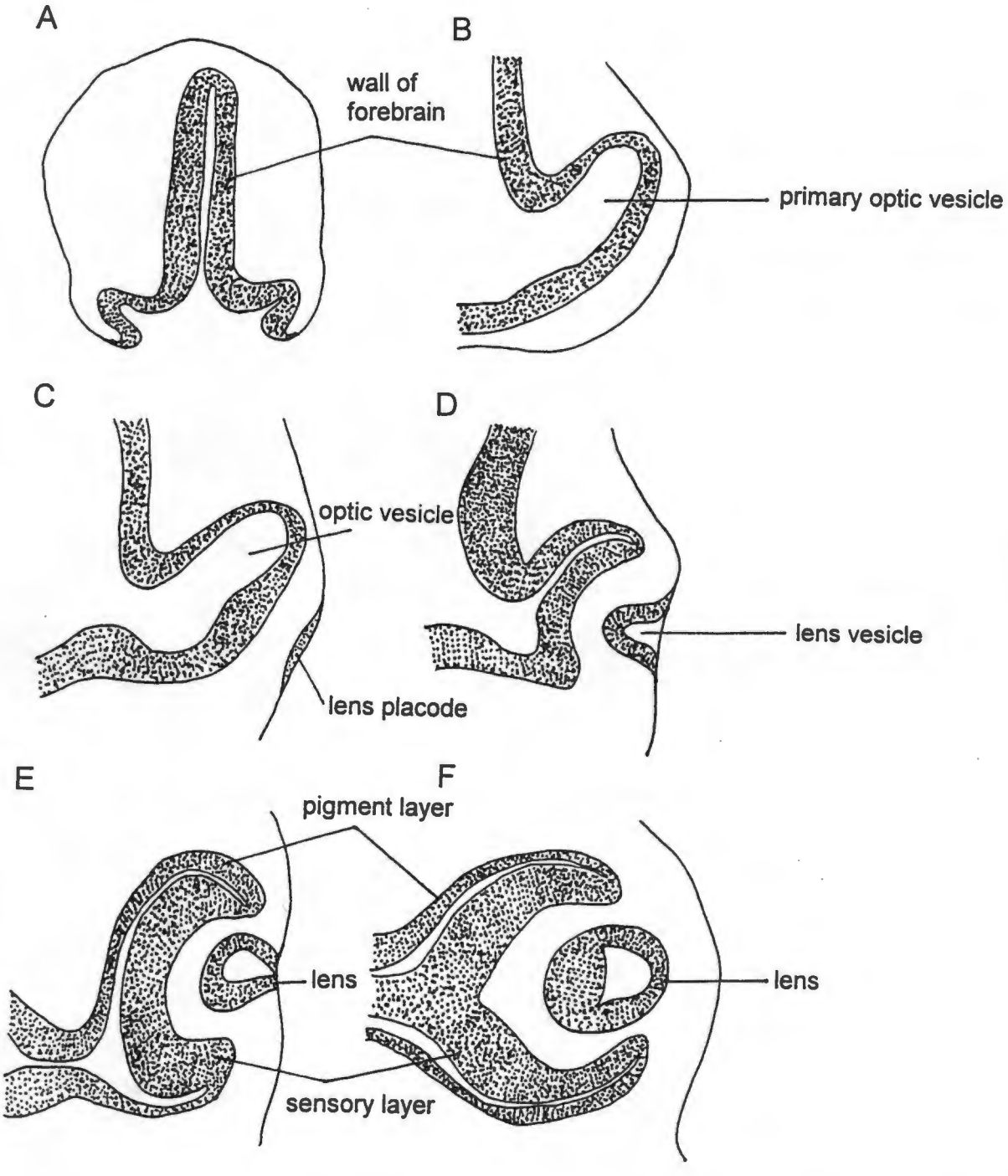


FIGURE 1.1. A general scheme of the early stages of development of the vertebrate eye (Balinsky, 1983).

1.2 Retinal pigment epithelial cell biology

This study focuses on the development of the RPE. As already mentioned the RPE develops from the outer layer of the optic cup (Fig.1.1). At the time that the space separating the inner and outer layers of the optic cup is being compressed, each layer is a pseudostratified neuroepithelium. As development proceeds, however, the outer layer thins into a single layer of roughly cuboidal cells (the future RPE), while the inner layer transforms into the complex, multi-layered NR. Cell death and cell division contribute to the transformation of the optic vesicle into the optic cup. In addition, it appears that microfilament bundles also play a role in this morphological transformation. Before invagination the cells of both layers possess bundles of microfilaments at their apices. However, at the onset of invagination the cells of the presumptive neural retina lose these filament bundles, whereas the RPE cells have thickened microfilament bundles and flattened apices (Camatini & Randi, 1976). These changes in microfilament bundles are the first cytological indication of the differences between presumptive RPE cells and presumptive NR cells.

The cells of the presumptive RPE are first polarised at the optic vesicle stage; the apical portion faces towards the presumptive NR and the basal portion towards the sclera. The apical surfaces are smooth and flat at first but later develop finger-like projections which eventually extend between and around the outer segments of the visual cells (rod and cone cells) of the sensory layer. Melanin granules can be found within these extensions. Even though the RPE is tightly apposed to the NR, there are no attachment junctions between these two layers and this area represents a site of retinal detachment which persists in the adult.

The basal surfaces of the RPE cells contain a series of relatively regular infoldings which increase the surface area of the cells, while the lateral surfaces are smooth and generally lack any invaginating or evaginating processes. A prominent junctional complex comprising a zonula adherens (towards the sclera) and a zonula occludens or tight junction (towards the vitreous) exists on these surfaces. The RPE cells rest on a basement membrane which forms part of Bruch's membrane, which itself may comprise as many as five different layers. This serves to separate the RPE cells from the capillaries of the choroid.

The RPE cells are mononucleate (except in rat and rabbit) and contain a single nucleolus (Ts'o and Friedman, 1967, cited Dunn, 1973). The cytoplasm is filled with extensive smooth and granular membranes, Golgi apparatus, mitochondria, free ribosomes and inclusion bodies (Fig.1.2). One type of inclusion body already mentioned is the melanin granule commonly known as the melanosome. Others include lysosomes, phagosomes, lipid droplets and myeloid bodies (of unknown function) (Dunn, 1973). Phagosomes represent phagocytosed

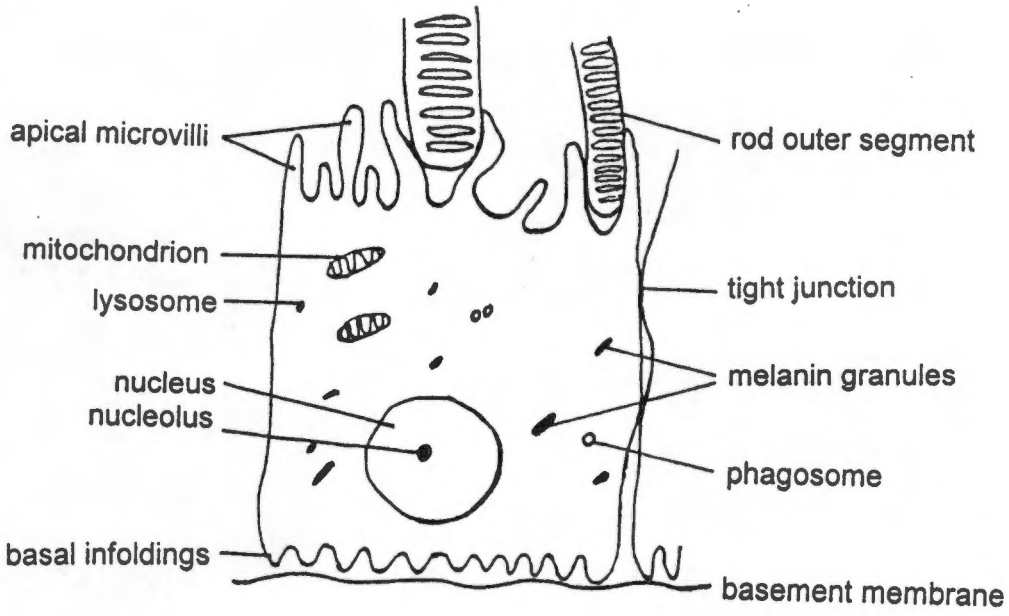


FIGURE 1.2. A schematic representation of a retinal pigment epithelial cell.

portions of outer segments of rod and cone photoreceptors. Rod and cone outer segments initiate shedding, where upon the apical projections of the RPE cells engulf and phagocytose the shed outer segments which are then incorporated into phagosomes (Dunn 1973; Young, 1978).

The focus of this study is the induction and development of the RPE in the chicken embryo. As described above, a distinguishing feature of RPE cells is the presence of pigment granules (melanosomes) within the cell cytoplasm. This study utilises this feature in order to determine when induction of the RPE occurs. Pigment synthesis is a complex process involving numerous biochemical steps and organelles. This will briefly be discussed below.

1.3 PIGMENT SYNTHESIS

Melanin granules or melanosomes of the retinal pigment epithelium vary in shape from short ovoid to long rod-shaped granules. Melanin itself is the product of numerous biochemical steps which begin with the amino acid tyrosine (Stanbury et al. 1983). The Golgi apparatus processes and transports the molecular complex required for the conversion of tyrosine into melanin, via coated vesicles. Only when the enzymatic components (within Golgi vesicles) and the matrix components (within vesicles from the smooth endoplasmic reticulum) join does melanin deposition within melanosomes occur.

There are four stages of development of melanosomes in RPE cells (Fig.1.3) (Hori et al. 1981; Toda and Fitzpatrick, 1972). The stage I melanosome or premelanosome is a spherical membrane bound vesicle that contains a few filaments of distinct periodicity. By stage II the melanosome is an ellipsoid structure containing numerous filaments of distinct periodicity. By stage III, the internal structure observed at stage II is partially obscured by electron dense material. At stage IV the melanosome is still ellipsoid but is totally electron dense. The electron dense substance is melanin which accumulates in the melanosome until it is totally filled.

The biochemical process of melanogenesis is complex and involves numerous genes: genes coding for enzymes (and perhaps inhibitors) involved in catalysing the conversion of tyrosine into melanin as well as genes coding for the structural components of the melanosome. The tyrosinase gene family controls the proximal steps in the melanogenic pathway while the less investigated pmel17 family is thought to control more distal steps (Kwon, 1993). At present it is generally believed that melanogenesis in cells of the RPE and the melanocytes of the skin follow a common pathway (Fig.1.4), even though these cells have different origins (Feeney et al. 1965, Hearing et al. 1973 and Sarna 1992, cited Schraermeyer 1993).

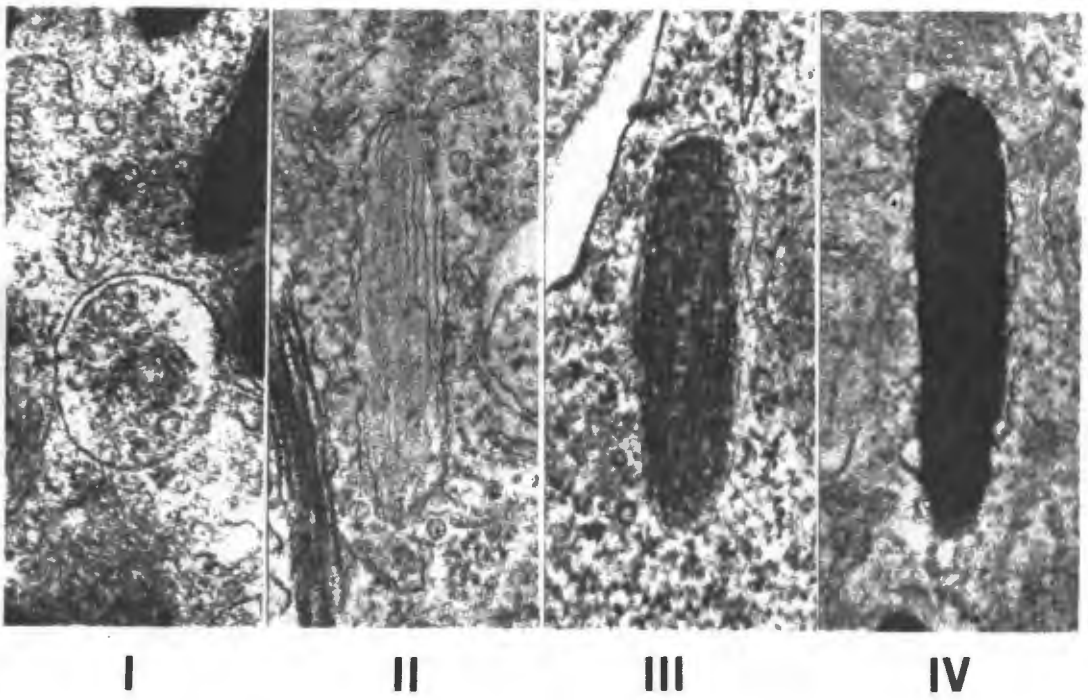


FIGURE 1.3. The four developmental stages of melanosomes. I = stage I melanosome; II = stage II melanosome; III = stage III melanosome and IV = stage IV melanosome. 50 000 x. (Stanbury et al. 1983).

The initial and rate-limiting step in melanin synthesis is the hydroxylation of tyrosine by tyrosinase to DOPA (del Marmol and Beermann, 1996). The subsequent oxidation of DOPA to DOPAquinone is also catalysed by the melanocyte-specific enzyme, tyrosinase. Tyrosinase is the critical enzyme for the synthesis of melanin and is sufficient to synthesise melanin. It is encoded by a gene at the c locus (albino) which is highly conserved between chicken, mouse and man (Hearing and Jimenez 1989, Kwon et al. 1987, 1989, cited Urabe et al. 1993; April et al. 1996). DOPAquinone then evolves spontaneously to DOPAchrome. At this point the pathway splits. DOPAchrome tautomerase or tyrosinase related protein-2 (TRP-2) converts DOPAchrome to stable 5,6-dihydroxyindole carboxylic acid (DHICA), and tyrosinase related protein-1 (TRP-1), another member of the tyrosinase gene family, is thought to oxidise DHICA for its final incorporation into melanin. This role for TRP-1 has not been confirmed and remains controversial. In the absence of TRP-2, DOPAchrome spontaneously converts to 5,6-dihydroxyindole (DHI), which is oxidised by tyrosinase into melanin. Tyrosinase, TRP-1 and TRP-2 have been cloned and characterised in mice and humans. In chickens, only tyrosinase has been fully characterised (Mochii et al. 1992, April et al. 1996). The chicken TRP-1 and TRP-2 cDNAs have recently been cloned and are currently being sequenced (April C., pers. comm.).

1.4 Induction of the RPE

In order to fully understand development of the RPE, it is important to determine **when** induction[1] of the presumptive RPE takes place, **what** factors are involved, from **where** these factors emanate and **how** they exert their effects. Most of the early studies in this field relied on experimental transplantation or explantation techniques and morphological analyses of the resulting tissues. However, with the advance of modern molecular techniques many of these experiments can be re-evaluated and early changes in cellular phenotypes can be determined independently of morphological changes.

As described above, at the optic cup stage of eye development, the presumptive RPE and presumptive NR layers form a pseudostratified neuroepithelium. While the RPE develops into a simple cuboidal epithelium, the NR becomes a multi-layered structure. In order to elucidate the mechanisms and tissues that are responsible for instructing part of the optic vesicle to become RPE, it is essential to establish when this instruction occurs. It has been reported that in chickens before invagination of the optic cup, the cells of the presumptive RPE appear slightly different to those of the presumptive NR - their nuclei are larger and they have less cytoplasm (Smith, 1920, cited Romanoff, 1960). This suggests that induction might have occurred even before the presumptive NR is apposed to the presumptive RPE.

[1] Refer to glossary for definition.

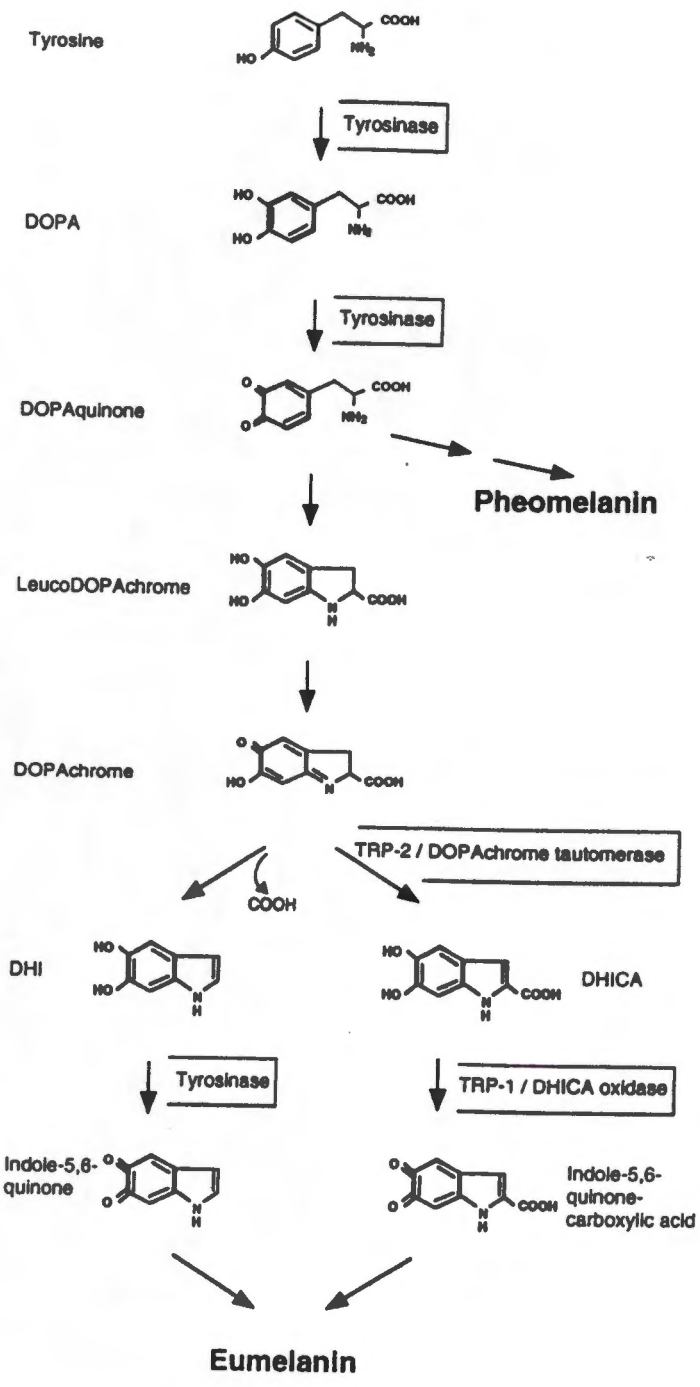


FIGURE 1.4. The melanin biosynthesis pathway. Enzymatic reactions attributed to tyrosinase, TRP-1 and TRP-2 are indicated. DOPA = 3,4 dihydroxyphenylalanine; DHICA = 5,6-dihydroxyindole-2-carboxylic acid; DHI = 5,6-dihydroxyindole. (del Marmol and Beermann, 1996).

It is reasonable to deduce that commitment[2] to the pigment-cell fate must occur before pigment is actually observed (because if the structural and enzymatic requirements for pigment synthesis are present then the cells must already have begun to differentiate). In the chicken unmelanised granules (premelanosomes) are present before pigment is visible and melanisation of premelanosomes takes 24 hours (Toda and Fitzpatrick, 1972). There is evidence that in mice the expression of the melanocyte-specific genes precedes the stage when pigment is visible by one to two days (Beermann et al. 1992, Steel et al. 1992, Cable et al. 1995). Thus the events directing commitment to the RPE cell fate must occur before the onset of expression of the pigment specific genes. In summary then, the sequence of events for pigment synthesis in the RPE is likely to be as follows: the presumptive RPE layer of the optic vesicle is instructed/induced to form the RPE. This is followed by expression of the genes required for pigmentation, followed by the formation of unmelanised pigment granules (premelanosomes). Finally melanin is synthesised and shortly thereafter pigment is visible to the naked eye.

The regulatory processes that are responsible for the induction of the RPE are obscure although a number of studies have been carried out. In 1951, Harrison cultured eye explants from chicken embryos of various ages on glucose media and observed pigment formation. The capacity for the explanted presumptive RPE tissue to form pigment increased from the 23 somite stage to the 27 somite stage. He concluded that this period (the 23-27 somite stage which is equivalent to Hamburger & Hamilton (1951), stage 15) was a critical period during which the pigment system of the eye possibly depended on some other region of the embryo. That is, at stage 15, when the optic vesicle begins to invaginate, some other tissue (not included in the explant) is required for induction of the presumptive RPE. In these cultures, embryos less than 22 somites (stage 14) had a poor survival rate. Therefore, prior to somite stage 23 dependance on other embryonic tissues could not be determined.

More recently, there have been a number of studies aimed at determining when RPE induction occurs and which tissue(s) are responsible for induction of the presumptive RPE in mice. In essence, there are two obvious candidate tissues, which by virtue of their position in the optic cup could be responsible for inducing the presumptive RPE - the mesenchyme underlying the optic cup and the presumptive NR (the inner layer of the optic cup).

Buse and de Groot (1991) cultured murine eye anlagen under a variety of different conditions and found that at stage 16 (and not before), the presumptive RPE is committed to a pigment cell fate. At this stage of murine eye development, the developing RPE is a monolayer. They cultured entire eye vesicles/cups (without surrounding mesenchyme) at stages 12-16 and observed that pigment only developed in the stage 16 explants. Thus they concluded that by

[2] Refer to glossary for definition.

stage 16, the RPE is committed. In addition, in order to determine the contribution of the surrounding mesenchyme on RPE induction, they cultured eye anlage alone and *in situ* (ie. within the head tissue) at stages 12-14. In all explants pigmentation occurred. Thus the mesenchyme (ie. the environment) surrounding the presumptive RPE appears to be crucial for its induction, since removal of the mesenchyme (before induction, ie. before stage 16) did not result in pigment formation. (Interestingly, the environment is still capable of inducing the explanted presumptive RPE in culture). In summary, Buse and de Groot (1991) established that in embryonic mice, commitment to the pigment cell fate is completed at stage 16 and up until this stage the environment (mesenchyme) is necessary for RPE induction. However, it should be noted that in all these cultures the presumptive NR was present and therefore whether or not the NR is involved in RPE induction could not be determined.

Two years later, Buse et al. (1993), investigated precisely this issue - is the presumptive NR involved in induction of the presumptive RPE?. They did this by removing the neural retina of the embryonic murine eye at stages 14-16 and then culturing the eye anlage within the head tissue (ie. with surrounding mesenchymal tissue). Removal of the NR was accomplished by puncturing it out with a glass capillary tube and then leaving the tube in the eye to maintain optic vesicle/cup shape during the culture period. In these studies, the authors observed that at stage 14, only 10% of the eye anlage were pigmented whereas at stage 15, 80% had developed pigment. They concluded that at stage 14, the NR is required for induction of the RPE but at stage 15, the RPE is already determined[3]. However, by leaving the capillary tube in the head tissue on culturing a diffusible tissue is being replaced by a non-diffusible synthetic substance which theoretically could block an inducible factor produced elsewhere in the eye (eg. in the lens vesicle/cup). Furthermore, the whole process of invagination is obstructed. During invagination the presumptive RPE thins to a monolayer while the presumptive NR thickens and therefore perhaps invagination itself is the inducing stimulus for RPE development. In summary, these experiments indicate that the NR is required for RPE induction and this requirement persists until the end of stage 14. These observations are in conflict with previous results (Buse and de Groot, 1991) in which the environment (ie. mesenchyme) surrounding the developing eye was shown to be necessary for RPE induction until stage 16.

This conflict in results lead Buse et al. (1993) to investigate whether the requirement for mesenchymal tissue (shown in the 1991 study) was peculiar to embryonic mesenchyme. They transplanted eye primordia (stage 13-15) without surrounding mesenchyme into (i) the mesenchyme of other eyes, (ii) the mesenchyme of limb buds, (iii) non-mesenchymal muscle tissue and (iv) into adult tissue. In all cases, pigmentation of the transplanted tissue occurred. From this study, it is clear that although the mesenchyme surrounding the presumptive RPE is necessary for induction of the RPE (Buse and de Groot, 1991), this role of the embryonic eye

[3] Refer to glossary for definition.

authors did not describe how the transplanted eye's mesenchyme was removed and one cannot exclude the possibility that the surrounding mesenchyme was not completely removed.

In summary, two independent studies have shown conflicting results. On the one hand, the RPE was shown to be committed at stage 16 of murine eye development and dependent on surrounding mesenchyme for this induction (Buse and de Groot, 1991). On the other hand, RPE induction was shown to be completed by the onset of stage 15 and to be dependent on the NR and not on the surrounding mesenchyme in murine eye development (Buse et al. 1993). The dependence on surrounding mesenchymal tissue as shown in the earlier study, was shown to be non-specific in the second study (as described above). Thus, it appears as if the pigment cell fate of the RPE begins to dominate over the neural fate between stages 14-16 in the embryonic murine eye. These are the stages of optic cup formation when the presumptive NR and presumptive RPE move into close apposition.

It is clear from the conflicting results presented here that the outcome of explant studies are difficult to interpret. Furthermore, the interpretation of the above results was dependent on the presence of pigment granules as an indicator of successful or completed induction. However, with the advance of molecular technology, the onset of expression of the pigment specific genes by RPE cells can now be used to more accurately determine when RPE induction occurs.

1.5 APPROACH AND AIM

The focus of this study is the induction and development of the avian RPE. The chicken RPE becomes pigmented from 70-72 hours (stage 20) of development as observed by light microscopy (Hamburger & Hamilton, 1951). Ultra-structural studies of embryonic chicken RPE cells have shown premelanosomes in the basal portion of the cells from stage 20. Shortly thereafter melanisation begins and by day 10 the total number of melanosomes has increased greatly and stage IV melanosomes dominate (Toda & Fitzpatrick 1972, Hori et al. 1981). One study however revealed conflicting data and report premelanosomes in the dorsal-lateral portion of the optic cup as early as stage 16 (51-56 hours) of chicken eye development (Toda 1969, cited Ide 1972). This study was conducted using a different white chicken breed and differences in breeds could therefore account for the different results obtained. In summary, the first premelanosomes are detected in the chicken RPE at stage 20, and therefore the RPE must have been instructed to pigment before this stage.

The specific aim of the present study was to determine (i) when induction of the chicken RPE occurs and (ii) whether contact with the NR is involved in RPE induction. In order to determine when induction of the RPE occurs, *in situ* hybridisation reactions were conducted to determine the temporal expression pattern of two genes specifically expressed in pigment cells, namely tyrosinase and TRP-2. Once the exact stage at which induction of the presumptive RPE has been determined, embryological manipulations could be conducted to determine which tissue(s) are responsible for inducing it. In order to investigate whether contact with the NR or a diffusible factor from the NR was responsible for induction of the RPE, various barriers were implanted into the eye vesicle before induction had occurred. Entire embryos were then cultured *in vitro* and morphological studies of the developing RPE conducted.

CHAPTER TWO

2.1 Introduction

In the embryonic murine eye, expression of all three members of the tyrosinase gene family has been observed (Steel et al. 1992, Beermann et al. 1992, Cable et al. 1995). Steel et al. (1992) detected TRP-2 mRNA in the optic vesicle at 9.5 days post coitum (dpc) (stage 15). Two days later TRP-1 expression is found in the optic cup, as well as evidence of pigment granules. Steel's group did not find tyrosinase expression until after pigment is visible at 13.5 dpc. They concluded that this was due to very low levels of expression, below the limit of their detection mechanism. Beermann et al. (1992) however, succeeded in detecting tyrosinase transcripts from 10.5 dpc in the murine RPE. No explanation for this difference in results was given.

The aim of this part of the present study was to determine when tyrosinase and TRP-2 are first expressed in the embryonic chicken RPE. Non-radioactive, digoxigenin-labeled antisense riboprobes (complementary to mRNA) were synthesised and used in *in situ* hybridisation (ISH) reactions. Initially RPE cell cultures were used in order to obtain a working ISH protocol for the chicken tyrosinase riboprobe. It was then necessary to test a variety of different protocols in order to map the expression of tyrosinase and TRP-2 in the RPE. This chapter presents the methods used to synthesise the riboprobes, the ISH protocols that were ultimately used and the final results that were obtained. In addition, some of the problems that were encountered as well as the steps taken to establish the ISH protocols are discussed.

2.2 MATERIALS AND METHODS

2.2.1 Gene constructs and preparation of template DNA

Four gene constructs were used for the synthesis of digoxigenin-labeled riboprobes: a chicken tyrosinase clone (B8.3, April et al.1996), a mouse tyrosinase related protein-2 (TRP-2) clone (TRP2A, Jackson et al. 1992) and two chicken TRP-2 clones (clones 196/7, April C.). Approximately 10-20µg of each clone was digested with the appropriate restriction enzyme (RE) for preparation of linear template DNA. Proteins were removed from the sample by standard phenol:chloroform isoamyl alcohol extraction and precipitated (Appendix II). Samples were then quantified by spectrophotometer reading and/or electrophoresed with known amounts of lambda DNA (Boehringer Mannheim) (Appendix II).

Chicken tyrosinase cDNA

The full length chicken tyrosinase cDNA (1.9 kb) was cloned into the EcoRI-XhoI site of pBluescript II SK (+/-) (2.96 kb) (Appendix I) (April et al. 1996) (Fig.2.1A). HincII was used to linearise the plasmid, producing a template of 636 bp for antisense riboprobe synthesis using T_7 RNA polymerase. For sense riboprobe synthesis using T_3 RNA polymerase, a template of 1.2 kb was produced.

Mouse TRP-2 cDNA

The mouse TRP-2 gene (1.75 kb) was cloned into the EcoRI site of pBS (+/-) (3.2 kb) (Appendix I) (Jackson et al.1992). XhoI was selected for the preparation of a 950 bp DNA template and T_7 RNA polymerase used to transcribe the antisense riboprobe (Fig.2.1B).

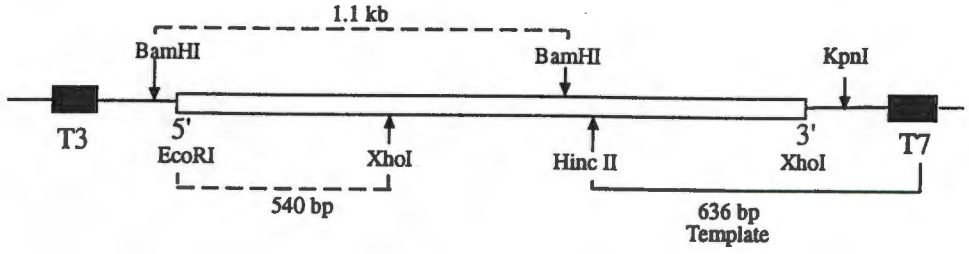
Chicken TRP-2 cDNA

Two clones containing restriction fragments encoding chicken TRP-2 were isolated during this study by strong hybridisation to mouse TRP-2 on a Southern blot (April C., unpublished). The two fragments, one partial (clone 196) and one (possibly) full length (clone 197), were cloned into the EcoRI-XhoI site of pBluescript II SK (+/-) (Appendix I) (Fig.2.1 C & D). Each of these clones will be described separately.

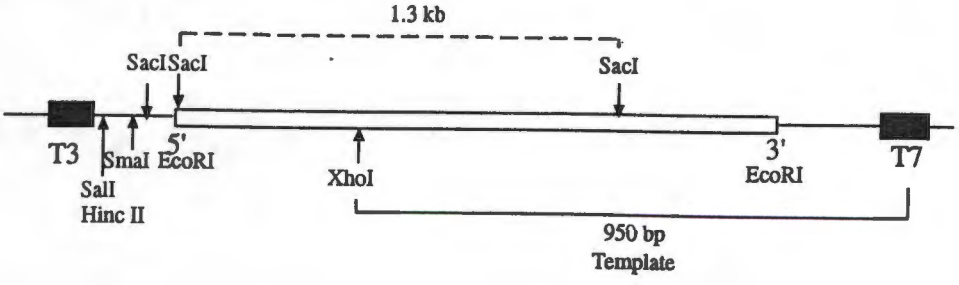
Clone 196:

Clone 196 contains a 2.1 kb fragment of chicken TRP-2 cDNA. At the outset of the present study, details regarding this clone were not known. However, it was later found to consist mainly of untranslated DNA by C.April. From the restriction enzyme map of clone 196 (created by C.April) (Fig.2.1D), BamHI and DraI were chosen for the preparation of template DNA for antisense and sense riboprobes respectively. BamHI digestion yielded a template of 1.4 kb for the synthesis of antisense riboprobe using T_7 RNA polymerase. Sense riboprobe was synthesised from a 1.3 kb DraI digestion fragment using the T_3 RNA polymerase.

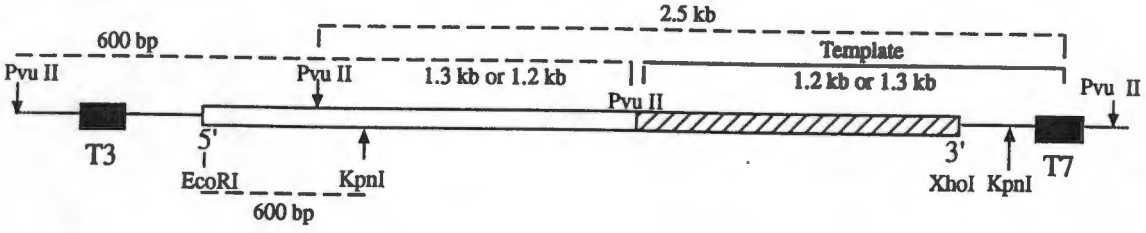
A. Chicken tyrosinase 1.9 kb



B. Mouse TRP-2 1.75 kb



C. Chicken TRP-2 clone 197 2.8 kb



D. Chicken TRP-2 clone 196 2.1 kb

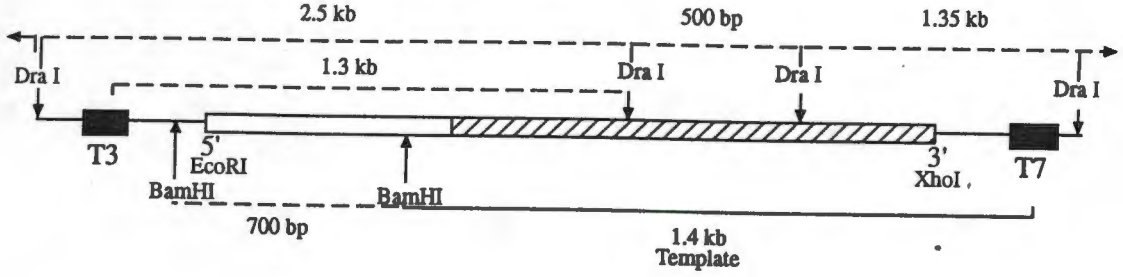


FIGURE 2.1. Restriction enzyme maps of the four gene constructs used in this study. Templates used for antisense riboprobe are indicated. The hatched region in C and D represent the untranslated portion of the cDNA.

Initially, because these digests yielded more than one fragment and in order to remove the non-template fragments, the samples were electrophoresed on 0.9-1% NuSieve GTG low melting point agarose gels (Appendix II). The gel fragments containing the template DNA (the 4.3 kb BamHI fragment and the 2.5 kb DraI fragment) were excised from the gel. Retrieval of DNA from the gel according to the manufacturers was poor in two separate attempts and for subsequent preparations of template DNA the entire digest sample was used.

Clone 197:
The second clone containing chicken TRP-2 cDNA, clone 197, was found by partial mapping (see results 2.3.1.3a) to contain a more full length cDNA, 2.8 kb in length (Fig.2.1C). It was unknown which way this EcoRI-XhoI chicken TRP-2 cDNA fragment had been cloned into the plasmid and since all other inserts using this plasmid had been cloned so that the 3' end of the insert was near the T_7 RNA polymerase binding site, the same was assumed to be true for this clone. A PvuII digest yielded DNA templates of between 0.4 - 1.3 kb and riboprobe was synthesised as described in section 2.2.2.1. However, the above assumption that the 3' end of the insert was near the T7 RNA polymerase binding site could not be confirmed (see results section 2.3.1.3a) and therefore the riboprobe synthesised from this clone was not used in ISH reactions.

2.2.2 Preparation of riboprobe.

2.2.2.1 Synthesis of riboprobe

Antisense and sense riboprobes were generated using T_7 and T_3 RNA polymerase respectively according to the following method. All solutions were treated with 0.01% diethyl pyrocarbonate (DEPC) (Appendix III) before use and gloves were worn throughout.

1µg linear template DNA
1µl rATP (100mM)
1µl rCTP (100mM)
1µl rGTP (100mM)
1µl rUTP mix (0,65µl rUTP + 0,35µl digoxigenin-11-UTP
(Boehringer Mannheim))
1µl RNAsin (25 Units)
2µl DTT (100mM)
4µl 5x transcription buffer

The reaction mixture was made to a final volume of 20µl by adding DEPC-treated water, mixing gently, and then adding 15-20 Units of T_7 or T_3 RNA polymerase (Boehringer Mannheim). The reaction mixture was then incubated in a 37°C waterbath for 2 hours and the reaction stopped with 2µl of 0,2M EDTA. Riboprobe was precipitated overnight at -20°C with 3µl 4M LiCl, 100µl absolute alcohol and 10µg tRNA, then pelleted by centrifugation and

resuspended in 50µl DEPC-treated water. Aliquots were stored at -20°C and at -80°C until required. (For template DNA samples in which more than one DNA fragment was present, the amount of template DNA used for riboprobe synthesis was over estimated).

In order to estimate incorporation of the dig-labeled-UTP and to determine the size of the riboprobe, 5µl of the riboprobe sample was electrophoresed on a 1.3% denaturing formaldehyde RNA gel with bacterial rRNA as markers (Appendix II). Northern transfers were carried out by one of two standard methods (see section 2.2.3.1) and the presence of dig-labeled transcripts determined by immunochemical detection of digoxigenin (see section 2.2.5).

2.2.2.2 Alkaline hydrolysis of riboprobe

Chicken tyrosinase antisense riboprobes were shortened according to instructions by Boehringer Mannheim (1994). In brief, 45µl of riboprobe sample was mixed with 30µl of 0.2M Na_2Co_3 and 20µl of 0.2M $NaHCO_3$. This sample was then incubated at 60°C for a calculated time which was estimated according to the following formula.

$$\text{time (minutes)} = \frac{\text{starting L - desired L}}{(0.11) \times (\text{starting L}) \times (\text{desired L})}$$

where L = length of the probe in kb. According to this calculation, with a starting length of approximately 640 bp and a required length of 250 bp, the desired time for incubation is 22 minutes. Hydrolysis was stopped by adding 3µl 3M Sodium Acetate (pH 6.0) and 5µl 10% glacial acetic acid. The RNA was then precipitated overnight and pelleted by centrifugation. Hydrolysis was verified by standard electrophoresis (Appendix II).

2.2.3 Transfer of nucleic acids onto membranes

2.2.3.1 Northern transfers

RNA was electrophoresed on denaturing formaldehyde gels as described in Appendix II and transferred to nylon membranes by one of two standard methods.
Long method: The gel was washed twice in 10 x SSC for 20 min and the RNA transferred onto a nylon membrane (Hybond N^+, Amersham) by capillary action using 10 x SSC as blotting buffer. The RNA was fixed onto the membrane by baking at 80°C for 2 hours. The blot was then ready for detection or hybridisation or stored at room temperature until required.

Short method: This method uses an alkali blotting buffer, 0.05M NaOH, which allows for a shorter transfer time of 2-3 hours. In addition, the gel does not have to be washed and the RNA does not have to be fixed to the membrane by baking. After blotting, the membrane was rinsed briefly in 2 x SSC with gentle agitation and was then immediately ready for detection or hybridisation.

2.2.3.2 Southern transfers

DNA electrophoresed on agarose gels was transferred onto nylon membranes for future hybridisations by one of two standard methods.
Long method: The gel was denatured and neutralised by washing twice in solution A (1.5M NaCl, 0.5M NaOH) for 20 min and twice in solution B (1M NaOAc, 20mM NaOH) for 20 min. The DNA was transferred onto a nylon membrane (Hybond N^+, Amersham) by capillary action using solution B as blotting buffer. The gel was blotted overnight and the DNA immobilised by baking at 80°C for 2 hours. The membrane was then ready for hybridisation or stored at room temperature until required.
Short method: The gel was not denatured or neutralised before blotting. 0.4M NaOH was used as the blotting solution and the gel was blotted for 2-3 hours only. After blotting, the membrane was rinsed briefly in 2 x SSC with gentle agitation and stored wrapped in plastic-wrap at 4°C until required.

2.2.4 Hybridisation techniques

2.2.4.1 Hybridisation with digoxigenin-labeled riboprobes.

Hybridisation of membranes (Southern and northern) to dig-labeled riboprobes was carried out according to Boehringer Mannheim (1994, Dig System User's Guide for Filter Hybridisation, p31-42). After rinsing, the membranes were prehybridised at 60°C for 2 hours (3.7 x SSC, 3.6 x Denhardt's solution, 0.36% sodium dodecyl sulphate (SDS), 500µg salmon sperm DNA). Riboprobe was then diluted to 1:400 in 2ml of fresh prehybridisation solution and the membranes hybridised at 60°C for 16 hours in bottles fitted to a rotator in a hybridisation oven (Hybaid). After hybridisation, the membranes were removed from the bottles and standard detection of the digoxigenin label was immediately carried out (2.2.5).

2.2.4.2 Hybridisation with a radioactive mouse TRP-2 cDNA probe.

A mouse TRP-2 cDNA probe (1.75 kb EcoRI-EcoRI fragment) labeled with [- ^{32}P]-dCTP by random priming (Boehringer Mannheim) was hybridised to a Southern blot. After wetting the membrane in 4 x SSC, it was prehybridised at 47°C for 16 hours (10ml prehybridisation solution contains 4 x SSC, 1.1g dextran sulphate (Sigma), 1 x Denhardt's solution, 20mM Tris

pH8.0, 40% deionised formamide, 200µg salmon sperm DNA). Denatured probe was added to the prehybridisation solution at a final concentration of 5 x 10^5 cpm/ml and hybridised at 47°C for 16 hours. The membrane was then washed first under low stringency (twice in a 2 x SSC, 0.1% SDS solution for 10 min at room temperature) and then under high stringency (0.1 x SSC; 0.1% SDS solution for 15 min). After washing, the membrane was exposed to autoradiographic film at -80°C for 2 hours, 17 hours and four days. The film was developed in Kodak D19 developer and fixed in Ilford fixer before drying at 37°C.

2.2.5 Detection of digoxigenin.

The method for detection of digoxigenin-labeled riboprobes that are fixed to membranes (section 2.2.3) and those hybridised to DNA fixed to membranes (section 2.2.4.1) is exactly the same and was carried out according to Boehringer Mannheim (1994). After rinsing, the membrane was blocked for 1 hour in blocking solution (3% milk powder, 2% normal sheep serum, 0.05% Tween) and then incubated in 1:5000 dilution of the anti-digoxigenin-alkaline phosphatase antibody (Boehringer Mannheim) for 1 hour. The membrane was washed twice in Tris buffered saline (TBS, pH 7.5) for 15 min to remove unbound antibody and then equilibrated in a high pH buffer (0.1M Tris, 0.1M NaCl, 0.05M $Mg_2Cl_2.6H_2O$, pH 9.5) for 2 min before detection. The detection solution contained 75 mg/ml nitroblue tetrazolium (NBT) and 50 mg/ml bromochloroindolyl phosphate (BCIP) in the above high pH buffer and was freshly made before use. The membrane was incubated with this solution (without agitation) in a dark cupboard for 5 min to 1 hour until a suitable signal was obtained. The detection reaction was stopped by washing the membrane in TE buffer (10mM Tris pH7.6, 0.1mM EDTA pH8.0) for 5 min and the blot photographed before drying.

2.2.6 RPE RNA extraction

The retinal pigment epithelium of 7 day old chicken embryos was dissected and RPE cells cultured to confluency in a humidified chamber with 5% CO_2 at 37°C. The culturing of RPE cells was conducted by T.Wiggins. Eagle's MEM (Sigma) containing 10% heat-inactivated foetal calf serum (Delta Bioproducts) and 100 µg/ml penicillin/streptomycin (Highveld Biological, SA) was used as culture medium. Total RNA from three confluent 10 cm dishes was extracted at any one time. Cells were trypsinised with 0.05% trypsin/0.02% EDTA and a cell suspension obtained by trituration. RPE cells were isolated by centrifugation and then resuspended in RNA extraction buffer (0.14M NaCl, 1.5mM $MgCl_2$, 10mM Tris pH 8.6, 0.5% Nonidet P40, 1mM dithiothreitol (DTT)). Cells were lysed by centrifugation at 12 000g at 4°C for 90 sec and the pellet containing unlysed cells discarded. The supernatant was then incubated in proteinase digestion buffer (0.2M Tris pH8.0, 25mM EDTA pH8.0, 0.3M NaCl, 2% SDS) for 15 seconds before adding proteinase K (20 mg/ml). Proteins were then removed by extraction with phenol:chloroform:isoamyl alcohol (49:1:1). The RNA was precipitated with

isopropanol and pelleted by centrifugation. The pellet was resuspended in 20-40µl 0.01% DEPC-treated water and the sample quantified by spectrophotometer reading (Appendix II).

2.2.7. Tissue preparation for *in situ* hybridisation

2.2.7.1 Preparation of embryos for sectioning

Chicken embryos (White Plymouth Rock X Pile Game) were incubated in a humidified chamber with 50% humidity at 37°C for 1.5-7 days. Embryos were staged according to Hamburger and Hamilton (1951)[1] and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, pH7.4) at 4°C overnight. Larger embryos were cut in order to make penetration of the fixative more effective or injected with fixative. Embryos were either processed for paraffin wax embedding (Appendix III) or cryoprotected (15% sucrose at 4°C for 2 hours, 30% sucrose overnight at 4°C) and embedded in 7% gelatin in 20% sucrose for 2 hours at 37°C. After setting the gelatin at 4°C the tissue was frozen in liquid nitrogen.
Sections (4µm) through the developing eye were cut and placed on aminopropyltriethoxy-silane (APTES)-coated glass slides (Appendix III). Wax sections were incubated at 60°C for 30 min or at 37°C overnight and were stored at 4°C until required. Frozen sections were heat fixed onto the slides at 50°C for 10-15 min before storage at -20°C or at -80°C.

2.2.7.2 Preparation of cell cultures for *in situ* hybridisation.

Retinal pigment epithelial cells and fibroblasts from 6 day old chicken embryos (White Plymouth Rock X Pile Game) were cultured to subconfluency by T.Wiggins as described earlier and then re-plated onto APTES-coated glass slides for further culturing. When the cells had sufficiently adhered to the slides they were washed in PBS and fixed in a 4% formaldehyde/5% acetic acid solution (in PBS) at room temperature for 22 min. Slides were stored under 70% alcohol at 4°C until required

2.2.8 *In situ* hybridisation

Numerous attempts were made to optimise the *in situ* hybridisation (ISH) reaction, details of which are described in the discussion of methodological issues (see section 2.4). The method described here represents the conditions that ultimately gave the best results.

---

[1] Embryos could not always be classed into a defined Hamburger and Hamilton (1951) stage and in these cases, half stages were used).

2.2.8.1 *In situ* hybridisation on cell cultures

ISH on RPE and fibroblast cell cultures grown on APTES-coated slides was carried out according Dirks et al. (1994) with some modifications. Cells were dehydrated in a graded series of alcohols, passed through xylol and rehydrated to 70% alcohol. After washing, the cells were permeabilised in 0.1% pepsin solution (in PBS) for 5 min at 37°C, washed twice in PBS and fixed in 1% formaldehyde in PBS for 5 min at room temperature. Riboprobe was prepared as described in 2.2.2 and diluted to 1:500 in hybridisation mix [1ml hybridisation solution contains 0.1g dextran sulphate (Sigma), 1 x Denhardt's solution (0.2% Ficoll, 0.2% polyvinylpyrolidone, 0.2% bovine serum albumin), 4 x SSC, 2mM EDTA, 50% deionised formamide and 500µg salmon sperm DNA]. The cells were hybridised at 55°C overnight in a moist chamber with a petri dish containing a 50% formamide/0.2 x SSC solution. Slides were then washed twice in 2 x SSC at 37°C for 2 min and then twice in a 60% formamide/0.2 x SSC solution at 42°C for 5 min and once for 10 min. The slides were then placed in 2 x SSC at 37°C for a final wash (10 min) before immunocytochemical detection. Normal sheep serum (2%) was used as a blocking solution and slides were blocked at 37°C for 40 min. Slides were then incubated in a moist chamber for 1 hour at 37°C with a 1:250 dilution of the anti-digoxigenin-alkaline phosphatase antibody (Boehringer Mannheim) and covered with parafilm. Unbound antibody was removed by washing twice in TBS after which the slides were equilibrated in a high pH buffer for 2 min and detected in 0.18 mg/ml BCIP and 0.34 mg/ml NBT in high pH buffer. Detection was carried out in the dark for 5-20 hours and monitored closely. The reaction was stopped by washing in TE buffer, after which the slides were rinsed and mounted in veronal buffered glycerol (0.5% sodium veronal barbiturate, 0.3% NaCl, pH8.6, 50% glycerol). Control slides were treated in exactly the same way as experimental slides except that no riboprobe was added to the hybridisation mix.

2.2.8.2 *In situ* hybridisation on sectioned tissue.

The protocol is essentially the same for wax and frozen tissue. Frozen sections were permeabilised in 0.005% pepsin (in 0.2M HCl) at 37°C for 20 min and wax sections in 0.015% pepsin solution (in 0.2M HCl) at 37°C for 20 min to allow penetration of the riboprobe. Sections were fixed in 4% paraformaldehyde (in PBS) for 5 min at room temperature, washed and then acetylated to increase the signal to noise ratio as follows. Slides were placed in a 0.1M triethanolamine solution for 2 min with agitation. Acetic anhydride to a final concentration of 0.25% was added and the solution stirred for a further 8 min. Acetylation was followed by dehydration through a graded series of alcohols and hybridisation. Riboprobe was diluted to 1:500 in the hybridisation solution (as described in 2.2.8.1) and hybridisation was carried out overnight at 55°C in a moist chamber with a petri dish containing

a 50% formamide/2 x SSC solution. Post-hybridisation washes and immunocytochemical detection was carried out as for the cell culture ISH protocol described in 2.2.8.1.

Controls: Negative controls were included in every ISH reaction carried out and were treated identically to the experimental samples except that no riboprobe was added to the hybridisation mix. In order to verify results obtained in the ISH reactions, for every stage embryo used a duplicate run with a different embryo at the same stage was also carried out.

2.3 RESULTS

2.3.1 Restriction enzyme digests, preparation of template DNA and synthesis of riboprobes

RE digests were performed with each of the gene constructs described in Materials and methods for two main reasons. 1) to verify the orientation of the insert (in order for the correct RNA polymerase to be used for antisense riboprobe synthesis) and 2) to determine a suitable RE for linearising the vector to ensure that the size of the RNA transcript would be between 200 bp - 1 kb. Short probes (200 - 500 bp) are recommended for the detection of low copy RNA molecules, such as tyrosinase. Since short probes have greater penetration into tissue but can cause high backgrounds in *in situ* hybridisation (ISH) reactions, a probe length of 500 bp was considered optimal for this study. In cases where restriction enzyme digestion of the gene construct yielded large templates, the riboprobes synthesised from these templates were hydrolysed by alkaline hydrolysis.

The RE digests that were performed and the subsequent synthesis of riboprobes from each of the gene constructs will be discussed separately.

2.3.1.1 Preparation of the chicken tyrosinase riboprobe

The 1.9 kb chicken tyrosinase cDNA was cloned into the EcoRI-XhoI site of pBluescript (2.9 kb) (Appendix I) as described in section 2.2.1 and diagnostic digests performed (Fig 2.1). The insert of 1.9 kb was released from the vector with an EcoRI/KpnI double digest (Fig.2.2, lane 2). XhoI could not be used to release the insert from the vector because the insert contains a XhoI site 540 bp from its 5' end (April et al. 1996) (Fig. 2.1). A BamHI digest was carried out to verify orientation of the insert and yielded a 1.1 kb and a 3.9 kb DNA fragment (Fig.2.2, lane 3) indicating that the 3' end of the insert was near the T7 RNA polymerase binding site of the vector (Fig.2.1). Antisense riboprobe, complementary to mRNA, would therefore be transcribed by T7 RNA polymerase. From the RE map of this construct (April et al. 1996), HincII was chosen for the preparation of template DNA (Fig.2.1). HincII yields a linear 4.8 kb fragment (Fig.2.2, lane 4) cutting the insert 636 bp from the T7 polymerase binding site. Because of the ideal position of the HincII RE site, this digest could be used as a template for the synthesis of both sense (1287 bp) and antisense (636 bp) riboprobes (Fig.2.1).

Antisense riboprobe using T7 RNA polymerase was subsequently synthesised and analysed on a 1.3% denaturing formaldehyde agarose gel (Fig.2.3). Northern transfer was then carried out and the blot containing the riboprobe detected by the digoxigenin detection method in order to determine the labeling efficiency of the riboprobe (Fig. 2.3B). This method of analysing the dig-labeled riboprobe was carried out for all the riboprobes synthesised in this

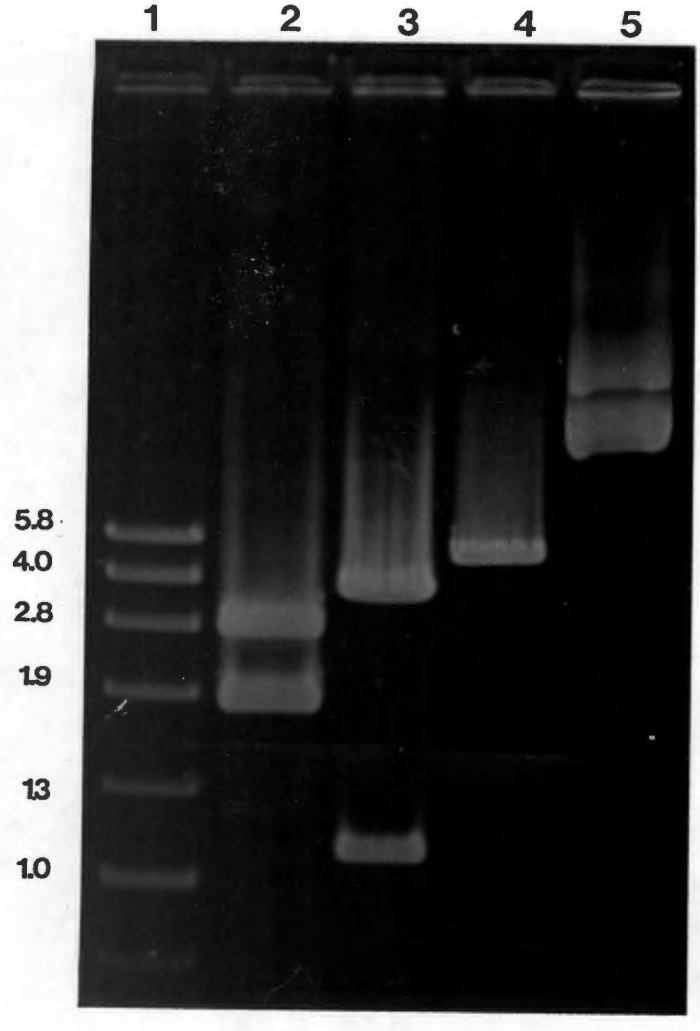


FIGURE 2.2. Diagnostic restriction enzyme digests of the chicken tyrosinase cDNA. The construct was digested with EcoRI and KpnI (lane 2), BamHI (lane 3) and HincII (lane 4). Lane 1, molecular weight marker; lane 5, undigested DNA.

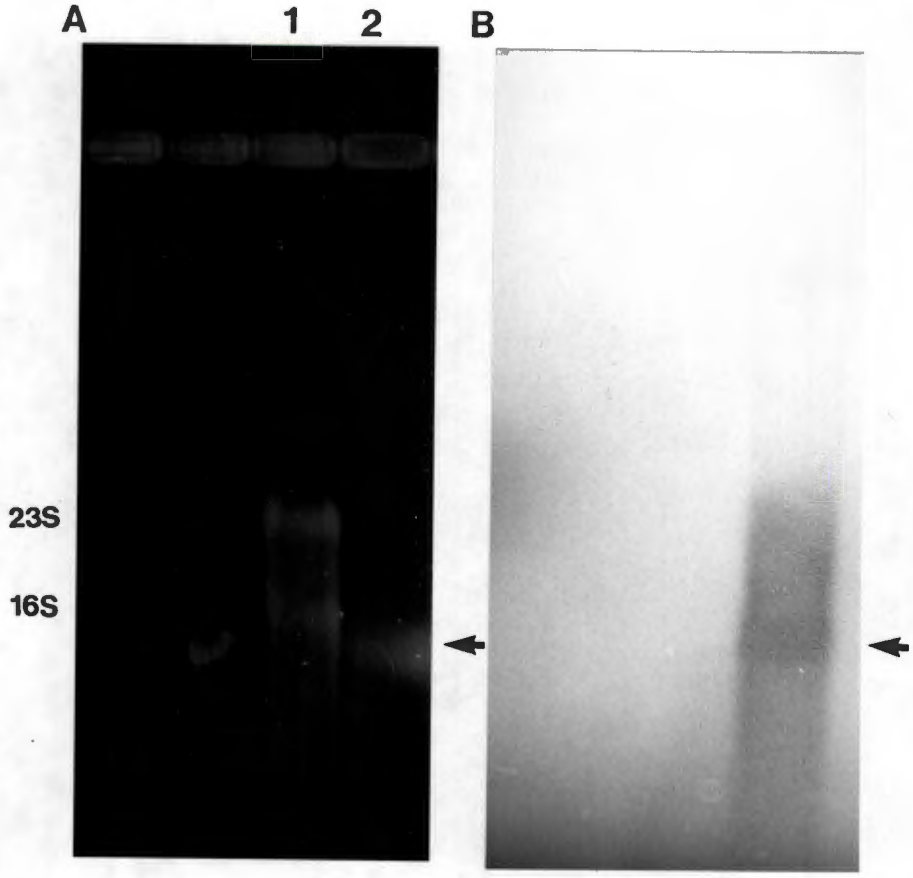


FIGURE 2.3. Chicken tyrosinase antisense riboprobe. (A) 5µl of riboprobe sample (lane 2, arrow) was electrophoresed with rRNA markers (lane 1) on a 1.3% denaturing formaldehyde agarose RNA gel. (B) Northern blot of A with the digoxigenin-labeled riboprobe (arrow) detected as described in 2.2.5.

study. All digoxigenin-labeled riboprobes synthesised did not migrate according to their molecular weights when electrophoresed and appeared larger than expected. The chicken tyrosinase antisense riboprobe of 636 bp (Fig. 2.3A, lane 2) is situated just below the 16S (1.7 kb) rRNA marker band (Fig 2.3A, lane 1) and therefore appears larger than 636 bp. This dragging was most likely due to the digoxigenin label which slows down the migration of the riboprobe during electrophoresis. The chicken tyrosinase sense riboprobe was similarly synthesised and analysed by electrophoresis and northern transfer (not shown).

In order to optimise dig-labeling of the riboprobe, various ratios of digoxigenin-11-UTP/UTP were tested. A ratio of 1:3 was found to efficiently label the riboprobe (Fig.2.3). In addition, a range of anti-digoxigenin antibody concentrations were used in order to determine the minimum amount of antibody required to detect the dig label. A concentration of 1:5000 was found to be sufficient for the detection of dig-labeled riboprobes fixed to northern blots.

The antisense chicken tyrosinase riboprobe (636 bp) was then used in ISH reactions on four day old chicken eye tissue. (The ISH protocols used are discussed in section 2.2.8). No positive results were obtained with this riboprobe initially and it was possible that this could be due to the probe size. Therefore in order to shorten the probe length to improve penetration into the tissue, alkaline hydrolysis was attempted. Both sense and antisense riboprobes were hydrolysed to 200 bp as described in 2.2.2.2. The hydrolysed sense riboprobe (lane 6) and the unhydrolysed sample (lane 5) can be seen in Figure 2.4. No full length riboprobe is visible in lane 6 (arrow) and a very faint smear is present in this lane. The greater intensity of the lower band of free nucleotides in this lane indicates that extensive hydrolysis had occured (arrowhead). The antisense riboprobe (lanes 2-4) only partially hydrolysed in a first attempt as evidenced by the faint smear below the riboprobe band in lane 4 compared to the unhydrolysed antisense riboprobe in lanes 2 and 3. This partially hydrolysed sample was rehydrolysed and the riboprobe was subsequently not visible when electrophoresed (not shown).

In order to determine whether the hydrolysed riboprobes had not been overhydrolysed, Southern blots containing the 4.8 kb chicken tyrosinase DNA template were hybridised with 1) the hydrolysed chicken tyrosinase antisense riboprobe (Fig. 2.5, lanes 4-5) and 2) the hydrolysed chicken tyrosinase sense riboprobe (Fig. 2.5, lanes 1-2). The very strong signal bands seen in lanes 2 and 4 of Figure 2.5B indicate that the hydrolysed RNA transcripts were able to hybridise to template DNA.

Both the hydrolysed and unhydrolysed antisense riboprobes (as prepared above) were initially used in ISH reactions on four day old chicken retinal tissue. Initially no positive results were obtained. The hydrolysed chicken tyrosinase antisense riboprobe continuously gave exceptionally high backgrounds which could not be reduced and therefore hydrolysed

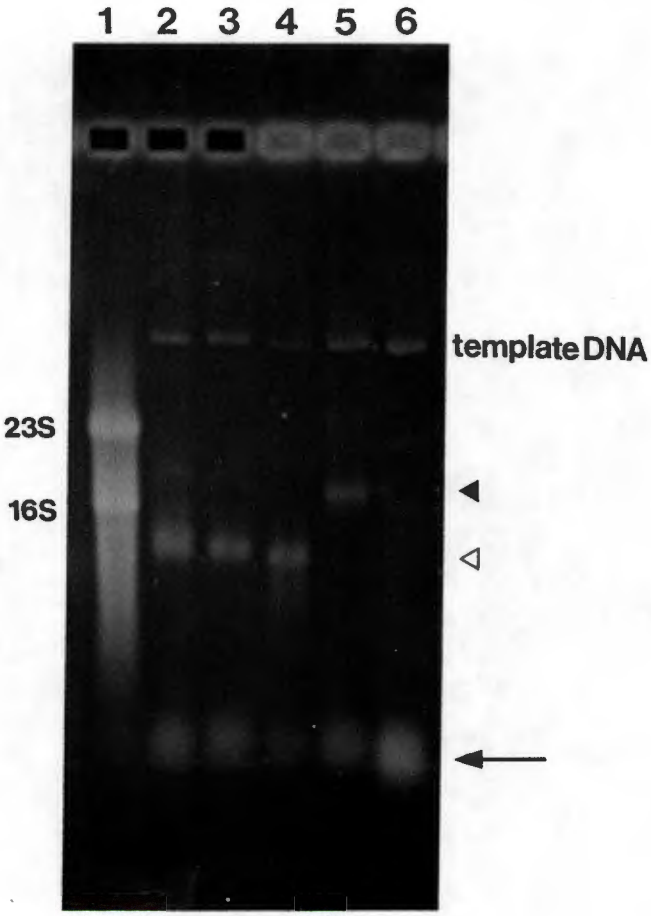


FIGURE 2.4. Electrophoretic analysis of hydrolysed and unhydrolysed sense and antisense chicken tyrosinase riboprobes. Lane 1, bacterial rRNA marker; lane 2 and 3, unhydrolysed antisense riboprobe; lane 4, partially hydrolysed antisense riboprobe; lane 5, unhydrolysed sense riboprobe; lane 6, hydrolysed sense riboprobe. Each lane contains 5µl of riboprobe sample. Filled arrowhead indicates the full-length sense riboprobe in lane 5 and the open arrowhead indicates the full-length antisense riboprobe in lanes 2, 3 and 4. The arrow indicates free nucleotides.

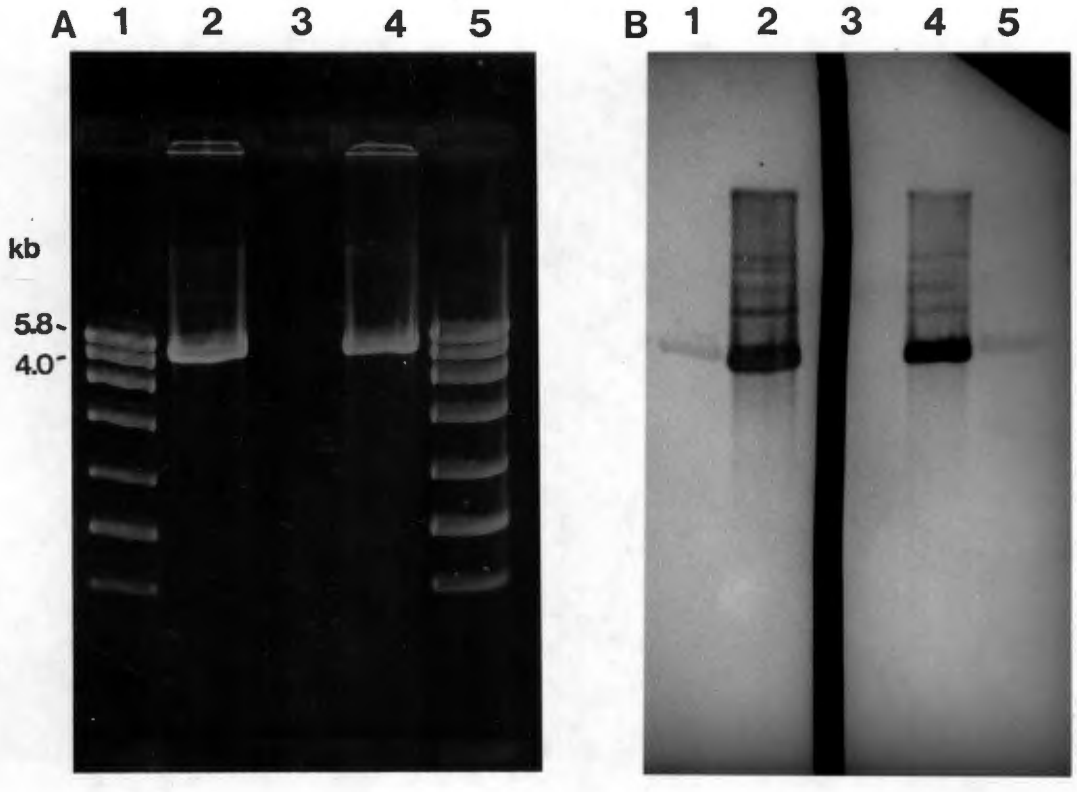


FIGURE 2.5. Analysis of the hydrolysed sense and antisense chicken tyrosinase riboprobes. (A) 0.5µg chicken tyrosinase template DNA (4.8 kb) (lane 2 and 5) was electrophoresed with the molecular weight marker (lane 1 and 5). (B) Southern blot hybridisation of template DNA in (A). Probe used for lanes 1 and 2 was the hydrolysed chicken tyrosinase sense riboprobe and for lanes 4 and 5, the hydrolysed chicken tyrosinase antisense riboprobe was used.

riboprobes were not used in future ISH reactions. After four months of experimentation with various ISH protocols and changing various parameters, an ISH protocol that yielded positive results on chicken RPE cell cultures and on wax and frozen chicken embryonic eye tissue was obtained using the unhydrolysed chicken tyrosinase antisense riboprobe (see section 2.2.8.2). This riboprobe was subsequently used to establish the temporal expression pattern of tyrosinase in the chicken embryonic eye (see section 2.3.2.2).

2.3.1.2 Preparation of the mouse TRP-2 riboprobe

At the initial stages of the present study, the chicken TRP-2 cDNA had not been isolated. Therefore in order to investigate expression of TRP-2 in the chicken embryonic eye, mouse TRP-2 cDNA was used for the generation of antisense transcripts. Diagnostic digests of the mouse TRP-2 cDNA gene construct were carried out in order to confirm that the correct plasmid had been obtained (Fig.2.6). HincII, SmaI and SalI all digested the plasmid once only and produced the expected linear 4.9 kb fragment (Fig.2.6, lanes 2-4). SacI digestion was used to verify the orientation of the insert (Fig.2.6A, lane 5) and the 3' end of the insert was found to be located near the T7 polymerase binding site of the vector (Fig.2.1). From the published sequence of the mouse TRP-2 cDNA (Jackson et al. 1992), XhoI, which cuts the insert at 950 bp from its 3' end was chosen for the preparation of template DNA (Fig.2.1). A XhoI digest thus yields a single 4.9 kb fragment and can be seen in Figure 2.6B, lane 3.

Antisense TRP-2 riboprobe was synthesised as described in 2.2.2.1 and electrophoresed on an RNA gel (together with the chicken tyrosinase antisense riboprobe) (not shown). The gel was blotted and the dig-labeled riboprobes detected (as described in section 2.2.5) (Fig.2.7). This was done in order to verify synthesis of the riboprobe and to determine the labeling efficiency of the riboprobe. The mouse TRP-2 antisense riboprobe (950 bp) (Fig.2.7, lane 2) is slightly larger than the chicken tyrosinase riboprobe (636 bp) (Fig.2.7, lane 3) and is therefore situated slightly higher on the blot.

ISH reactions with the mouse TRP-2 antisense riboprobe did not yield positive results and therefore in order to verify that the riboprobe was indeed antisense and not sense, a northern blot containing RNA from various mouse cell lines was hybridised with the mouse TRP-2 dig-labeled antisense riboprobe (as described in 2.2.4.1) (Fig.2.8). (This blot was generated by S.Prince). Two positive signals were obtained for B16 RNA (lane 2) and for the pigmented mouse melanocyte cell line, DMEL-3 (Prince et al.1996, unpublished) (lane 5) which confirmed that antisense riboprobe had been synthesised. The quality of the RNA extracted from the B16 cells was poor (not shown) and hence the signal in lane 2 was not as strong as that in lane 5.

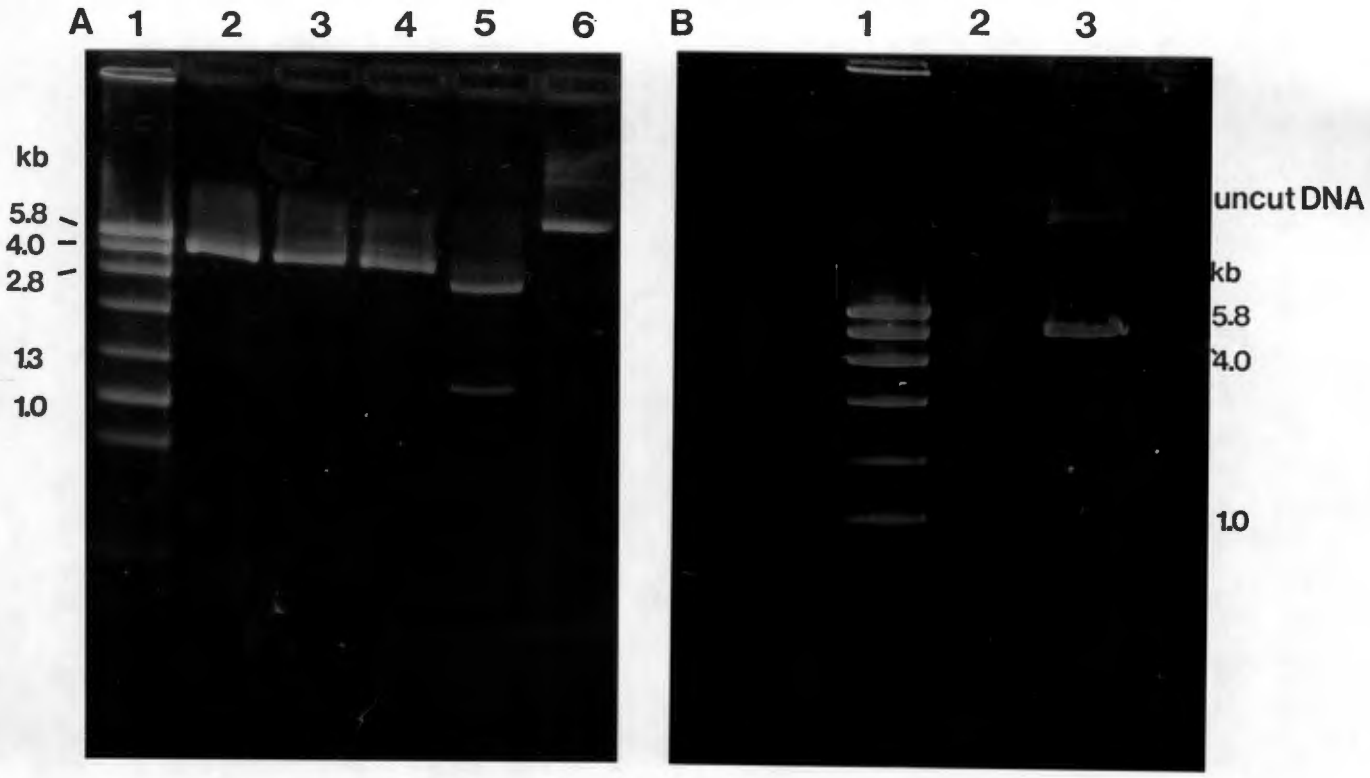


FIGURE 2.6. Restriction enzyme analysis of the mouse TRP-2 cDNA. (A) Lane 2-5 contain samples digested with HincII (lane 2); SmaI digestion (lane 3), SalI digestion (lane 4) and SacI digestion (lane 5). Lane 6 contains uncut DNA and lane 1 the molecular weight marker. (B) XhoI digestion produced a 4.8 kb DNA template (lane 3) for riboprobe synthesis. No DNA was loaded in lane 2. Lane 1, molecular weight marker.

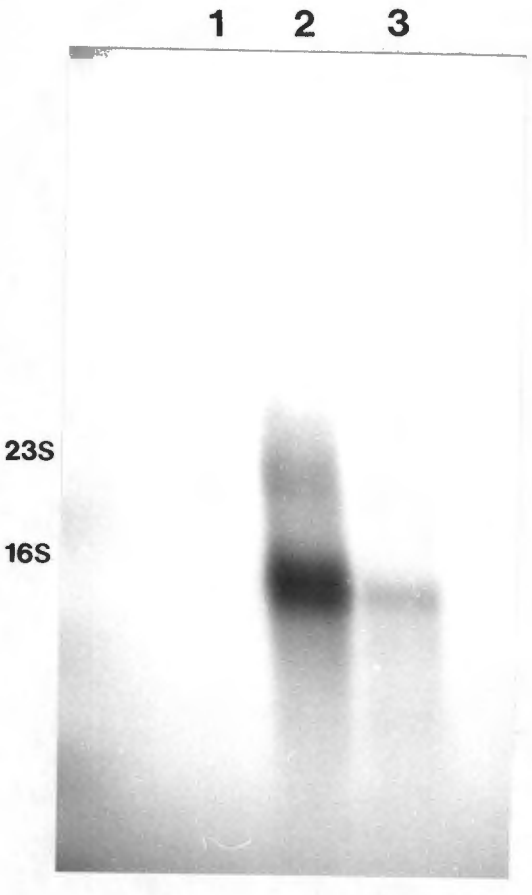


FIGURE 2.7. Northern transfer and detection of dig-labeled mouse TRP-2 antisense riboprobe (lane 2) and chicken tyrosinase antisense riboprobe (lane 3). Lane 1, bacterial rRNA marker.

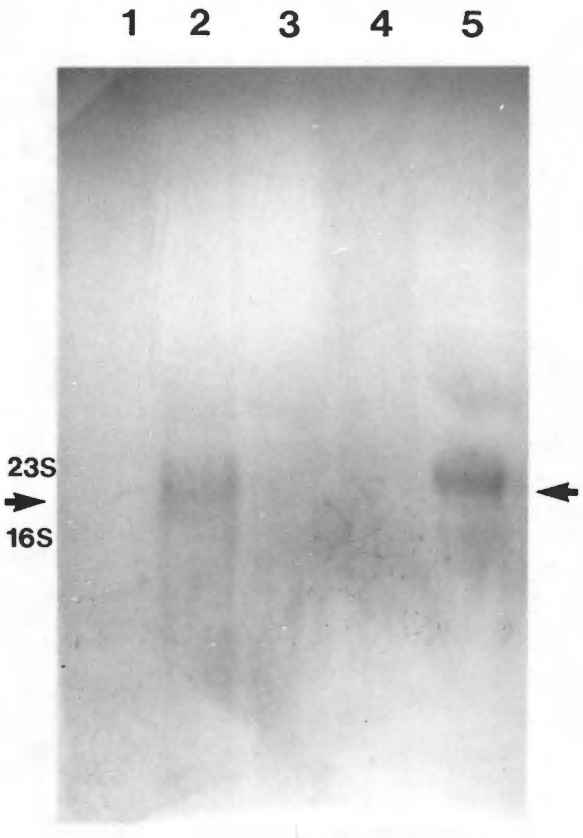


FIGURE 2.8. Northern hybridisation of RNA derived from B16 mouse melanoma cells (lane 2), 3T3 mouse fibroblasts (lane 3) and a pigmented mouse melanocyte cell line, DMEL-3 (lane 5). Probe used was the mouse TRP-2 dig-labeled riboprobe. No RNA was loaded in lane 4. Lane 1, bacterial rRNA marker. Arrows indicate the hybridisation signal in lanes 2 and 5.

Further ISH reactions with the mouse TRP-2 riboprobe were carried out on RPE cell cultures and on wax sections through the embryonic chicken eye without success (discussed later). It is likely that this was due to a stringency problem because of cross-species hybridisation.

At this stage in the study, however, seven chicken TRP-2 clones were isolated by C.April making it possible to synthesise a chicken TRP-2 antisense riboprobe.

2.3.1.3 Preparation of the chicken TRP-2 riboprobe

Of the seven clones containing chicken TRP-2 cDNA that were isolated from the chicken cDNA library (April et al.1996), only two (clone 196 and clone 197) were evaluated in this study. For the ease of discussions, the two clones will be discussed separately although the investigations were conducted concurrently at times.

2.3.1.3 a) Clone 197:

Since clone 197 (obtained from C.April) had not been previously mapped, it was first necessary to map this clone to determine its size and to locate an appropriate restriction enzyme site for the preparation of template DNA for riboprobe synthesis.

In order to determine the size of the EcoRI - XhoI cDNA fragment, double and single digests with EcoRI and XhoI were carried out. The single digests both linearised the clone to a 5.8 kb fragment[1] (Fig.2.9, lanes 3-4, arrows) indicating that neither enzyme cuts the insert. The double digests produced a strong 2.8 kb fragment (Fig.2.9, lanes 5-7, arrows). The extra bands in lanes 3-7 represent a recurring contaminant[1] which was essentially ignored. (Non-contaminant fragments are indicated with arrows). Since no vector band was found in the double EcoRI/XhoI digests, it was concluded that the insert was 2.8 kb in length and was co-running with the vector fragment during electrophoresis. The insert size was confirmed with an EcoRI/KpnI digest and a KpnI site found in the insert 2.2 kb from its 3' end (Fig.2.1).

In order to map clone 197, a series of diagnostic digests were performed (not shown). However, partial digestion was common and therefore in order to determine which digestion fragments contained large amounts of insert, gels containing partial digests of clone 197 were transferred by Southern blotting and hybridised with a ^{32}P-mouse TRP-2 cDNA probe

[1] In some instances, when samples of clone 196/7 were electrophoresed, anomalous bands would appear. These extraneous bands were of various sizes as can be seen in Fig. 2.9 (lanes 3-7). The contaminant could not be cloned out and numerous attempts by myself and others were made to remove it. Previous reports of a contaminant within DNA preparations when using Stratagene's pBluescript plasmid with E.coli host strain XL1-Blues have been made (Hengen, 1994). The source of these bands is unknown but has been found not to interfere with further DNA manipulations and is therefore mostly ignored.

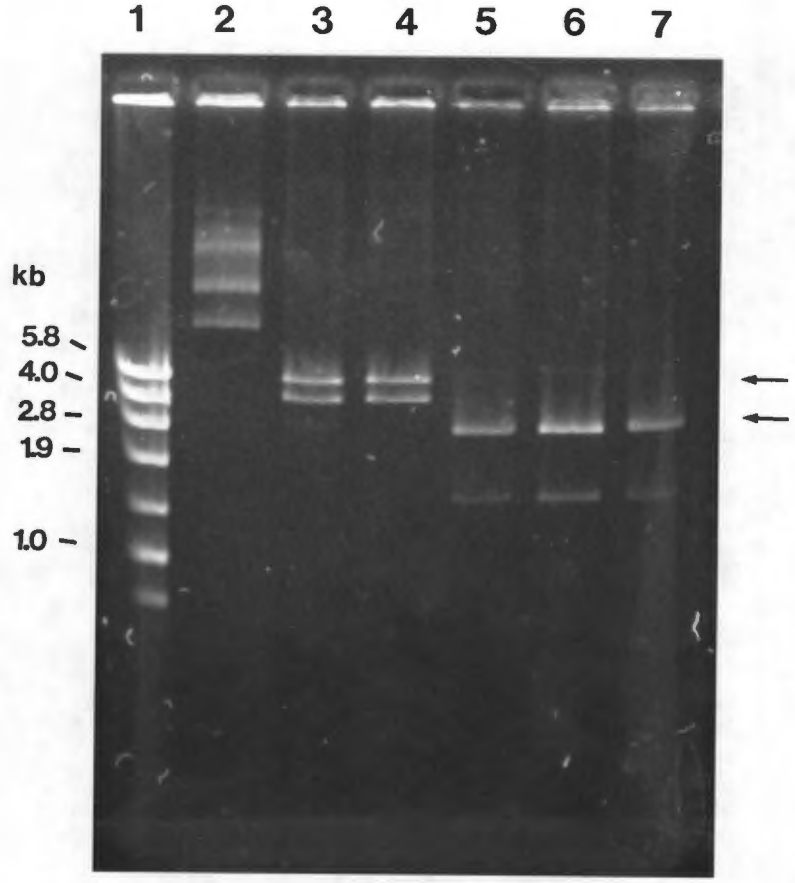


FIGURE 2.9. Restriction enzyme analysis of clone 197. (A) Lane 1, molecular weight marker; lane 2, uncut DNA; lane 3, EcoRI digestion and lane 4, XhoI digestion. Lanes 5-7 contain EcoRI/XhoI double digest samples. Non-contaminant bands are indicated (arrows).

(described in 2.2.4.2). Analysis of these results were tricky and none of the restriction enzyme sites could be confirmed (data not shown).

Digestion of clone 197 with PvuII however, yielded four fragments (2.5 kb, 1.3 kb, 1.2 kb, 0.4 kb) (Fig.2.10, lane 1). pBluescript has two PvuII sites 2.5 kb apart (Appendix I). The other three digestion fragments therefore must consist largely of chicken TRP-2 cDNA. It was deduced that T7 RNA polymerase would be required for the synthesis of antisense chicken TRP-2 riboprobe (using clone 197) because of the way the chicken cDNA library had been constructed (April et al. 1996). (Directional cloning had been used to ensure that the XhoI site at the 3' end of the cDNA insert would be next to the T7 polymerase binding site of the vector). Using T7 RNA polymerase, either of the three template DNA fragments (0.4 - 1.3 kb) obtained by PvuII digestion were long enough to serve as DNA templates for riboprobe synthesis. Therefore the entire PvuII digestion sample was used and chicken TRP-2 riboprobe synthesised (section 2.2.2.1). The riboprobe was electrophoresed and analysed on a northern blot (as described earlier) (Fig.2.11). Since the dig-label hinders migration of labeled probe during electrophoresis (as previously described), and from the position of the riboprobe (arrow) between the two rRNA marker bands, it is likely that the riboprobe was synthesised from either the 1.2 kb or the 1.3 kb template DNA fragments.

In order to attempt to determine whether antisense (as opposed to sense) riboprobe had indeed been synthesised from this clone (since errors during directional cloning can occur), northern blots containing chicken RPE RNA were hybridised to the chicken TRP-2 riboprobe synthesised from clone 197 (Fig 2.12). Hybridisation to both RPE rRNA bands and to the lower bacterial rRNA band were obtained, making the distinction between background and true signal difficult (Fig.2.12B). It could not therefore be unequivocally determined whether the riboprobe synthesised from clone 197 was antisense (ie. complementary to mRNA).

During this investigation of clone 197, another clone containing chicken TRP-2 cDNA (clone 196) had been partially mapped by C.April. It was decided to synthesised riboprobe from this clone instead of testing the riboprobe synthesised from clone 197 in ISH reactions since ISH results with sense riboprobes can yield strange results (Boehringer Mannheim). Thus the chicken TRP-2 riboprobe from clone 197 was abandoned and attention was shifted to clone 196.

2.3.1.3 b) Clone 196:

From the partial restriction enzyme map of this clone created by C.April it was established that the 3' end of the cDNA was closest to the T7 RNA polymerase binding site (Fig. 2.1). Thus T7 RNA polymerase was required for antisense riboprobe synthesis. The vector was linearised by BamHI digestion, which yields two fragments of 4.3 kb and 0.7 kb (Fig. 2.13). Because of a concern that the 0.7 kb fragment could interfere with riboprobe synthesis,

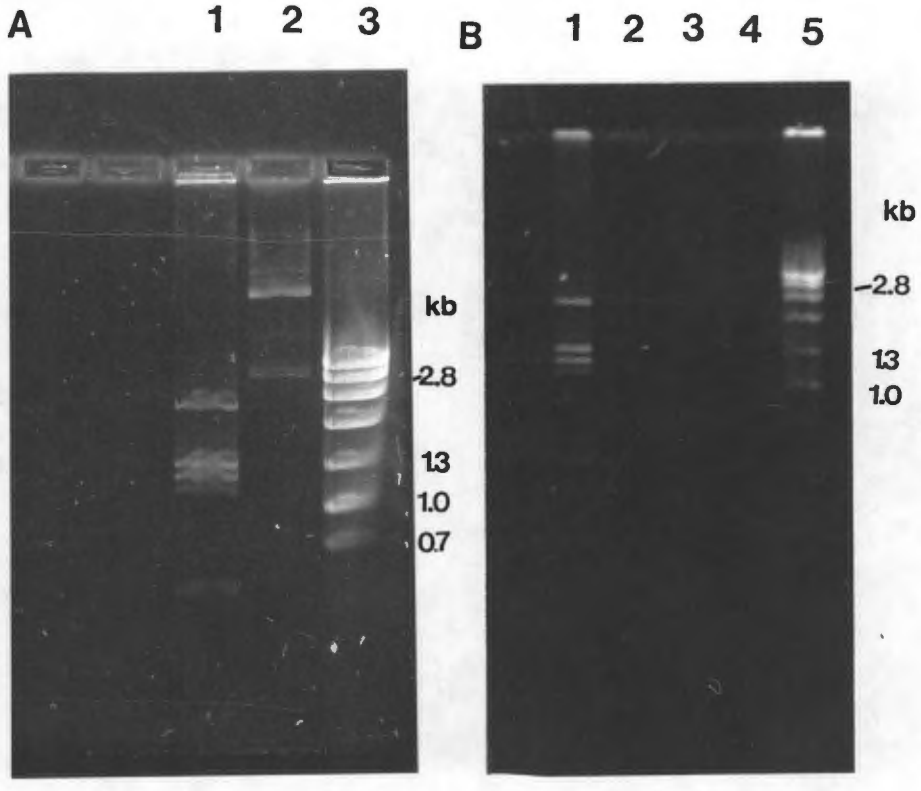


FIGURE 2.10. PvuII digestion of clone 197 (lane 1) of A and B. (A) Lane 2, EcoRI digestion of clone 196; lane 3, molecular weight marker. (B) Lane 5, molecular weight marker; lanes 2,3 and 4 are empty.

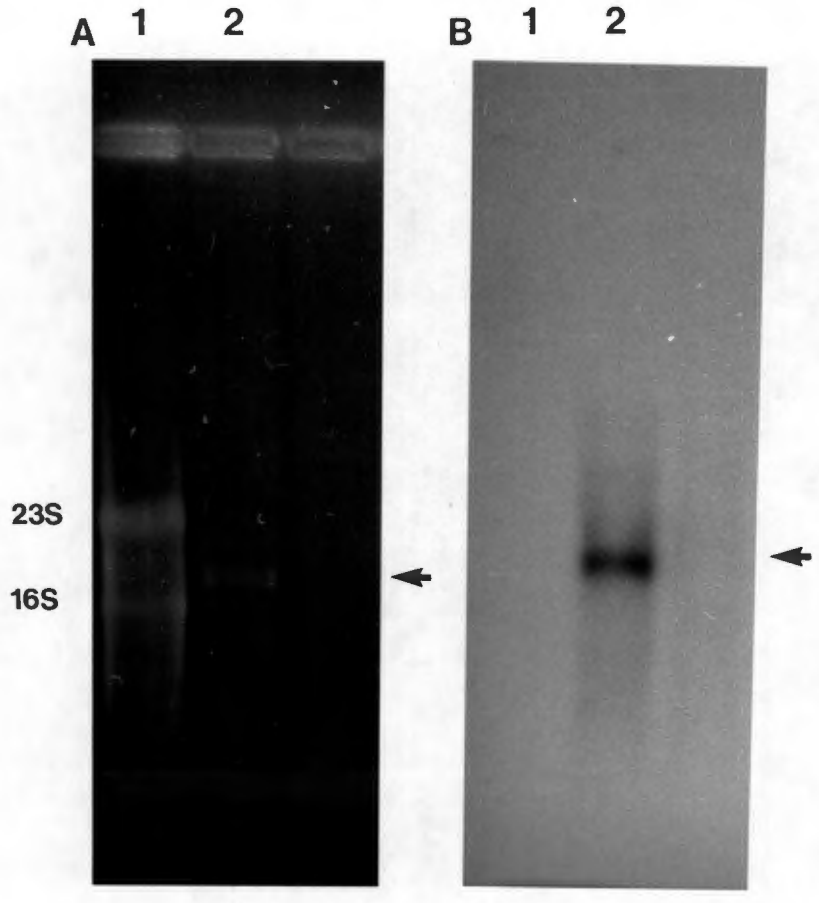


FIGURE 2.11. Chicken TRP-2 antisense riboprobe (arrow) synthesised from clone 197. (A) 5µl of the riboprobe sample (lane 2) was electrophoresed with bacterial rRNA markers (lane 1). (B) Northern transfer of (A) with the digoxigenin detected riboprobe.

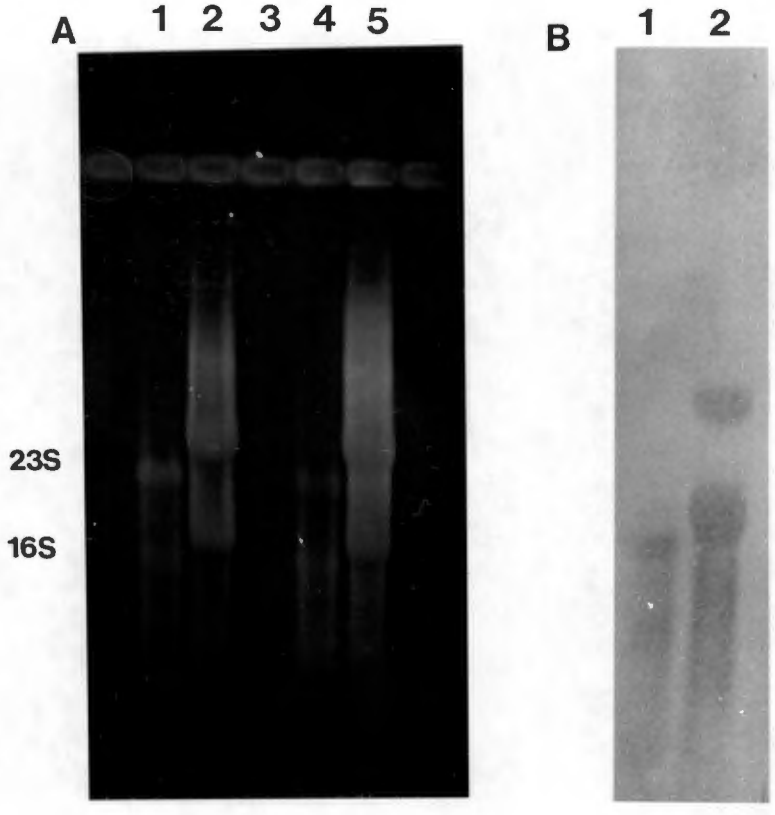


FIGURE 2.12. 20µg of chicken RPE RNA (lane 2 and 5) electrophoresed with rRNA markers (lane 1 and 4). No RNA was loaded in lane 3. (B) Northern hybridisation of lanes 1 and 2 in (A). Probe used was the chicken TRP-2 riboprobe from clone 197.

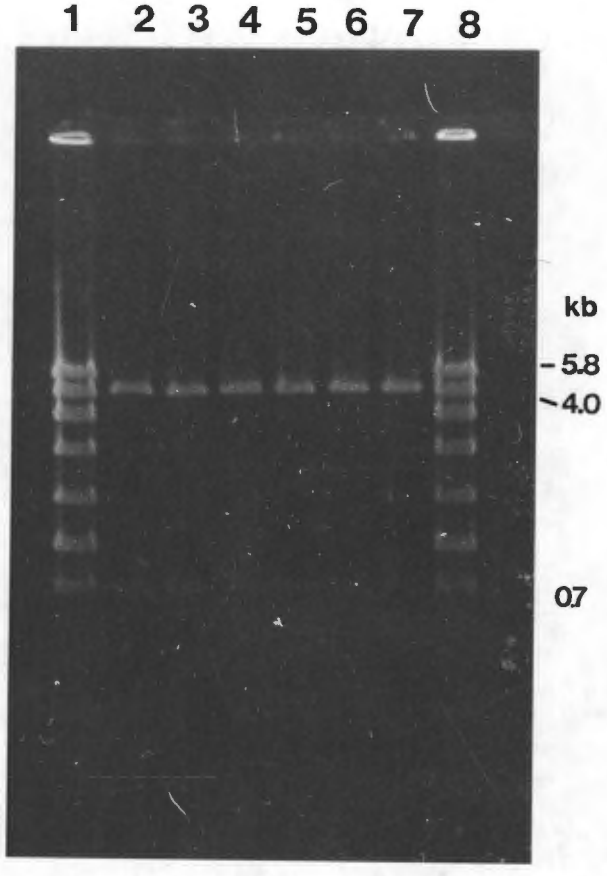


FIGURE 2.13. 10-20µg of clone 196 digested with BamHI (lane 2-7) and electrophoresed on a low melting point agarose gel. The upper fragment contains the DNA template. Lanes 1 and 8 contain the molecular weight marker.

attempts to remove it were made. In order to remove the 0.7 kb fragment from the digestion sample, the sample was electrophoresed on a low melting point agarose gel (LMP), the 4.3 kb band excised and the DNA extracted. Recovery of DNA from the agarose fragment was low in two separate attempts. For this reason, the 0.7 kb fragment was subsequently left in the digest sample and the entire digest sample used for the synthesis of antisense riboprobe (as described in 2.2.2.1). A similar procedure was used to prepare a DNA template for sense riboprobe synthesis (from DraI digestion) but was also unsuccessful (not shown).

At this point in the study, repeating BamHI digestion of clone 196 yielded extra DNA fragments (not previously observed) (not shown). These were due to the contaminant described earlier. A purified stock of clone 196 was (finally) prepared and used for the preparation of template DNA for riboprobe synthesis. In addition, from PCR investigations, a large portion (67%) of the insert of clone 196 was found to consist of untranslated DNA (April C., pers. comm.) (Fig 2.1, hatched region)[2]. The template DNA (1.4 kb) for antisense riboprobe prepared by BamHI digestion contained the entire 1.3 kb untranslated DNA region. Although this untranslated region is spliced out before the protein is made, it is transcribed and therefore could be used to detect transcripts *in situ*. This template was therefore used for the subsequent synthesis of antisense chicken TRP-2 riboprobe.

Initial attempts at synthesising this riboprobe (1.4 kb) were however poor. It was thought that the reason for this was the presence of the 0.7 kb BamHI digestion fragment which had the effect of reducing the actual amount of template DNA available for the synthesis of riboprobe. The amount of template DNA used in the riboprobe synthesis reaction was therefore increased (to 1.5 x the previous volume used) and chicken TRP-2 antisense riboprobe was successfully synthesised (Fig.2.14). This riboprobe was subsequently used in ISH reactions to map the temporal expression of TRP-2 in the chicken embryonic eye (see section 2.3.2.3).

[2] This untranslated region appears to be present at the 3'end of clone 197 as well (April C., pers. comm.).

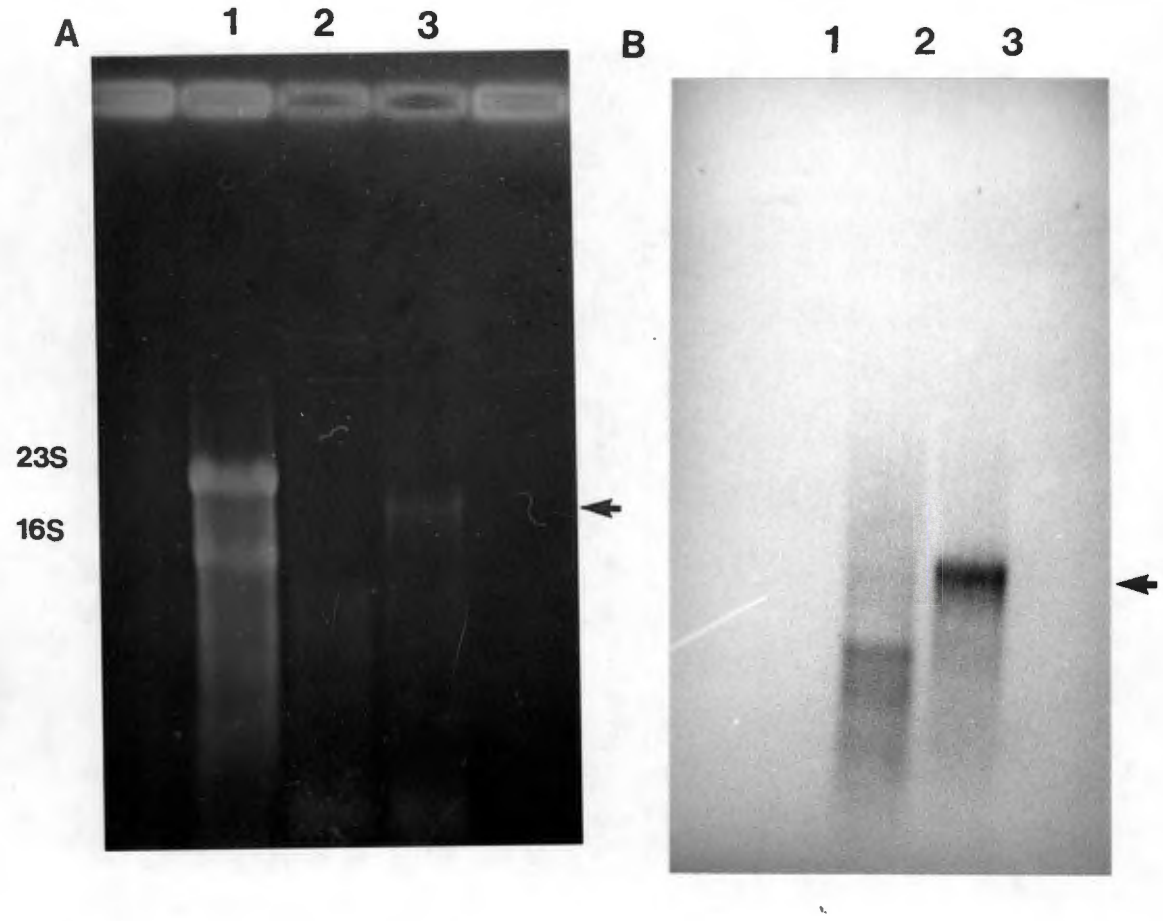


FIGURE 2.14. Analysis of chicken TRP-2 riboprobe synthesised from clone 196. (A) 5µl of antisense chicken tyrosinase riboprobe (lane 2) (636 bp) and 5µl of antisense chicken TRP-2 riboprobe (1.3 kb) (lane 3, arrow) were electrophoresed on an agarose gel. (B) Northern transfer of (A) with the dig-labeled riboprobe detected by standard methods.

2.3.2 *In situ* hybridisation

The temporal and spatial expression of tyrosinase and tyrosinase related protein-2 (TRP-2) in the chicken embryonic eye was investigated by *in situ* hybridisation (ISH). It was necessary to carry out numerous pilot studies in order to establish an ISH protocol suitable for wax sections through the chicken embryonic eye. The steps used and problems encountered while establishing this protocol are discussed in section 2.4. The conditions that were finally used to detect tyrosinase and TRP-2 mRNA in the retinal pigment epithelium (RPE) of the chicken embryo are described in the Materials and methods section 2.2.8.

2.3.2.1 Pilot studies

Initial attempts were made to establish an ISH protocol on wax sections through the four day old chicken eye using the tyrosinase antisense dig-labeled riboprobe (636 bp). (The conditions that were used are discussed in section 2.4). However these attempts failed. Since permeabilisation of cells for probe penetration is easier to establish on cell cultures than on sectioned tissue, attempts to detect tyrosinase mRNA by ISH on RPE cell cultures was made.

Expression of tyrosinase mRNA in cultured cells

Pigmented RPE cells from four day old chicken embryos were cultured as described in Materials and methods section 2.2.7.2, seeded onto APTES-coated glass slides, fixed and used for ISH reactions (section 2.2.8.1) (Fig.2.15 A & B). A strong positive signal representing tyrosinase mRNA transcripts can be seen in every RPE cell in Figure 2.15A. This signal is perinuclear. All the RPE cells in the negative control without probe were clear staining and positive signals were not detected within these cells (Fig.2.15B). Background staining was very low in both positive and negative slides.

Figure 2.16 shows another view of a pigmented RPE cell culture on which the ISH reaction was conducted. Brown pigment granules (arrowheads) can be seen in the cytoplasm of most of the RPE cells in view. The pale area within each cell represents the cell nucleus. A strong blue/black perinuclear signal (arrows) representing tyrosinase mRNA can be seen in most of the RPE cells and in some cases this signal masked the pigment granules within the cell cytoplasm.

Chicken fibroblast cells (from seven day old chicken embryonic limb buds) which do not produce pigment were similarly prepared and used as a negative control cell line. In slides to which riboprobe had been added no positive signals or background staining was observed (Fig.2.17), indicating that tyrosinase mRNA is not expressed within these cells. (The negative control without probe also had a very low background (not shown)).

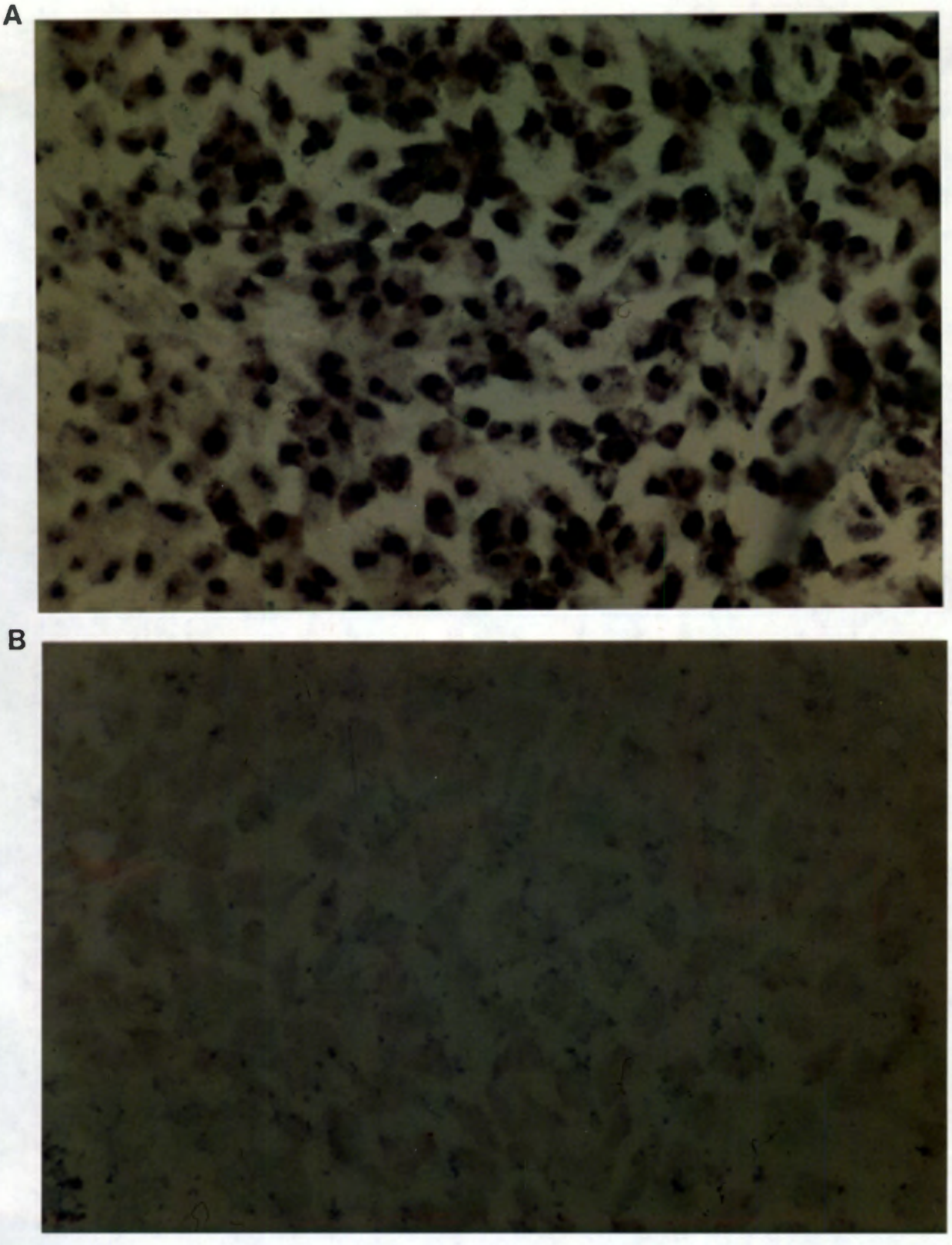


FIGURE 2.15. Expression of tyrosinase mRNA in chicken RPE cell cultures. (A) Probe used was a 636 bp digoxigenin labeled riboprobe (440 x). (B) Negative control with no riboprobe added during hybridisation. (440 x).

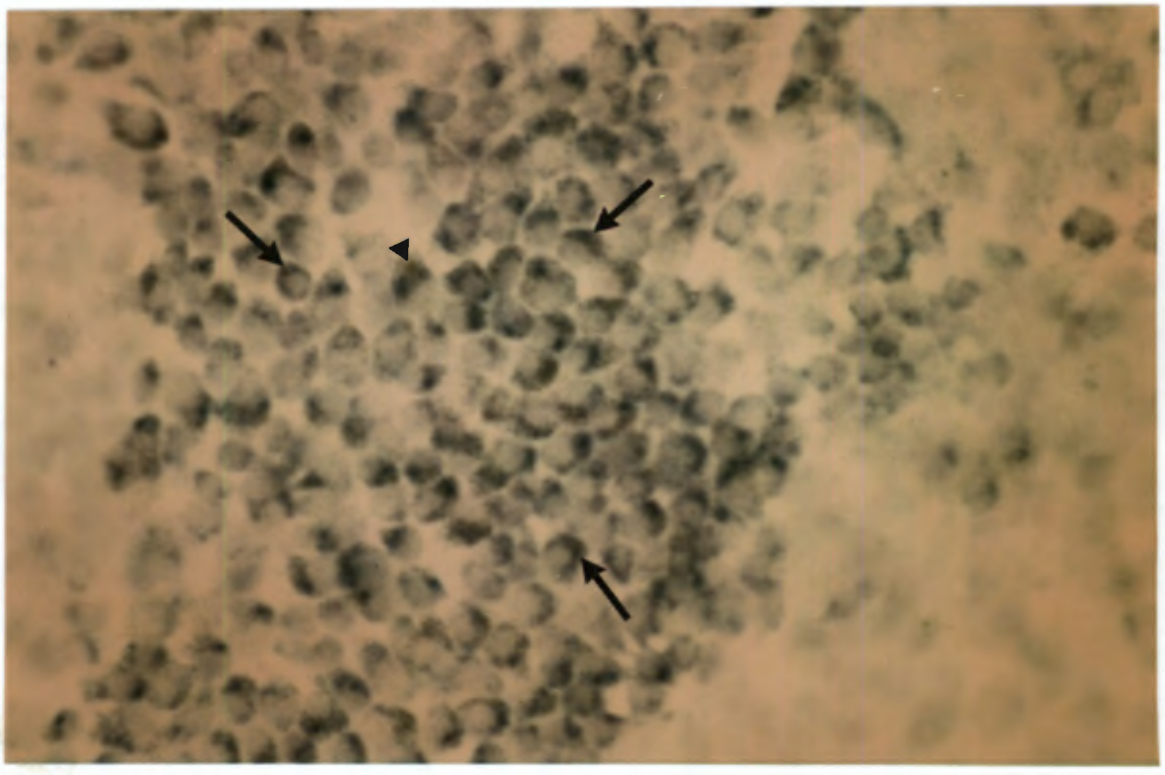


FIGURE 2.16. Expression of tyrosinase mRNA in chicken RPE cells. Probe used was a tyrosinase dig-labeled riboprobe. Brown pigment granules (arrowhead) can be distinguished from the blueish/black cytoplasmic signal (arrows) in most cells. (355 x).

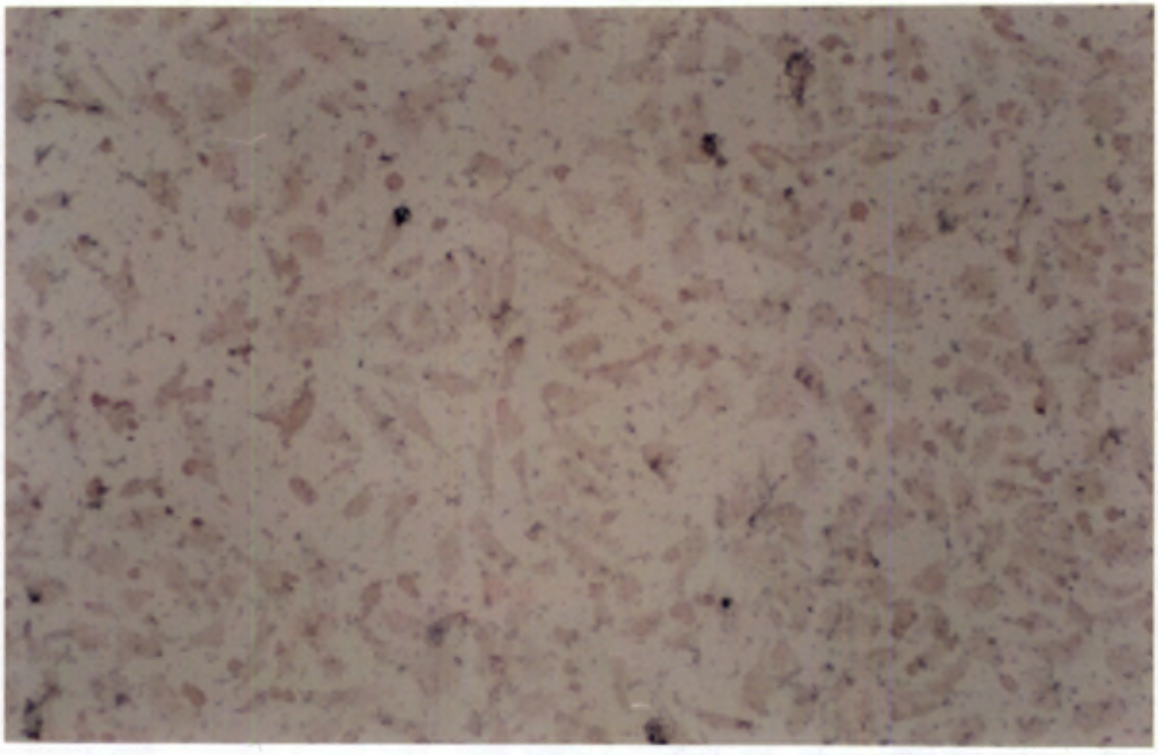


FIGURE 2.17. Fibroblasts cultured from seven day old chicken limb buds do not express tyrosinase mRNA. Probe used was a tyrosinase riboprobe. No positive signal is present within any of the cells in view. The few dark deposits represent background precipitate and not a positive result. (355 x).

Similar results were obtained in three independent ISH reactions using the dig-labeled tyrosinase antisense riboprobe. These results indicate that pigmented RPE cells express tyrosinase mRNA in culture and that fibroblasts do not.

Expression of tyrosinase mRNA in frozen sections of the chicken embryonic eye.

Four day old (stage 24) chicken embryonic eyes were frozen and sectioned (4µm) for ISH. The reason for attempting to perform ISH reactions on frozen tissue instead of on wax embedded tissue was that frozen tissue ISH is more sensitive. Dijkman et al. (1995) report as much as a 30% loss of RNA during tissue processing for wax embedding. The conditions for ISH on frozen tissue were established by modifying steps in the cell culture ISH protocol. (These are discussed in section 2.4 and the frozen tissue ISH protocol is described in section 2.2.8.2).

Positive ISH results using the dig-labeled tyrosinase antisense riboprobe were obtained in two independent ISH reactions on frozen sections through the stage 24 (four day old) chicken embryonic eye (Fig.2.18). At this stage of development, the optic cup is well-developed. The neural retina (NR) has thickened substantially and the RPE is a monolayer containing basally situated pigment granules (arrowheads). The NR disintegrated somewhat during sectioning and is located above the RPE. The mesenchymal tissue below the RPE is visible in the figure. A strong blueish/black signal in the apical part of the RPE (Fig.2.18A, arrows) represents tyrosinase mRNA expression and is easily distinguishable from the brown pigment granules. In controls with no riboprobe added during hybridisation, no tyrosinase expression was observed in the RPE (Fig.2.18B). Background staining was low in both positive and negative sections. (A precipitate which should have been washed off before mounting the slides is present in the negative control shown). Tyrosinase mRNA was thus localised to the apical part of the RPE at stage 24 of chicken embryonic development, with pigment granules located basally.

Since sectioning and preservation of frozen tissue is more difficult than for wax embedded tissue, it was desirable to determine the conditions for ISH on wax sections. This was achieved by adjusting the permeabilisation step in the frozen tissue ISH protocol (as described in 2.2.8.2). This new ISH protocol was then used for all the further studies of tyrosinase and TRP-2 expression in the chicken embryonic eye.

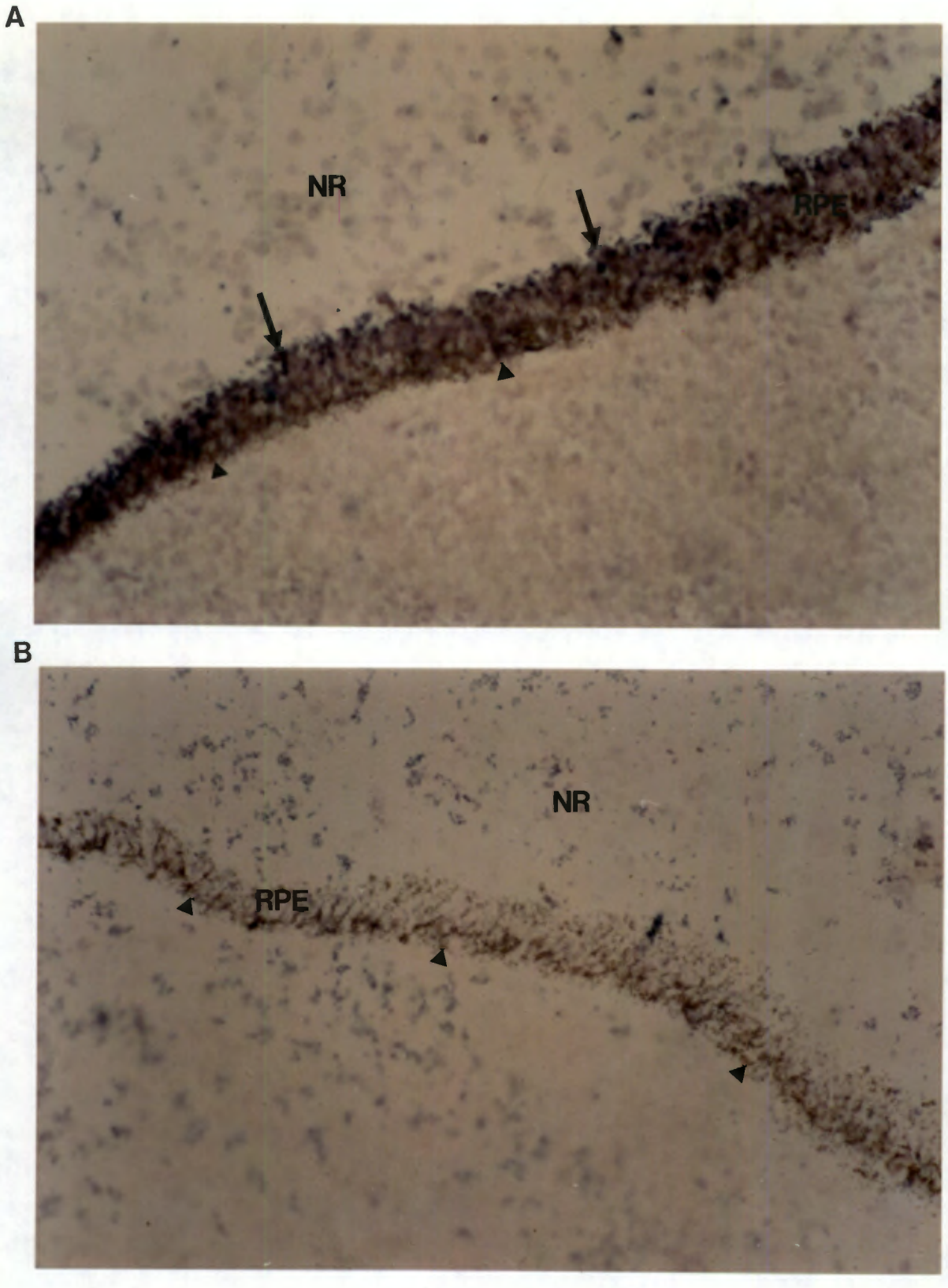


FIGURE 2.18. Tyrosinase expression in frozen sections (4µm) through the four day old chicken embryonic eye. (A) Probe used was a dig-labeled tyrosinase riboprobe (636 bp) (275 x). (B) Negative control with no riboprobe added during hybridisation. (275 x)

2.3.2.2 Temporal expression of tyrosinase mRNA in wax sections of the chicken embryonic eye.

The ISH protocol used to detect tyrosinase mRNA in frozen sections of the chicken eye was modified slightly for wax sections (as described in 2.2.8.2). In order to determine the tempora expression pattern of tyrosinase mRNA in the RPE of the chicken embryo, a stage at which pigment is visible and tyrosinase is expressed was chosen as a starting point (ie. stage 24). The stage at which tyrosinase mRNA was no longer present in the RPE could then be determined by examining younger and younger embryos. The results are therefore presented in the sequence of older (stage 24) to younger (stage 18.5) embryos.

STAGE 24 (four day)
At this stage of development the optic cup is well developed. The RPE is a simple cuboidal epithelium and pigment granules are present basally within this layer (Fig.2.19, arrowheads). (The NR is situated above the RPE and the eye mesenchyme is located below the RPE). The morphology of both the NR and the mesenchyme was excellent when compared to frozen sections of the same age tissue (compare Fig 2.18 and Fig 2.19). A small space was observed between the NR and RPE and it is not clear whether this is a sectioning artifact or a real representation of the contact between these two layers.

Tyrosinase mRNA was detected in the RPE of the stage 24 chicken embryonic eye and can be seen as a fuzzy black/blue signal within the RPE cells (Fig.2.19). The entire RPE was positive for tyrosinase expression and some background staining was present within the NR. The negative control without riboprobe is totally clear of staining and only pigment granules at the base of the RPE are visible in this section (Fig.2.19B, arrowheads). These results confirm that tyrosinase mRNA is expressed in the RPE of the four day old (stage 24) chicken embryonic eye.

Figure 2.20 shows another section of tyrosinase mRNA detected in the RPE of the four day old chicken embryonic eye. Brown pigment granules in the basal part of the simple RPE are indicated (arrowheads) and each RPE cell contains a large, pale staining nucleus. Tyrosinase mRNA expression was detected in the apical portion of the RPE (Fig.2.20, arrows). It is not clear why this signal is apical in Figure 2.20 and more fuzzy in Figure 2.19, but this may be related to the detection times of the ISH reactions.

In all studies of earlier stages of eye development, a slide of the four day old chicken embryonic eye was included as a positive control. Therefore the results described above for the stage 24 (pigmented) RPE were consistently obtained in numerous independent reactions.

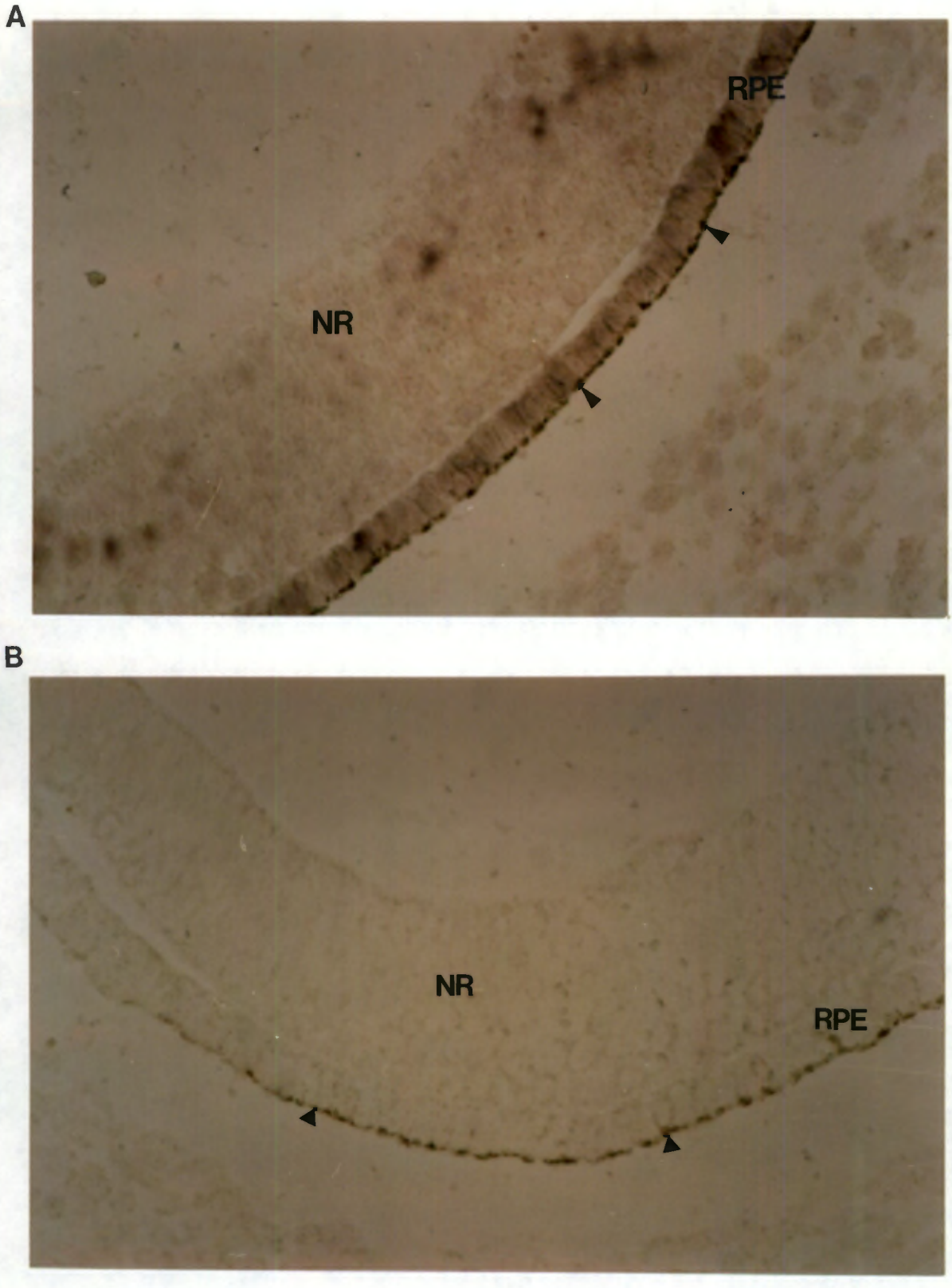


FIGURE 2.19. Tyrosinase mRNA in wax sections (4μm) through the four day old chicken embryonic eye. Distinct pigment granules can be seen in the basal part of the RPE (arrowheads). (A) Probe used was a tyrosinase antisense dig-labeled riboprobe (636 bp) (275 x). (B) Negative control with no probe added (275 x).

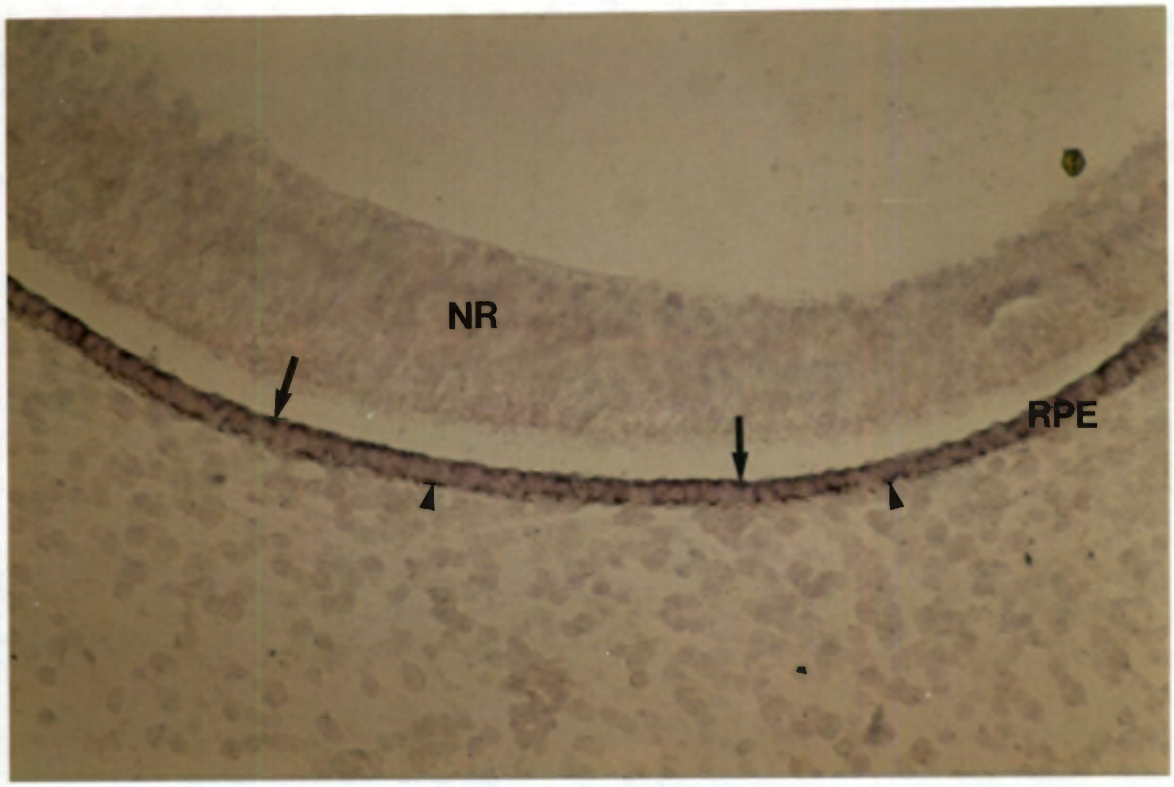


FIGURE 2.20. Tyrosinase mRNA detected in a wax section (4µm) through the four day old chicken embryonic eye. Probe used was a 636 bp dig-labeled riboprobe. Signal is located in the apical part of the retinal pigment epithelium (RPE) (arrows) and pigment granules can be seen basally (arrowheads). The neural retina (NR) is situated above the RPE. (220 x).

STAGE 20.5 (approx. 71-78 hours)

The ISH protocol described in section 2.2.8.2 was used to detect tyrosinase mRNA in wax sections through the stage 20.5 (approx. 71-78 hours) chicken embryonic eye (Fig.2.21A). At this stage of development, the thickened NR has made contact with the simple cuboidal RPE for most of its length, with only the margins of the RPE/NR unopposed. Therefore the space seen in the figure in the optical axis of the eye between these two layers probably represents a processing artifact and the space present at the edges of the eye cup is real. In addition, the lens vesicle has formed and separated from the surface ectoderm. A portion of the brain epithelium is also in view. No distinct pigment granules could be seen in the RPE in this section at this magnification. However, at higher magnifications a few granules were observed in the optical axis of the eye (Fig.2.21A, arrowhead).

Tyrosinase mRNA was detected in the RPE of the stage 20.5 chicken embryonic eye in three separate reactions on different embryos at this stage of development. In all cases, a positive signal (arrows) was localised to the central part of the RPE (the optical axis of the eye, Fig.2.21A). (The dark staining in the brain epithelium (asterisks) is due to a fold in the section and is not a positive result). Background staining was relatively low in both the positive and negative sections.

Tyrosinase mRNA was thus detected in the RPE of the stage 20.5 chicken embryonic eye when only a few pigment granules are present.

STAGE 19.5 (approx. 70-71 hours)

At this stage of development, the lens has formed and detached from the surface ectoderm. The NR is thickened and closely appeased to the simple cuboidal RPE. The space present between the NR and the RPE in the optical axis of the eye in Figure 2.21B is therefore likely to be an artifact (as is the space below the RPE). However, the space between the NR and RPE at the margins of the optic cup is probably real since this is the last area of the optic cup to make contact. No pigment granules were present at this stage of development.

Tyrosinase mRNA was detected in the RPE of the stage 19.5 chicken embryonic eye and can clearly be seen in Figure 2.21B (arrows). Again, the signal was strongest in the optical axis of the eye and very little background staining was observed. In addition, negative controls without probe added during hybridisation were totally clear of any staining (not shown).

These results were obtained in three independent ISH reactions using different embryos at this stage of development. Thus tyrosinase mRNA is expressed at stage 19.5 of chicken embryonic development and this expression is strongest in the optical axis of the eye.

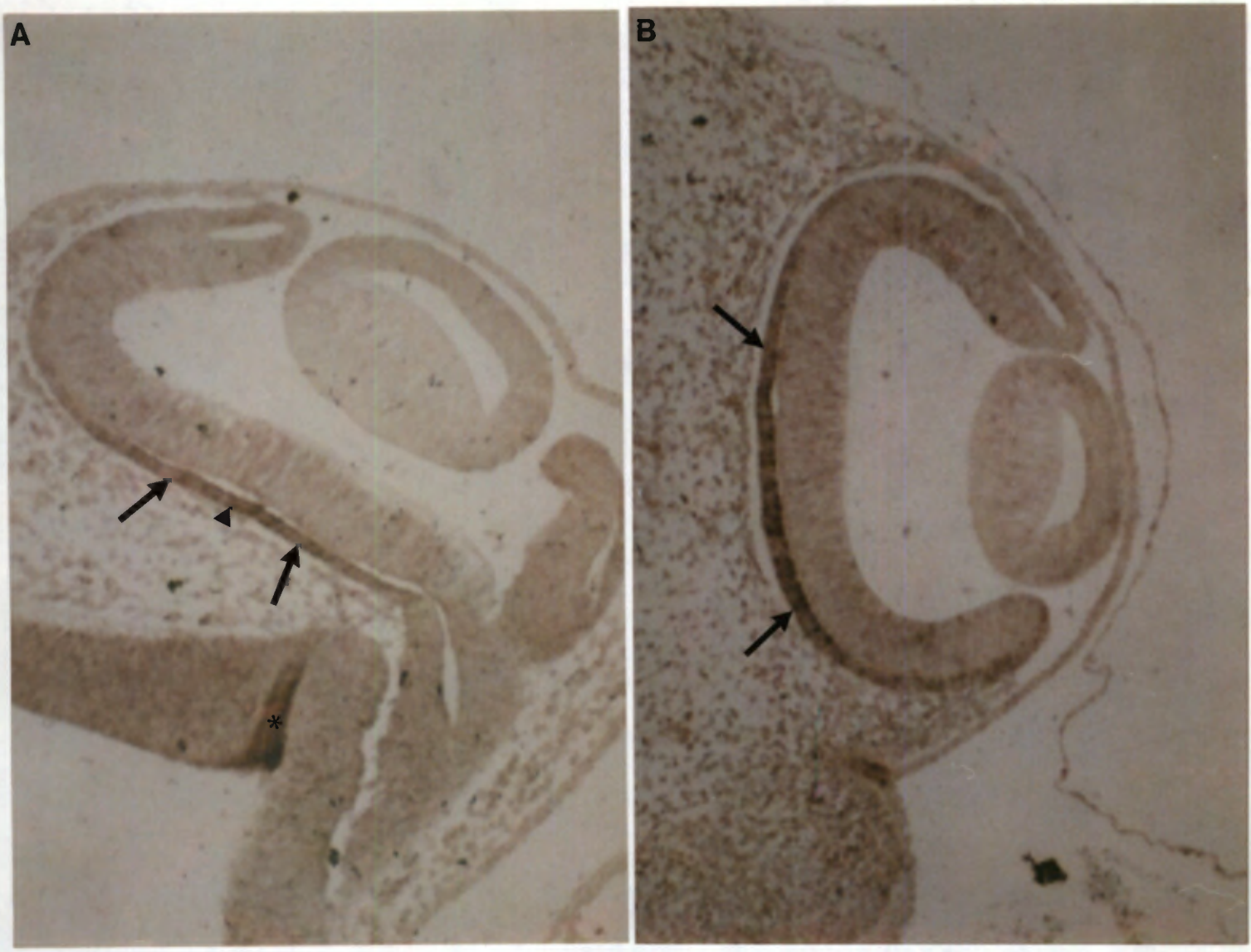


FIGURE 2.21 Analysis of the temporal expression pattern of tyrosinase in the chicken embryonic eye. Probe used was a tyrosinase dig-labeled riboprobe (636 bp). Arrows indicate tyrosinase expression in the RPE. (A) Stage 20.5; the arrowhead indicates the position of pigment granules and an asterisk indicates the brain epithelium. (170 x). (B) Stage 19.5 (170 x).

STAGE 18.5 (approx. 67-69 hours)

At this stage of eye development, the optic cup has invaginated and the lens vesicle has formed. Pigment granules are not present. No positive signals were detected in either the negative control (without riboprobe) or the experimental sample (with riboprobe) (data not shown). In addition, background staining was low in the negative control and slightly higher in positive slides. The same results were obtained in three independent ISH reactions using different embryos and suggest that tyrosinase mRNA is NOT expressed in the RPE at stage 18.5 of chicken eye development.

In an attempt to verify this result, the above hybridisation experiment was carried out on fixed frozen sections. No tyrosinase mRNA expression was detected in stage 18.5 eyes. This leads to the conclusion that either tyrosinase mRNA is not present at this stage or that it is present but at levels below the limit of this detection system.

Summary

The above results demonstrate that tyrosinase mRNA is present in the RPE of the chicken embryonic eye from stage 19.5 onwards. Pigment granules are visible from approximately stage 20, although the eye is only distinctly (visibly) pigmented at stage 21 (3.5 days). Thus tyrosinase expression precedes pigment formation. From these results one can deduce that the tyrosinase gene is switched on at or before stage 19.5

The spatial pattern of tyrosinase expression was also elucidated in this study. Tyrosinase expression was detected in the entire RPE of pigmented eyes whereas in younger embryos, expression was localised to the optical axis of the eye with very little expression detected at the margins of the RPE. This suggests that pigmentation of the RPE appears to begin at the optical axis of the eye and progress outwards towards the margins of the eye cup. In support of this, pigment granules were first observed in the optical axis of the RPE.

These results are summarised in Table 1. and their significance in relation to induction of the RPE will be discussed in chapter 4.

TABLE 1. The temporal expression of tyrosinase and TRP-2 mRNA and pigment granules in the chicken embryonic eye.

		STAGE [1]		
	62-66 h [2] 17.5	67-69 h 18.5	70-71 h 19.5	71-78 h 20.5
pigment	-	-	-	+
tyrosinase	N/D [3]	-	+	+
TRP-2	-	+	+	+

[1] Hamburger and Hamilton (1951) stage of chicken embryonic development.
[2] h = hours of incubation.
[3] N/D = not determined.

2.3.2.3 Temporal expression of TRP-2 mRNA in wax sections of the chicken embryonic eye.

In order to determine when and where TRP-2 mRNA is first expressed in the RPE of the chicken embryo, ISH reactions using the chicken TRP-2 riboprobe (1.3 kb) (from clone 196) were conducted. It was however first necessary to optimise the permeabilisation step of the ISH protocol for the TRP-2 riboprobe, which was larger than the tyrosinase riboprobe. For eye tissue from stage 21 to stage 24 of embryonic development a pepsin concentration of 0.03% was used. However, when this concentration was used on younger embryos, tissue morphology was adversely affected. Therefore, for younger eye tissue (stage 17 - stage 19) a pepsin concentration of 0.015% was used. (This is the same concentration that was used for ISH reactions with the tyrosinase riboprobe).

STAGE 23 (3.5 - 4 days)
The morphology of the chicken embryonic eye at stage 23 of embryonic development has been described. TRP-2 transcripts were detected in the RPE of the chicken embryonic eye at stage 23 of development in three independent ISH reactions with different embryos (Fig.2.22A). At this stage of development the thickened NR is in close contact with the thin RPE. Each RPE cell contains a large pale staining nucleus which fills the entire cell. Brown pigment granules (arrowheads) are basally situated within these cells. TRP-2 mRNA was detected in the apical part of the RPE as can be seen by the very strong black/blue signal within this region (Fig.2.22A, arrows). Negative controls without probe were totally clear of any positive signal (not shown) and both positive and negative slides had low backgrounds.

STAGE 19.5 (approx. 70-71 hours)
At this stage of development, the lens has detached from the surface ectoderm and rests within the optic cup. The NR is thickened compared to the thin RPE and these two layers contact one another. The space visible between the NR and the RPE in the optical axis of the eye in Figure 2.22B is therefore likely to have been created during sectioning. As described earlier, the margins of the RPE are the last to make contact with the NR during eye development and therefore the space in this region could be real. (It is not clear at exactly what stage these two layers appose one other).

TRP-2 transcripts were detected in the entire RPE of stage 19.5 of eyes as seen in Figure 2.22B (arrows). The boundary between the developing RPE and the developing NR can be seen at the margins of the eye cup where TRP-2 transcripts are no longer detected (arrowheads). The NR and brain epithelium both had some background staining. Negative controls without riboprobe added during hybridisation were clear of any positive staining and backgrounds were low in these slides (not shown).

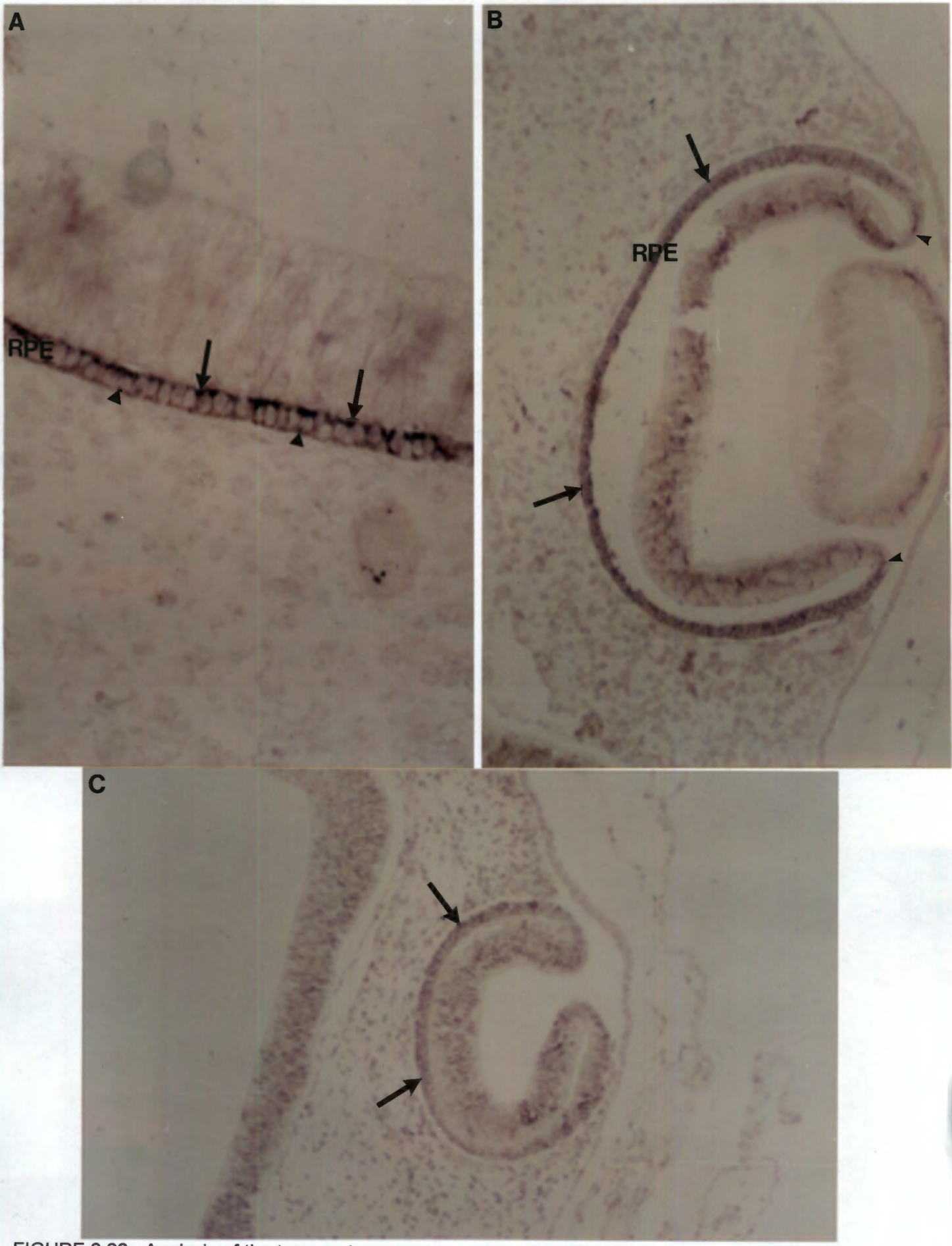


FIGURE 2.22. Analysis of the temporal expression pattern of TRP-2 in the chicken embryonic eye. Probe used was a 1.3 kb dig-labeled TRP-2 riboprobe. Arrows indicate TRP-2 expression in the RPE. (A) Stage 23; the arrowhead indicates pigment granules (550 x). (B) Stage 19.5 (170 x). (C) Stage 18.5 (170 x).

These results were repeated in three independent ISH reactions using different embryos at stage 19.5 of development and indicate that TRP-2 is expressed in the entire RPE at this stage of chicken embryonic development.

STAGE 18.5 (approx. 67-69 hours)
At this stage of development, the optic cup is newly formed and the thickened NR is tightly apposed to the thin RPE (Fig.2.22C). The lens, which has detached from the surface ectoderm is not visible in the figure. A portion of the telencephalon is also in view.

TRP-2 transcripts were detected in the RPE of the stage 18.5 embryonic eye as indicated by a blueish signal in this layer (Fig.2.22C, arrows). However, background staining in the NR and brain epithelium was higher at this age than previously observed, making the distinction between true signal and background difficult. Negative controls without probe added were entirely clear of any staining and had very low backgrounds. These results were obtained in two independent ISH reactions.

STAGE 17.5 (approx. 62-66 hours)
The morphology of the chicken embryonic eye at this stage of development is similar to that at stage 18.5. Background staining in positive slides was high, making the distinction between a possible positive signal in the RPE and background staining very difficult to determine (Fig.2.23). Negative controls without probe added had no background staining (not shown). Thus it could not be unequivocally determined whether TRP-2 is expressed at stage 17.5 of chicken embryonic development and it is possible that low levels of TRP-2 mRNA are present in the RPE at this stage of development. No earlier stages of chicken embryonic development were investigated by ISH since background staining was likely to mask possible positive signals at these stages as well.

Summary
The above results demonstrate that TRP-2 transcripts are present from stage 18.5 onwards. Low levels of TRP-2 mRNA may be present at stage 17.5 or earlier but these could not be detected because of increased background staining. Pigment granules are only present from stage 20 and therefore TRP-2 expression precedes this stage. In addition, TRP-2 expression precedes tyrosinase expression since tyrosinase mRNA was first detected at stage 19.5 and was undetected at earlier stages when TRP-2 transcripts were present. These results are summarised in Table 1. and will be discussed in chapter 4.

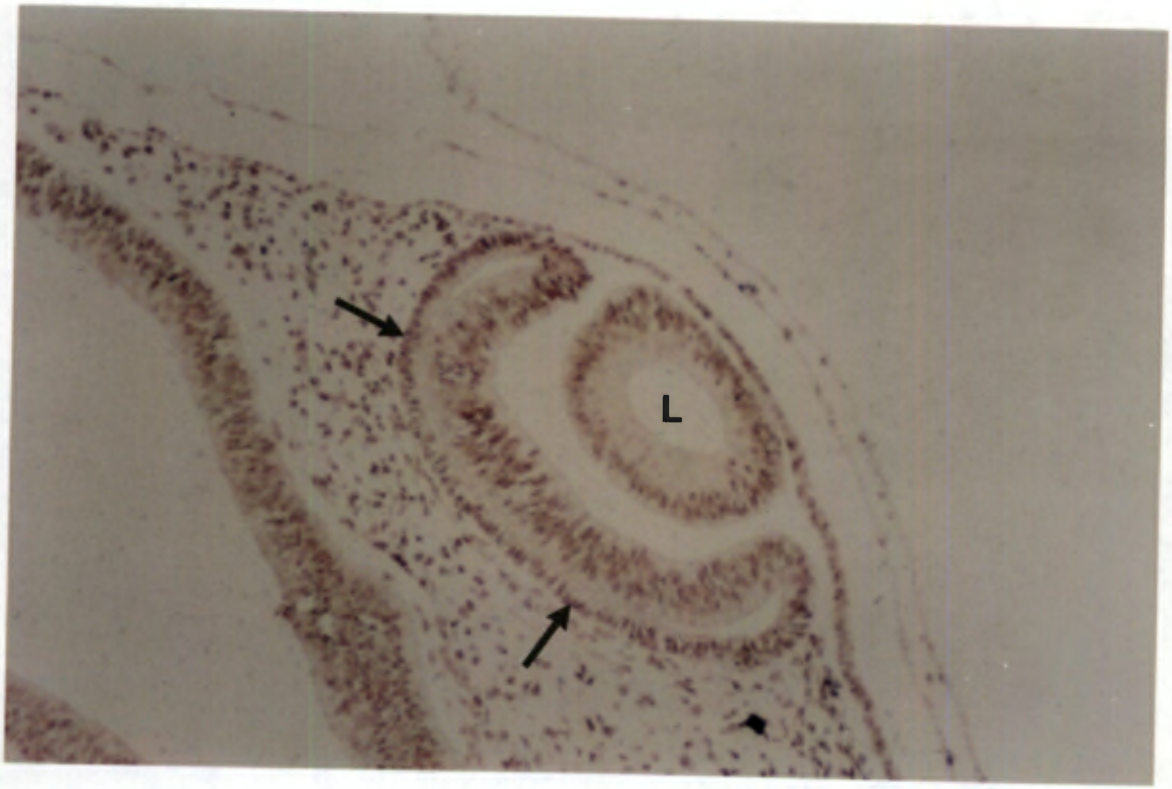


FIGURE 2.23. A stage 17.5 chicken embryonic eye. Probe used was a 1.3 kb TRP-2 riboprobe. Arrows indicate the presumptive RPE and the lens vesicle (L) is indicated. (170 x).

2.3.2.4 Other areas expressing tyrosinase mRNA

While investigating tyrosinase expression in the chicken RPE, six other areas of the chicken embryo also showed positive signals for tyrosinase expression (at various stages of development). These included the neural tube, the mesenchyme surrounding the neural tube, the limb buds, the ectoderm and the embryonic gonad. Table 2. summarises the number of times a positive signal was obtained for each of these areas, given as a percentage of the total number of independent ISH reactions carried out in the area.

The neural tube and mesenchyme surrounding the neural tube.

Tyrosinase mRNA was detected in cells of the neural tube of stage 23 (4.5 days old) and stage 30 (6.5 day old) chicken embryos (Table 2.). Figure 2.24 (arrows) shows cells expressing tyrosinase in the dorsal part of the neural tube in an area which could be the dorsal root ganglion or the mesenchyme adjacent to the tube. At higher magnification the signal appears to be perinuclear (Fig.2.24C). These results were obtained in eight (out of 15) independent ISH reactions at stages 24 and 30. Negative controls (without riboprobe added during hybridisation) were clear of any staining, suggesting that these results were real.

The ectoderm

At stage 24 (four day old) and stage 30 (6.5 day old) tyrosinase mRNA was detected (on occasion) in the ectoderm of the chicken embryo (Fig.2.25A). This expression was found only at these two stages and in four out of seven (57%) ISH reactions conducted at stage 30, tyrosinase mRNA was detected in the ectoderm of the embryo (Table 2.). The expression in the ectoderm was not consistent and was detected 33% of the time at stage 24 and 57% of the time at stage 30. No ectodermal staining was detected at stage 23. In addition, the ectodermal staining occured in isolated patches and was not detected through out the ectoderm of the sectioned embryo. It is possible that the positive cells within the ectoderm represent early skin melanocytes. (Fig.2.25A, arrows). Positive cells were also detected in the mesenchyme below the ectoderm and these could represent migrating melanoblasts (originating from the neural crest) (Fig.2.25A, arrowheads). Negative controls (without probe) were clear of staining in all but one ISH reaction. This makes the results of tyrosinase expression in the ectoderm questionable.

The limb buds

Tyrosinase positive cells were frequently found within the mesenchyme of stage 23 and 24 limb buds (Fig.2.25B, arrows). No expression was detected at stage 30. Negative controls were clear of any background staining. In some cases a trail of positive cells extending from the limb bud to the nearby neural tube was detected (not shown). These positive cells could

TABLE 2. Areas where tyrosinase expression was detected in the chicken embryo, given as a percentage of the number of times a positive result was obtained out of the total number of attempts made.

AREA	STAGE [1]			
	23	4 day old 24	30	7 day old 32
neural tube	0%(n=2) [2]	50%(n=6)	55%(n=9)	N/D
mesenchyme	N/D [3]	100%(n=4)	100%(n=1)	N/D
limb bud	40%(n=5)	80%(n=5)	0% (n=1)	N/D
ectoderm	0%(n=6)	33%(n=6)	57%(n=7)	0% (n=3)
gonad	N/D	0%(n=1)	25%(n=4)	N/D
RPE	100%(n=6)	100%(n=6)	100%(n=1)	100%(n=1)

[1] Hamburger and Hamilton (1951) stage of chicken embryonic development.
[2] The numbers in parentheses (n=...) refer to the total number of *in situ* hybridisation reactions carried out in the area.
[3] N/D = not determined.

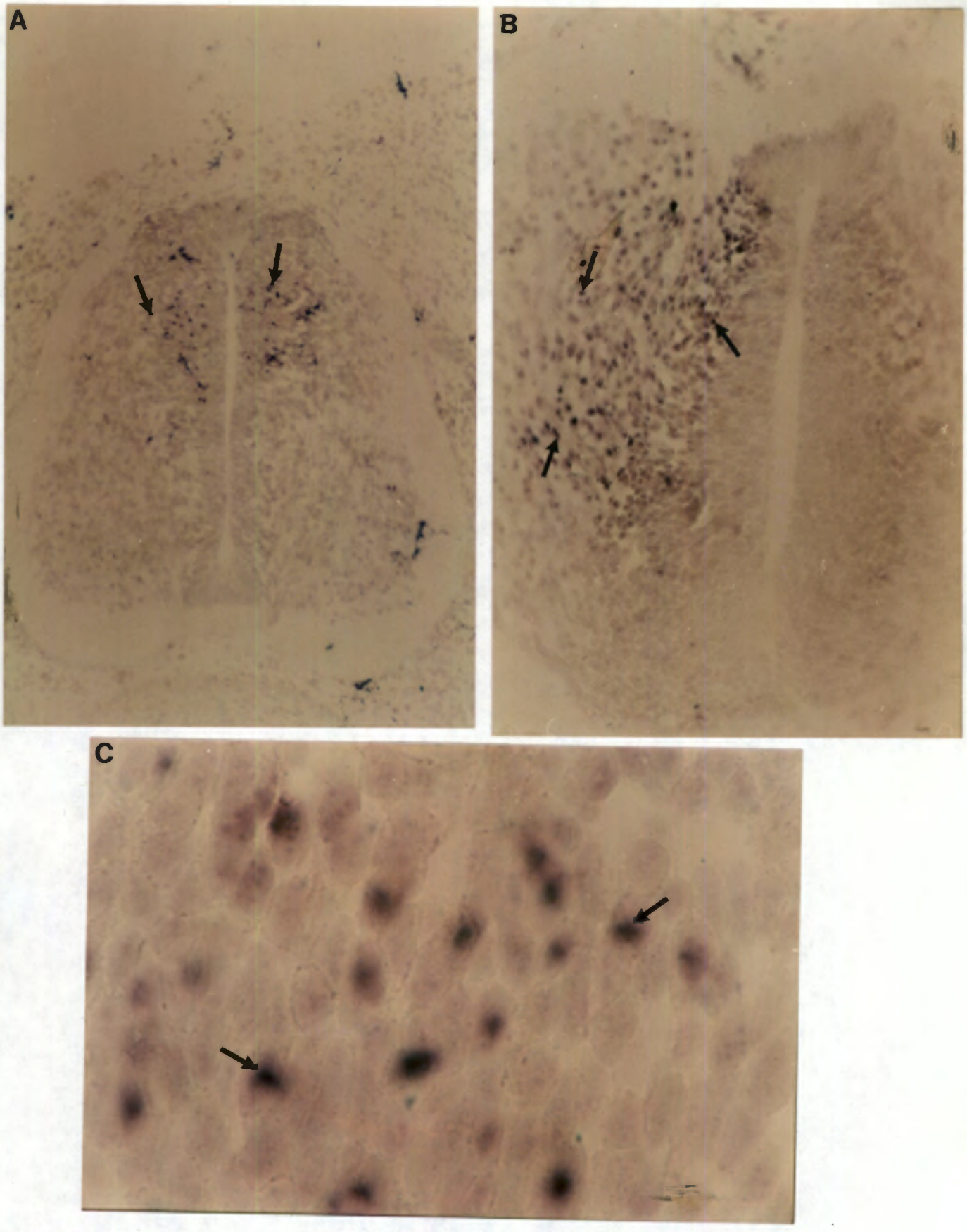


FIGURE 2.24. Tyrosinase expressing cells (arrows) in the neural tube of a stage 30 chicken embryo. (A) Trunk neural tube (170 x); (B) Neck neural tube (170 x); (C) High magnification of tyrosinase positive cells in the neural tube (1715 x).

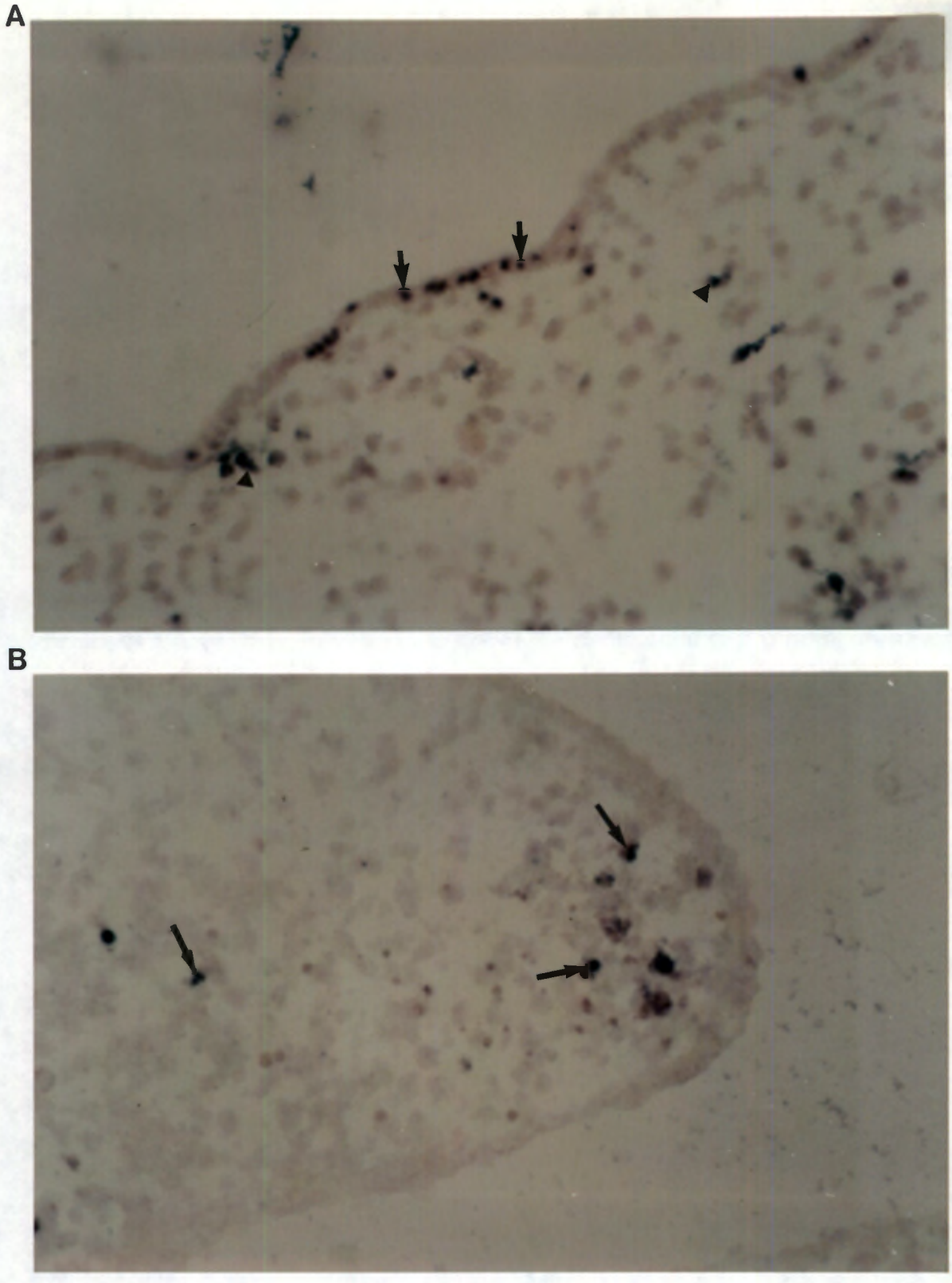


FIGURE 2.25. Tyrosinase mRNA detected in the ectoderm and limb bud of the chicken embryo using a dig-labeled riboprobe. (A) Tyrosinase positive cells (arrows) in the ectoderm and underlying mesenchyme of a stage 30 chicken embryo (444 x). (B) Tyrosinase positive cells (arrows) in the developing limb bud of a stage 24 chicken embryo (444 x).

possibly represent migrating melanoblasts destined to pigment the skin or feathers of the limb bud.

The developing gonad

In one experiment, tyrosinase mRNA expression was detected in the gonadal primordium at stage 30 (6.5 days old) of embryonic development (Fig.2.26A). These labeled cells were located both at the margins of the primordial gonad and within the mass of mesenchymal cells. This result was obtained in a few sections in this ISH run but was not repeatable in three further attempts (Table 2.). Negative controls without probe added were clear and no background staining was observed in these sections.

The surrounding mesenephronic tubules (asterisks) and chromaffin cells (arrowheads) (Romanoff, 1960) were free of staining in the positive slides. Thus chromaffin cells (which are of neural crest origin) and are present at this stage of development did not express tyrosinase mRNA.

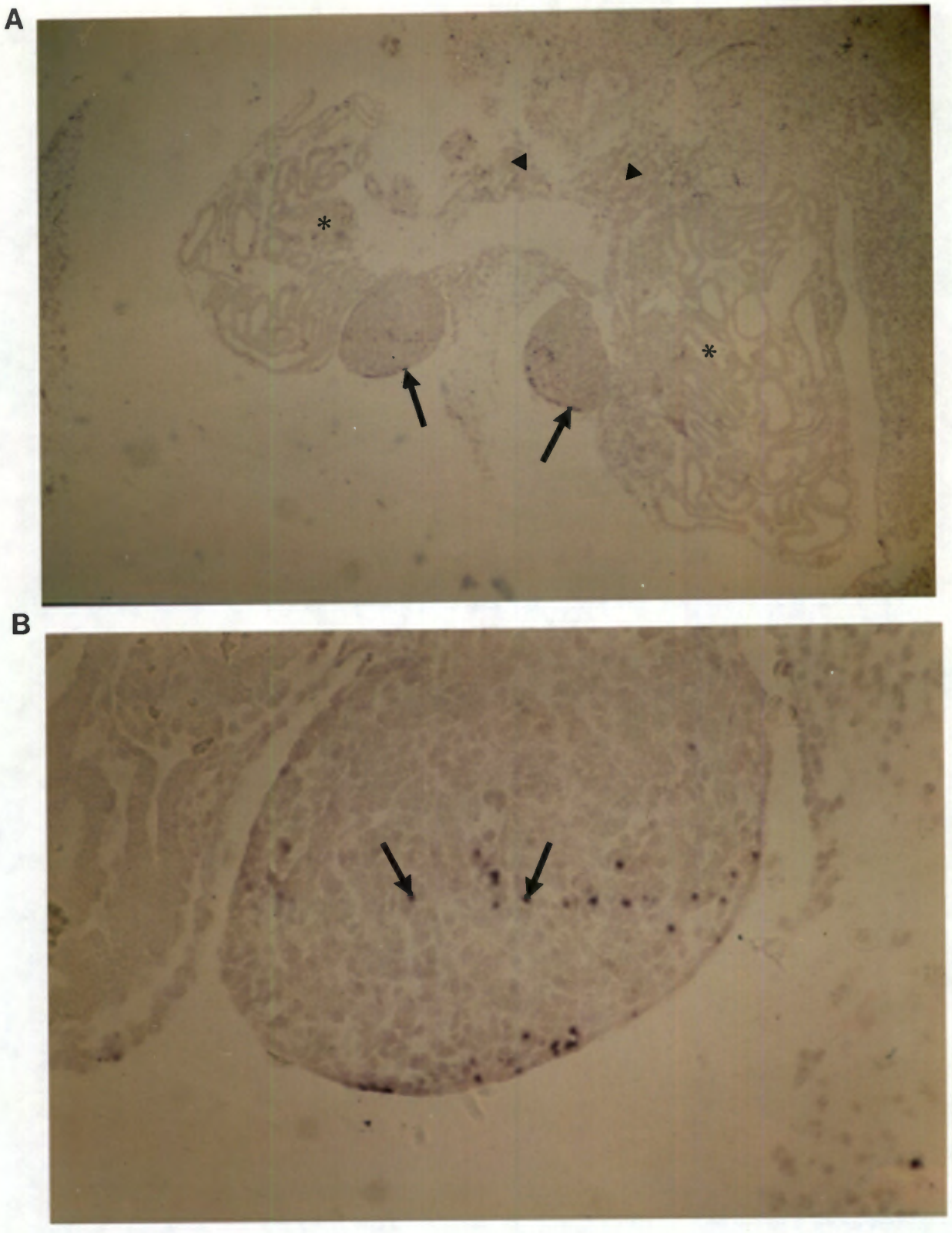


FIGURE 2.26. Tyrosinase expression in cells of the chicken embryonic gonad (stage 30). Probe used was a dig-labeled tyrosinase riboprobe (636 bp). (A) Chromaffin cells (arrowhead), mesenephronic tubules (asterisks) and the gonadal promordia (arrows) are indicated (71 x). (B) The embryonic gonad containing some tyrosinase positive cells (arrows) (355 x).

2.4 DISCUSSION

Brief comments of ISH methodology and problems encountered with this technique.

The ISH protocol that was finally used for investigating the temporal expression of tyrosinase and TRP-2 in the chicken embryonic eye is described in Materials and methods section 2.2.8. Numerous pilot studies were conducted with the avian tyrosinase antisense riboprobe (636 bp) in order to optimise this protocol. Initially, ISH reactions with modified protocols were performed on wax sections, however no positive results were obtained. Since ISH reactions on frozen tissue is more sensitive than on wax sections (Dijkman et al. 1995), frozen sections through the chicken embryonic eye were prepared and the ISH reaction carried out. No positive results were obtained using this procedure. Finally an ISH protocol was established using chicken RPE cell cultures. Once established, this protocol was then adapted for frozen and wax embedded tissue. Each parameter that was changed during ISH pilot studies was altered separately in order to observe its effect. The most important of these parameters is discussed below.

Initial ISH reactions on wax sections

An ISH protocol based on that of Angerer and Angerer (1991) was initially carried out on wax sections through the chicken embryonic eye. No signal was detected but background staining in the negative controls (without riboprobe) were low compared to slides with riboprobe added; this indicated that the background staining was due to non-specific binding of the riboprobe and not due binding of the anti-digoxigenin antibody. The stringency of post-hybridisation washes was therefore increased by including further higher stringency washes. This reduced background but still no signals could be detected with certainty.

Additional modifications recommended by Dijkman et al. (1995), such as varying the probe concentration used in the hybridisation step and treating with RNAse after hybridisation were attempted. Post-hybridisation RNAse treatment is reported to reduce background staining by digesting single stranded RNA molecules (i.e. unbound probe). No positive results were obtained when these changes were made. It appeared as if the probe was not penetrating the tissue and that RNAse treatment was degrading all the (unbound) riboprobe resulting in no background staining. Therefore, in order to enhance penetration of the riboprobe into the tissue, a higher concentration of proteinase K and a riboprobe shortened to 250 bp by alkaline hydrolysis was used. However, none of these changes gave positive results. In addition, the hybridisation temperature was increased from 42°C to 55°C (as recommended by Leitch et al. 1994), and post hybridisation washes were modified to include a 50% formamide: 2 x SSC wash (2 x 30 min, 50°C). Some protocols recommend a prehybridisation step and this was included but appeared to have no effect on the results obtained.

At this point in the study, none of the variations described above had given a positive result on wax sections although backgrounds were sufficiently low. According to Dijkman et al. (1995), to detect low copy RNA molecules (such as tyrosinase), frozen tissue should be used. They report up to a 30% loss of RNA during processing for wax embedding. For this reason, the frozen tissue ISH protocol recommended by Dijkman et al. (1995) was carried out.

Initial ISH on frozen tissue

Fresh frozen sections (4µm) of four day old chicken embryonic eye were prepared and the basic ISH protocol described by Dijkman et al. (1995) with some minor changes was performed. This protocol does not contain a proteinase K digestion step or an acetylation step. Permeabilisation with proteinase K can adversely affect tissue morphology and therefore this step was omitted. The reason Dijkman et al. (1995) do not include an acetylation step is unclear since acetylation is reported to reduce background staining (Leitch et al. 1994). The post-hybridisation washes of the wax ISH protocol previously optimised for low background staining was incorporated into this protocol. When performing this protocol, slides with riboprobe added gave high backgrounds and no positive signal in the RPE was observed. According to Leitch et al. (1994), frozen tissue should be fixed prior to freezing to inactivate RNAses and to decrease diffusion of target sequences. Tissue was therefore fixed in either 100% methanol or in 4% paraformaldehyde in PBS overnight at 4°C. After fixation, the tissue was cryoprotected by passing it though a series of sucrose solutions and then embedded in gelatin (Stander, 1991). The tissue was then frozen in liquid nitrogen or in N-pentane frozen in liquid nitrogen and sectioned. Independent preparations indicated that the quality of the tissue that had been fixed in paraformaldehyde and frozen in liquid nitrogen was the best, and that less breakage occurred during sectioning of this tissue. Therefore this tissue preparation method was subsequently used for all preparations of frozen sections for ISH reactions.

After further consultation with the literature and personal communication with Prof. Larsson (State Serums Institute, Denmark), additional changes to the ISH protocol were made. The fixative was injected into the eye to ensure good fixation and cryoprotection was achieved in two steps (as described in Materials and methods section 2.2.7.1). No prehybridisation step was performed since rRNA in the prehybridisation mix can bind to sections and the probe can stick to this RNA resulting in high backgrounds (Prof. Larsson, pers. comm.). In addition, the stringency of the hybridisation step was increased to 4 x SSC and 50% formamide and hybridisation was carried out at 55°C. More stringent post-hybridisation washes were also performed. Furthermore, it was recommended that detection be carried out overnight especially for low abundant messages and not only for 3 - 5 hours. Various concentrations of hydrolysed and unhydrolysed riboprobes were used ranging from 1:10 to 1:1000 and a probe

concentration of 1:500 was found to be optimal. Reasonably low backgrounds in all slides were obtained but still no positive signals were observed.

Since attempts at using modified ISH protocols on both wax and frozen sections through the chicken embryonic eye had failed, it was decided that chicken RPE cell cultures should be used instead. The main reason for this change was that it is easier to determine the conditions for permeabilisation of cells in culture than for sectioned tissue.

ISH on cell cultures

ISH on chicken RPE and fibroblast cell cultures were carried out according to the protocol described in Dirks et al. (1994). Essentially the same hybridisation mix and washes as for the original frozen tissue protocol were used but initial steps of the protocol were altered as recommended. The most significant of these changes was the use of pepsin instead of proteinase K for permeabilisation. In addition, hybridisation was carried out at 55°C and a 1:500 probe concentration was used. Positive signals were observed in all cells on the experimental slide with riboprobe added only, while the control slide without riboprobe added was clear of staining (Fig.2.15). This ISH reaction is described in Materials and methods section 2.2.8.1. Attempts were subsequently made to optimise conditions for ISH on frozen sections through the chicken embryonic eye, since ISH reactions on this tissue is reported to be more sensitive than on wax sections (Dijkman et al. 1995).

ISH on frozen sections

In order to get the ISH reaction to work on frozen tissue, minor changes to the cell culture ISH protocol were made. The pepsin concentration was altered as recommended by Prof. Larsson (pers. comm.) and an acetylation step was re-introduced. (Acetylation prevents non-specific binding of the riboprobe to the slide and thereby reduces background staining). Finally a positive result on frozen sections of a four day chicken embryonic eye was detected in the RPE (Fig.2.18). Further attempts to obtain complete frozen sections through the eyes of chicken embryos in order to map tyrosinase expression over time were not successful. Sections frequently broke and did not adhere to the APTES-coated slides sufficiently. For these reasons, an attempt to perform ISH reactions on wax sections was made.

ISH on wax sections

Essentially the same protocol that was used for frozen tissue was used on wax sections except that the pepsin concentration was increased as recommended by Prof. Larsson (pers. comm.). Positive results were obtained and since wax sections are easily prepared, they were used in all subsequent ISH reactions.

Initial attempts to map TRP-2 expression in the chicken eye with the mouse TRP-2 riboprobe were unsuccessful. Neither increasing the permeabilisation step nor lowering the stringency of the hybridisation and post-hybridisation washes produced a positive result. However, at this point in the study, the chicken TRP-2 cDNA was isolated and a 1.3 kb riboprobe synthesised. By altering the permeabilisation step to accommodate the larger probe, a positive result in the RPE of a stage 21 chicken embryo was detected. Results are presented in section 2.3.2.3.

CHAPTER THREE

3.1 INTRODUCTION

The aim of this study was to investigate whether induction of the presumptive RPE depends on contact with the presumptive NR or whether a diffusible factor from the presumptive NR i responsible for RPE induction during chicken embryonic eye development.

Experimental manipulations involving barrier implantations were conducted to attempt to answer this question. However, since manipulations are easier to conduct *in vitro* than *in ov* it was necessary to first establish the conditions for culturing chicken embryos *in vitro*. Feasibility studies were conducted to determine whether synthetic barriers could be inserted into the optic vesicle. The aim was to trap the synthetic barrier between the presumptive NR and the presumptive RPE of the optic cup by allowing invagination of the optic vesicle to proceed in culture. In this way the effect (if any) of contact between the developing NR on RPE induction could be established. Results of these experiments will be presented in secti 3.3 and the technical problems encountered will be discussed together with these results in section 3.4.

3.2 MATERIALS AND METHODS

3.2.1 Culture medium

3.2.1.1 Glucose-albumin agar culture medium

Thin albumin from hen's eggs containing 150 Units/ml mycostatin and 1% penicillin/streptomycin was heated to 50°C and mixed with an equal volume of sterilised 2% bacto-agar, 1% D-glucose solution in chick saline (125mM NaCl, 5mM KCl, 1mM $CaCl_2.2H_2O$). The culture medium was poured into sterilised culture dishes, allowed to set and then stored at 4°C until required.

3.2.1.2 Thin albumin culture medium

Thin albumin from hen's eggs containing 1% penicillin/streptomycin was freshly prepared before use, heated to 37°C and poured into sterilised culture dishes.

3.2.2. Preparation of embryos for culture

All dissecting instruments were sterilised before use. Chicken eggs (White Plymouth Rock X Pile Game) were incubated for 40-50 hours in a humidified chamber (50% humidity) at 37°C. Eggs were carefully opened into a sterile bowl containing chick saline heated to 37°C and the yolk mass turned so that the embryo was uppermost. By holding one end of the yolk mass with a pair of forceps, a ring was cut just above the equator of the yolk mass, separating the area opaca (and the embryo) from the yolk. This procedure must be done rapidly to ensure that the embryo does not tear with release of the yolk. The embryo was transferred to a petri dish and rinsed several times in chick saline to remove adhering yolk. The vitelline membrane was carefully removed by gently pulling it off the embryo and the embryo orientated the correct way up (as it is in the egg). The embryo was then staged according to Hamburger and Hamilton (1951).

3.2.3 Culturing

Embryos were carefully transferred to the culture dish and covered with saline. Sterilised filter paper and plastic rings cut to size (1 cm diameter) were used to aid culturing. A ring was placed around the embryo and the edges of the area opaca flipped over the rim of the ring to prevent the membrane from pulling towards the embryo as the embryo grows (Fig. 3.1). For thin albumin cultures, the embryo-ring construct was prepared in a watchglass flooded with saline and the construct carefully lifted into the culture dish. Culture dishes were covered and sealed with parafilm. Two to three dishes were placed in a larger glass petri dish containing

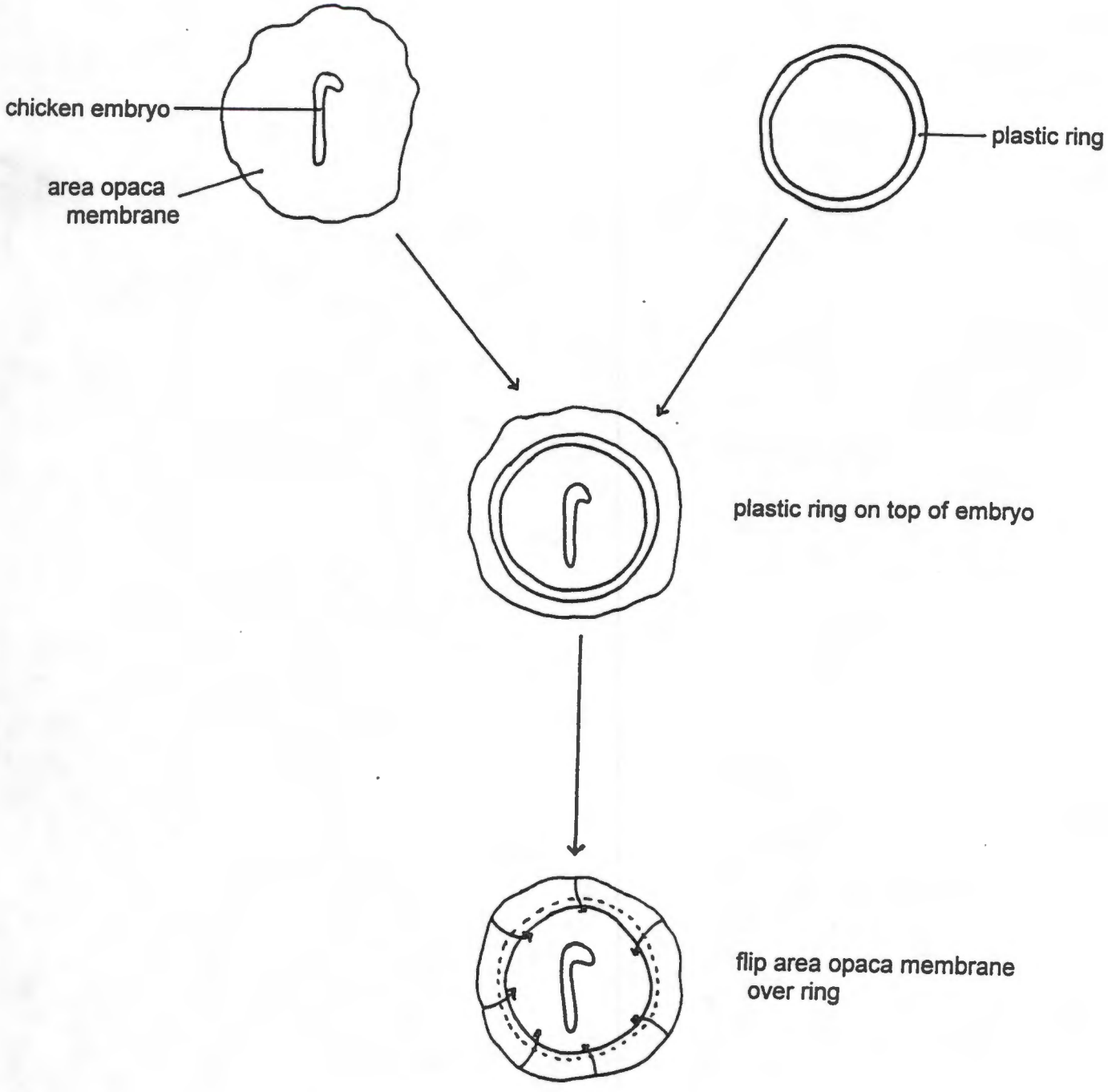


FIGURE 3.1. A schematic diagram of the ring-culturing system used to culture chicken embryos *in vitro*.

moist cottonwool. This petri was also covered and sealed with parafilm and then placed in an incubator at 37°C (50% humidity). Survival of the embryos was monitored on a daily basis.

Normal embryos were cultured on both types of culture media to observe their survival rate and to optimise culturing conditions before implantation studies were conducted.

3.2.4 Implantation of barriers into the optic region

3.2.4.1 Feasibility study

In order to test the feasibility of inserting barriers into the optic vesicle (at stage 11 of chicken embryonic development), chicken eggs were incubated for 40-45 hours as described previously. The embryos were removed and prepared as described in sections 3.2.2 and 3.2.3. Sterilised synthetic barriers of the appropriate size were cut out of aluminium foil, Hybond N^+ (Amersham) and Millipore filter paper (0.22µm) under a dissecting microscope. Once the embryos were rinsed and the vitelline membrane removed, they were pinned down in a wax-filled dish containing saline. Under a dissecting microscope and using two very fine tungsten needles, a slit was made in one of the optic vesicles. The implant barrier was then pushed/forced into the optic vesicle using tungsten needles. Embryos were fixed immediately in 4% paraformaldehyde (in PBS) overnight at 4°C, processed and wax embedded (Appendix III).

3.2.4.2 Implanting barriers into the optic vesicle

The implantation technique described above was used to implant barriers into embryos ready for culture. These embryos were cultured on thin albumin as described (3.2.3) for 40-45 hours (stage 11) and were monitored for survival. Twenty-one separate attempts to insert barriers into the optic vesicle were conducted. In addition, five attempts were made to insert barriers into the optic vesicle *in ovo*. Eggs were incubated for 40-45 hours (stage 11) and a window cut in the eggshell above the embryo. A few drops of chick saline solution were placed on top of the embryo before and after insertion of the barrier to prevent desiccation. The eggs were then sealed with tape before incubating for a further 24-48 hours.

After culturing or when the embryos had died, they were removed from the egg or culture dish, rinsed in saline and fixed in 4% paraformaldehyde (in PBS) overnight at 4°C before processing and wax embedding (Appendix III).

Controls: The optic vesicle of control embryos were slit but no implant was implanted. These embryos were cultured under the same culture conditions and for the same culture period as

experimental embryos. They were then fixed and processed together with experimental embryos.

3.2.4.3 Feasibility of implanting barriers into the eye mesenchyme

Attempts (five) were made to insert aluminium foil barriers into the mesenchyme surrounding the developing eye *in ovo* at day 3.5 (stage 21) of chicken embryo development. These experiments were conducted using the same techniques described for implantations into the optic vesicle (3.2.4.1).

3.2.5. Morphological studies

3.2.5.1 Processing, embedding and sectioning of tissue

After fixing, embryos were processed for paraffin wax embedding as described in Appendix III Embryos were orientated before embedding in order to obtain sections of the appropriate eye and blocks stored at room temperature. Sections (4µm) through the embryonic eye were cut, placed on glass slides and incubated at 60°C for 30 min or at 37°C overnight to allow the sections to adhere to the slides.

3.2.5.2 Staining of tissue

Selected sections were dewaxed in xylene, hydrated through a graded series of alcohols to tap water and stained in Mayer's haematoxylin for 10 min. After washing in tap water, slides were dipped three times in 1% acid alcohol, rinsed in water and stained lightly in eosin for 5 min. Sections were then dehydrated through a graded series of alcohols and mounted in Entellan. Sections were viewed in order to locate the implant and then photographed.

3.3 RESULTS

3.3.1 Culture media

Manipulations of chicken embryos are easier to perform *in vitro* than within the egg because *in vitro* the yolk mass beneath the embryo is removed and the embryo is thereby prevented from moving around during manipulation. It was therefore necessary to establish a culture medium and culture conditions for embryo development *in vitro*. Since manipulations were to be conducted on stage 11 or 12 (40-48 hours) chicken embryos when the optic vesicle has developed as an outpocketing of the wall of the diencephalon, eggs were incubated to this stage before the embryo was explanted for culturing. Two different culture media were tested - a glucose-albumin-agar culture medium and a thin albumin culture medium.

In order to determine the feasibility of culturing embryos on a glucose-albumin-agar culture medium, five eggs were incubated for 48 hours, the embryos were removed from the eggs, rinsed in chick saline solution and placed on culture dishes containing the glucose albumin agar culture medium. The embryos were cultured for a further 24-48 hours in a humidified chamber (50% humidity, 37°C) and survival and development of the embryos were closely monitored. One embryo died after 24 hours and two appeared abnormal after 48 hours in culture. The remaining two embryos had developed to the equivalent of a further 6-8 hours (ie. to stage 14) in a period of 48 hours. Thus normal development on this culture medium was greatly retarded and appeared to be hindered by the semi-solid glucose-albumin-agar culture medium. Normal chicken embryos turn onto one side at stage 13 (48 - 52 hours of incubation) and it appeared as if embryos on the glucose-albumin-agar culture medium was unable to turn over. For this reason, a less solid, thin albumin culture medium was tested.

Ten embryos were incubated for 48 hours (to stage 11 or 12 of chicken embryonic development), removed from the egg and transferred to a thin albumin culture medium (as described in section 3.2.3). Embryos were cultured for 48 hours and monitored daily. Two embryos died and the other eight developed for a further 5-7 hours in a 24 hour period, reaching approximately stage 16. After a total incubation time (*in vitro*) of 48 hours, two of these embryos had reached stage 20/21 of development . These two embryos appeared normal and had faintly pigmented eyes. Pigment is first visible at stage 20 (70-72 hours) in normal chicken embryos and by stage 21 (3.5 days) the eyes are distinctly pigmented.

In summary, even though development was stunted *in vitro*, all the embryos had developed beyond the optic cup stage (stage 15) of chicken embryonic development (which was critical for subsequent implantation experiments) and some embryos developed further to the pigmented eye stage (stage 21). Thus the thin albumin culture medium was used in further studies.

Optimisation of culturing conditions:

Once a culture medium had been established, steps to optimise embryonic development on this medium were undertaken. For example, the chick saline solution and the culture medium were preheated to 37°C before transferring the embryos from the 37°C incubated egg. This was done in order to prevent the embryos from being exposed to low environmental temperatures during transfer. In addition, a mini-humidity chamber was made to ensure that the percentage humidity was maintained and to prevent the embryos from drying out during the incubation period. This chamber was made by placing the culture dishes in a larger petri dish containing some moist cottonwool and then sealing the culture dishes (and the large petri dish) with parafilm. At stage 13 (48-52 hours) of chicken embryonic development when the embryo turns onto one side, growth and development of many organs begins and therefore there is a dramatic increase in the size of the embryo after this stage. In order to prevent the area opaca membrane from pulling towards the growing embryo and interfering with the growth process, a ring system for culturing embryos was developed. Plastic rings were placed around the embryos and the area opaca membrane flipped over the ring (as described in 3.2.3). This was sufficient to prevent the membrane from pulling towards the embryo during growth. Figure 3.2 shows a chicken embryo at stage 16 of embryonic development which was cultured in the ring-culture system. (The ring is indicated with an asterisk in the figure). The blood-ring surrounding the embryo from which bloodvessels later extend towards the growing embryo can also be seen in the figure (arrowheads).

After optimisation, embryos consistently developed for 18-22 hours during a 48 hour culture period *in vitro* reaching stage 18-20 (65-72 hours) of chicken embryonic development. Therefore although the eyes of *in vitro* cultured embryos did not pigment, the optic cup was well developed (Fig 3.2, arrow). These culturing conditions were used in all subsequent manipulation/implantation experiments.

3.3.2 Feasibility of implanting barriers into the optic vesicle

Feasibility studies were conducted in order to determine whether synthetic barriers could be inserted into the optic vesicle at stage 11/12 of chicken embryonic development.

At stage 11 (40-45 hours) of chicken embryonic development, the optic vesicle is approximately 100µm in diameter, distinct and clearly visible in the chicken embryo. Sterilised synthetic barriers were cut using a blade under a dissecting microscope to the appropriate size (approx. 500µm) in order to fit into the optic vesicle. (The cutting of barriers proved to be time-consuming and a more efficient way of cutting them was not found). Once the embryos had been incubated to the appropriate stage and transferred to the culture dish, tungsten needles were used to make a slit in the optic vesicle. The barrier was then manoeuvered to above the optic vesicle with tungsten needles and forced inside the eye cavity. Figure 3.3

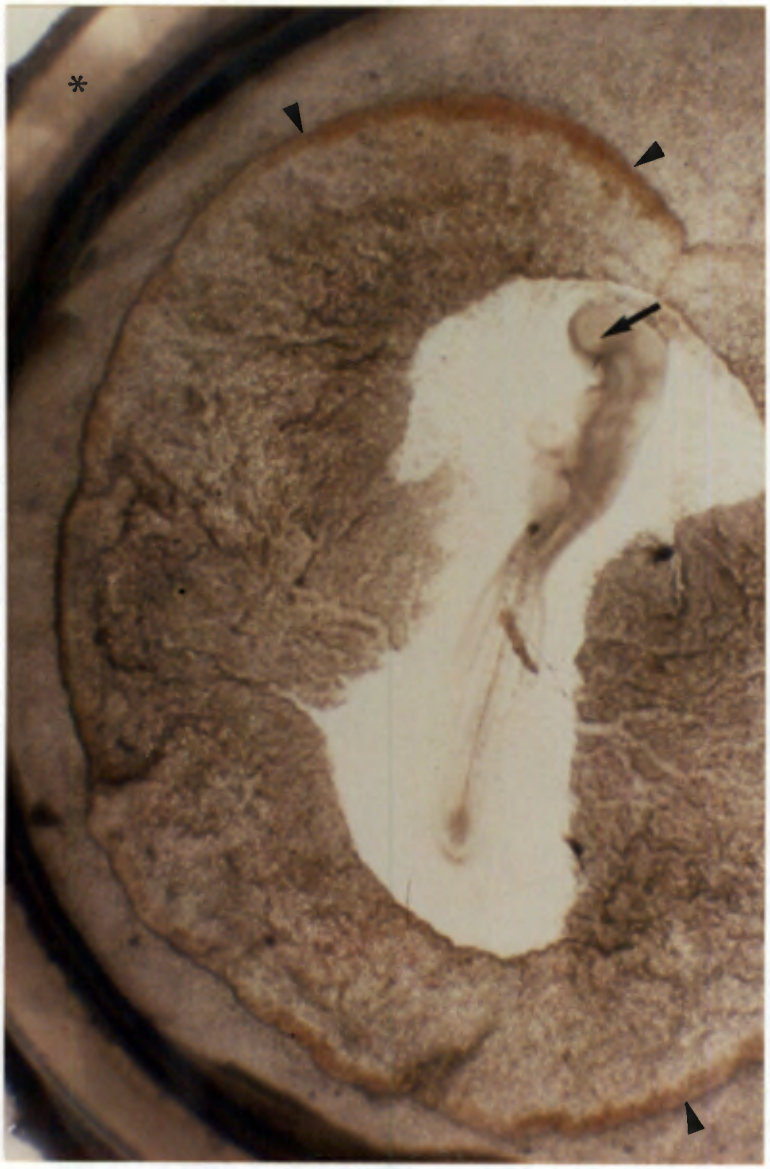


FIGURE 3.2. A living stage 16 (51-56 hours) chicken embryo in a culture dish containing thin albumin. The arrow indicates the optic cup. The ring (asterisk) used to aid culturing and the blood ring surrounding the embryo (arrowheads) are indicated (85 x).

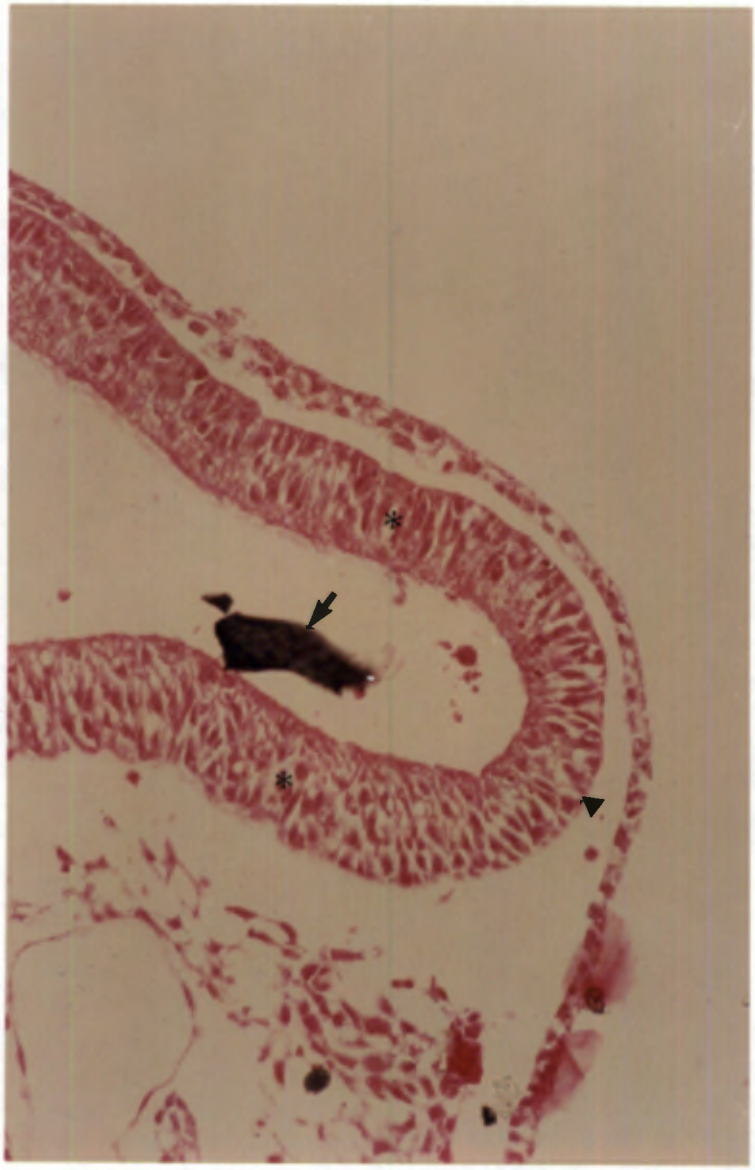


FIGURE 3.3. An aluminium foil barrier (arrow) implanted into the optic vesicle of a stage 11 (40-45 hours) chicken embryo. The presumptive retinal pigment epithelium (asterisk) and the presumptive neural retina (arrowhead) are indicated. (282 x).

shows an aluminium foil implant (arrow) in the optic vesicle of a stage 11 chicken embryo. Aluminium foil barriers were easier to manoeuver and insert because of their rigidity and shiny colour compared to paper filters (Hybond and Millipore) which were difficult to locate above the embryo. Attempts to stain the embryos with neutral red in order to increase contrast with the filter paper were made but did not markedly improve visibility.

From this feasibility study, it was determined that implantation of synthetic barriers into the optic vesicle at stage 11 of chicken embryonic development was possible. With practice this became easier to achieve.

3.3.3 Implanting barriers and culturing in culture dishes

Once feasibility studies were completed, the aim was then to culture the embryos (with implants) *in vitro* and allow invagination of the optic vesicle to occur. It was hoped that the synthetic barrier would become trapped between the developing NR and the developing RPE layers of the optic cup. The arrows in Figure 3.4 indicate the space in which the implant was aimed at being trapped. By implanting the barrier at an early stage when the NR and RPE layers are undifferentiated and both are neuroepithelia, the effect of contact between the developing NR and the developing RPE on RPE induction could be established. The following results were obtained.

Twenty-one attempts at implanting synthetic barriers into the optic vesicle of stage 11 (40-45 hours) chicken embryos and culturing *in vitro* for 48 hours were made. However, in only two embryos were the implants found in the invaginated optic vesicle (Fig.3.5). In Figure 3.5A, the embryo had died at some point before fixation as evidenced by the numerous necrotic cells and the diffuse nature of the mesenchyme. The effect of the aluminium foil implant (arrowhead) is therefore, unfortunately, impossible to determine. In Figure 3.5B, a Hybond N^+ (Amersham) filter (arrowhead) was successfully inserted into the optic vesicle of a stage 11 chicken embryo and the embryo was cultured further. In this embryo, the implant prevented invagination of the optic vesicle from taking place. Invagination normally occurs at stage 14 (50-53 hours) of chicken embryonic development. In addition, the mesenchyme is diffuse indicating that some necrosis had occurred. However, the presumptive RPE and presumptive NR layers do appear different in morphology, suggesting that by preventing contact between these two layers normal induction of the RPE was unaffected. However this result was only achieved once and therefore its validity is questionable.

In most of the other attempts at implanting barriers in the optic vesicle and allowing invagination to occur, the barriers could either not be found on sectioning the optic region or

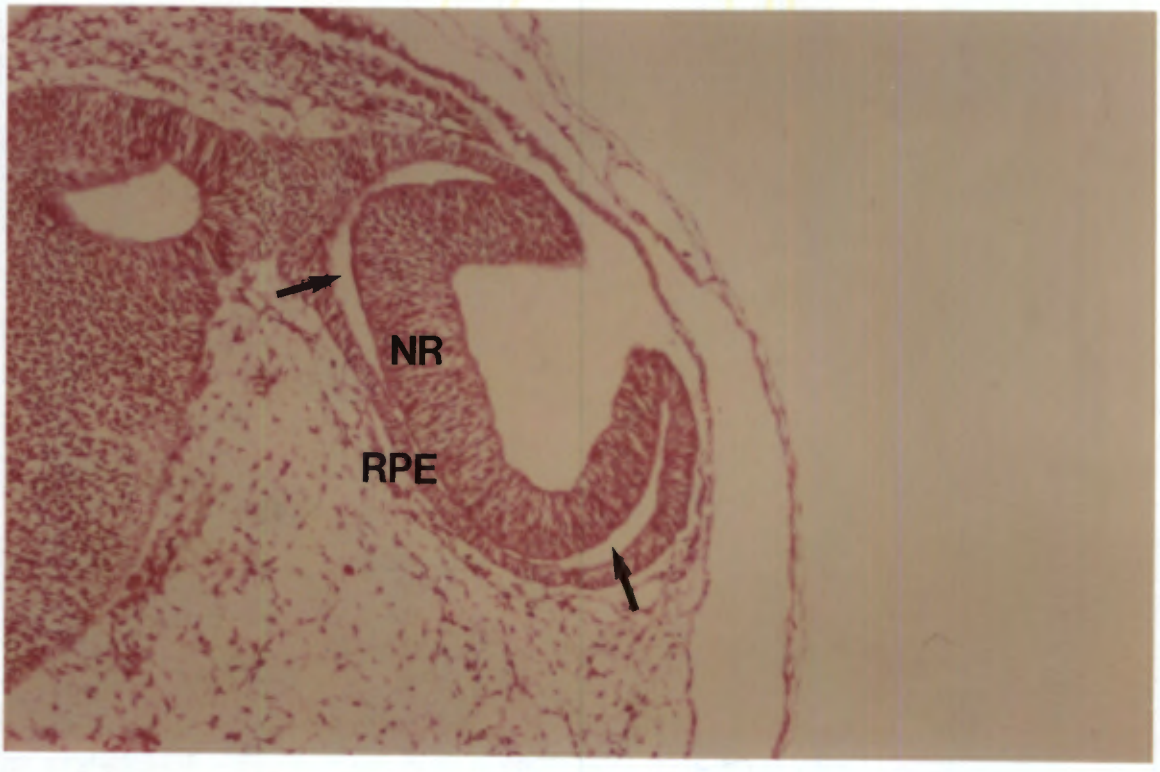


FIGURE 3.4. Optic cup formation in the stage 18 (65-68 hour) chicken embryo. The retinal pigment epithelium (RPE) and the neural retina (NR) are not completely apposed to one another. Arrows indicate the space between these two layers. (282 x).

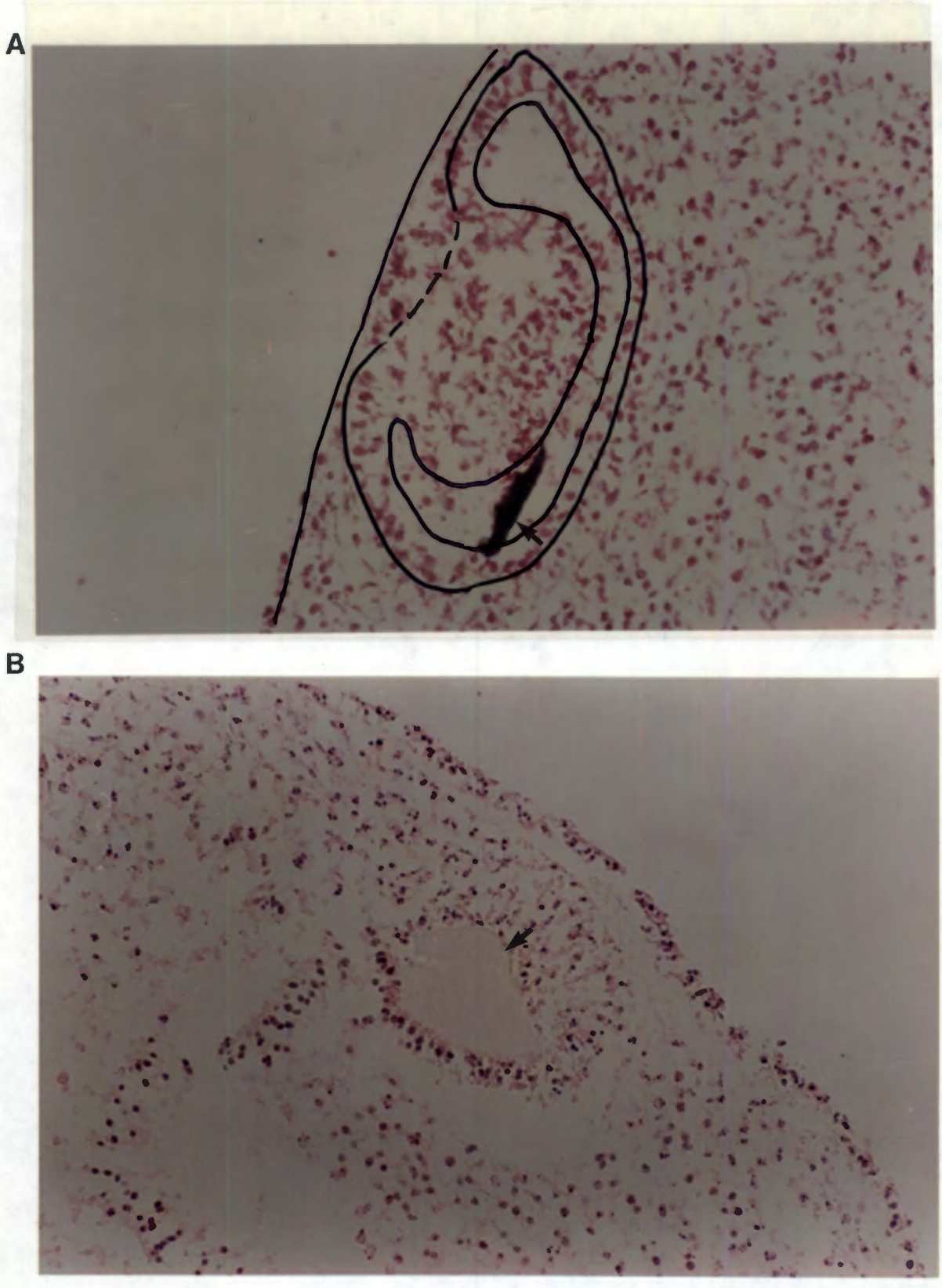


FIGURE 3.5. Synthetic barriers in the optic cup region of chicken embryos after implantation at stage 11 (40-45 hours) and *in vitro* culturing. (A) Aluminium foil barrier (arrowhead) in the optocoele of the invaginated optic cup at stage 18 of embryonic development. The outline of the optic cup is indicated in the overlay. (282 x). (B) A Hybond N^+ filter barrier (arrowhead) sandwiched between the presumptive RPE and the presumptive NR layers during invagination of the optic vesicle at stage 14 of chicken embryonic development (282 x).

were found in the brain cavity or mesenchyme surrounding the eye (Fig.3.6, arrowheads). Thus the implants had a tendency to "pop out" of the optic vesicle either during invagination or during further growth of the embryo. In addition, aluminium foil barriers although easier to implant, were difficult to section and tendered to sheer on sectioning.

In summary, the effect of the implant on RPE induction could unfortunately not be unequivocally determined from these studies. However in a single experiment it appeared as if the implant prevented invagination which in turn did not appear to effect the normal development of the presumptive RPE or presumptive NR. Determining whether induction had occurred was difficult from morphological studies and it is unfortunate that *in situ* hybridisation reactions to detect expression of the pigment specific genes could not be performed on these embryos, since the tissue in these experiments had not been prepared appropriately. Furthermore, cultured embryos generally developed to stage 16/17 (51-64 hours) of chicken embryonic development from stage 11 (40-45 hours) in 24-48 hours after manipulation but frequently did not survive the entire 48 hour culture period, or showed no further development/growth in the last 24 hours *in vitro*. Compared to results from the studies done here, the manipulated embryos did not develop as successfully. The reason for this was not entirely clear. It may be due to prolonged exposure of the embryo to room temperatures while the manipulation was carried out or due to the culture medium which may not have been ideal. In addition, development of the vascular system was hindered by the ring used during culturing and this could also have affected the rate of development of the embryo.

3.3.4 *In ovo* implantations

Implantation into the optic vesicle

Embryos cultured *in vitro* could not be cultured routinely to the pigmented eye stage (stage 21) of embryonic development (see discussion section 3.4.1). Since pigment in the RPE is an indicator that RPE induction has occurred, these conditions were not ideal. Therefore, attempts at inserting barriers into the optic vesicle of stage 11 (40 -45 hours) chicken embryos *in ovo* were made. All five attempts were unsuccessful and the embryos died after a 24 hour incubation. None of the implants were found on sectioning (not shown).

Implantation into the mesenchyme below the optic cup

Five attempts at inserting implants *in ovo* into the mesenchyme below the optic cup at stage 21 (3.5 days) of embryonic development were also made. It is thought that a diffusible factor emanating from the mesenchyme below the developing optic vesicle could be involved in RPE induction (Buse and de Groot, 1991). Therefore the feasibility of inserting barriers into this region *in ovo* (when induction of the RPE occurs) was established by determining whether barriers could be inserted at stage 21 (3.5 days) of embryonic development when the embryo is large and the optic region readily accessible.

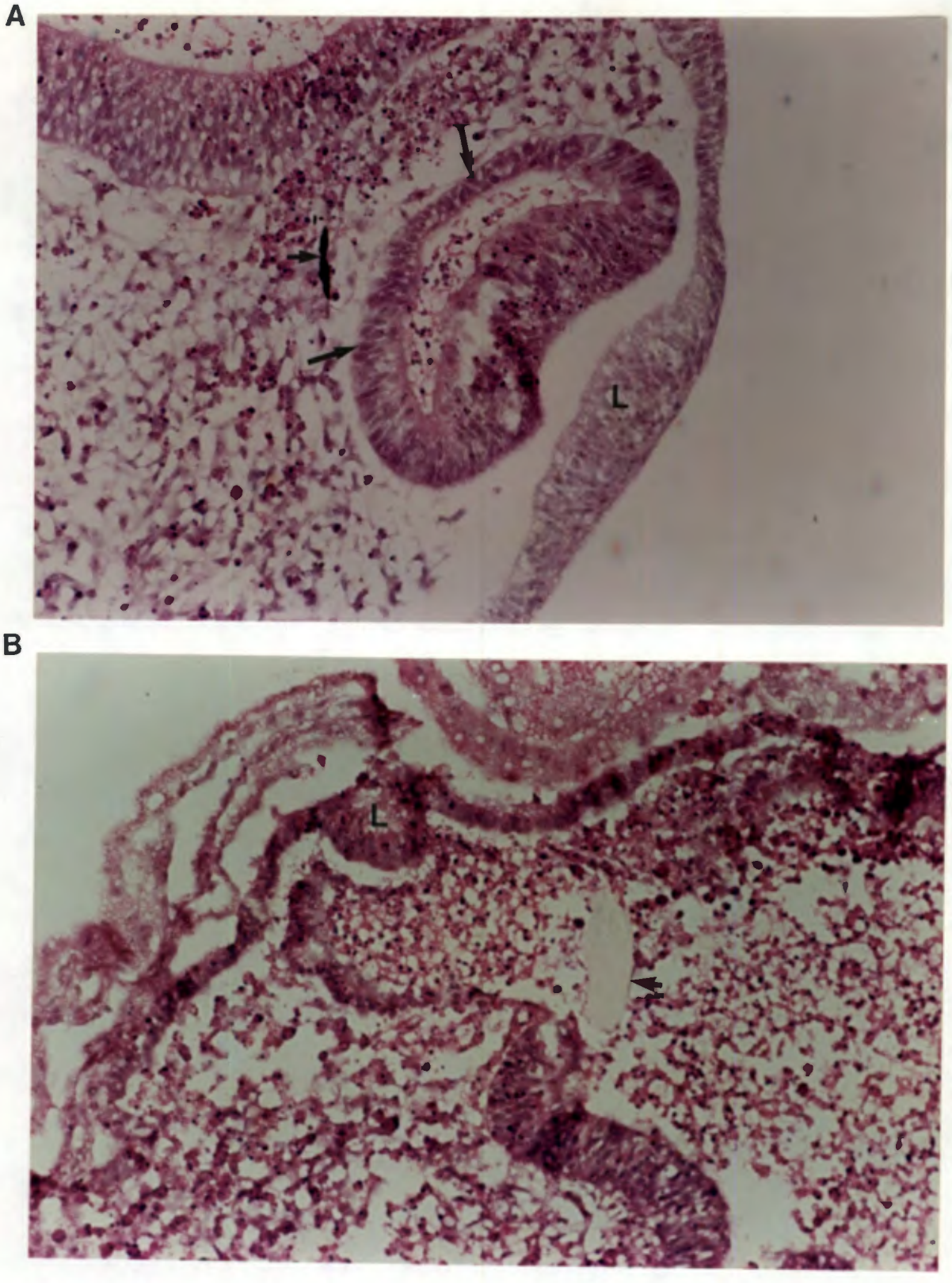


FIGURE 3.6. Synthetic barriers in the mesenchyme surrounding the optic cup (A) and in the brain cavity (B) of chicken embryos. Barriers were implanted at stage 11 (40-45 hours) of chicken embryonic development and the embryos were then cultured *in vitro* for 24 hours. The lens placode is indicated (L). (A) An aluminium foil barrier (arrowhead) in a stage 14 (50-53 hours) embryo. Arrows indicate the presumptive retinal pigment epithelium. (282 x). (B) A Hybond N^+ filter barrier (arrowhead) in a stage 13 (48-52 hours) embryo (282 x).

All five attempts were however unsuccessful. One embryo died after 24 hours and the other three embryos survived a further 48 hours incubation. These embryos appeared normal when compared to control embryos (not shown). However when sectioned none of the implants could be found.

3.4 DISCUSSION

3.4.1 METHODOLOGY: Problems and criticisms

3.4.1.1 Culturing Technique

An *in vitro* system for culturing chicken embryos was successfully established and optimised. A reasonable rate of development of manipulated embryos *in vitro* was attained in this study but perhaps could be further optimised since non-manipulated embryos still developed at a faster rate than normal embryos. Most embryos cultured *in vitro* in the ring system did not develop beyond a certain stage (stage 17). The reason for this was that the ring prevented further development of the vascular system. Therefore culturing to the pigmented eye stage (stage 21) was rare and perhaps larger rings could be used in future. Furthermore, the optic vesicle is accessible *in ovo* and therefore manipulating the embryo when it is still in the egg may be possible. However, this technique needs to be optimised.

3.4.1.2 Implants/barriers

Implantation of barriers into the optic vesicle of stage 11 chicken embryos was possible. However, determining the optimum size of the implant was difficult. Too large an implant hinders further eye development as seen in Fig.3.5B, in which invagination was prevented, and a very small implant makes handling with tungsten needles impossible. The implant must be a suitable size to be trapped between the two layers of the optic cup in at least a portion of the developing eye in order to observe its affect on RPE induction (as in Fig.3.5A).

3.4.2 Induction results

3.4.2.1 *In vitro* manipulations and culturing

In this study synthetic barriers were implanted into the stage 11 optic vesicle, the embryos were cultured *in vitro* to beyond the optic cup stage and morphological studies on sectioned tissue were then conducted. The results were however difficult to interpret because the embryos could not be cultured to the pigment eye stage (stage 21). In one experiment in which invagination was prevented, the presumptive RPE layer appeared different from the presumptive NR (Fig.3.5B) suggesting that RPE induction had occured. However, the barrier used was porous (a Hybond N^+ (Amersham) filter of 0.45µm). Thus there is the possibility that if a diffusible factor from the presumptive NR is produced that it could have been responsible for the RPE induction and differentiation observed. No clear conclusions could be obtained from these studies.

3.4.2.2 *In ovo* manipulations and culturing

All *in ovo* attempts (10) at implanting barriers into either the optic vesicle or the mesenchyme below the eye cup (section 3.2.4.2 and 3.2.4.3) were unsuccessful. The main reason was

because the yolk mass below the embryo is very unstable offering no resistance to manipulations of the embryo. In addition, the yolk mass is easily pierced making implantations (especially in younger embryos) tricky. Implantations into the mesenchyme below the optic cup (stage 21) were easier to perform because of the larger embryo size. However, at this stage of chicken embryonic development, blood vessels into and surrounding the eye are present and have to be avoided.

Thus *in ovo* implantations into the optic vesicle or mesenchyme below the optic cup, although unsuccessful in this study are possible but conditions need to be optimised and further practice is required.

CHAPTER FOUR

4.1 DISCUSSION

During development of the vertebrate eye, the RPE develops from an outgrowth of the forebrain, the optic vesicle, which invaginates to form the optic cup (Balinsky, 1983). The outer layer of the optic cup differentiates into the RPE while the inner layer differentiates into the NR or sensory layer of the eye. As invagination progresses, the inner layer comes into contact with the developing RPE (the outer layer) and apical microvilli of the RPE cells begin to interdigitate with the cells of the presumptive NR. In order to fully understand induction and development of the RPE, one needs to know when induction takes place, what factors are involved in this inductive event, from where these factors emanate and how they exert their effect. The present study directly investigates two of these questions - namely when does RPE induction occur and what tissue(s) could be responsible for this induction. The first question was addressed by determining the temporal expression pattern of two genes specifically expressed in pigment cells, namely tyrosinase and TRP-2. The second question was addressed by implanting barriers into the uninvaginated optic vesicle thereby preventing contact between the presumptive RPE and the presumptive NR during development.

4.1.1 Temporal expression of tyrosinase and TRP-2 as indicators of RPE differentiation.

The temporal expression pattern of the tyrosinase gene family in the RPE has not previously been investigated in avian embryos. In this study, it was shown that both tyrosinase and TRP-2 transcripts are present in the RPE prior to the appearance of pigment granules. TRP-2 transcripts were first detected at stage 18.5 (67-69 hours) and tyrosinase transcripts were detected a few hours later at stage 19.5 (70-71 hours). Pigment granules are faintly visible in the RPE of this chicken breed at stage 20 (70-72 hours). Thus, in the chicken, TRP-2 and tyrosinase are expressed before pigment is visible and TRP-2 expression precedes that of tyrosinase. Since more is known about development of the murine RPE, comparing the expression patterns of tyrosinase and TRP-2 in the chicken RPE with that observed in the murine RPE might provide further insight into the development of the chicken RPE.

Three studies investigating the temporal expression pattern of tyrosinase and TRP-2 in the developing murine RPE have been conducted (Steel et al. 1992; Beermann et al. 1992; Cable et al. 1995). In the studies of Steel et al. (1992) and Cable et al. (1995), TRP-2 transcripts were first detected at stage 15 (9.5 dpc) and pigment granules were detected two days later (11.5 dpc). Using radioactively-labeled riboprobes in ISH reactions, Steel's group detected tyrosinase transcripts only after pigment granules were observed at 13.5 dpc and did not detect tyrosinase mRNAs prior to this stage. This group suggested that very low levels of tyrosinase mRNA may be present earlier than 13.5 dpc but that these transcripts were not

detectable with the detection methodology used in their study. Beermann et al. (1992), however, using similar methodology to Steel et al. (1992), detected tyrosinase mRNA in the murine RPE at 10.5 dpc, shortly after TRP-2 expression was first detected and before pigment granules were present. No explanation for this difference in early tyrosinase expression was given. In summary, in the mouse embryo, TRP-2 is expressed first at 9.5 dpc and tyrosinase transcripts were first detectable at 10.5 dpc. Pigment granules are present in the developing murine RPE a day later. Thus, it appears that in both the chicken and the mouse, TRP-2 is expressed before tyrosinase.

It is not however clear why TRP-2 transcripts are detected prior to tyrosinase transcripts? The TRP-2 gene product, dopachrome tautomerase (DCT), is involved in converting toxic DOPAchrome, the final product of tyrosinase oxidation, into a colourless, less toxic, intermediate 5,6-dihydroxyindole-carboxylic acid (DHICA) (Tsukamoto et al. 1992). TRP-2 was found to be expressed before tyrosinase transcripts were detectable in the RPE of the chicken embryo (in this study) and in the mouse embryo (Steel et al. 1992, Cable et al. 1995). The reason for this early expression of TRP-2 is unclear since there is no evidence for an earlier role of TRP-2 in the melanogenic pathway. Perhaps DCT must be present and active before DOPAchrome is produced (ie. before the melanogenic pathway begins) in order to immediately degrade the toxic DOPAchrome products after they are produced.

Induction of the RPE must occur before overt differentiation of the pigment epithelial cells. By determining the onset of expression of the pigment-specific genes, the timing of induction of the presumptive RPE may be more precisely determined. In the present study, chicken TRP-2 transcripts were first detected in the RPE at the optic cup stage (stage 18.5) of eye development, shortly after invagination of the optic vesicle had taken place. These results could suggest that contact of the presumptive NR with the presumptive RPE is crucial for RPE induction. (In support of this, results of the present study indicate that the morphology of the cells of the presumptive RPE and the presumptive NR are similar at the optic vesicle stage of eye development which suggests that induction has not taken place.) These results contrast with the studies of Steel et al. (1992) and Cable et al. (1992) in which TRP-2 expression was first detected in the RPE at the optic vesicle stage of eye development, before optic cup formation. These results suggest that contact with the NR may not be involved in RPE induction since the NR is not in close proximity to the RPE at the optic vesicle stage of development. It is, however, possible that these differences in TRP-2 expression in the mouse and chicken RPE are due to slight differences in methodology. According to Boehringer Mannheim (1992), non-radioactively labeled probes used in ISH reactions (as in the present study) are less sensitive than radioactively-labeled probes and therefore the detection methodology used in the present study may not have been sensitive enough to detect the first TRP-2 transcripts in the presumptive RPE. One cannot therefore exclude the possibility that these transcripts may have been present earlier than the optic cup stage (stage

18.5) of chicken embryonic development. This discrepancy would be answered by carrying out further ISH studies using radioactively-labeled probes on chicken eye tissue.

The next question to address is whether the greater intensity of the hybridisation signal observed (for both tyrosinase and TRP-2) in younger embryos when compared with older embryos reflects real quantitative differences in expression levels during development? For example, the tyrosinase expression signal detected at stage 19.5 appeared more intense than that detected at stage 20.5 (Fig.2.21) in each section examined. Similarly, Beermann et al. (1992) report that in their study of tyrosinase expression in the murine RPE, the intensity of the tyrosinase hybridisation signal was weaker in older embryos. There are several possible explanations for these differences in signal intensity. Two of these explanations are technical. First, the density of the tissue at each stage might differ and therefore probe penetration into the tissue could be responsible for these differences in strength. The second explanation for the different intensities of the hybridisation signals observed is that different riboprobe stocks were used for different experimental runs and because the labelling of probes can never be identical, this may have affected signal intensity. If, on the other hand, the intensity of the hybridisation signal does reflect quantitative differences in the expression levels of tyrosinase (and TRP-2) during development, then this would suggest that as development progresses and the RPE differentiates further, tyrosinase (and TRP-2) and perhaps all the pigment-specific genes are down regulated. This would have bearing on melanogenesis in the adult RPE. It is however felt that the technical issues raised above are more likely to be responsible for the difference in signal intensity observed.

The temporal expression pattern of tyrosinase and TRP-2 within the optic cup not only reflects the timing of induction of the RPE but may also provide insight into the direction of pigmentation and differentiation of the RPE. In the chicken, at stage 19.5, TRP-2 transcripts were detected throughout the entire RPE, whereas tyrosinase transcripts were only detected in the central part of the RPE (ie. in the optical axis of the eye cup). At stage 20.5, the first visible pigment granules were also localised to this region. Thus, both tyrosinase and TRP-2 transcripts were first detected in the chicken RPE at the optical axis of the eye and the expression of both these genes later spread to the margins of the eye cup. These results indicate that pigmentation of the chicken RPE occurs in a centre to periphery direction. In the mouse embryo, a similar localisation of tyrosinase and TRP-2 expression was observed (Steel et al. 1992; Beermann et al. 1992; Cable et al. 1995) with both gene transcripts first detected in the optical axis of the eye and later spreading outwards to the margins of the eye cup. Thus, these results not only demonstrate the temporal order of expression of TRP-2, tyrosinase and pigment production but also indicate the direction in which pigmentation and perhaps also differentiation of the RPE proceeds.

This leads to the question of whether the differentiation pattern of the RPE (centre to periphery) can be used to deduce information about which tissues might be involved RPE induction. It has been reported that in chickens, the cells located in the central part of the RPE exit the cell cycle before the peripheral cells (Coulombre, 1955; Coulombre et al. 1963). Furthermore, a withdrawal of cells from the mitotic cycle is reported to be a prerequisite for melanisation (Coulombre et al. 1963). Therefore, since the cells at the centre of the chicken RPE exit the cell cycle first (Coulombre, 1955; Coulombre, 1963), these cells would be predicted to be the first to pigment. This prediction has been confirmed in the present study and is in agreement of Leplat (1914) who observed pigmentation in the RPE of the chicken embryo to proceed from the optical axis outwards. It is interesting to note that differentiation of the neural retina also proceeds in the centre to periphery sequence (Romanoff, 1960). Thus, both the neural retina and the RPE differentiate in the same direction. Since the NR is a possible candidate for inducing the presumptive RPE to differentiate (Buse and de Groot, 1991; Buse et al. 1993), the centre to periphery pattern of differentiation of both these layers may reflect the direction of induction of the RPE. Specifically it implies that the central presumptive RPE cells are induced earlier than those at the periphery. However, differentiation of the RPE is completed two days before differentiation of the NR begins (Romanoff, 1960) and therefore NR differentiation lags behind RPE differentiation as it progresses from the centre of the eye cup to the periphery (Romanoff, 1960). This difference in timing of differentiation does not exclude the presumptive NR from inducing the RPE.

In the mouse embryo, the direction of pigmentation of the RPE appears to be similar to that of the chicken, since expression of the pigment-specific genes (tyrosinase, TRP-1, TRP-2) were first detected in the centre of the RPE (Steel et al. 1992, Beermann et al. 1992, Cable et al. 1995). In other mammals however, pigmentation has been reported to proceed in the opposite direction from the periphery to the centre of the eye cup (in man, Mann 1949; in hamster and ferret, Strongin and Guillery, 1981).

4.1.2 Is the NR involved in RPE induction?

From the preliminary implantation studies performed in the second part of the present study, it was suggested that direct contact with the neural retina may not be required for RPE induction. This result contradicts previous work in the mouse embryo in which the NR was found to be required for RPE induction (Buse et al. 1993). The entire NR was removed in this study and therefore both contact with the NR and the potential production of a diffusible factor(s) by the NR were prevented. It was not determined which of these two events/factors was required for RPE induction in the mouse embryo. Thus, perhaps production of a diffusible NR factor and not contact with the NR is a requirement for RPE induction.

Previous studies have shown that the environment (ie. the mesenchyme) surrounding the developing eye is necessary for induction of the murine RPE (until stage 16 of optic cup development) (Buse and de Groot, 1991). Therefore, explanting the presumptive RPE and presumptive NR layers from the underlying mesenchyme and culturing with a barrier sandwiched between the two layers will not answer the question of whether contact with the NR is required for RPE induction. Perhaps a more defined way of investigating the effect of contact between the presumptive NR and the presumptive RPE on RPE induction would be to implant a barrier into the optic vesicle *in vivo* and then culture the embryos *in vitro* to the optic cup stage (stage 15). RPE induction could then be determined more precisely using ISH by monitoring TRP-2 gene expression.

In summary, RPE induction may occur as early as the optic vesicle stage, stage 11 of chicken embryonic development, as it does in the mouse embryo even though no tyrosinase or TRP-2 transcripts were detected in the present study at these early stages. Both TRP-2 and tyrosinase transcripts could only be detected from the optic cup stage of eye development onwards. Furthermore, contact with the NR appears not to be involved in RPE induction however a diffusible factor from the NR may be responsible for this inductive event.

4.1.3 The potentially ectopic expression of tyrosinase in the chicken embryo and its significance.

Digoxigenin-labeled cells were frequently detected in the neural tube, the mesenchyme surrounding the neural tube, the ectoderm, the limb buds and the gonadal primordia when using the tyrosinase riboprobe in ISH reactions (Table 2.). Whether this signal represents tyrosinase expression will be discussed below.

Melanocytes of the skin, feathers and internal organs arise from neural crest cells (NCC), a population of multipotent cells situated dorsal to the newly formed neural tube (Lallier and Bronner-Fraser, 1988) . These NCC migrate along defined pathways to eventually reach their target tissues. Since dig-labeled cells detected in the present study were found in the neural tube and not in the neural crest, it is unlikely that these cells represent migratory melanoblasts. However, consistent with these results, Tief et al. (1996) report tyrosinase expression in cells situated along the entire length of the neural tube of the mouse embryo, after NCC migration has taken place. In their study, the part of the tyrosinase gene promoter (270 bp) that drives transcription of tyrosinase in melanocytes was fused with a lac Z reporter gene. Transgenic mice were generated and tyrosinase expression was investigated at various ages of murine development. Thus it appears as if tyrosinase expression is not restricted to pigment cells. Clearly, further investigations would be needed to confirm the

expression of tyrosinase in cells of the neural tube and to determine the function of this gene product within these cells.

When using the tyrosinase riboprobe in ISH reactions, digoxigenin-labeled cells were also detected at stages 23 to 32 in the mesenchyme surrounding the neural tube, the ectoderm, and the limb buds. In the mouse embryo, tyrosinase expression has been found to label migratory melanoblasts at 14 dpc, before pigment is present within these cells (Steel et al. 1992). The onset of neural crest cell migration in chicken embryos is stage 15 (50-55 hours) and those cells destined to pigment the skin reach the dermis at approximately day 5 of embryonic development (Weston , 1963). Since the timing of NCC migration in the chicken embryo coincides with the stages at which dig-labeled cells were detected in the ectoderm, mesenchyme and limb buds in the present study, these cells could represent migratory melanoblasts. These results could be confirmed by performing co-expression studies with an antibody specific to NCC.

The gonads of avians begin forming as a mass of mesenchymal cells from day three to four of embryonic development (Romanoff, 1960). Digoxigenin-labeled cells were detected in the gonadal primordium at stage 30 (6.5 days) in the present study. Since the gonads of avians are pigmented, the tyrosinase expression detected in the gonadal promordia was not unexpected. However, this result was only obtained in one ISH reaction and the reason for this may be related to the difficulty in locating and sectioning the embryonic gonad. In addition, precursor chromaffin cells of the adrenal medulla which are of neural crest origin and which are present in the mesenchyme surrounding the aorta from stage 27 (five day old) onwards (Romanoff, 1960) were not detectable with the tyrosinase riboprobe used in the present study.

Choroidal and iris melanocytes are also derived from the neural crest (Ris, 1941). In the murine eye, these melanocyte precursors have been detected in the mesenchyme surrounding the eye at 11 dpc, just prior to the presence of pigment in the RPE (Cable et al. 1995). In the present study, in the chicken embryo, no tyrosinase expression were detected in this area. Thus, no migratory melanoblasts associated with the eye (ie. the future choroidal and iris melanocytes) were present at stages 23-32 (3.5-7 days). However, choroidal melanocytes are only reported to be present from day eight of chicken embryonic development (Romanoff, 1960) therefore this result is not unexpected. This late stage of eye development was not investigated in the present study. In addition, in the chicken embryo, migration of melanoblasts does not appear to correlate with eye development as it does in the mouse embryo.

The areas in which tyrosinase expression was observed in the present study were not investigated to determine whether they express TRP-2. However, at stage 18.5, digoxigenin-

staining was detected in the forebrain of the chicken embryo when using the TRP-2 riboprobe in ISH reactions. Steel et al. (1992) report TRP-2 expression in the murine forebrain from 10.5 dpc to 14.5 dpc and suggest that if TRP-2 is expressed in the same cells as tyrosine hydroxylase (TH) then DCT may play a role in the detoxification of melanin precursors which spontaneously accumulate from DOPA, the product of TH.

4.2 FUTURE PROSPECTS

It has been reported that the pigmentation pathways in the RPE and the skin, although similar, may not be identical (Schraemeyer, 1993; Schraermeyer and Stieve, 1993). Therefore the temporal expression pattern of TRP-1, the third member of the tyrosinase gene family, needs to be determined in order to more fully understand pigmentation of the RPE in the chicken embryo.

There have been relatively few studies investigating the factors regulating transcription of the genes involved in melanogenesis. Recently, a transcription factor thought to be responsible for the melanocyte specific transcription of the tyrosinase gene in the mouse embryo was identified (Hodgkinson et al. 1993). Embryos homozygous for some mutations in this gene have small eyes and this gene was therefore named microphthalmia (*mi*) (Hertwig, 1942). The microphthalmia gene product appears to regulate expression of tyrosinase and TRP-1 (Hodgkinson et al. 1993). A specific domain, the M box, is present in the promoters of all three of the tyrosinase gene family members and mi is thought to bind to this domain (Shibahara et al. 1991; Lowings et al. 1992; Yazuver and Goding, 1994; Yasumoto et al. 1995). The human TRP-1 gene promoter has been shown to be transactivated by mi through the M box (Yazuver and Goding, 1994) whereas in the human tyrosinase gene promoter, binding of MITF (the human homologue of mouse mi) to the initiator E box is responsible for pigment cell specific expression (Yasumoto et al. 1995). TRP-2 is the first of the tyrosinase gene family members to be expressed and the regulation of this gene is unclear. Vachtenheim et al. (1996) suggest that microphthalmia does regulate TRP-2 expression since a strict correlation between *MITF* and TRP-2 expression levels were observed on northern blots of human melanoma cell lines. However, Yasumoto et al. (1996) suggest that transcripts of the TRP-2 gene are regulated in a different manner from that of tyrosinase and TRP-1.

Mi expression in embryonic (13 dpc to 16 dpc) and adult mouse tissues has been investigated by northern blots and *in situ* hybridisation by Hodgkinson et al. (1993). These authors only investigated expression of *mi* from 13 dpc and found that it co-localised with TRP-2 expression found in the mouse RPE (Steel et al. (1992)). It would be of interest to investigate

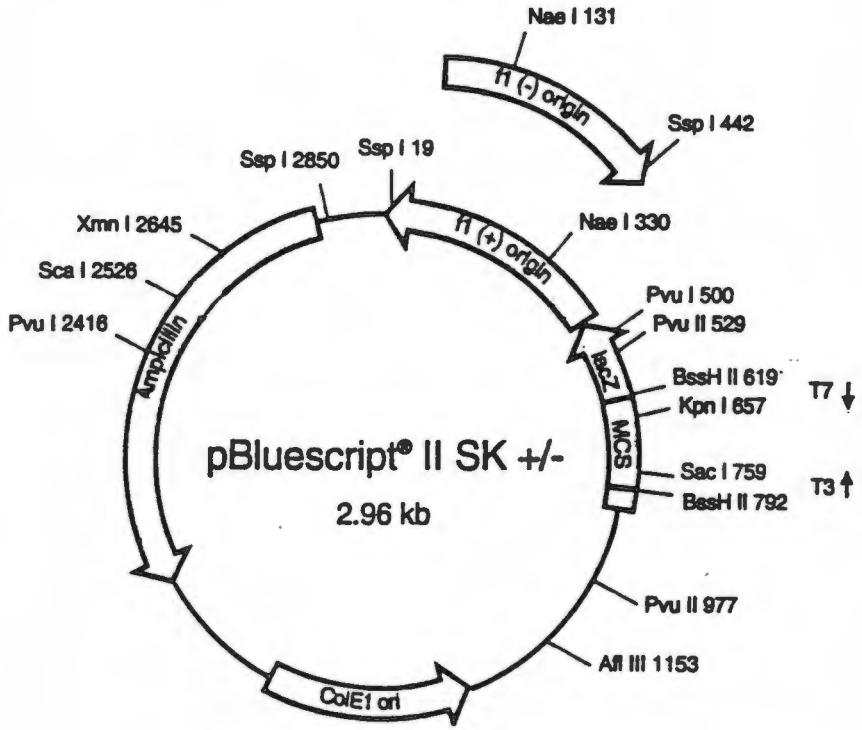
expression of *mi* in the developing RPE to determine whether there is a correlation between *mi* expression and TRP-2 expression *in situ*.

The chicken homologue of the mouse mi gene has only recently been isolated (April C., pers. comm.) and the temporal expression of this gene is currently being investigated. If microphthalmia expression is found to precede TRP-2 expression in the RPE of the chicken embryo then this would suggest that induction of the RPE occurs earlier than stage 18.5 when TRP-2 transcripts were first detected in this study. This would assist in the understanding of timing of RPE induction as well as the role of the NR in this process.

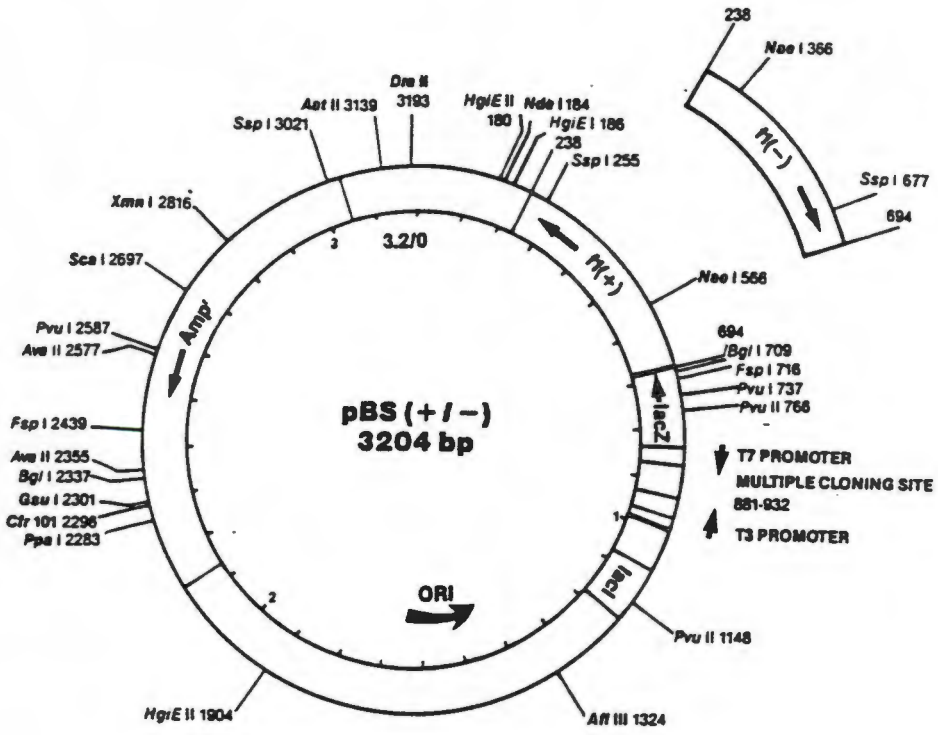
APPENDIX I
Cloning vectors

The maps of the vectors used in this study. A, pBluescript II S/K (+/-) (2.96 kb) and B, pBS (+/-) (3.2 kb).

A



B



APPENDIX II
Additional methods

Standard electrophoresis:

RNA and DNA agarose gels were prepared by standard methods according to Sambrook et al. (1989). Agarose (0.9% - 1%) (Sigma) gels for DNA samples were electrophoresed in 0.5 x TBE buffer (45mM Tris-borate, 1mM EDTA). For RNA samples, 1.3% denaturing formaldehyde agarose gels (15ml contains 1.5ml 10 x MOPS (0.2M MOPS, 0.05M sodium acetate, 0.01M EDTA pH 7.0, filtered); 0.17g agarose (Sigma); 0.75ml 40% formaldehyde) were used. The electrophoretic tank was prepared according to Sambrook et al. (1989) and 1 x MOPS used as the running buffer. Voltages used for electrophoresis in both cases were as recommended by Sambrook et al.(1989). Low melting point GTG agarose (LMP) (0.9% - 1%) (NuSieve) gels were prepared and electrophoresed as recommended by the manufacturers. Nucleic acid markers were used in every electrophoretic run: Pox DNA digested with EcoRI produces seven bands of known sizes (5.8 kb, 4.0 kb, 2.8 kb, 1.9 kb, 1.3 kb, 1.0 kb, 0.75kb) and was used as a DNA marker. Bacterial rRNA which runs as two distinct bands (23 S 3.7 kb, 16 S 1.7 kb) was used as an RNA marker. Samples were dyed (before electrophoresis) with a bromophenol blue buffer (Sambrook et al. 1989). After electrophoresis, the gels were stained in 0.01% ethidium bromide, viewed and photographed.

Phenol/chloroform extraction of DNA

Tris-saturated phenol and chloroform: isoamyl alcohol (24:1) were used for DNA extractions. In general, the DNA sample was made up to a volume of 200µl with sterile water. An equal volume of phenol was added, the sample vortexed and then centrifuged at 10 000g for 2 min. The number of phenol extractions performed depended on the presence of proteins at the interface. The bottom layer was removed with a drawn out pipette before proceeding to the next extraction step. A phenol:chloroform isoamyl alcohol extraction (1:1) was then carried out in the same way as the phenol extraction described. This was followed by a chloroform:isoamyl alcohol extraction after which the aqueous phase was transferred to a fresh sterile eppendorf. Samples were then precipitated.

Precipitation of DNA

Precipitation of DNA was performed by adding 3M sodium acetate at 1/10th of total sample volume and absolute alcohol at 2.5 x total volume. The sample was mixed and then placed at -20°C overnight or at -70°C for 30 min. The precipitated DNA was collected by centrifugation and the DNA pellet washed in 70% alcohol to remove residual salts and centrifuged again.

The sample was then vacuum dried and finally resuspended in a suitable volume of TE buffer depending on the size of the DNA pellet.

Spectrophotometer reading

DNA and RNA samples were quantified by spectrophotometer (Shimadzu) reading. The absorbance of nucleic acid samples were read at wavelengths of 260nm and 280nm. Samples were diluted to 1:1000 or 1:500 in sterile or DEPC-treated water and the diluent used was also used as a blank to zero the spectrophotometer at 260nm. (The absorbance of the blank at 280nm was also read). The concentration of DNA samples was calculated as follows:

OD_{260} sample x 50 (µg/ml DNA) x dilution used

For RNA samples the following formula was used:

OD_{260} sample x 40 (µg/ml RNA) x dilution used

Cloning of plasmid DNA

5µl of the subclone was used to innoculate 2mls of Luria Broth (LB) (10% bacto-tryptone, 5% bacto-yeast extract, 170mM NaCl) containing ampicillin (100 µg/ml) and allowed to shake at 37°C overnight. This 2ml culture was then used to innoculate a larger volume of LB (150 - 200mls) and incubated with agitation at 37°C overnight. When an OD_{600} greater than 0.4 was reached, 100 µg/ml chloroamphenicol was added to the LB to prevent bacterial growth and the LB again incubated overnight while shaking at 37°C. A maxi preparation of the plasmid DNA was carried out according to Davis et al. (1986) or the Wizard Maxiprep Kit for DNA purification (Promega) was used.

APPENDIX III

Preparation of tissue, slides and solutions for *in situ* hybridisation.

Processing for wax embedding

After fixation, the tissue was passed through a graded series of alcohols and finally to molten wax as follows:

50% alcohol for 30 min
70% alcohol for 1 hour
90% alcohol for 45 min
100% alcohol for 3 x 1 hour
xylene for 2 x 30 min
molten paraffin wax for 30 min

The tissue was then orientated and embedded in paraffin wax on an embedding machine. The blocks were set and stored at 4°C until required.

Aminopropyltriethoxysilane (APTES)-coated glass slides

Slides were washed in 10% Extran overnight, rinsed in water and dried. Once the slides had cooled down they were coated in 2% 3-aminopropyltriethoxysilane in acetone, dipped twice in acetone and once in sterile water before drying at 42°C. Slides were then DEPC-treated as described below.

Diethyl pyrocarbonate (DEPC)-treatment of slides and solutions

All solutions and slides were DEPC treated for RNA work.
Solutions were DEPC-treated by adding diethyl pyrocarbonate at a final concentration of 0.01% and shaken for 4-6 hours, after which the solutions were autoclaved.
Slides were DEPC-treated before use by placing them in freshly made 0.01% DEPC-treated water and allowing them to gently shake for 4-6 hours. Slide racks were then autoclaved and stored in sealed bags until required.

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