STUDY OF THE EXPRESSION OF TWO RXR GENES IN THE CHICKEN

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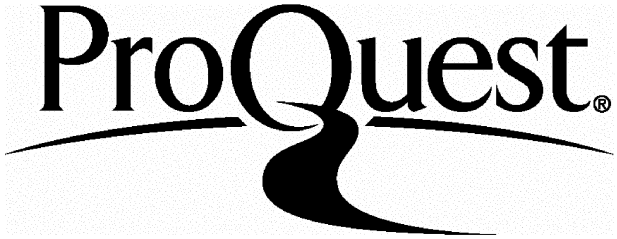

ABSTRACT

The retinoid X receptors (RXRs), together with the retinoic acid receptors (RARs), have important roles in mediating the effects of retinoids during development and differentiation. They are ligand-dependent transcription factors belonging to the steroid/thyroid hormone receptor superfamily. The RXRs have been shown to activate the expression of target genes in the presence of their ligand 9-cis-retinoic acid, but their main function seems to be as auxiliary proteins, that form heterodimers with other members of the superfamily, and enhance their DNA-binding and transcriptional activities.

In this work, the expression of two RXR genes in the chicken was characterised:

The chicken RXRγ gene was shown to give rise to two major mRNA species of different sizes (RXRγ1 and RXRγ2), that have a tissue specific distribution in embryonic and adult tissues: RXRγ1 is expressed predominantly in the eye, and is also expressed at lower levels in some neural crest derived tissues, such as the peripheral nervous system and the adrenal glands; RXRγ2 is expressed exclusively in the liver. The major sites of expression of the chicken RXRγ gene both in the embryo and in the adult are the retina and the liver, suggesting a possible involvement of this gene in the metabolism of vitamin A.

Ribonuclease protection analysis of these two distinct chicken RXRγ mRNAs, following the isolation of the corresponding cDNA clones, showed that they differed from each other in their 5' regions, and have the potential to encode two distinct protein isoforms of RXRγ that, like the RAR isoforms, differ only in their amino-terminal A domains. Examination of a genomic clone for chicken RXRγ, suggested that the two RNA isoforms may be generated by the use of alternative promoters.

A cDNA clone for chicken RXRα was also isolated. Preliminary analysis of the expression pattern of the chicken RXRα gene, showed that a single major transcript of this gene is found in most embryonic and adult tissues, with only slight variations in its levels of expression in different tissues. In particular this gene is expressed in the limb buds, at the stages when the patterning events are occuring, make it a possible candidate for mediating the proposed effects of 9c-RA as a positional signalling molecule.

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ABREVIATIONS

9c-RA - 9-cis retinoic acid
A/P - anterio-posterior
AER - apical ectodermal ridge
at-RA - all-trans retinoic acid
BMP - bone morphogenetic protein
bp - base pairs
CNS - central nervous system
cpm - counts per minute
CRABP - cellular retinoic acid binding protein
cRAR - chicken retinoic acid receptor
CRBP - cellular retinol binding protein
cRXR - chicken retinoid X receptor
D/V - dorso-ventral
DRG - dorsal root ganglia
ER - estrogen receptor
GR - glucocorticoid receptor
Hox - homeobox containing protein or gene
hRAR - human retinoic acid receptor
HRE - hormone response element
hRXR - human retinoid X receptor
IPTG - isopropyl b-D-thiogalactopyranoside
kb - kilobase
mRAR - mouse retinoic acid receptor
mRXR - mouse retinoid X receptor
p.f.u. - plaque forming unit
P/D - proximo-distal
PDGF - platelet derived growth factor
PPAR - peroxisome proliferator activated receptor
PR - progesterone receptor
RA - retinoic acid
RAR - retinoic acid receptor
RARE - retinoic acid receptor response element
RXR - retinoid X receptor
RXRE - retinoid X receptor response element
SG - sympathetic ganglia
TGF - transforming growth factor
TR - thyroid hormone receptor

TTNPB - p-([E]-2-[5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphtyl-6,7-propenyl]) benzoic acid

VDR - vitamin D_3 receptor

X-GAL - 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I
INTRODUCTION

1.1 - Foreword

The understanding of how an organism can be derived from an egg, and ultimately the understanding of how we, ourselves, are formed, has fascinated mankind, and has been addressed in all cultures with more or less naïve explanations. The serious study of embryological development and of the mechanisms by which these processes are regulated, is however relatively new, and it has only been recently, with the use of techniques of cellular and molecular biology and genetic manipulation, that theories and models proposed previously could be put to the test.

Several different organisms, as varied as amoebas, nematodes, echinoderms and insects, have contributed important clues to our understanding of these problems. The transposition of these findings to vertebrate, and ultimately to human development, has not always been straightforward, but the general mechanisms and ideas that have been established in these systems have provided valuable starting points.

Amongst the vertebrates, amphibians, birds and, more recently (with the introduction of genetic manipulation techniques) fish and rodents, have been widely used in developmental studies. Each of these organisms offers some particular advantage for the study of each specific problem, but so far, most conclusions made with one animal model have been extrapolated with relative success to the other systems, demonstrating that most general developmental processes and mechanisms are broadly conserved within the vertebrate kingdom.

1.2 - The morphogenesis of the chick wing bud

The description of the morphological changes that occur during normal embryonic development falls outside the scope of this introduction. However, one model system will be refered to extensively throughout this work: the development of the chick wing bud. Therefore, a brief description of its early development seems appropriate.

In all amniote embryos, the limb buds arise at late neurula stages, as protuberances of undifferentiated flank mesenchyme. These mesenchymal cells then induce the overlying ectoderm to thicken along the limb margin, forming a specialised structure, the apical ectodermal ridge (AER). The AER will in turn induce the rapid proliferation of the mesenchymal cells directly underneath it, generating a region of actively mitotic undifferentiated cells, referred to as the progress zone (fig. 1.1).

As the limb bud grows distally, mesenchymal cells leave the progress zone and differentiate to form the cartilaginous condensations that will later give rise to the bony elements of the limb. During this time, cells from the somites and from the neural crest migrate into the bud, giving rise to the musculature and the nerves, respectively.

The limb structures are therefore specified and formed in a proximo-distal manner, first the more proximal structures, such as the humerus or femur, and later the more distal ones. At later stages, other morphogenetic events will take place that will play some role in the shaping of the limb, but it is during these early stages, while the mesenchymal cells are in the progress zone, that spatial patterning occurs. Cells in the progress zone somehow "sense" their position across the different axes of the limb bud, become specified, and differentiate, generating a predetermined pattern.

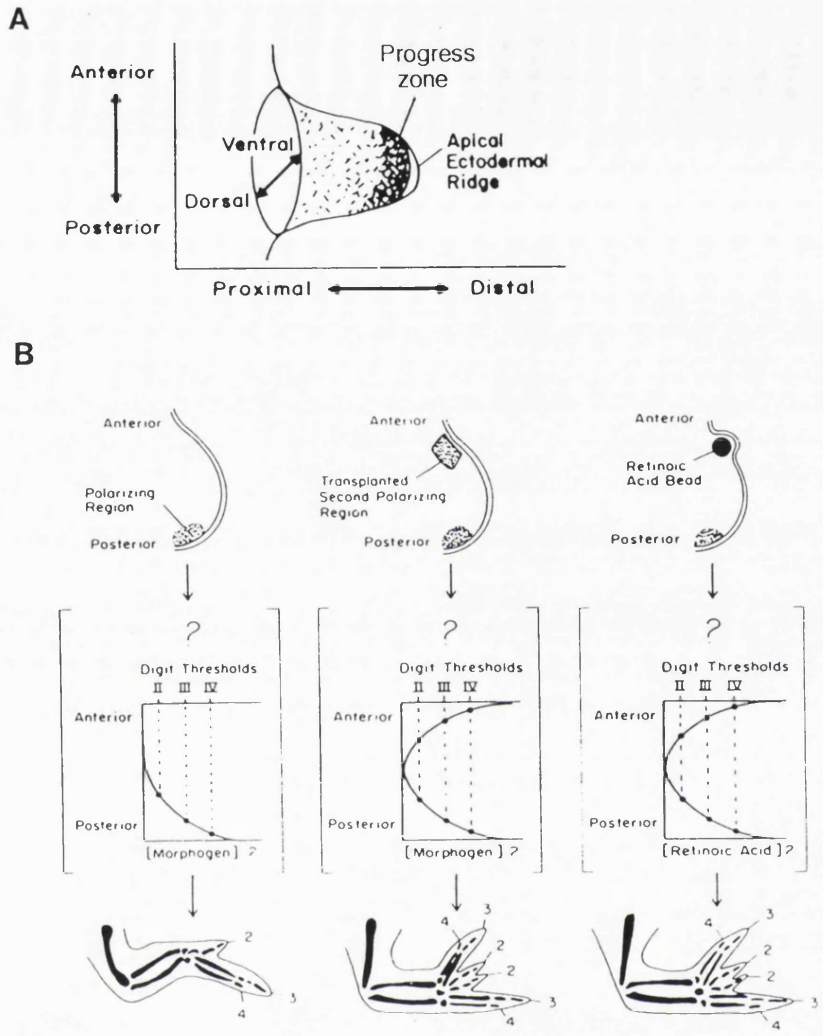


Figure 1.1

A - Schematic representation of a developing limb bud

B - According to the morphogen gradient model, the positional information across the anterio-posterior axis of the developing limb bud, would be generated by the production in the polarizing region of a diffusible molecule whose gradient decreases anteriorly. As the concentration falls through certain threshold levels, the limb bud tissue is instructed to form particular digits. Grafting a second polarizing region at the anterior margin would create a second source of the morphogen. changing the concentration profile of the postulated morphogen such that each threshold would be reached twice in a symmetrical pattern. Implanting a retinoic acid bead has the same effect on limb pattern as a transplant of polarizing region, consistent with the proposed role of retinoic acid, as being or inducing the putative endogenous morphogen (reproduced from Tabin, 1991).

1.3 - Generation of positional information

The mechanisms responsible for the generation of positional information, i. e., the mechanisms by which the cells within an embryonic field are able to "sense" their position in relation to their neighbours, and respond to this information by differentiating into the appropriate structures, are just now beginning to be understood.

Extensive transplantation experiments identified specific regions in the embryo that seem to be responsible for the establishment of positional information and polarity in different morphogenetic fields, and that are able to induce new axis in these fields when grafted to ectopic positions.

The first example of these so called inducers/organizers to be identified was Speman's organiser, the dorsal lip of the blastopore of *Xenopus* embryos (the equivalent of Hensen's node of amniote embryos), which establishes anterio-posterior (A/P) polarity in the major body plane, and which is able to induce a new axis when transplanted to another region of the blastula/blastoderm (Spemann and Mangold, 1924).

Later, in 1968, Saunders and Gasseling identified the zone of polarizing activity or polarizing region, in the posterior margin of the emerging limb bud. When transplanted from its normal position to the anterior margin of another limb bud, the polarizing region causes mirror image duplications of the digit pattern, indicating that this region is responsible for the establishment of A/P polarity in the developing limb (fig. 1.1).

More recently, the notochord has been identified as the structure which is responsible for the induction of the floor plate of the neural tube, which in turn is responsible for dorso-ventral (D/V) patterning of the neural ectoderm. Implantation of an ectopic notochord induces the formation of an ectopic floor plate, and the ectopic expression of D/V positional markers in the neural tube (Placzek *et al.*, 1991).

It is probable that other regions in the embryo, that have yet to be identified, are acting in the same way in the patterning of other morphogenetic fields.

A remarkable observation, is that the mechanisms for the generation of positional information by these inducers/organizers, must be at least partly similar, since they are able to mimic the actions of each other in some systems. For example, Hensen's node, the notochord and the floor plate, can all mimic the actions of an ectopic anterior graft of polarizing region in the developing limb bud system (Hornbruch and Wolpert, 1986; Wagner *et al.*, 1990).

The nature of the "signals"produced by these inducers/organizers, must also be more or less general in all amniotes, since transplantation of these same regions across species generates similar results (Kintner and Dodd, 1991; Hogan *et al.*, 1992).

Several theories and models have been proposed to try to explain how these positional cues are produced and interpreted. Only two of them, however, the polar coordinate model and the morphogen gradient model, seem to hold their value in the light of recent molecular and genetic data.

1.3.1 - The polar coordinate model

Briefly, this model postulates that the cells situated along an axis would have an intrinsic positional character, the so called positional value, that would determine their subsequent differentiation potentials, and that each cell would remain in its respective position by constantly recognising the positional values of its neighbours. Any discontinuity in positional values, such as that generated by polarizing region grafts, would be evened out by intercalary growth of new cells that would assume intermediate positional values, therefore generating mirror image duplications of the digit patterns (Bryant and Muneoka, 1986).

There is not a great deal of evidence to support this theory. In particular, the putative cell surface molecules responsible for these cell-cell interactions, have never been identified. It is also difficult to explain, without having to make any more assumptions, why only very specific regions of the limb bud can produce these results; why for instance, transplanting tissue from the anterior margin to the posterior margin of the limb bud, does not generate duplication of the digit pattern.

1.3.2 - The morphogen gradient model

This model postulates that the cells situated in organiser/inducer regions produce a signalling molecule, or morphogen, that diffuses along the axis and is metabolized or removed by the other tissues, thereby forming a concentration gradient. Cells at different positions along the axis will therefore be exposed to different concentrations of the morphogen, and it is by responding to these different concentrations of morphogen that patterning across the axis is established.

Each different structure formed across an axis, is determined by upper and lower thresholds of concentration of the morphgen, within which all cells would be specified in the same differentiation pathway (Wolpert, 1969).

In the case of the limb buds, grafting of the polarizing region to the anterior margin, would generate a "U" shaped gradient of this putative morphogen resulting in the mirror image duplication of the pattern along the A/P axis (Summerbell *et al.*, 1973).

The relevance of this model for the establishment of positional identity has been clearly demonstrated in *Drosophila*, where several endogenous morphogens have been identified, and shown to operate in this way:

The anterio/posterior and dorso/ventral polarity of the *Drosophila* egg is initially determined by the graded distribution of maternal and

zygotically expressed proteins. The gradient of distribution of the products of the *bicoid* and *nanos* genes, determine the anterior and posterior regions of the egg, respectively (Driever *et al.*, 1988), whilst the gradient of distribution of the product of the *dorsal* gene, along with the products of other genes, such as the *Toll* protein, seems to determine the dorsal and ventral regions of the embryo (Roth *et al.*, 1989).

These morphogen proteins are transcription factors, which act by directly regulating the expression of other genes in a concentration-dependent manner. These target genes are frequently themselves transcription factors, therefore generating a cascade of gene regulation that progressively refines the initial pattern of concentration of the morphogen, to the final complex pattern of the embryo.

Positional signalling of this type has never been unequivocally shown in higher animals, however, this model received great support following the discovery of a possible endogenous morphogen in the chick limb bud, retinoic acid (RA).

1.4 - RA as a possible morphogen

In the early eighties, Tickle and collaborators discovered that the implantation of an all-trans retinoic acid (at-RA) soaked bead at the anterior margin of chick limb buds, produced symmetrical duplications identical to those obtained with grafting of the polarizing region (Tickle *et al.*, 1982 and 1985) (fig. 1.1).

Apart from the morphological similarities of these duplicated limbs, the mechanisms of generating duplications by grafting polarizing region cells or by local application of at-RA seem to share other striking features:

- in both cases, there is a similar narrow developmental-stage window (stages 18-20) during which the grafting of polarizing region, or the

application of at-RA soaked beads, will produce limb duplications (Tickle *et al.*, 1985)

- application of increasing concentrations of at-RA, had the same effect as the grafting of increasing amounts of polarizing region tissue: a gradual increase in the extent of the digit duplications, from 2 2 3 4 to 3 2 2 3 4 and finally to 4 3 2 2 3 4 (Tickle *et al.*, 1985)

- both procedures exhibit the same biphasic induction pattern, starting with a reversible "priming" phase, lasting approximately 8 h after application, followed by a "duplication" phase of 3-6 h during which the cells on the anterior end of the limb bud become irreversibly committed to form the duplicated structures (Eichele *et al.*, 1985)

- finally, they both show similar positional dependency: when polarizing region grafts or at-RA-soaked beads are placed on the posterior margin of the limb bud, no effect is observed; when placed at the apex of the bud they both induce the formation of a symmetrical digit 4 posteriorly (Tickle *et al.*, 1975) and when implanted in the anterior margin of anterior half wings, which will normally give rise to truncated limbs, they will both generate a complete set of limb structures although with the reverse of the normal polarity (Eichele 1989)

1.4.1 - Retinoids and limb duplications

All-trans retinoic acid, is a member of a large family of compounds, the retinoids, that includes its precursor vitamin A, as well as several other vitamin A derivatives, and a growing number of synthetic compounds. Retinoids, and Vitamin A in particular, have a well documented range of strong teratogenic effects in various developmental systems (see below).

Local application of radiolabelled retinoids, specifically at-RA or one of its active synthetic analogs, such as p-([E]-2-[5,6,7,8-tetrahydro-

5,5,8,8-tetramethyl-2-naphtyl-6,7-propenyl]) benzoic acid (TTNPB), showed that these retinoids behave much like the proposed morphogens in the morphogen gradient model, generating an exponential concentration gradient across the limb bud (Tickle *et al.*, 1985; Eichele and Thaller 1987). Moreover, it was also shown that the maintenance of this gradient is necessary to produce the limb duplications (Eichele *et al.*, 1985). This dependence on a localised source of RA to induce duplications, is further stressed by the observation that systemic treatment of mice with RA at the time of limb bud outgrowth, produces only truncation of the limbs (Kochhar, 1973).

The proposal that RA could be an endogenous morphogen responsible for the patterning on the A/P axis of the chick limb, was further boosted by the finding, that RA could be synthesized in the limb buds from its precursor, retinol, and that at-RA was present endogenously at concentrations within the range of those capable of causing limb duplications (10^{-6} M) (Thaller and Eichele, 1987). Furthermore, it was also found that endogenous at-RA was not distributed evenly across the A/P axis, but was enriched some 2.5 times in the posterior margin of the limb bud, a difference of concentration that seemed to be sufficient to produce duplications in experiments with ectopically applied at-RA (Thaller and Eichele, 1987).

Since the discovery of the effects of at-RA, several other natural and synthetic retinoids, have been shown to cause limb duplications with varying degrees of efficiency. In particular two other vitamin A derivatives, were also shown to be present in limb buds at the right stages: 3,4-didehydro retinoic acid (3,4-dd-RA) (Thaller and Eichele, 1990) and 9-cis retinoic acid (9c-RA) (Leid *et al.*, 1992) (fig. 1.2). 3,4-dd-RA, is as potent as at-RA in inducing limb duplications, and is present in the limb buds at concentrations approximatelly 6 times of that of at-RA (Thaller and Eichele, 1990); 9c-RA is aproximally 25 times more potent

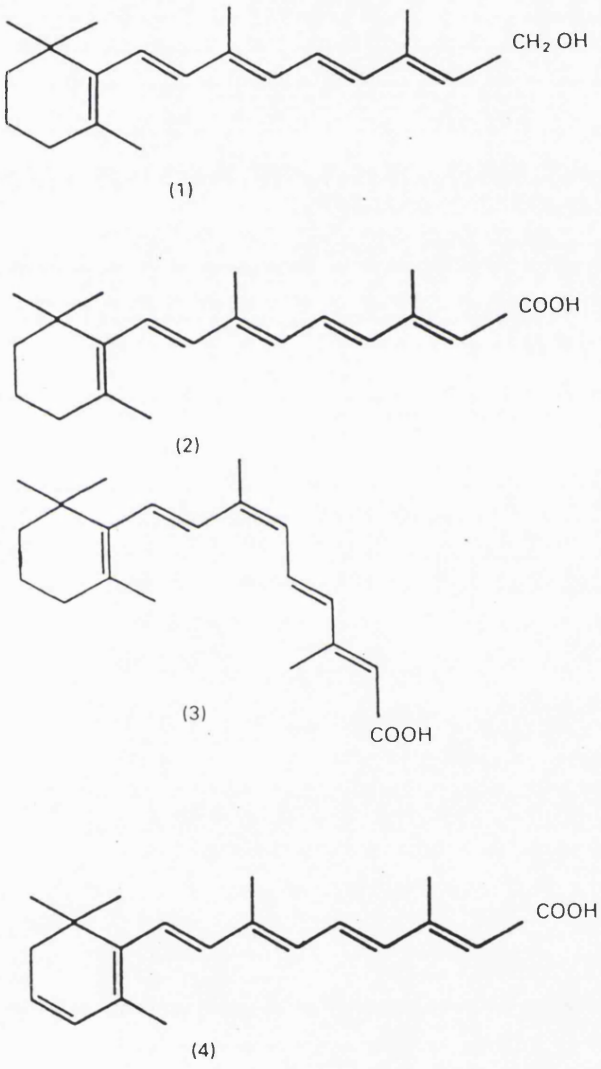


Figure 1.2

The structure of Retinol (1) and of some of its naturally occuring metabolites: (2) all-trans-retinoic acid (at-RA); (3) 9-cis-retinoic acid (9c-RA); (4) 3,4-didehydroretinoic acid (reproduced from Rowe and Brickell, 1993).

in inducing limb duplications than its all-trans isomer (Thaller *et al.*, 1993). These and other yet unknown retinoids, may also play important roles in the patterning of the chick wing bud.

1.4.2 - RA as an unlikely morphogen

The demonstration that exogenously applied RA can produce duplications of the digit pattern similar to those induced by polarizing region grafts, and that RA is present in the limb bud at the right developmental stages, with a graded distribution that is compatible with that which has been predicted for a hypothetical morphogenetic molecule, is not sufficient to prove that RA is in fact responsible for establishing positional information along the A/P axis of the developing limb bud.

Several lines of evidence seem to indicate that RA might not be the true morphogen in the developing limb bud. Wanek and collaborators (1991) demonstrated that wedges of limb bud tissue from regions adjacent to beads soaked with RA, were able to induce limb duplications when transplanted to the anterior margin of a host limb bud (fig. 1.3). They ruled out carryover of RA as the cause of this effect, by incubating the wedges of tissue for a sufficient time for the exogenous RA to be cleared. Furthermore, they observed that the duplicating activity of these RA activated wedges of limb tissue, increased as the length of time of exposure to the RA soaked beads increased, long past the time point at which the concentration of exogenous RA in the limb tissues is highest (12 hours after bead implantation).

These results seem to suggest that RA is not directly involved in establishing positional information across the A/P axis of the limb bud. Instead, a local high concentration of exogenously applied RA could act by inducing the mesenchymal cells around it to become polarizing region

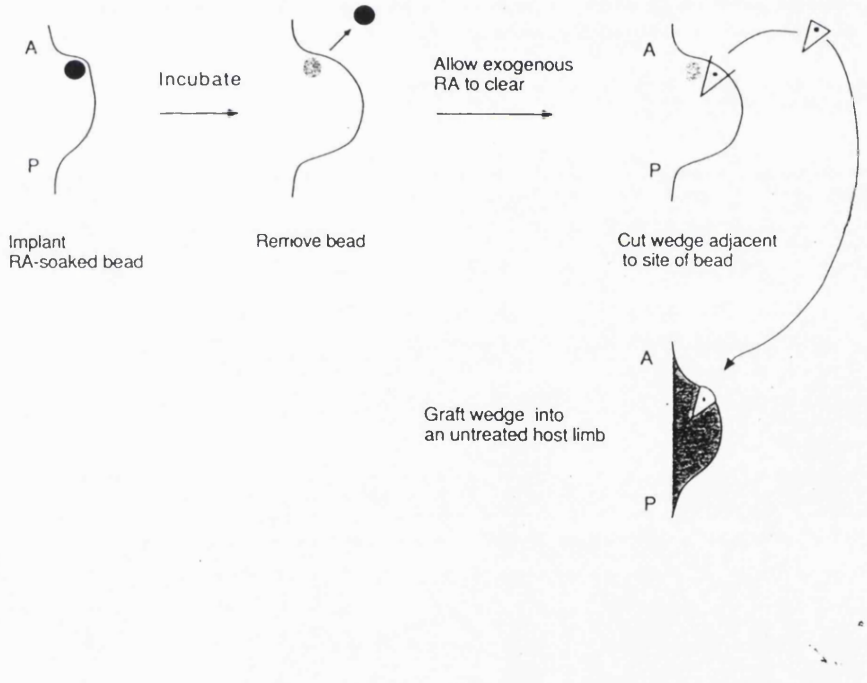


Figure 1.3

Schematic representation of the experiment carried out by Wanek and collaborators (1991). An at-RA soaked bead was implanted in the anterior margin of a limb bud in a donor embryo, and incubated for 12-24 hours, after which the bead was removed and the embryos incubated for a further 90 minutes to allow for the clearance of the exogenous retinoic acid. A wedge of tissue adjacent to the region where the bead had been implanted was then removed and grafted into an untreated host limb inducing the mirror image the duplication of the digit pattern (reproduced from Wanek *et al.*, 1991).

cells, which will then specify the positional information along the A/P axis by some mechanism independent of RA.

The similarities between the limb bud duplications generated by grafting polarizing region cells or by local application of at-RA, such as the dose-dependency of the response, can simply be interpreted as a result of increasing numbers of neighbouring cells being converted to polarising region cells by increasing doses of RA.

An alternative explanation of these results, postulates that exogenous RA acts by inducing mesenchymal cells to become polarizing region cells, but that these cells in turn produce themselves more RA, that will function as proposed previously, providing spatial cues to the other cells of the limb bud. This explanation has however a fundamental flaw. If it is true that RA can induce normal mesenchymal cells to become polarizing region cells, and if these in turn produce more RA, then they would induce their neighbours to become polarizing region cells, and so on, which is obviously incompatible with the establishment and maintenance of a gradient of RA. For this autocatalytic model to function, there would have to be some inhibitory molecule that would inactivate or sequester RA outside of the polarizing region, so that its concentration would be enough to specify positional information, but not high enough to start the autocatalytic cycle. One such possible group of inhibitors are the cellular retinoic acid binding proteins (CRABPs), and their reported distribution across the A/P axis of the chick limb bud seems to allow for such a function. A detailed analysis of the functions and distribution of these proteins will be presented in section 1.5.

Other reports however, seem to dismiss both the direct action of RA, and this rather complex autocatalytic mechanism of action. Noji and collaborators (1991), showed that whilst the application of RA soaked beads in the anterior margin of developing limb buds induced the expression in the mesenchyme of transcripts of a retinoic acid responsive

gene, the retinoic acid receptor β (RARβ) gene, the expression of this gene was not induced by polarizing region grafts. These observations were interpreted as indicating that, although the end results of the two procedures were identical, the mechanism of generation of positional information by the ZPA had to be independent of RA.

This interpretation has however been strongly questioned, since it has been shown that in transgenic mouse embryos expressing the β-galactosidase gene under the control of the promoter of RARβ, maternal administration of RA induced β-galactosidase activity in a variety of limb bud tissues, as in the marginal ectoderm of the limb bud and in the polarising region, but no β-galactosidase activity was ever detected in the mesenchyme (Mendelsohn *et al.*, 1991). In other transgenic mouse embryos in which β-galactosidase expression was placed under the control of only a small region of the promoter of RARβ responsible for its RA inducibility, exogenous RA treatment produced widespread β-galactosidase activity (Rossant *et al.*, 1991). The promoter of RARβ seems therefore to have some elements responsible for a very strong tissue specific repression of the expression of this gene. It is possible therefore, that the induction of RARβ expression by exogenous application of RA observed by Noji and collaborators, might be due to the abnormally high levels of RA in the mesenchyme of the limb bud, and that the polarising region could still be producing low levels of RA that are enough for patterning, but not to overcome the local block of these tissues on RARβ expression (Mendelsohn *et al.*, 1992).

It is therefore impossible to completely rule out an intrinsic function of RA in the establishment of positional information across the A/P axis of the limb bud, however it is now clear that its role is not that of a classic morphogen molecule.

1.4.3 - RA in other developmental processes

The fact that similar signalling mechanisms seem to act in different morphogenetic fields, and that the regenerating blastema (McCormic *et al.*, 1988), Hensen's node (Hogan *et al.*, 1992) and the floor plate of the neural tube (Wagner *et al.*, 1990), have been shown to be able to synthesize at-RA from its precursor retinol, prompted several groups to look for other effects of RA in axial specification and pattern formation.

1.4.3.1 - RA and urodele limb regeneration

Urodele amphibians have the capacity of regenerating amputated limbs and facial structures. Following amputation, the skin from the edges of the wound, rapidly grows to cover it, and the underlying tissues give rise to a mass of undifferentiated cells, the so called blastema, from which regeneration proceeds (Thornton, 1968). Blastemal cells must therefore have a mechanism of "sensing" their position on the limb, ensuring that only the correct structures, distal to the point of amputation, are regenerated.

RA has dramatic effects in respecifying positional identity along the proximo-distal (P/D) axis of the regenerating urodele limb. Exposure of the regenerating blastema to RA proximalizes its positional information, so that for instance a complete limb will form distally to a limb which has been amputated at the level of the wrist (Niazi and Saxena, 1978). In other experiments, RA has been shown to affect the positional identity also in the other axes of the regenerating blastema (Stocum, 1991).

1.4.3.2 - RA and the A/P patterning of the main body axis

Exposure of *Xenopus* embryos to RA during early blastula/gastrula stages, resulted in respecification in the A/P axis of the major body plan, of both ectodermal and endodermal structures. These effects ranged from reduction in the size of the eyes and brain, to complete truncation of the structures anterior to the spinal cord, depending on the dose of RA administered (Durston *et al.*, 1989; Sive *et al.*, 1990; Ruiz and Jessel, 1991). A more detailed analysis of these effects,revealed that RA affects the development along the A/P axis of the *Xenopus* central nervous system (CNS), by transforming anterior neural tissues to more posterior identities (Durston *et al.*, 1989).

In avian and mamalian embryos, truncations of this severity have not been reported, due to the high toxicity of RA at high concentrations. However, treatment with RA during particular times of development, induced anterior and posterior homeotic transformations of some vertebrae of mouse embryos (Kessel and Gruss, 1991; Kessel, 1992).

In contrast with the dramatic effects of RA in the developing *Xenopus* CNS, in which fore- mid- and hindbrain structures can be completely truncated, in other vertebrates the effects of RA treatment in the developing CNS are more subtle and seem to be restricted to the posterior midbrain and hindbrain: RA treatment caused the disorganisation of the midbrain-hindbrain boundary in zebrafish embryos, as judged by the disappearance of the domain of expression of the *engrailed* protein in the neural tube (Maden and Holder, 1991); in mouse embryos, RA treatment caused a reduction of size of the hindbrain and the disorganisation of the rhombomere boundary formation (Morriss-Kay *et al.*, 1991; Papalopulu *et al.*, 1991), together with the anteriorisation of the pattern of homeobox gene expression in the rhombomeres (Morriss-Kay *et al.*, 1991; Marshal *et al.*, 1992).

1.4.3.3 - RA and the patterning of the neural crest

The neural tube defects generated by RA treatment around neurulation stages, are often accompanied by a series of craniofacial and cardiac abnormalities. In human embryos exposed during the first weeks of development to the retinoid 13-cis retinoic acid, as a result of the treatment of the mothers for acne, abnormalities of the CNS were commonly observed, together with defective or absent ears, small or absent jaws, cleft palate, thymic deficiencies and aortic arch abnormalities (Lammer *et al.*, 1985). In RA treated mice, a large reduction in the size of the first and second pharyngeal arches was detected (Goulding and Pratt, 1986), giving rise to abnormalities of the mandible, including cleft palate, and of the maxillae and hyoid bones (Morriss and Thorogood, 1978; Sulik *et al.*, 1988). In the chick RA treatment leads to partial or complete absence of the upper beak (Tamarin *et al.*, 1984), and to heart defects such as cardia bifida, and defects of the aortico-pulmonary septation (Bockman *et al.*, 1989; Kirby *et al.*, 1989).

All of these structures share a common embryonic origin, being derived from cranial neural crest cells. Neural crest cells are a population of pluripotent cells that migrate from the margins of the neural plate at the time of neural tube closure. Neural crest cells from the mesencephalon migrate through the space under the ectoderm, completely surrounding the neural tube and the optic vesicles, later contributing to the meninges of the fore- and midbrain, as well as originating several of the structures of the eye. Some neural crest cells from the mesencephalon and rhombencephalon, stop migrating soon after they leave the neural folds, and condense in discrete regions, directly underneath the ectodermal placodes that have been formed adjacent to each pharyngeal cleft, together giving rise to the cells of the cranial ganglia and nerves.

The remaining cranial neural crest cells have a different fate and migrate into the facial primordia and pharyngeal arches, and give rise to the cartilage and bones of the face and base of the skull, to the dermis of the face and to the odontoblasts, as well as to the connective tissue of the parathyroid, thyroid and thymus and to part of the branchial arches which will later form the cardiac outflow tract (LeDouarin, 1982 Kirby *et al.*, 1989) (fig. 1.4).

Neural crest cells from the trunk region have a more restricted developmental potential, and only give rise to the cells of the trunk peripheral nervous system, both sensory and autonomic, to the chromaffin cells of the adrenal glands and to the melanocytes of the skin.

It is the subset of cranial neural crest cells, that originate mainly from the midbrain and hindbrain regions, that migrate to the facial primordia and pharyngeal arches and undergo mesenchymal differentiation, that seem to be affected by RA. This large range of effects, might therefore be more or less directly associated with the RA induced respecification of the A/P pattern in the anterior neural tube described in the previous section.

1.4.3.4 - RA and skin development

RA and other retinoids have also been shown to affect the development and differentiation of the skin, and of various epidermal structures. The role of vitamin A in the differentiation and maintenance of several epithelia has been well established.

In vitamin A deficient rats, the development of the eye is affected, not only at the level of the production of visual pigments, but also as a result of the keratinisation of the conjunctiva and cornea. In addition, the internal epithelia of these rats undergo widespread squamous metaplasia and become keratinised (Underwood, 1984). On the other hand, in

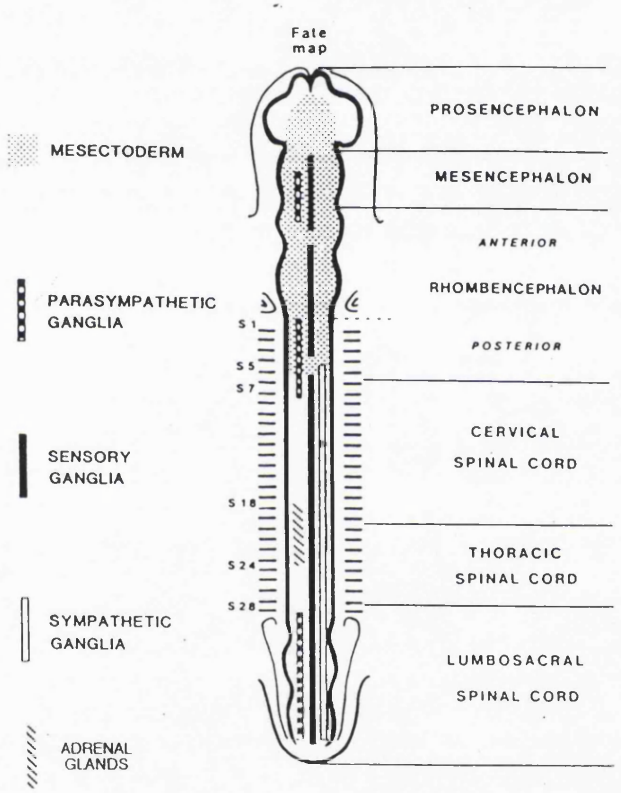


Figure 1.4

Fate map of the neural crest established by quail-chick isotopic transplantation, showing the presumptive territories that originate the mesectoderm, the sensory and autonomic ganglia, and the adrenal glands in the avian embryo. S, somite (modified from LeDouarin *et al.*, 1993).

animals treated with high doses of vitamin A, certain anomalies of the development of the skin and of the hair follicles and feather buds are also observed (Hardy, 1967).

1.4.4 - Effects of RA on gene expression

Retinoids, and in particular RA, seem therefore to be able to cause a range of different effects during development and differentiation, depending on the time of treatment and on the tissues on which they are acting upon, suggesting that this group of molecules are somehow able to influence the expression of a multitude of different genes.

The ability of RA to induce the differentiation of certain cell types in culture, has provided valuable models for the study of its mechanisms of action. RA treatment causes HL60 human promyelocytic leukemia cells to differentiate to granulocytes (Collins *et al.*, 1990), F9 murine embryonal carcinoma cells to differentiate to parietal endoderm cells (Strickland and Mahdavi, 1978) and NT2/D1 human embryonal carcinoma cells to differentiate to several cell types including neurons (Simeone *et al.*, 1990).

The RA induced differentiation of embryonal carcinoma (EC) cell lines is particularly interesting, because it may reflect the normal effects of RA in the specification of differentiation pathways during development. These systems have therefore been the subject of extensive studies of the patterns of RA-induced gene activaton.

In these cells, RA has been shown to stimulate rapidly the transcription of a variety of genes, including homeobox containing genes (Hox genes) and other classes of regulatory genes which have been implicated in the regulation of distinct developmental processes.

In F9 cells, RA treatment up-regulates expression of the Hox A1 (previously known as Hox 1.6) (LaRosa and Gudas 1988) and Hox A5

(previously known as Hox 1.3) (Murphy *et al.*, 1988) genes, but down-regulates the expression of Rex1, another homeobox-containing gene (Gudas, 1991).

In NT2/D1 cells, RA treatment rapidly induces the expression of genes from all four homeobox gene clusters in a coordinated way. The genes positioned at the 3' end of each cluster, are sequentially activated by RA in a time and concentration dependent fashion, in an order that is colinear with their 3' to 5' position in the cluster (Mavilio *et al.*, 1988; Simeone *et al.*, 1990 and 1991). Similar results have been reported for several other EC cell lines (Kessel and Gruss, 1991).

This pattern of induction is reflected *in vivo*, in the expression patterns of the Hox genes in several developing axial systems. Genes from the Hox A and Hox B clusters (previously known as Hox 1 and Hox 2 clusters), are expressed in overlapping domains along the main body axis, in the developing CNS and axial skeleton. Genes that lie progressively more 5' in each cluster, have successively more caudal (anterior) boundaries of expression (Wilkinson *et al.*, 1989 and 1990; Kessel and Gruss, 1991) (fig. 1.5). In the developing limb, genes of the Hox D cluster (previously known as Hox 4 cluster), are expressed in the mesenchyme, also in progressively more restricted posterior domains according to their 3' to 5' positions in the genome (Dollé *et al.*, 1989b; Izpisua-Belmonte *et al.*, 1991).

The similarities between the pattern of expression of these Hox genes in the embryo, and the pattern of activation of the same genes by RA, have important implications for the proposed role of this molecule in the specification of positional information along various embryonic axes. Moreover, RA treatment has been shown to affect the *in vivo* expression patterns of some of these homeobox containing genes. For example:

- in early *Xenopus* embryos, RA treatment up-regulates the expression of Xhox.lab 1 and 2, which are thought to be involved in the specification of anterior mesoderm (Sive and Cheng, 1991), and anteriorises the

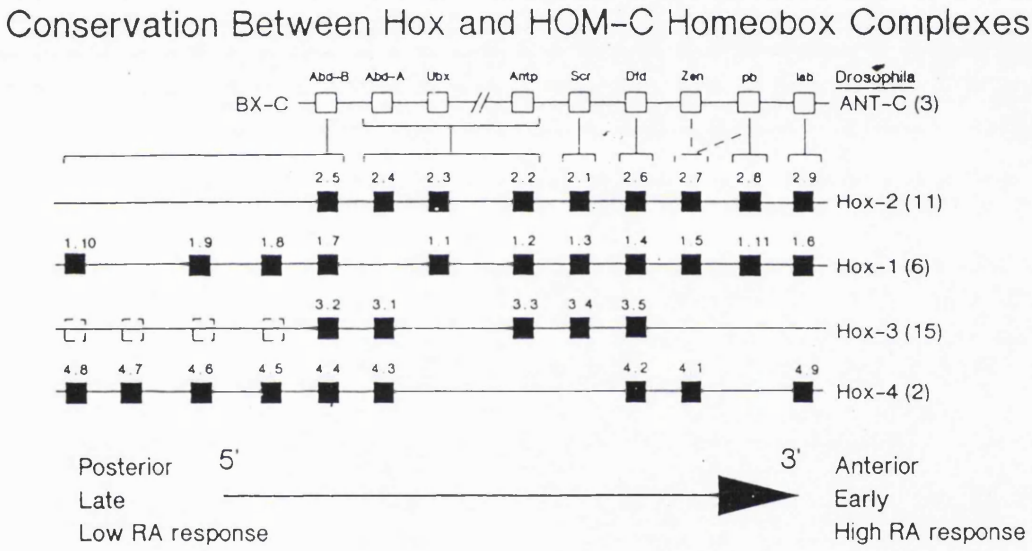


Figure 1.5

The structure of the murine *Antennapedia* class homeobox gene complexes, illustrating the relationship with their *Drosophila* equivalents. Members of a subfamily or paralogous group are vertically aligned with each other. The bracketed numbers to the right of the figure indicate chromosomal location of the gene clusters. The arrow at the base of the diagram indicates that the various properties of the genes listed next to it, vary with the position of the genes within their clusters in the direction indicated: the most 3' genes are the most anteriorly expressed, are expressed at the earliest times, and and are most sensitive to retinoic acid. Solid boxes represent characterised genes, and the dashed boxes represent the position of genes predicted on the basis of their presence in the human HOX clusters. (reproduced from Hunt *et al.*, 1991).

domains of expression of other homeobox containing genes in the brain (López and Carrasco, 1992). These effects in gene expression are probably directly or indirectly connected with the posteriorisation of anterior structures induced by RA in these organisms;

- RA treatment of mouse embryos at the time of neurulation, causes the shift of the expression patterns of a range of homeobox containing genes in the hindbrain, spinal cord and in the surrounding vertebrae (Morriss-Kay *et al.*, 1991 Kessel and Gruss, 1991; Kessel, 1992). This is strikingly reminiscent of the RA induced defects in mid- and hind-brain formation (Wilkinson *et al.*, 1989 and 1990), to the abnormalities in craniofacial structures derived from the neural crest which originated form these areas of the brain (Hunt and Krumlauf, 1991), and to the homeotic changes of the axial skeleton (Kessel and Gruss, 1991) observed in the litters of RA treated mice;

- local application of RA to the anterior margins of developing limb buds, causes the formation of an anterior nested pattern of expression of Hox D genes (previously known as Hox 4), in a mirror-image of their normal pattern of expression on the posterior side of the limb bud, with low doses of RA generating the up regulation of only the 3' most genes of the Hox D cluster (Nohno *et al.*, 1991; Izpisua-Belmonte *et al.*, 1991).

During RA-induced differentiation of F9 cells, the levels of transcription of a number of genes encoding proteins that bind RA or other retinoids are also rapidly increased, suggesting the existence of mechanisms for auto-regulation of retinoid action. These are genes encoding some retinoid binding proteins (CRBP and CRABP), as well as genes encoding nuclear retinoid receptors, such as some of the retinoic acid receptor (RAR) genes (Hu and Gudas, 1990 Zelent *et al.*, 1989 and 1991; Leroy *et al.*, 1991a Mangelsdorf *et al.*, 1991). A detailed description of these genes and of their RA inducibility *in vitro* and *in vivo*, will be given in later chapters.

In the cases described above, the action of RA on gene expression is rapid and independent of protein synthesis. In these cases RA is thought to act by directly promoting the expression of these early genes. In other cases however, induction occurs several days after RA treatment, and is probably the consequence of a cascade of gene activation induced by the early genes.

Some of these late RA inducible genes include other classes of developmentally important genes, such as the genes coding for some peptide growth factors, such as PDGFα (Mercola *et al.*, 1990), and members of the transforming growth factor β (TGFβ) family (Mahmood *et al.*, 1992), as well as several extracellular matrix molecules such as the laminin A, B1, B2 and type IV collagen genes (Wang and Gudas, 1983; Barlow *et al.*, 1984; Kleinmam *et al.*, 1987; Vasios *et al.*, 1991).

1.4.5 - Mechanisms for the interpretation of the retinoid signals

RA therefore, seems to meet many of the criteria required by the morphogen gradient model for a natural morphogen molecule in the developing limb bud, such as:

- exogenously applied RA closely mimics the effects of polarising region grafts, in terms of dose/length of exposure, position and stage dependency of the response;
- endogenous RA is present in the limb bud at a concentration that is sufficient to generate duplications if applied locally to the anterior margin;
- RA is present in a graded distribution, with higher concentrations in the posterior margin;
- locally applied retinoids generate a graded distribution, which seems to be necessary for the induction of duplications. When this gradient is disrupted no duplications occur;

- RA affects gene expression *in vitro* and *in vivo*, and at least some of these effects are concentration dependent.

The final requirement of this model, a mechanism through which cells along an axis could interpret the local concentration of retinoids and respond to it, was provided with the discovery of two classes of molecules that bind to retinoids.

The first group includes the cellular retinol binding proteins (CRBPs) and cellular retinoic acid binding proteins (CRABPs), which bind retinoids in the cytoplasm (Ong *et al.*, 1982). Their precise function is not yet clear, but they seem to be involved in retinoid metabolism and in regulating the concentration of free retinoids.

The second group of retinoid-binding molecules are the retinoic acid receptors (RARs), and the retinoid X receptors (RXRs). These are ligand-dependent transcription factors, belonging to the steroid/thyroid hormone superfamily of nuclear receptors, that regulate transcription of specific sets of target genes. These receptors provide a direct mechanism for converting different concentrations of RA into different patterns of gene expression.

The following sections will provide an overview of the data collected over the past few years on these retinoid binding molecules, and on their involvement in the control and modulation of the morphogenetic effects of retinoids.

1.5 - The cellular retinoid binding proteins

The cellular retinoid binding proteins are small cytoplasmic molecules of approximately 15-16 kD. They are unrelated to other retinoid binding proteins such as the serum retinol binding protein (RBP) and the nuclear retinoid receptors (RARs and RXRs), and have no counterparts in the mechanism of steroid and thyroid hormone action (reviewed by Blomhoff *et al.*, 1990).

Two families of cellular retinoid binding proteins have been identified on the basis of their specific binding affinities for different retinoids: the cellular retinol binding proteins (CRBP I and II), and the cellular retinoic acid binding proteins (CRABP I and II) ((Ong *et al.*, 1982; Saari *et al.*, 1982).

Although the members of these two families are related, they do not show a very high degree of sequence identity. For example, there is only 44 % amino acid sequence identity between rat CRBP I and II (Sundelin *et al.*, 1985 Li *et al.* 1986) and 73 % amino acid sequence identity between mouse CRABP I and II (Giguére *et al.*, 1990b). These differences are reflected in different binding affinities and therefore possibly in different functions: CRBP I has approximately 3 times higher affinity for all-trans retinol than CRBP II ((Ong *et al.*, 1982; MacDonald and Ong 1987), and CRABP I has approximatelly 15 times higher affinity for all-trans retinoic acid than CRABP II (Bailey and Siu, 1988).

Both CRBP I and CRABP II have been shown to be RA inducible (Levin *et al.*, 1987; Smith *et al.*, 1991). Analysis of the promoters of these genes has identified the presence of two independent retinoic acid response elements, one of which in the case of CRABPII, seems to be specifically activated by RXR homodimers (Mangelsdorf *et al.*, 1991).

CRBP I and CRABP I are the two predominant forms in most adult tissues. They are both widely distibuted in the adult rat, and are

particularly abundant in regions of known retinoid storage and activity: CRBP I expression is abundant in the liver, lungs, kidneys and testes, whilst CRABP I is more abundant in the testes, skin and eyes (Ong *et al.*, 1982; Chytil and Ong, 1984;). Both of these proteins are therefore thought to be involved in the general metabolism of retinoids.

CRBP II has a very restricted distribution in the adult rat, occurring almost exclusively in the absorptive cells of the small intestine, suggesting a role in the absorption and transport of retinol, from the intestinal lumen. In the embryo it is only found in the liver and the lung (Crow and Ong, 1985).

CRABP II has not been detected at high levels in adult tissues. In the chicken embryo it has a limited pattern of expression, being present in skin, muscle, bone and in the developing limb, where its graded distribution seems to suggest a role in the establishment of a gradient of free RA (Bailey and Siu, 1988; Maden *et al.*, 1988). However this graded distribution of CRABP II in the limb has not been seen in the mouse embryo (Giguére *et al.*, 1990b; Mendelsohn *et al.*, 1992).This will be described in greater detail in the following section.

1.5.1 - Expression of CRBPs and CRABPs during development

The distribution of proteins and of the transcripts for these genes during development, has been carefully studied both in the chick and in mouse. They have different patterns of expression that change during the successive developmental stages, and are often associated with sites of normal retinoid action or with structures susceptible to retinoid induced defects, suggesting important roles in modulating the effects of retinoids during development.

In early mouse embryos expression of the CRBP I gene is found in the epiblast, in the primitive streak and in the mesenchyme adjacent to it, suggesting a possible role in mesoderm formation. At later stages, expression of this gene is found in cranial neural crest cells and the neural epithelium, with the exception of the floor plate (Ruberte *et al.*, 1991). CRBP I transcripts are only found in the mouse limb bud after day 11.5, when limb patterning has been completed. They are present in the distal limb mesenchyme, mainly in the interdigital spaces, in a region overlapping but more extended than the domain of expression of RARβ (see below) (Mendelsohn *et al.*, 1992). At later stages the CRBP I gene is widely expressed, although not ubiquitous (Maden *et al.*, 1990; Dollé *et al.*, 1990). Its domains of expression are often associated with those of RARβ and RARγ and complementary to those of CRABP I (Dollé *et al.*, 1990).

This pattern of expression of CRBP I, together with its proposed role in the metabolism of retinol, has led to the suggestion that this protein might be involved in maintaining high levels of retinol, in the tissues where this retinoid is being actively converted to retinoic acid (Dollé *et al.*, 1990; Ruberte *et al.*, 1991; Ruberte *et al.*, 1993). However, the absence of CRBP I from regions with known polarizing activity, such as the floor plate and the polarizing region of the limb bud, which are believed to generate RA, seem to contradict this idea. Note however, that the patterns of expression of CRBP I described above for the mouse embryo differ significantly from the ones described in chick embryo, where one of the few regions where CRBP protein is present at high levels is the floor plate of the neural tube, and it is thought that this distribution could generate a gradient of free RA that could have an important role in D/V patterning of the neural tube (Maden *et al.*, 1989).

In the early mouse embryo, the CRABP I gene is expressed throughout the mesoderm but is absent from the streak itself or from the epiblast. During neurulation, it is expressed in the neural crest cells and it is switched on in the neural plate as the neural crest cells start migrating from it, suggesting a possible role in the formation and migration of these cells (Ruberte *et al.*, 1991).

At later stages of mouse development, the distribution of CRABP I transcripts becomes progressively more restricted. Interesting relations between the patterns of expression of this gene and of some retinoid receptors, are found in the mesenchyme of the limb, the craniofacial structures and the genital tubercle: in the early stages of the development of these structures, CRABP I transcripts are mainly superficial, whilst RARβ transcripts are limited to the central and/or proximal areas, and RARγ transcripts are widespread. As these structures develop, the domains of expression of CRABP I and of these two RAR genes become strictly separated: CRABP I transcripts become restricted to the more distal regions, whilst the RARβ and RARγ transcripts are only present in the proximal regions (except for the dermis where both RARγ and CRABP I transcripts are expressed) (Dollé *et al.*, 1990; Ruberte *et al.*, 1991).

These characteristics of the expression pattern of CRABP I, its strictly complementary distribution throughout development in relation to CRBP I, and to RARβ and RARγ during the morphogenesis of structures that are sensitive to RA treatment (the developing limb bud, the craniofacial primordia, the genital tubercle), support the idea that CRABP I could act as a local "sink" for RA, limiting its availability to the nuclear receptors, in regions where gene activation by retinoids must be tightly controlled. The defects induced in these regions by RA treatment could therefore be due to the saturation of CRABP I, and consequent activation of RARs, resulting in abnormal gene activation patterns

(Maden *et al.*, 1988; Smith *et al.*, 1989; Dollé *et al.*, 1990; Ruberte *et al.*, 1991; Ruberte *et al.*, 1993).

The function of CRABP II is not so easy to understand. During early chick limb bud formation, CRABP II protein was found to be distributed in a graded fashion, with highest levels on the anterior side of the progress zone. It was suggested that this distribution could have the function of steepening the postulated gradient of RA generated by the polarizing region (Bailey and Siu, 1988; Maden *et al.*, 1988). In the mouse, a similar graded distribution of CRABP II transcripts has also been reported (Perez-Castro *et al.*, 1989). However, other groups have reported different results. Mendelsohn and collaborators (1992) studied in detail the distribution of both CRABPs in developing mouse limb buds. They found CRABP I transcripts expressed uniformly throughout the progress zone (opposite to the distribution of CRBP I), and in the most dorsal cells of the AER, whilst CRABP II transcripts were present throughout the limb bud, with higher levels being found dorsally, anteriorly and proximally. Therefore, they did not find any evidence supporting the existence of a graded distribution of these proteins across the A/P axis of the limb bud, even when the patterns of expression of both genes were superimposed.

The fact that CRABP II is inducible by RA (Mangelsdorf *et al.*, 1991), would make it a good candidate for being a back-up mechanism for the proposed control of the abundance of free RA by CRABP I. However, its lower affinity for RA, together with its expression pattern which often overlaps with the expression domains of some of the retinoid receptors, seem to point more to a function of local fine regulation of RA availability (Mendelsohn *et al.*, 1992).

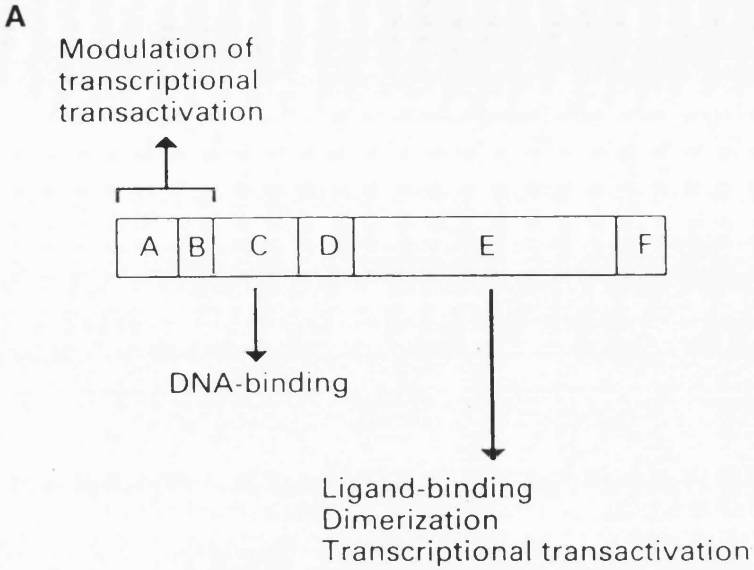
1.6 - The steroid/thyroid hormone superfamily of nuclear receptors

These are a group of ligand-dependent transcription factors, that share strong structural and functional similarities. All steroid/thyroid hormone receptors contain a region of high homology, 60-80 amino acid long, that is involved in DNA binding, the DNA-binding domain. Upon dimerisation, these receptors bind to specific cis-acting elements, the so called hormone response elements (HREs), present in the promoter regions of target genes, stimulating or, in some rarer cases, inhibiting transcription (Green and Chambon, 1988; Evans, 1988; Beato, 1989).

The response elements for this group of receptors consist of two or more repeats of a 6 nucleotide core sequence motif, the so called response element half sites, that can be arranged as palindromes, or as direct- or inverted repeats, separated by a range of spacer sizes. It is the sequence of these half sites, together with their orientation and spacing, that determines the specificity of receptor binding. The stucture of the HREs, and its implications on receptor binding and activity, will be analysed in detail in the following sections.

All members of this superfamily share a similar modular structure, and can be divided into six functional domains, A-F (fig. 1.6-A):

- The A and B domains have been shown to have a constitutive transcriptional activation function (AF1) in the steroid and thyroid hormone receptors (Green and Chambon, 1988; Gronemeyer 1991), and are involved in modulating transcriptional activity in a cell type and promoter specific fashion, probably by interacting with the cellular transcription machinery (Berkenstam *et al.*, 1992). There were some doubts as to the presence of this independent transcriptional activation function on the A and B domains of the RARs and RXRs (Nagpal *et al.*, 1992a), but recent reports have identified it in the corresponding regions of both groups of receptors (Nagpal *et al.*, 1993).



B

Type I receptors

Receptor	P-box	Half-site
GR	GSCKV	AGAACA
PR	GSCKV	
ER	EGCKA	AGGTCA

Type II receptors

Receptor	P-box	Half-site
RAR	EGCKG	AGG/$_T$TCA
T_3R		
VD_3R		
RXR		
PPAR		

Figure 1.6

A - General domain structure of the steroid thyroid hormone nuclear receptor proteins (reproduced from Rowe and Brickell, 1993).

B - Comparison of the amino-acid sequence of the P-box and the consensus DNA binding site (half-site) recognised by steroid hormone receptors (Type I receptors) and thyroid hormone/retinoid receptors (Type II receptors). The underlined animo acids in the P-box have been implicated in determining specificity of binding site recognition (reproduced from Stunnenberg, 1993).

- The C domain or DNA binding domain, is the most conserved region amongst these proteins. This high degree of conservation accounts for the binding of all the different members of the superfamily to closely similar response elements (fig. 1.6-B)(Umesono and Evans, 1989; Mader *et al.*, 1989). It consists of a region of approximately 70 amino acids, containing two sets of four highly conserved cysteine residues that coordinate a Zn ion, forming a structure similar to the Zn finger motifs present in other transcription factors, through which these receptors bind to the DNA (Evans, 1988; Green and Chambon, 1988). Each of the Zn finger-like structures present in these receptors, is not capable of independently binding DNA, unlike the true Zn fingers found in other DNA binding proteins, instead both structures have a cooperative effect in DNA binding (Hollenberg and Evans, 1988; Luisi *et al.*, 1991).

The C domain also plays a role in the dimerization of the steroid receptors bound to their ligands (Luisi *et al.*, 1991). In the remaining receptors it is not clear whether this dimerization function is still present in this domain, or whether the dimers are simply stabilised by binding to the DNA (see below).

- The functions of the D domain are not clear. In the steroid hormone receptors it is important for nuclear localisation (Picard and Yamamoto, 1987; Guichon-Martel *et al.*, 1989), however its major function seems to be that of providing a flexible link between the DNA and the ligand binding domains, hence it is sometimes called the hinge region (Green and Chambon, 1988).

- The E domain is mainly responsible for ligand binding, and is therefore conserved within each subfamily. It contains large numbers of hydrophobic residues which have been suggested to form an hydrophobic "pouch" in which the ligand can fit (Evans, 1988; Green and Chambon, 1988). The carboxy-terminal regions of the E domain, and the F domain, are also involved in dimer formation and contain the ligand-dependent

transcriptional activation function (AF2) present in all these receptors (Green and Chambon, 1988; Nagpal *et al.*, 1992a).

1.6.1 - General mechanisms of action of the nuclear receptors

Based on their functional characteristics, the members of the steroid/thyroid hormone receptor superfamily can be divided into two major subclasses. One contains the typical steroid hormone receptors such as the glucocorticoid receptor (GR), the progesterone receptor (PR) and the estrogen receptor (ER), known as the type I receptors, whilst the second subgroup includes the thyroid hormone receptor (TR) the vitamin D_3 receptor (VDR), the retinoid receptors (RARs and RXRs) and the peroxisome proliferator activated receptor (PPAR), known as the type II receptors (Stunnenberg, 1993).

The major difference between the two types of receptors is in their general mechanisms of DNA binding:

In type I receptors, binding of their respective ligands induces an allosteric change in the receptor, that releases it from a multiprotein complex with which it is associated in solution. This complex includes several regulatory proteins, such as hsp90 and p59. The receptors are then free to dimerise and bind to their cognate HREs, stimulating the transcription of their target genes (Beato, 1989; Allan *et al.*, 1991).

In contrast, type II receptors are not associated with other proteins, and so ligand binding is not necessary for dimerisation and DNA binding, although it is still required for transcriptional activation (Damm *et al.*, 1989; De Thé *et al.*, 1990). Moreover, type II receptors only bind their cognate response elements with high affinity, in the presence of auxiliary nuclear factors (Glass *et al.*, 1991; Yang *et al.*, 1991; Näär *et al.*, 1991). Characterization of these auxiliary factors revealed that they are in fact the RXRs. Further studies showed that the RXRs can form heterodimers

in solution with any of the other type II receptors (Hamada *et al.*, 1989; Yu *et al.*, 1991; Leid *et al.*, 1992; Zhang *et al.*, 1992; Nagpal *et al.*, 1992a and 1993; Kliewer *et al.*, 1992a; Carlberg *et al.*, 1993). It is believed therefore, that *in vivo*, type II receptors bind DNA only as heterodimers with one of the RXRs.

Mutational analysis has identified two regions in the Zn fingers of the C domains of the steroid/thyroid hormone receptors that are critical for the correct recognition of the response elements. These are the so called P-box in the amino-terminal (proximal) Zn finger, and D-box in the carboxy-terminal (distal) Zn finger (Umesono and Evans, 1989; Mader *et al.*, 1989; Danielsen *et al.*, 1989).

X ray crystallography of the GR-DNA binding domain/GR-response element complex, showed that the amino acids in the P-box region make contacts in the major groove of DNA helix (Luisi *et al.*, 1991). These results confirm the previous observations that have implicated the P-box amino acid sequences, in determining the specific HRE half site sequences recognised by each receptor (Umesono and Evans, 1989; Mader *et al.*, 1989) (fig. 1.6-B).

The same X ray crystallographic studies of the GR-DNA binding domain/GR-response element complex, have also shown a "head to head" orientation of the GR proteins, and a close contact between the D-box regions of the two elements of the dimer. The D-box provides therefore a strong DNA-dependent dimerisation interface in this type of receptors (Luisi *et al.*, 1991).

Response elements for type I receptors, are invariably constituted by palindromes separated by 3 nucleotides, which results in both half sites being positioned on the same side of the DNA helix, with opposite orientations. This arrangement of the half sites, is compatible with the

observations of the X ray crystallographic studies. The presence of a strong dimerization interface in the region that binds the DNA is probably responsible for the invariable arrangement of the response elements recognised by type I receptors. Other spacings and orientations of the half sites could not be accomodated without major distortions of the DNA helix (Stunnenberg, 1993).

The D-box sequences present in type II receptors differ significantly from those found in the type I receptors, and are thought not to have a role in dimerization (Schwabe and Rhodes, 1991; Stunnenberg, 1993). The absence of the constraints imposed by a dimerization interface so near to the sites of DNA binding, has clear implications in the ability of these receptors to recognise different orientations and spacings of the half sites of different HREs, and also probably in the choice of dimerization partners to form homodimers or heterodimers (Stunnenberg, 1993).

Type II receptors recognise response elements that are composed of two or more repeats, of the same more or less degenerate core motif ($AG^{G}/_{T}TCA$) (Beato, 1989), separated by a range of spacer sizes. These "half sites" are arranged as direct repeats in the majority of promoters naturally responsive to type II receptors, but in some cases, particularly in a number of artificial HREs, these receptors can also recognise palindromic or inverted repeats of the same core sequences (Nagpal *et al.*, 1992aMader *et al.* 1993a and b).

In the case of the type II receptors, the region that seems to be responsible for dimerization is situated in the carboxy-end of the E domain. Truncated type II receptors lacking part or the whole of the E domain, have been shown to be unable to form homodimers or heterodimers in solution, or in the presence of their respective response

elements (Green and Chambon, 1988 Yu *et al.*, 1991; Kliewer *et al.*, 1992a; Leid *et al.*, 1992; Zhang *et al.*, 1992).

Truncated receptors which have lost only the most carboxy-terminal regions of the E domain, can in most cases still form dimers and bind to DNA, but are unable to transactivate the expression of reporter genes placed under the control of their respective response elements, due to the loss of the carboxy-terminal, ligand-dependent transcriptional activation function (AF2) (Nagpal *et al.*, 1992a and 1993; Yu *et al.*, 1991; Kliewer *et al.*, 1992a; Leid *et al.*, 1992; Zhang *et al.*, 1992).

These truncated receptors, also act as dominant negative inhibitors, blocking the function of the normal receptors by forming dimers with them that lack transcriptional activation activity. A naturally occurring example of this type of repression of the function of type II nuclear receptors is the oncogene v-erbA.

v-erbA is one of the two viral oncogenes of the avian erythroblastosis virus (AEV), and is responsible for potentiating the oncogenic phenotype induced by the product of the other viral oncogene, v-erbB. The v-erbA gene is a mutated copy of the host thyroid hormone receptor gene, c-erbA, lacking part of the amino-terminal and carboxy-terminal regions, and with 13 internal amino acid changes (Weinberger *et al.*, 1986). The deletion at the carboxy-terminus caused the loss of the transcription activation function present in this region (AF2). v-erbA is therefore thought to act by forming inactive dimers with the normal host receptors, that bind to the response elements in their target genes, but cannot activate their transcription (Damm *et al.*, 1989). Another interesting observation about v-erbA action, is that the change of one of the amino acids in its P-box, has changed its DNA binding affinity, so that its oncogenic effects seem to result from interference with retinoid receptor function, rather than with its cellular progenitor, the thyroid hormone receptor (Sharif and Privalsky, 1991).

The extremely high degree of conservation in the P-box regions of all the steroid/thyroid hormone receptors, particularly between the ER and all the type II receptors, means that all these receptors bind to very similar sequences ($AG^{G}/_{T}TCA(X_n)AG^{G}/_{T}TCA$). This provides a possible explanation for the overlapping physiological effects often observed with some of these receptors, but at the same time raises the question of how different receptors distinguish between different HREs, and activate only their own specific sets of target genes. Other characteristics of the response element, such as the orientation and the spacing of the half sites, must be involved in the generation of the specificity of receptor binding.

As referred earlier, most of the natural type II receptor response elements identified in the promoters of the respective responsive genes, are in the form of direct repeats separated by a range of spacer sizes. When these differentially spaced direct repeats were examined in heterologous promoters, the spacing between the half sites seemed to determine to a certain extent, the specificity of receptor binding (Glass *et al.*, 1989; Umesono *et al.*, 1991; Mangelsdorf *et al.*, 1991; Näär *et al.*, 1991).

Based on these observations, Evans and collaborators proposed a model in which the spacing between the two half sites in a direct repeat could be responsible for determining the binding of that HRE by different receptors, the so called 3-4-5 rule (Umesono *et al.*, 1991). They proposed that homodimers of VDR, TR or RARs, would specifically bind response elements containing direct repeats spaced by 3, 4 and 5 nucleotides respectively.

This rule was later extended to accomodate RXR homodimers and heterodimers, with the discovery of a response element in the promoter of the CRBP II gene, composed by a direct repeat separated by a single nucleotide, which confered RXR specific transactivation (Mangelsdorf *et*

al., 1991; Yu *et al.*, 1991). It became the 1-3-4-5 rule: response elements containing direct repeats spaced by 1 nucleotide would be specifically bound by RXR homodimers, whilst response elements containing direct repeats spaced by 3, 4 and 5 nucleotides would be specifically bound by VDR- TR- or RAR-RXR heterodimers. Furthermore, it was proposed that the spacings that had not yet been assigned, could correspond to the binding sequences for other combinations of receptors (Kliewer *et al.*, 1992a).

In an extensive study of the binding specificities of the different combinations of receptors to natural and artificial response elements, with different arrangements and spacings, Nagpal and collaborators (1992) showed that such simplifications have only a very restricted value. First, each combination of receptor heterodimers was found to bind with high affinities to several response elements that do not comply with these rules. Moreover, the extent of the activation of transcription in each combination of receptors and response elements, is also influenced by the isoforms of the receptors involved, by the sequences flanking the response element, and by cell specific factors (Nagpal *et al.*, 1992a and 1993 Mader *et al.* 1993a and b).

So far, an obvious mechanism for the generation of the specificity of the response to the different steroid/thyroid hormone receptors has not been found. Clearly, detailed X ray crystallographic studies of the interactions of normal and truncated type II receptors with each other, and with their response elements, are necessary to clarify the mechanisms of action of this class of molecules.

1.7 - The nuclear retinoid receptors

Two families of nuclear retinoid receptors have been identified on the basis of their different amino acid sequences and of their different binding affinities. These are the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs).

Although both the RARs and the RXRs bind the same, or structurally similar ligands, they only share a noticeable degree of amino acid sequence homology, in their DNA-binding domains, and they do not share any considerably higher homology to each other, than to any other member of the steroid thyroid hormone receptor superfamily, suggesting that they must have diverged at an early point in the evolution of this superfamily.

The RARs are efficiently activated by physiologic levels of at-RA, 3,4dd-RA or 9c-RA, but can also respond to a wide range of synthetic retinoids with varying efficiency (Thaller and Eichele, 1990; Cretaz *et al.*, 1990; Heyman *et al.*, 1992; Allenby *et al.*, 1993)

The RXRs were first implicated in the retinoid response pathway, on the basis of their ability to activate gene expression in the presence of high concentrations of at-RA. Later it was shown that their natural ligand is 9c-RA and that they are not able to bind at-RA (Levin *et al.*, 1992; Heyman *et al.*, 1992 Allenby *et al.*, 1993). The activity of the RXRs in the presence of at-RA is probably due to its isomerisation to 9c-RA in culture and *in vivo* (Thaller *et al.*, 1993).

1.7.1 - The retinoic acid receptors

Three different members of the RAR family, designated RARα, β and γ, have been identified so far in humans (Petkovich *et al.*, 1987; Brand *et al.*, 1988; Krust *et al.*, 1989) mice (Brand *et al.*, 1988; Benbrook *et al.*,

1988; Zelent *et al.*, 1989) and newts (Ragsdale *et al.*, 1989). Two of these receptors have also been identified in the chicken (RARβ and RARγ) (Rowe *et al.*, 1991; Nohno *et al.*, 1991; Rowe and Brickell, unpublished results) and *Xenopus* (RARα and RARγ) (Blumberg *et al.*, 1992). All three RAR types have high degree of amino acid sequence identity in their DNA- and ligand-binding domains, and to a lesser extent also in their B domains. In contrast the A, D and F domains are less, or not at all conserved within a given organism. However, when compared across species, these same regions show almost complete conservation in individual members of the family (Krust *et al.*, 1989; Zelent *et al.*, 1989; Ragsdale *et al.*, 1989 Rowe *et al.*, 1991), indicating that these regions may be important for the functional specificity of each receptor.

This evolutionary conservation, together with the specific patterns of expression found in embryonic and adult tissues, suggest that each RAR type must play a specific role during development and in the adult animal.

1.7.1.1 - Structure of the transcripts of the RAR genes

Analysis of the expression of the RAR genes, revealed that each of these receptors is expressed as two or more different mRNAs, with a tissue and developmental stage specific distribution.

Isolation and characterisation of several cDNA clones corresponding to the different transcripts of each RAR gene, showed that they only differed from each other in their 5' untranslated regions and in the region coding for the A domain. Each RAR gene is therefore able to encode multiple protein isoforms, that differ only in their A domains (Kastner *et al.*, 1990; Leroy *et al.*, 1991a; Zelent *et al.*, 1991; Nagpal *et al.*, 1992a) (fig. 1.7). The role of the A/B regions in the regulation of transcriptional activity is well established (Green and Chambon, 1988;

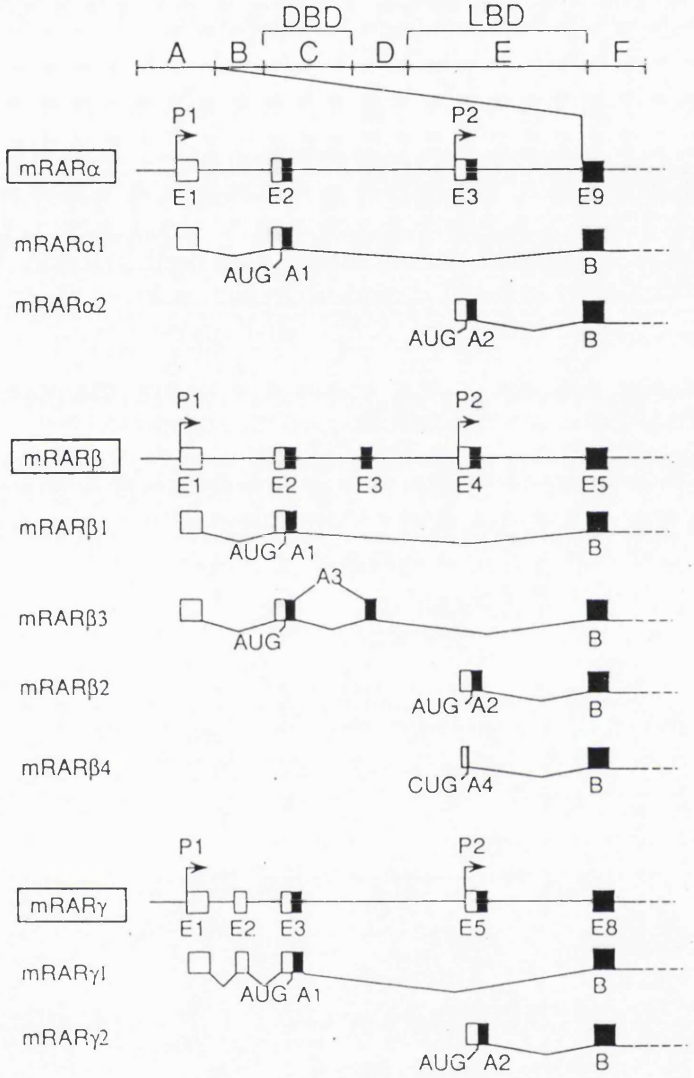


Figure 1.7

Schematic organisation of the 5' regions of the three mouse RAR genes and of their major isoforms. For each RAR gene, the genomic organisation of the 5' region is displayed and the various exons (E) indicated by boxes. Black boxes represent translated sequences and white boxes represent 5'-untranslated (5'UT) sequences. In each case, P1 and P2 correspond to the upstream and downstream promoters respectively. For agivenRAR subtype (α, β and γ) A1, A2, A3 and B correspond to the A1, A2, A3 and B regions, respectively (reproduced from Lohnes *et al.*, 1992).

Nagpal *et al.*, 1992a and 1993). The different isoforms, may therefore have an important role in the generation of the diversity of RA responses, allowing the same receptor in different tissues, to regulate the expression of the same genes in a different way, or even to regulate the expression of different sets of genes.

The mouse RARα gene, encodes at least two protein isoforms (mRARα1 and mRARα2), that are generated as a result of transcription from two distinct promoters (mRARα-P1 and mRARα-P2, respectively) (Leroy *et al.*, 1991a and 1991b). The mouse RARβ gene encodes at least four protein isoforms (mRARβ1 to mRARβ4) that are generated by alternative splicing of two primary mRNA transcripts, transcribed from two independent mRARβ gene promoters (mRARβ1 and mRARβ3 are generated by transcription of the more 5' promoter mRARβ-P1, whilst mRARβ2 and mRARβ4 are generated by transcription of the downstream promoter mRARβ-P2) (Zelent *et al.*, 1991; Mendelsohn *et al.*, 1991; Nagpal *et al.*, 1992a). Finally, the expression of the mouse RARγ gene is similar to that of mRARα, encoding at least two protein isoforms (mRARγ1 and mRARγ2) (Giguére *et al.*, 1990a; Kastner *et al.*, 1990), that are also transcribed from two promoters (mRARγ-P1 and mRARγ-P2, respectively) (Lehmann *et al.*, 1992b). Although the characterisation of the expression and structure of the various isoforms of each of the RAR genes, has been most extensively done in the mouse, several isoforms of human and chick RARs have also been isolated, which also differ only in their amino-terminal A domains (Krust *et al.*, 1989; Nohno *et al.*, 1991).

Comparison of the sequences of the different RAR isoforms across species, shows a very high degree of conservation not only in the region coding for the A domain but also in the 5' untranslated regions, suggesting that each protein isoform might have specific conserved

functions, and also that the expression of each of the different RAR isoforms may be differentially regulated at the post transcriptional level (Leroy *et al.*, 1991a).

Strong homologies are also observed in the spatial organisation of the 5' exons and promoters of each of the three mouse RAR genes. The isoforms that are transcribed from the second promoter (P2) of the mouse RARα, RARβ and RARγ genes, respectively, mRARα2, mRARβ2 and β4, and mRARγ2, show some degree of RA inducibility (the two other RARβ isoforms also have a week RA inducibility). Analysis of these promoter sequences, has revealed in all three cases the presence of a closely similar RA response element (Leroy *et al.*, 1991a; Nagpal *et al.*, 1992b; Lehmann *et al.*, 1992a and b).

These results strongly support the idea that the different RAR genes might have evolved by duplication of a common ancestor (Leroy *et al.*, 1991a).

1.7.1.2 - Gene activation by the RARs

The mechanism of action of the RARs is similar to that of other members of the type II class of steroid/thyroid hormone receptors. They form heterodimers with the RXRs, and regulate the transcription of a variety of genes containing retinoic acid response elements (RAREs) in their promoter regions.

RAREs have been identified in the promoters of several RA responsive genes (fig. 1.8). Functional RARE consensus sequences have also been identified in the promoters of genes that do not seem to respond to RA *in vivo*, including phosphoenolpyruvate carboxykinase gene (Lucas *et al.*, 1991), oxytocin gene (Richard and Zingg, 1991), apolipoprotein A1 gene (Zhang *et al.*, 1992), major histocompatibility complex class I (Kralli *et al.*, 1992), osteocalcin gene (Schule *et al.*, 1990) human alcohol

Gene	Sequence of the retinoic acid response element
mRARα2	5′ GGCG**AGTTCA**GCAAG**AGTTCA**GCCGA 3′
hRARα2	5′ GGCG**AGTTCA**GCGAG**AGTTCA**GCCGA 3′
mRARβ2	5′ GAAG**GGTTCA**CCGAA**AGTTCA**CTCGC 3′
hRARβ2	5′ GTAG**GGTTCA**CCGAA**AGTTCA**CTCGC 3′
mRARγ2	5′ GGCC**GGGTCA**GGAGG**AGGTGA**GCGCG 3′
mCRBPI	5′ TAGT**AGGTCA**AAA**AGGTCA**GACAC 3′
rCRBPII	5′ GCT**GTCA**C**AGGTCA**C**AGGTCA**C**AGGTCA**C**AGTTCA** 3′
hADH3	5′ ACAG**GGGTCA**TTCAG**AGTTCA**GTTTT 3′
mLB1	5′ G**AGGTGA**GCTA**GGTTAA**GCCCTTAGAAAAA**GGGTCA**A 3′
h-apoAI	5′ **AGGGCA**G**GGGTCA**AG**GGTTCA**GTGGG 3′
mCP-H	5′ CAGC**AGGTCA**CTGAC**AGGGCA**TAGTA 3′

Figure 1.8

Nucleotide sequence of some naturally occuring retinoic acid response elements. mRARα2, hRARα2 - mouse and human RARα2 promoter (Leroy *et al.*,1991a and b); mRARβ2, hRARβ2 - mouse and human RARβ2 promoter (de Thé *et al.*, 1990; Zelent *et al.*,1991); mRARγ2 mouse RARγ2 promoter (Lehmann *et al.*,1992); mCRBPI mouse CRBP I gene (Smith *et al.*,1991); rCRBPII - rat CRBP II gene (Mangelsdorf *et al.*, 1991); hADH3 - human alcool dehydrogenase 3 gene (Duester *et al.*, 1991); mLB1 - mouse laminin B1 gene (Vasios *et al.*, 1991); h-apoAI - human apolipoprotein A1 gene (Zhang *et al.*, 1992); mCP-H - mouse complement factor H gene (Muñoz-Canoves *et al.*, 1990). Each core element is shown in bold, and its orientation with respect to the gene promoter is shown with a half-arrow-head. The consensus core sequence is:

5' $(^{A}/_{G})G(^{T}/_{G})T(^{C}/_{G})A$-X(0-3)-$(^{A}/_{G})G(^{T}/_{G})T(^{C}/_{G})A$ 3'.

dehydrogenase 3 gene (Duester *et al.*, 1991) and mouse complement factor H gene (Muñoz-Canoves *et al.*, 1990).

Generally, the RARs function by activating transcription in the presence of RA. However, in some cases they have also been shown to down-regulate the expression of certain genes. For example, CRABP I is down-regulated in RA-treated mouse teratocarcinoma cell lines (Stoner and Gudas, 1989). RARs were also shown to down-regulate the expression of the metalloproteinase genes, stromelysin and collagenase (Nicholson *et al.*, 1990;). The mechanism of this down-regulation, does not involve the interaction with RAREs. Instead, it was shown that the RARs can form complexes with Jun-Jun or Jun-Fos dimers, and prevent their binding to AP-1 sites in the promoters of the stromelysin and collagenase genes, therefore indirectly inhibiting their expression. Conversely, Jun-Jun or Jun-Fos dimers, can also inhibit the RA dependent transcription of RARE-containing genes, by sequestering the RAR molecules, therefore introducing another level of complexity in the retinoid signalling pathway (Lehmann *et al.*, 1992a and b).

1.7.1.3 - RAR gene expression during development

Because of the teratogenic effects of RA and of its proposed functions as a possible natural morphogen, the distribution of transcripts for the different RARs during development has been extensively studied.

In mouse embryos, the RARβ gene is the first to be expressed, and RARβ transcripts are found during gastrulation in the newly formed mesoderm lateral to the primitive streak. In the early neurula stages, the RARβ gene is expressed in the lateral plate mesoderm but not in the somites, and in the closed trunk neural tube from the caudal limit of the hindbrain to the caudal neuropore, whilst RARγ transcripts are found in the caudal streak and unsegmented caudal mesoderm, and in the open

neural plate, posteriorly from the caudal neuropore. RARα transcripts are not clearly detected until late neurula stages, when they are found in the forebrain and at high levels in the neural folds and migrating neural crest cells of the caudal hindbrain (Ruberte *et al.*, 1990 and 1991).

At later stages of development, the RARα gene is expressed almost everywhere, making it difficult to predict its specific functions. RARγ transcripts are widely distributed in the mesenchyme of the frontonasal mass, pharyngeal arches, limb buds, genital tubercle and also in the sclerotome. RARβ transcripts are found along the length of the spinal cord, around the developing eye, and have a distinctive distribution in the facial primordia, limb buds and genital tubercle, where they are restricted to the most proximal regions of the mesenchyme and ectoderm (Dollé *et al.*, 1990; Ruberte *et al.*, 1990 and 1991).

As noted earlier, an interesting relationship is seen in the mesenchyme of the developing facial primordia, limb buds and genital tubercle, between the distribution of the transcripts of RARβ, RARγ and CRABP I genes. In the initial phases of the development of these structures, RARβ transcripts are found in central and/or proximal areas, and RARγ transcripts are more or less widespread, but both become progressively restricted to the more proximal regions of the mesenchyme, whilst CRABP I transcripts,whichare initially expressed everywhere, become gradually restricted to distal regions of these structures (Dollé *et al.*, 1990; Ruberte *et al.*, 1990 and 1991; Mendelsohn *et al.*, 1992).

At later stages, the expression domains of the RARβ and RARγ genes become more restricted and separated.

The expression of RARβ transcripts becomes very closely associated with regions that are undergoing programmed cell death. In the developing limb, RARβ transcripts become restricted to the opaque zone of the forelimb cartilaginous condensation, that will later divide it into radius and ulna (Dollé *et al.*, 1990; Mendelsohn *et al.*, 1991) and to the

interdigital mesenchyme (Dollé *et al.*, 1989a; Mendelsohn *et al.*, 1991; Rossant *et al.*, 1991). Furthermore, doses of RA that produce limb truncations, result in an increase in cell death in the core mesenchyme and in a concomitant expansion of the domain of expression of RARβ in the same region (Mendelsohn *et al.*, 1991). All these results point to a relationship between the expression of RARβ and programmed cell death in these regions. However, it is not possible to determine clearly, which is the cause and which is the effect (Mendelsohn *et al.*, 1991).

The strong RA-inducibility of RARβ transcripts, make this gene a prime candidate to mediate at least some of the teratogenic effects of retinoids. The observation of pronounced changes in the pattern of expression of RARβ transcripts, in the developing limb buds and in facial primordia of chicken embryos, as a result of local application of RA (Noji *et al.*, 1991; Rowe *et al.*, 1991) seems to support this idea. However as pointed out previously (section 1.4.2), polarising region grafts do not induce this same change in the pattern of expression of RARβ (Noji *et al.*, 1991), and in transgenic mice with an RARβ-promoter/lacZ or β2-RARE/lacZ reporter genes, although systemic treatment with RA did caused changes in lacZ expression, these were not strictly associated with the areas where endogenous RA treatment has been reported to show a teratogenic effect (Mendelsohn *et al.*, 1991 Rossant *et al.*, 1991).

As development progresses, RARγ transcripts in the mouse become restricted to newly formed cartilage and to the dermis. The expression of the mouse RARγ gene in the developing cartilaginous elements is detected from the point where they are just mesenchymal condensations to their differentiation into the bony elements of the skeleton. This pattern of expression therefore, strongly suggests that RARγ may be responsible for the concentration-dependent effects of RA in promoting or inhibiting cartilage formation both *in vitro* and *in vivo*, and also for the effects of

RA in the development and maintenance of squamous epithelia (Dollé *et al.*, 1989a and 1990; Ruberte *et al.*, 1992; Mendelsohn *et al.*, 1992).

However, it is interesting and somewhat worrying to note, that in a recent report on transgenic mice in which the expression of all isoforms of the RARγ gene had been disrupted (Lohnes *et al.*, 1993), no general defects were detected on cartilage or bone formation, or on the skin. RARγ-deficient mice appeared normal, although they exhibited retarded growth, early lethality, male infertility (due to squamous metaplasia of the seminal vesicles and prostate glands), some very localised defects in cartilage formation (malformation of the tracheal rings), partial or total agenesis of the Harderian glands of the eye, and some homeotic transformations of the proximal axial skeleton similar to those observed with RA treatment (Kessel and Gruss, 1991; Kessel, 1992). Some of these defects, such as the retarded growth, higher lethality and the squamous metaplasia of the seminal vesicles and prostate glands, are often seen in vitamin A deficient mice, suggesting that RARγ does indeed mediate some of the effects of retinoids in normal development (Lohnes *et al.*, 1993).

Interestingly, RA treatment of RARγ-deficient transgenic mice, never produced some of the most common teratogenic effects of RA, such as the production of lumbosacral truncations, suggesting the specific involvement of RARγ in these teratogenic effects (Lohnes *et al.*, 1993). However, the fact that these same regions develop normally in the untreated RARγ-null mutants, shows that RA malformations do not seem to reflect a direct interference with the normal functions of each receptor, but rather, that they are a completely artificial effect due to ectopic activation of RA responsive genes.

These, and other results of an apparently "normal" phenotype of transgenic mice in which the expression of the RARα gene had been disrupted (Lufkin *et al.*, 1993) raise some very serious doubts, on the relevance of all the predictions made over the last few years, about the

functions of these receptors, based on the normal distribution of their transcripts.

1.7.2 - The retinoid X receptors

Three different members of the RXR family, which have been designated RXRα, β and γ, have been identified in mice (Hamada *et al.*, 1989; Yu *et al.*, 1991; Mangelsdorf *et al.*, 1992). Of these, only two have been identified so far in humans (RXRα and RXRβ) (Mangelsdorf *et al.*, 1990; Leid *et al.*, 1992; Fleischhauer *et al.*, 1992); chicken (RXRγ and RXRα) (Rowe *et al.*, 1991; Seleiro and Brickell, unpublished results); and xenopus (RXRα and RXRγ) (Blumberg *et al.*, 1992). An RXR homolog has also been identified in *Drosophila*, the gene product for the ultraspiracle locus (Oro *et al.*, 1990), suggesting that the RXR gene family of retinoid receptors is probably more ancestral than that of the RARs.

Again, as with the RARs, the different RXR types within a given organism only share a high degree of sequence identity in their B, C and E domains, while the remaining regions are highly conserved across species for each individual member of the family (Mangelsdorf *et al.*, 1992).

1.7.2.1 - Structure of the transcripts of the RXR genes

We have only a very limited knowledge of the expression of the RXR genes. The structure of their transcripts, their genomic organisation, and the regulation of their transcription, have only recently started to be investigated.

Several of the RXR genes show a complex pattern of expression, similar to that of the RARs, with several transcripts of different sizes

being expressed in a tissue specific manner. To the present date, different RXR isoforms have only been reported for the hRXRβ (Fleischhauer *et al.*, 1992) and mRXRγ (Liu and Linney, 1993). In addition, the isolation and analysis of several cRXRγ isoforms is described in this thesis.

1.7.2.2 - Genes activated by the RXRs

The effects of the RXRs in modulating gene expression seem to be more general than those of any other member of the nuclear receptor superfamily, due to their ability to form heterodimers and act synergistically with either the VDRs, THRs, PPARs or the RARs (Zhang *et al.*, 1992; Kliewer *et al.*, 1992a and b; Durand *et al.*, 1992; Nagpal *et al.*, 1992a; Carlberg *et al.*, 1993 Mader *et al.*, 1993a and b). As noted earlier, heterodimerization seems to be independent of the presence of DNA or of ligand. Furthermore, although full transcriptional activation only occurs in the presence of both ligands, the presence of the RXRs alone seems to be sufficient to increase the levels of transcription activation of the other members of the heterodimer, which explains why the effects of vitamin D3 and thyroid hormone seem to be RA independent (Zhang *et al.*, 1991; Kliewer *et al.*, 1992a; Carlberg *et al.*, 1993 Nagpal *et al.*, 1993).

Recent reports demonstrated that the RXRs are also capable of functioning effectively as homodimers in the presence 9c-RA. Such homodimers can activate the transcription of reporter genes containing certain RAREs, including the RAREs present in the promoters of the ApoAI and CRBP II genes (Mangelsdorf *et al.*, 1991; Zhang *et al.*, 1992; Lehmann *et al.*, 1992b; Nagpal *et al.*, 1993).

The RXRs, acting either as homodimers or as heterodimerisation partners for other nuclear receptors, seem therefore to play some role in the regulation of the expression of a large number of genes. More

importantly, due to their heterodimerization properties, they are at the center of a network of signalling pathways, and could even be part of the mechanisms through which cross-regulation of these different regulatory signals is accomplished (Leid *et al.*, 1992).

1.7.2.3 - RXR gene expression during development and in the adult

The distribution of all three RXR types has only been partially characterized in rodents (Mangelsdorf *et al.*, 1992).

The RXRα gene gives rise to a single transcript of approximately 5.6 kb in the mouse embryo and in the adult rat, which is abundantly expressed in a range of embryonic and adult tissues. In the mouse embryo, RXRα is expressed at very high levels in the epithelia of the gut, in the liver, and in the skin, and at lower levels in the central nervous system and in the skeleton. This pattern of expression is similar to that observed in northern blots of adult rat tissue RNA, where RXRα transcripts are abundantly expressed in the visceral tissues such as the liver, kidney, lung, muscle and spleen, less abundant in the heart, and expressed at very low levels in the adrenals, brain, intestine and testes. This pattern of expression of the RXRα gene, particularly its strong expression in embryonic gut epithelia and in the liver, strongly suggests a possible role of this receptor in retinoid absorption and metabolism (Mangelsdorf *et al.*, 1991).

The RXRβ gene gives rise to two major transcripts, of 2.8 and 3.4 kb in the mouse embryo, and of 2.7 and 3.0 kb in the adult rat, which are relatively abundant in all tissues analysed. In the adult rat the expression of RXRβ transcripts is fairly widespread, with only the intestine, liver and testes expressing lower levels of these transcripts. Expression of RXRβ transcripts in the mouse embryo is also almost ubiquitous, which makes this gene the only member of the RXR family to be expressed at

fairly high levels in the developing central nervous system of the mouse, and therefore, the only of the RXRs that is likely to be involved in the modulation of the retinoid effects in the development of these structures (Mangelsdorf *et al.*, 1991).

Finally, the RXRγ gene gives rise to two major transcripts, of 1.9 and 2.3 kb in the mouse embryo, and of of 2.0 and 2.5 kb in the adult rat. These mRXRγ transcripts are expressed at considerably lower levels than the transcripts of the other mRXR genes, and have a fairly complex pattern of expression, with each of the transcripts having a specific tissue distribution. In northern blots of adult rat tissue RNA, the highest levels of both RXRγ transcripts are detected in the heart and muscle, whilst smaller levels of the 2.0 kb transcript are also expressed in the adrenals, kidney and liver, and the 2.5 kb transcript is also expressed in the brain and lung (Mangelsdorf *et al.*, 1991; Liu and Linney, 1993).

In the early mouse embryo RXRγ transcripts are detected predominantly in the somites and in two very localised regions of the developing brain, the pituitary anlage and the corpus striatum (caudate putamen). Smaller levels of expression are also found in the ventral horns of the spinal cord, where the motor neurons are developing. At later stages, high levels of expression of this gene become restricted to the pituitary and the corpus striatum, whilst weaker expression can still be detected in some skeletal muscle (Mangelsdorf *et al.*, 1991).

Interestingly, the distribution of RXRγ transcripts in the mouse embryo is considerably different from that previously described in the chick (Rowe *et al.*, 1991). In the early chicken embryo (stage 16), RXRγ transcripts are strongly expressed in migrating neural crest cells. At later stages (stages 24 to 27), the expression of this gene seems to be restricted to the liver, and to the neural crest derived elements of the peripheral nervous system.

Expression of the RXRγ gene in the chicken embryo, seems therefore to be associated with migrating neural crest cells and their derivatives, with the exception of the subset of cranial neural crest cells that will undergo mesenchymal differentiation, and originate amongst other structures, the bones and connective tissue of the face and of the basis of the skull, where expression of this gene was never observed.

These differences in the distribution of RXRγ transcripts in the chicken and in the mouse are hard to explain. Clearly, more information is needed, about the expression of this gene and of the other members of the RXR family in both of these animals, before we could start to understand more about their functions in embryonic development and in the adult animal.

1.8 - Aims of this thesis

The recent discovery of the Retinoid X Receptors, and of their ligand 9c-RA, has prompted a series of very detailed studies of their function and expression. These studies have however concentrated almost exclusively in mammals, and not a lot was known about the structure and the expression of this group of molecules in other organisms, in particular in the chicken, one of the most important systems for the study of the functions of retinoids in development.

We decided therefore to examine the expression of at least some of the RXR genes in embryonic and adult chicken tissues.

The preliminary study of the expression pattern of the chicken RXRγ gene, carried out by Dr. Annie Rowe in our laboratory, had revealed a complex pattern of expression in the embryo, strongly associated with neural crest cells and derivatives. The first part of this work consists of a continuation of the characterisation of the expression pattern of this gene,

extending it to cover most of the stages of development and into the adult animal, and analysing in particular detail the expression of this gene in derivatives of the neural crest (Chapter 3).

During this part of the work, it became apparent that the chicken RXRγ gene gives rise to two distinct mRNAs with tissue specific distributions, that are expressed both in the embryo and in the adult. We decided therefore to isolate and characterise the expression of these different transcripts of the chicken RXRγ gene, and to study how they compare in structural terms, with the different isoforms reported for the other RAR and RXR genes (Chapter 4).

Finally, during the screening for the different isoforms of the chicken RXRγ gene, a novel cDNA clone for the chicken RXRα gene was isolated. The preliminary characterisation of the expression pattern of this gene in embryonic and adult chicken tissues was also carried out (Chapter 5).

CHAPTER II
MATERIALS AND METHODS

2.1 - Materials

2.1.1 - Chicken tissues

Adult White Leghorn chicken and fertilised White Leghorn chicken eggs were obtained from Polyndon Farm (Waltham Cross, Hrts.). The eggs were incubated at approximately 38°C for the appropriate length of time.

2.1.2 - Bacterial strains

The strains of *Escherichia coli* used in the λgt10 and λgt11 library screenings, namely JM83 and JM101 were available in the laborarory.

The strain DH5α was purchased from New England Biolabs (Beverley, USA).

The strains of *Escherichia coli* used in the λ-Zap library screenings, XL-1 Blue and SOL-R, were purchased from Stratagene (La Jolla, USA).

2.1.3 - Oligonucleotides

The primers used for sequencing and PCR amplifications were synthesized in our laboratory, in an Applied Biosystems 381A DNA Synthesizer.

All ribonucleotides and deoxyribonucleotides were obtained from Pharmacia (St. Albans, Herts.).

2.1.4 - Cell culture

Dissociated dorsal root ganglia and sympathetic ganglia cells were cultured in Ham's F14 medium, supplemented with 1.176 g/l of sodium bicarbonate (to give a final concentration of 0.2%), 1 mM glutamine and 100 units/ml of penicillin and streptomycin (Gibco/BRL, Paisley, Scotland). The cultures were incubated at 37°C in 5% CO_2.

Tissue culture plastics, media and solutions were purchased from Gibco/BRL (Paisley, Scotland).

2.1.5 - Enzymes

All restriction endonucleases, DNA modifying enzymes, DNA and RNA polymerases used in this work were purchased from Gibco/BRL (Paisley, Scotland) unless otherwise stated.

2.1.6 - Radiochemicals

The following radioisotopes were obtained from New England Nuclear Inc. (Boston, U.S.A.): [α-^{32}P] dCTP (3000 Ci/mmol); [α-^{32}P] CTP (800 Ci/mmol); [α^{35}S] CTP (1500 Ci/mmol) and [α-^{35}S] dATP (500 Ci/mmol).

2.1.7 - Miscellaneous

Gene Screen Plus membranes were obtained from Du Pont (Dreieich, Germany), and Hybond-N from Amersham International plc. (Aylesbury, Bucks.). Acrodisc filters were purchased from Gelman Sciences Ltd. (Northampton). Film was obtained from the following: Polaroid 667

(Polaroid, U.K., St. Albans, Herts.), Kodak X-omat AR (Kodak Ltd., Hemel Hempstead, Herts.), Fuji RX (Fuji Photofilm Co., London).

General use chemicals of the highest standard of purity available, were obtained from standard suppliers unless otherwise stated.

2.2 - Methods

2.2.1 - General procedures

All solutions used in the methods described here were prepared with double deionised water, and sterilized by autoclaving, unless this was not recommended. Plasticware and glassware were sterilized by autoclaving or by baking at 180°C for 2 hours, prior to using.

Separate stocks of solutions and plasticware were kept for RNA work. RNase free solutions were made up in autoclaved water, and either autoclaved or sterilized through a 0.45 μm filter.

2.2.2 - List of general use buffers and culture media

Agarose gel loading buffer:

0.25% (w/v) bromophenol blue, 25mM EDTA, 50% (v/v) glycerol

100X Denhardt's solution:

20% (w/v) BSA, 20% (w/v) polyvinylpyroloidone,
20% (w/v) Ficoll

20X MEA:

400 mM MOPS (morpholinopropanesulfonic acid),
100 mM sodium acetate, 20 mM EDTA; pH 7.2

Phosphate buffered saline (PBS):

135mM NaCl, 27mM KCl, 10mM Na_2HPO_4, 15mM KH_2PO_4

Polyacrylamide Gel Loading Buffer:

0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol,
25mM EDTA, 50% (v/v) glycerol

SM buffer:

100 mM NaCl, 10 mM $MgSO_4$, 0.01% (w/v) gelatin,
50 mM Tris-HCl; pH 7.5

20X SSC:

3M NaCl, 0.3M trisodium citrate.

50X TAE:

2M Tris, 50mM EDTA; adjusted to pH 8.0 with glacial acetic acid

10X TBE:

1M Tris, 1M boric acid, 20mM EDTA; pH 8.35

1X TE:

10mM Tris-HCl, 1mM EDTA; pH 8.0

Bacterial growth media:

LB-broth:

1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl.
Adjusted to pH 7.2 with NaOH.

LB-Amp:

LB-broth or LB-Agar with 0.1 mg/ml of ampicillin

LB-agar:

1.5% (w/v) technical agar in LB-broth.

LB-agarose:

0.8% (w/v) agarose in LB-broth.

2.2.3 - Phenol/chloroform extraction of nucleic acids

Unless otherwise stated, DNA and RNA samples were phenol/ chloroform extracted according to the following procedure:

An equal volume of equilibrated phenol/chloroform/isoamyl alcohol (50:49:1 v/v) was added to the DNA or RNA solution, and the mixture vortexed until it formed a homogeneous emulsion. After spinning in a microfuge, or in a Sorval RC 5B centrifuge at 6,000 rpm for 5 minutes, the upper aqueous layer was removed to a fresh tube taking care not to transfer any debris from the interface. This solution was extracted again with an equal volume of equilibrated chloroform, following the same procedure. The resulting solution was then usually ethanol precipitated.

2.2.4 - Ethanol precipitation of nucleic acids

Ethanol precipitations of DNA and RNA samples, were performed according to the following procedure:

First, the concentration of anionic salt in the DNA or RNA solution, was adjusted to the appropriate levels (final concentration of 0.2 M sodium chloride or 0.3 M sodium acetate pH 5.2). After mixing with the salt the nucleic acids were precipitated by adding 2.5 volumes of ice-cold ethanol, and incubating at -20 or -70°C for at least 20 minutes. The nucleic acids were then recovered by centrifuging at 4°C in a microfuge or in a Sorval RC 5B centrifuge at 12,000 rpm for 5 minutes. The supernatant was removed and the pellet and the walls of the tube washed with ice-cold 70% (v/v) ethanol. The tube was spun again under the same conditions, the supernatant removed, and the pellet air dried at room temperature for 5 minutes, before being resuspended in an appropriate volume of water, Tris-EDTA (TE) buffer, or another appropriate buffer.

2.3 - DNA methods

2.3.1 - Restriction enzyme digestion

Restriction digests were carried out in the buffers recommended by the manufacturer. Typically, 1 μg of DNA was digested with 1 unit of enzyme, for at least 1 hour, in a total volume greater than 10 times the volume of enzyme used.

2.3.2 - Agarose gel electrophoresis of DNA.

Normal, or low melting point agarose (SIGMA, Poole, Dorset) was used for the preparation of the gels, depending on the subsequent applications of the DNA fragments.

Restriction digests were analysed on horizontal agarose gels containing between 0.7 and 1.2% (w/v) agarose, in 1x Tris-Borate-EDTA (TBE) or Tris-Acetate-EDTA (TAE) buffer with 0.5 mg/ml ethidium bromide. Samples were mixed with 10% (v/v) agarose gel loading buffer, loaded on to the gel, and electrophoresed at 10 volts/cm, in the same buffer used to prepare the gel, for the required length of time. Finally, the DNA was visualised on a short wave ultraviolet transilluminator (254 nm) and photographed with Polaroid type 667 film.

2.3.3 - Isolation of DNA fragments from TAE/agarose gels

Three methods were used:

A - Mini spin-column

This method was originally described by Gannon and Powell (1990).

The relevant fragments were cut out of the gel, mashed up, and transferred to a punctured 0.5 ml microfuge tube, previously plugged with 2-3mm of siliconised, sterile glass wool. This was placed inside a 1.5

ml microfuge tube, and centrifuged at 6,000 rpm for 10 minutes. Elution of the DNA was checked on a transilluminator. The eluate was extracted with phenol/choroform, and then with choroform and finally ethanol-precipitated. Typically 70 to 90% of the DNA was recovered using this method.

B - "GeneClean"

This procedure was used to isolate low abundance DNA fragments of up to 5 kb from agarose gels, following the method recommended by the manufacturer of the kit (Bio-101, La Jolla, USA)

The relevant fragments were cut from the gel, and the agarose dissolved by mixing with 3x its volume of (4 M) NaI, and incubating at 50°C for 5 minutes with intermittent shaking.

Once the gel was completely dissolved, 5 μl of "glass milk" were added to the tube, vortexed and incubated on ice for 10 minutes to allow the binding of the DNA to the glass particles. The tube was then spun in a microfuge for 15 seconds, and the pellet washed twice with ice-cold "new wash" before being resuspended in 10 μl double deionised water and incubated at 50°C for 5 minutes. The "glass milk" was then pelleted by spinning in a microfuge for 30 seconds, and the supernatant containing the DNA removed. An aliquot of this solution was then run on a mini-gel, to check quality and concentration of the DNA.

C - "Magic PCR columns"

Small DNA fragments (50-1000 nucleotides) generated by PCR amplification, were purified from low melting temperature agarose gels using "Magic PCR columns", following the method recommended by the manufacturer of the kit (Promega, Madison, USA)

The relevant bands were cut from the gel, transferred to a microfuge tube and incubated at 70°C for 2-5 minutes. Once the agarose was

completely melted, it was mixed with 1 ml of "magic PCR resin" and filtered through a "magic mini-column" using a 3 ml syringe. The filtered resin was then washed twice in the column, with 2 ml of 80% (v/v) isopropanol, and spun dry in a microfuge. The DNA was eluted by incubating the resin in the "mini-column" with 50 μl of TE for 1 minute at room temperature, and microfuging it for 20 seconds. Finally, an aliquot of the DNA solution spun through the column was run on a mini-gel to check its quality and concentration.

2.3.4 - Southern blotting

Southern blots were performed using the method described in the Gene Screen Plus (Du Pont, Dreieich, Germany) manual.

After running and photographing the gel, the DNA was denatured by soaking the gel in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 30 minutes, followed by neutralising solution (0.5 M Tris-HCl pH 8.0, 1.5 M NaCl) for 30 minutes, both with gentle shaking.

During this time, a sheet of Gene Screen Plus was cut to size, wetted in double deionised water, and soaked in 10x SSC for approximately 15 minutes. The blot was assembled in the usual way (Sambrook *et al.*, 1989) with 10x SSC, and the DNA allowed to transfer overnight.

After blotting, the membrane was removed, rinsed in 2x SSC to remove any residual agarose, and the position of the wells marked with a pencil. Finally, the DNA was crosslinked to the membrane in a U.V. Stratalinker (Stratagene, La Jolla, USA), and the blot was dried and stored at -20°C, until it was hybridized.

2.3.5 - Labelling of DNA probes

DNA probes were prepared by random priming (oligolabelling) of DNA templates using a modification of the method described by Feinberg and Vogelstein (1984).

Recombinant plasmids containing the template sequences were digested with the appropriate restriction enzymes and run on a 1% (w/v) low melting point agarose gel in 1x TAE. The relevant fragments were cut out of the gel, placed in a microfuge tube with 3x their volume of double deionised water, and melted by heating to 65°C for 10 minutes. Templates prepared in this way were stored at -20°C until used.

Just prior to use, the DNA template was denatured by boiling for 5 minutes and quickly quenching on ice. An aliquot corresponding to 50 - 100 ng of DNA was combined with 5 μl of 5x oligonucleotide labelling buffer, 2 μl (20 μCi) of [α-^{32}P] dCTP, 2 μl of Klenow fragment of DNA polymerase I (Gibco/BRL, Paisley, Scotland) and double deionised water making up the volume to 25 μl. The reaction was incubated at 37°C for 4 hours, or at room temperature overnight.

5 x O.L.B.: 100 μl Solution A
250 μl 2 M HEPES pH 6.6
150 μl 90 units/μl random 6-mers

Solution A: 1 ml (1.25 M Tris-HCl pH 8.0, 0.125 M $MgCl_2$)
18 μl 2-mercaptoethanol
5 μl (0.1 M dATP, 0.1 M dTTP, 0.1 M dTTP)

After the labelling reaction was complete, unincorporated nucleotides were removed by spinning the reaction mixture through a Sephadex G-50 (Pharmacia, St. Albans, Herts) column. The column was prepared by pipeting approximately 1 ml of Sephadex G-50 beads, resuspended in TE, into a 1 ml syringe plugged with of siliconised, sterile glass wool. The column was then centrifuged at 3000 rpm for 10 minutes to remove the

buffer and compact the beads. The volume of the labelling reaction mixture was increased to 200 μl with TE, loaded on to the column and centrifuged at 3000 rpm for 10 minutes. The eluate was collected inside a 15 ml Falcon tube.

The purified probe was transferred to a microfuge tube, and 1 μl was taken into a scintillation vial containing 8 ml of Ecoscint (National Diagnostics, Atlanta, USA), to determine the activity of the probe. The probes prepared in this way, were stored at -20°C for up to two weeks until they were used.

2.3.6 - Hybridization of random primed DNA probes

Hybridization of Southern blots and colony or plaque lifts with oligolabelled DNA probes, was performed according to the method described in Sambrook *et al.* (1989)

The membranes, interleaved with sheets of nylon mesh, were placed in hybridization bottles with prehybridization mixture (6x SSC, 5x Denhardt's solution, 1% (v/v) SDS and 100 mg/ml of heat-denatured sheared herring sperm DNA), and incubated at 65°C in a Hybaid rotary hybridization oven, for at least 1 hour.

Hybridization was carried out at 65°C overnight, in a mixture identical to the prehybridization mixture with extra 10% (w/v) dextran sulphate, and 10^5-10^6 cpm/ml of ^{32}P labelled DNA probe. The probe was combined with the herring sperm DNA, and denatured by boiling for 5 minutes, before adding to the hybridization mixture.

After hybridization, depending on the number of membranes in the hybridization bottles, the membranes were kept in the bottles or transferred to sandwich boxes and washed as follows:

- 3x SSC, 1.0 %(w/v) SDS, 20 minutes at 65°C
- 1x SSC, 0.5 %(w/v) SDS; 20 minutes at 65°C
- 0.5x SSC, 0.5 %(w/v) SDS; 20 minutes at 65°C
- 0.1x SSC, 0.1 %(w/v) SDS, 30 minutes at 65°C

Finally the membranes were wrapped in Saran wrap, without allowing them to dry, and exposed to Fuji-RX film at -70°C for an appropriate length of time.

2.3.7 - Screening cDNA bacteriophage libraries

Bacteriophage libraries were screened according to the method outlined in Sambrook *et al.* (1989).

Host bacteria of the appropriate strain, were prepared by inoculating 50 ml of LB-broth, supplemented with 10 mM $MgSO_4$ and 0.2% (w/v) maltose, with a single bacterial colony. This was incubated by shaking overnight at 37°C. The cells were then pelleted at 3,000 rpm in a Beckman J-6B RC 5B centrifuge for 10 minutes at 4°C. The bacterial pellet was resuspended in 25 ml of ice-cold 0.01M $MgSO_4$ and stored at 4°C for up to two weeks.

Prior to screening, the libraries were titered by plating serial dilutions of the bacteriophage stock. 10-fold serial dilutions were prepared in SM buffer, and equal volumes of each dilution were added to 100 μl of the prepared host bacterial suspension and incubated at 37°C for 20 minutes. These were then mixed with 3 ml of LB-agarose (kept liquid at 47°C), and quickly poured on to set LB-agar in Petri dishes. After incubating overnight at 37°C, the number of plaque forming units (p.f.u.) per dish was counted, and the titer of the original library calculated.

Screening was performed using 22 x 22 cm plates, that were sterilized with 70% (v/v) ethanol for 4-6 hours before pouring. Two to four plates were poured for each screen. For each plate, an aliquot of the library containing approximately 2 x 10^5 p.f.u., was mixed in a 15 ml Falcon tube with 1 ml of the prepared host bacterial suspension, and incubated at 37°C for 20 minutes. It was then mixed with 50 ml of LB-agarose (kept liquid at 47°C), poured onto a 22 x 22 cm LB-agar plate, left to cool to room temperature for 15 minutes, and then incubated at 37°C overnight.

Lifts were taken from the plates on to Gene Screen Plus membranes (Du Pont, Dreieich, Germany) according to the manufacturer's instructions. After chilling at 4°C for 30 to 60 minutes to harden the top

agarose, the plates were overlayed with 20 x 20 cm membranes, avoiding trapping any air bubbles, and orientation marks were made, by puncturing the membranes on the agarose with a sterile syringe needle. After 1 minute, the membranes were peeled off. For each plate, lifts were taken in duplicate by placing a second membrane on the plate and marking it in the same places with a sterile syringe needle. The second membrane was left on the plate for 5 minutes and then peeled off.

The transferred DNA was denatured on the membranes, by floating them DNA side up, on denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 5 minutes, and then neutralised in neutralising solution (0.5 M Tris-HCl pH 8.0, 1.5 M NaCl) twice for 5 minutes. Finally, the membranes were rinsed in 3x SSC, air dried, after crosslinking the DNA to the membrane in a U.V. Stratalinker (Stratagene, La Jolla, USA), and stored at room temperature until hybridized.

Preparation of the oligolabelled DNA probes, hybridization, and washing of the membranes were performed using the standard conditions described in sections 2.3.5 and 2.3.6. Prehybridization and hybridization were performed at 65°C in a Hybaid rotary hybridization oven (with at least of 10^6 cpm of probe/ml of hybridization mixture) and the washing was performed in sandwich boxes, as described earlier, down to 0.5x SSC, 0.5% (w/v) SDS at 65°C for 30 minutes.

After washing, membranes were wrapped in Saran wrap and exposed to X-ray film. Signals which were duplicated on both membranes were taken as identifying positive clones. The autoradiograms were then aligned with the plates, and the positive plaques picked into 0.5 ml SM buffer, to which were added 2 drops of chloroform. Dilutions of these positive clones were made in SM buffer, mixed with plating bacteria and plated out on to Petri dishes. By successive rounds of plating, lifting, hybridizing and picking, pure positive plaques were selected. These were finally stored in SM buffer at 4°C.

2.3.7.1 - In vivo excision of p-Bluescript from λ–ZAP

The procedure summarised here, is based on the protocol recomended in the λ-ZAP-cDNA synthesis kit manual (Strategene, La Jolla, USA).

A single pure plaque of each of the λ–ZAP recombinant clones was picked as described earlier, and transfered into a sterile microfuge tube with 500 μl of SM buffer, and 20 μl of chloroform. The tubes were briefly vortexed, and incubated overnight at 4°C to allow the complete resuspension of the phage particles.

The next morning, 50-100 μl of the phage suspension were mixed with 200 μl of freshly grown XL1-Blue cells (OD_{600} approximately 1.0), and 1 μl of ExAssist helper phage, and then incubated at 37°C for 15 minutes, to allow the simultaneous infection of the host bacteria by the two phages. After adding 3 ml of LB-broth, the infected cells were further incubated for 2.5-3 hours at 37°C with shaking, to allow the rescue and packaging of the pBluescript phagemid. The cultures were then heated to 70°C for 20 minutes, and spun at 4,000 rpm for 15 minutes at 4°C in a Beckman J-6B centrifuge, to destroy and remove the debries of the host bacteria. The supernatant containing the packaged pBluescript phagemid, was collected and stored at 4°C.

The pBluescript clones were recovered by mixing 1-5 μl of the rescued phagemid stock, with 200 μl of freshly grown SOL-R cells (OD_{600} approximately 1.0), and incubating at 37°C for 15 minutes. Finally, 1, 10 and 100 μl of this culture were plated on LB-Amp plates, and incubated overnight at 37°C. The resulting colonies were minipreped, and glycerol stocks were made of the clones found to contain the desired inserts.

2.3.8 - Large scale plasmid preparation

The procedure for plasmid maxi-preps outlined here is a simplified version of one of the methods described in Sambrook *et al.*, (1989).

An overnight culture, was set up by inoculating 500-700 ml of LB-Amp with a bacterial glycerol stock, or with a colony containing the desired plasmid, and grown for approximately 18 hours at 37°C with shaking.

The following day, the culture was chilled on ice for 30 minutes, and spun at 3,000 rpm for 20 minutes at 4°C in a Beckman J-6B centrifuge. The bacterial pellet was resuspended in 20 ml of ice-cold solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA), then gently mixed with 40 ml of freshly prepared solution II (0.2 N NaOH, 1% (v/v) SDS), and incubated at room temperature for 10 minutes to allow full bacterial lysis. Next, 20 ml of ice-cold solution III (3 M potassium acetate, pH 4.3) were added, thoroughly mixed, and incubated on ice for 10 minutes to precipitate the bacterial debris.

The precipitate was removed by centrifuging at 3,000 rpm for 20 minutes at 4°C in a Beckman J-6B centrifuge, and by filtering the supernatant through several layers of nylon gauze.

The nucleic acids obtained at this point were precipitated with 0.6 volumes of propan-2-ol, incubated at room temperature for 10 minutes, and then centrifuged at 6,000 rpm for 15 minutes in a Sorvall GS3 rotor at room temperature. The pellet was washed with 70% (v/v) ethanol, briefly air dried at room temperature and resuspended in 3 ml of autoclaved, double deionised water.

The nucleic acid solution was transferred to a 10 ml Sarstedt tube, and high molecular weight RNA precipitated by adding the same volume of ice-cold 5 M LiCl, and centrifuging at 10,000 rpm for 10 minutes at 4°C in a Sorvall SS34 rotor. The supernatant was transferred to a 35 ml

Sorvall centrifuge tube, and the plasmid DNA precipitated at room temperature for 10 minutes with 2.5 volumes of ethanol. After centrifuging again in a Sorvall SS34 rotor at 10,000 rpm for 10 minutes at 4°C, the pellet was washed with 70% (v/v) ethanol, briefly air dryed, and resuspended in 750 μl of TE.

The remaining contaminating RNA was removed by digesting with 1 μl of 50 mg/ml RNase-A (previously boiled for 10 minutes to remove any DNase contamination), at 37°C for 30 minutes. After this incubation the DNA was precipitated by adding the same volume of ice-cold PEG buffer (1.6 M NaCl, 13% (w/v) polyethylene glycol 8000) and microfuging at 4°C for 10 minutes.

The DNA/PEG pellet was resuspended in 400 μl of 1x TE/200 mM NaCl, and extracted twice with phenol/chloroform, once with chloroform, and precipitated with 2.5 volumes of ethanol at room temperature for 5 minutes. Following centrifugation for 5 minutes in a microfuge, the DNA pellet was washed with 70% (v/v) ethanol, briefly dried at room temperature and finally resuspended in 1 ml of TE.

The DNA was quantitated by measuring the absorbance of an aliquot of the plasmid solution at 260 and 280 nm (1 $O.D._{260}$ of DNA=50 μg/ml; the ratio $O.D._{260/280}$ for DNA is approximately 1.8), and its quality was checked by agarose gel electrophoresis, after which it was finally stored at -20°C.

2.3.9 - Small scale plasmid preparation

This procedure for plasmid mini-preps was modified from the method described by Ish-Horowicz and Burke (1981).

A single bacterial colony was used to inoculate 2.5-5 ml of LB-Amp in a sterile Universal tube, and grown overnight at 37°C with shaking.

The following day, 1.5 ml of the culture were transferred into a microfuge tube, spun for 5 minutes, whilst the remaining of the culture was stored at 4°C. The culture medium was removed by spinning for 5 minutes in a microfuge, and the resulting bacterial pellet was resuspended in 250 μl of solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA), followed by 500 μl of freshly prepared solution II (0.2 N NaOH, 1% (w/v) SDS), and the bacterial debris were precipitated with 250 μl of solution III (3 M potassium acetate, pH 4.3) on ice for 10 minutes.

After spinning in a microfuge for 5 minutes, the supernatant was removed into another microfuge tube, taking care not to transfer any of the debris, and the nucleic acids precipitated with 0.6 volumes of propan-2-ol at room temperature for 10 minutes. This was spun again in a microfuge for 5 minutes, and the pellet washed with 70% (v/v) ethanol, briefly air dried, and resuspended in 100 μl of TE.

Most of the contaminating proteins were then removed by precipitation with 100 μl of 5 M ammonium acetate, at room temperature for 10 minutes. After spinning for 2 minutes in a microfuge, the supernatant was transferred to a fresh microfuge tube and the nucleic acids precipitated, this time with 500 μl of ethanol. After microfuging again for 2 minutes, the pellet was washed with 70% (v/v) ethanol, air dried for 5 minutes, and finally resuspended in 20 μl of TE.

2-5 μl of the DNA solution were digested with an appropriate restriction enzyme(s) in a total volume of 10-20 μl (in the presence of 1-2 μg of boiled RNase-A) for 1 hour, and analysed on an agarose gel.

Finally, glycerol stocks of the clones of interest, were made with the aliqout of the culture that had been kept at 4°C.

The plasmid DNA obtained by this method was usually clean enough for restriction digest analysis, but in some cases, or if the isolated DNA was to be used for sequencing, a further phenol/chloroform and chloroform extraction, and ethanol precipitation were performed.

2.3.10 - Construction of recombinant plasmids

2.3.10.1 - Preparation of the insert and vector DNA

1-5 μg of the vector, and an equivalent amount of the insert DNA, were digested with the appropriate restriction enzymes. The digests were incubated at 37°C for 2-4 hours, and checked by running approximately 10% of each reaction on an agarose gel. If the digestion was complete, the remaining DNA was separated on a TAE agarose gel, and the desired bands purified by one of the methods outlined in section 2.3.3.

When a single restriction enzyme was used, the vector was not gel purified, but was instead phosphatased (to prevent recircularization), by digesting with 10 units of calf intestinal phosphatase at 37°C for 30 minutes, followed by phenol/chloroform extraction and ethanol precipitation.

Vector and insert fragments were resuspended in small volumes of autoclaved double deionised water, and their concentrations estimated by running a small aliquot of each on an agarose gel, next to known quantities of DNA markers.

2.3.10.2 - Ligation reactions

A standard ligation reaction contained 100-200 ng of vector DNA, 10-20 ng of insert DNA, 1-5 units of T4 DNA ligase and 2 μl of 5x T4 DNA ligase buffer, in a total volume of 10 μl. These components were mixed briefly and incubated for 6-24 hours at room temperature, after which, they were transformed into freshly prepared competent cells.

For each set of ligations, two or three reactions with different amounts of insert and vector DNA were set up (usually 2:1, 1:1 and 1:2), ensuring that suitable molar ratios of the two were obtained. In parallel, several control reactions were also set up, to monitor the efficiency of ligation.

2.3.11 - Transformation of *E. coli* with with plasmid DNA

2.3.11.1 - Preparation of competent cells

Competent cells were prepared using a modification of the method described in Sambrook *et al.*, (1989).

A single bacterial colony of the appropriate bacterial strain was grown in a small volume of LB-broth overnight at 37°C with shaking. The next morning, a 1:100 dilution of this culture was made in 50-100 ml fresh LB-broth, and grown for 1-2 hours to an $OD_{(600)}$ of 0.3-0.4. The culture was then chilled on ice for 15 minutes and harvested by centrifugation at 2,500 rpm for 10 minutes at 4°C, in a Beckman J-6B centrifuge. The pellet was resuspended in half of the original volume, in ice-cold 50 mM $CaCl_2$ and incubated on ice for 20 minutes. The bacteria were centrifuged once more, as before, and resuspended in one-tenth of the original volume, in ice-cold 50 mM $CaCl_2$. After a further incubation of 1 hour on ice, the competent bacteria were ready for transformation.

2.3.11.2 - Transformation of competent cells

One third of each ligation reaction, usually 3 μl, was diluted 5x with autoclaved double deionised water, and briefly chilled on ice, before adding to 200 μl of freshly prepared competent cells. This mixture was incubated on ice for 1-2 hours, and heat-shocked at 42°C for 2 minutes. After cooling down to room temperature for 10 minutes, 1 ml of LB-broth was added to each tube, and the culture was incubated at 37°C for 45 minutes, to allow the expression of the antibiotic resistance gene. After centrifuging for 5 minutes at low speed in a microcentrifuge, most of the supernatant was discarded, and the bacterial pellet gently

resuspended in the remaining medium. For vectors with blue/white selection, 8 μl of 100 mg/ml IPTG (isopropyl β-D-thiogalactopyranoside) and 8 μl of 100 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were added, and the transformed bacteria were then plated on LB-agar plates with the appropriate antibiotic, and incubated overnight at 37°C.

2.3.12 - Colony screening

Recombinant colonies were screened according to an adaptation of the method described by Grunstein and Hogness (1975).

A 0.5 cm grid, was drawn with a pencil on circular Gene Screen Plus membranes of the size of the Petri dishes. Colonies were chosen, and plated both on the membrane circle laid on an LB-Amp. plate, and on a replica LB-Amp. plate with an equivalent grid drawn on the back. Both plates were then incubated overnight at 37°C, after which the replica plate was stored at 4°C.

The membrane with the colonies grown on it, was peeled from the agar plate, and prepared for hybridization by floating it, colony side up, on denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 5 minutes, and then in neutralising solution (0.5 M Tris-HCl pH 8.0, 1.5 M NaCl) twice for 5 minutes. Next, the bacterial debris were removed by wiping the surface of the membrane several times with tissues soaked in 2 X SSC, 0.1% (w/v) SDS. Finally, the membrane was rinsed in 2x SSC, and air dried after crosslinking the DNA in a U.V. Stratalinker (Stratagene, La Jolla, USA), and stored at room temperature until hybridized.

2.3.13 - DNA sequencing

DNA sequencing was performed by the dideoxy chain termination method, according to the method recommended by the manufacturer of the "Sequenase-2" kit (United States Biochemical Corporation, Cleveland, USA).

Double stranded DNA templates were denatured by the alkaline-denaturation method, before proceeding with the sequencing reactions: 2-10 μg of double stranded DNA were denatured, by incubating in 0.2 M NaOH for 10 minutes at room temperature, then neutralised with 0.4 volumes of 5M ammonium acetate, and precipitated with 2.5 volumes of ethanol at -70°C for 15 minutes. The denatured DNA was recovered by spinning in a microfuge for 5 minutes, and the resulting pellet washed with 70% (v/v) ethanol, air dried at room temperature for 5 minutes, and resuspended in a small volume ($\leq$ 7 μl), of double deionised water. The normal sequencing procedure was then followed.

For each set of reactions, 2-3 μg of DNA were mixed in a microfuge tube with 2 μl of 5x "Sequenase buffer", and approximately 2 p moles of primer (typically, 12 ng of an 18-mer oligonucleotide) in a final volume of 10 μl. This mix was then warmed to 75°C for 2 minutes, and allowed to slowly cool down to room temperature, by floating the tube in a small beaker with water from the waterbath at 75°C.

Once the temperature had dropped below 35°C, the annealing was complete, and the mix was stored at 4°C for a maximum of 4 hours until it was used.

To the annealed template and primer mix were added, 1 μl 0.1M DTT, 2 μl of "labelling mix" (diluted 1:5 with double deionised water), 0.5 μl of [α-^{35}S] dATP and 2 μl of Sequenase enzyme (diluted 1:8 in ice-cold

enzyme dilution buffer). This was mixed and incubated at room temperature for 5-10 minutes.

If it was intended to sequence close to the primer, 1 μl of "Mn buffer", was also added. If, on the contrary, it was intended to sequence far from the primer, the labelling mix was less diluted (1:2) or even used undiluted.

During the labelling incubation, 2.5 μl of each ddNTP termination mix were added to four tubes and prewarmed for 1 minute at 37°C. Once the labelling step was complete, 3.5 μl of the labelling mixture were added to each of the four tubes containing the different termination mixes, mixed and incubated at 37°C for 5 minutes. Finally the reaction was stopped by adding 4 μl of stop solution, and the mixtures stored at -20°C for up to 1 month, before being denatured at 70°C for 5 minutes, and loaded on a sequencing gel.

2.3.14 - Separation of DNA and RNA samples in polyacrylamide denaturing gels

Polyacrylamide/urea gels were prepared using "Sequagel" (National Diagnostics, Atlanta, USA) reagents, according to the manufacturer's instructions.

The two gel stock solutions were mixed to give a final concentration of: 5.7% (w/v) acrylamide, 0.3% (w/v) bis-acrylamide, 7 M urea, in 1x TBE. The polymerization was started by adding 1 ml of 10% ammonium persulphate and 25 μl of TEMED (N,N,N',N' tetramethyl-ethylene-diamine) for each 100 ml of gel mixture, and the gel was poured between two 40 x 20 cm glass plates, separated by 0.4 mm thick spacers, with an inverted "sharks tooth comb" on the top.

Once it had set, the gel was placed on a sequencing tank with 1x TBE and pre-run at 30 mA for 30 minutes. The samples were denatured at 75°C for 2 minutes, loaded on to the gel and run at 25-30 mA for the required length of time. Once electrophoresis was complete, the two glass plates were prised apart, and the gel fixed in 1:1:8 methanol/acetic acid/water (v/v) for 20 minutes, before being transferred to a sheet of Whatman 3MM paper and dried for 20 minutes in a vacuum dryer at 80°C. Finally, the dried gel was exposed to Fuji RX film overnight at room temperature.

2.3.15 - Standard PCR amplification conditions

PCR amplification was used for the rapid characterization of clones isolated from λgt10 and λgt11 cDNA libraries, based on the method described by (Hamilton *et al.*, 1991)

A typical 100 μl PCR reaction contained, 5 n moles of each dNTP, approximately 20 p moles of each primer (120 ng for an 18-mer oligonucleotide), 50-500 ng of template DNA, and 2 units of Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, USA) in 1x "PCR buffer" (supplied by the manufacturer). Reactions were prepared in a 0.5 ml microfuge tube, and overlayed with 75 μl of liquid paraffin to minimize evaporation.

Amplification was carried out in a Hybaid thermal cycler, using the following temperature profile: 30 seconds at 94°C; 45 seconds at the annealing temperature; 1 minute at 72°C, for 25 to 35 cycles, followed by a final extension at 72°C for 5 minutes. The reactions were then stored at 4°C and an aliquot analysed on an agarose gel.

The annealing temperature for each PCR reaction, was determined on the basis of the length and nucleotide composition of the primers, and was usually 5°C below the lowest of the melting temperatures (T_m) of the two primers. The T_m of each primer was calculated using the following formula.

$$T_m \text{ (in °C)} = 4x(G+C \text{ content}) + 2x(A+T \text{ content})$$

2.3.16 - Sequencing of PCR products

DNA fragments derived from PCR reactions were sequenced directly with Sequenase, following the method described by (Casanova *et al.*, 1990).

PCR products were run on an agarose gel, to remove unincorporated nucleotides and primers, and the bands of interest isolated using "Magic PCR preps" as described in section 2.3.3.

Unlike the normal sequencing protocol, these templates were not alkaline-denatured before the annealing step. Instead, 200-500 ng (approximately 1 p mole) of the isolated PCR product, was mixed in a microfuge tube with 2 μl of 5x "Sequenase buffer", and approximately 1 p mole of primer, in a final volume of 10 μl. This mix was denatured by boiling for 10 minutes, and rapidly annealed by snap-freezing in liquid nitrogen for 20 seconds. The mixture was kept on ice for 2 minutes, during which time the following mixture was added to the cap of the microfuge tube: 1 μl 0.1M DTT, 2 μl of labelling mix (diluted 1:5 with double deionised water), 0.5 μl of [α-^{35}S] dATP and 2 μl of Sequenase enzyme (diluted 1:8 in ice-cold enzyme dilution buffer). After mixing by briefly spinning in a microfuge, extension was allowed to take place on ice for 3 minutes. The remaining procedures were performed as described in section 2.3.13.

2.4 - RNA methods

2.4.1 - Isolation of total RNA

Total RNA was isolated using the acid guanidinium - phenol/ chloroform method, as described by Chomczynski and Sacchi (1987).

Tissue samples were collected, rinsed in ice-cold PBS, snap-frozen in liquid N_2, and stored at -70°C until processed. An aliquot of up to 0.5 g of tissue was cut without allowing it to thaw, and immediately placed in 5 ml of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% (w/v) sarcosyl and 0.1 M 2–mercaptoethanol) in a 10 ml Sarstedt tube, homogenized with a glass homogeniser, and left to stand at room temperature for 15 minutes. The following were then added, mixing after each addition: 500 μl of 0.2M sodium acetate pH 4.0, 5.0 ml of water saturated phenol and 1.0 ml of chloroform/isoamyl alcohol (49:1 v/v). The tubes were vortexed for 10 seconds, cooled on ice for 15 minutes, and centrifuged at 10.000 rpm. for 20 minutes at 4°C in a Sorval SS35 rotor, in a Sorval RC 5B centrifuge,

The upper aqueous layer was carefully removed to another 10 ml Sarstedt tube, and the RNA precipitated by adding an equal volume of isopropanol, incubating at -20°C for at least 1 hour, and centrifuging at 10.000 rpm. for 20 minutes at 4°C in a Sorval SS35 rotor, in a Sorval RC 5B centrifuge.

The resulting pellet was resuspended in 600 μl of solution D, transferred to a microfuge tube, and again precipitated by adding an equal volume of isopropanol, incubating at -20°C for one hour, and microfuging at 4°C for 10 minutes. This pellet was then washed with 70% (v/v) ethanol, briefly air dried at room temperature, and resuspended in 400 μl of autoclaved double deionised water, by heating in a 65°C waterbath. The RNA obtained at this point, was often not pure

enough, and was cleaned by a further phenol/chloroform and chloroform extraction, followed by ethanol precipitation.

Finally, the RNA solution was stored at -20°C, after being quantitated by measuring the absorbance at 260 and 280 nm (1 $O.D._{260}$ of RNA=40 μg/ml; the ratio $O.D._{260/280}$ for RNA is approximately 2.0). The quality of the RNA was checked, by running a small aliquot of the RNA preparation on a small formaldehyde denaturing agarose gel.

2.4.2 - Selection of poly-(A)$^+$ RNA

Poly-(A)$^+$ RNA was isolated from total RNA, by affinity chromatography on oligo(dT)-cellulose as described by Sambrook *et al.* (1989).

An appropriate amount of oligo-(dT) cellulose (Pharmacia, St. Albans, Herts.) was resuspended in 0.1 N NaOH, and poured into a sterile Dispocolumn (Bio-Rad, Richmond, USA) column, to give a packed volume of approximately 0.5 ml. The packed column was then washed with 3 volumes of autoclaved double deionised water, followed with approximately 3 volumes of loading buffer (20 mM Tris-HCl pH 7.6, 0.5 M NaCl, 1 mM EDTA and 0,1% (w/v) sodium lauryl sarcosinate) until the pH of the eluate was less than 8.0.

Typically, 200 to 500 mg of total RNA were mixed with an equal volume of 2x loading buffer, denatured at 65°C for 5 minutes, and loaded on to the column. Poly-(A)$^-$ RNA was removed by washing with 10 column volumes of loading buffer, and the eluate collected in a sterile 15 ml Falcon tube.

Next, poly-(A)$^+$ RNA was eluted with 3 column volumes of elution buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA and 0,05% (w/v) SDS) collecting the eluate in another sterile 15 ml Falcon tube.

The poly-(A)$^+$ RNA was ethanol precipitated with 0.3 M sodium acetate (pH 5.2) at -70°C for 30 minutes, and centrifuged at 4.000 rpm. for 20 minutes at 4°C in a Beckman J-6B centrifuge. The pellet was washed with ice-cold 70% (v/v) ethanol, air dried for 5 minutes at room temperature, resuspended in a small volume of sterile double deionised water, and stored at -20°C, after being quantitated by measuring the absorbance of a small aliquot of the solution at 260 and 280 nm.

The oligo-(dT) cellulose resin, was regenerated and used again by resuspending it in 0.1 N NaOH and storing at -20°C.

2.4.3 - Agarose gel electrophoresis of RNA

Formaldehyde denaturing agarose gels for the separation of RNA samples, were prepared according to the method outlined in Sambrook *et al.* (1989).

Typically, 20 μg of total RNA or 2 μg of poly-(A)$^+$ RNA were mixed with two volumes of RNA loading buffer (75% (v/v) deionised formamide, 9% (v/v) formaldehyde, 1.5x MEA and 10 μg/ml ethidium bromide) denatured by heating at 65°C for 5 minutes, and quenched on ice. In addition to the samples, the recommended amounts of RNA size markers (Boehringer Mannheim, Lewes, Sussex) were treated in the same way.

The samples and the size markers, were then mixed with gel loading dye, and loaded on to a denaturing agarose gel containing, 1.1% (wv) agarose, 1x MEA and 6% (v/v) formaldehyde. The gel was then run at 5 volts/cm, in 1x MEA running buffer, for the required length of time. Finally, the RNA was visualised on a short wave ultraviolet transilluminator (254 nm), and photographed with a Polaroid type 667 film.

2.4.4 - Northern blotting

Northern blots were performed using the method described in the Gene Screen Plus (Du Pont, Dreieich, Germany) manual.

After running and photographing the RNA agarose gel, excess formaldehyde was removed by washing 4 times for 5 minutes, in double deionised water. During this time a sheet of Gene Screen Plus was cut to the size of the gel, briefly wetted in double deionised water, and soaked in 10x SSC for approximately 15 minutes. The blot was then assembled in the usual way (Sambrook *et al.*, 1989) with 10x SSC, and the RNA was allowed to transfer overnight.

After this period, the membrane was removed, rinsed in 2x SSC to remove any residual agarose, and the position of the wells marked with a pencil. After drying at room temperature, the membrane was baked at 80°C for 2 hours to reverse the formaldehyde, and crosslink the RNA. The blots were then stored at -20°C until they were hybridized.

2.4.5 - Labelling RNA probes

Full length, strand-specific RNA probes were prepared according to the following protocol based on the method described in Sambrook *et al.*, (1989).

Plasmids containing the DNA templates were linearized, phenol/ chloroform and chloroform extracted, and ethanol precipitated. They were then resuspended at a concentration of approximately 1 mg/ml in autoclaved double deionised water, and stored at -20°C. Depending on the vector, and on which strand of the cDNA fragment was to be transcribed, T3, T7 or SP6 RNA-polymerase were used.

The reaction contained, 1x RNA-polymerase buffer (T3/T7 or SP6), 20 mM DTT, 0.5 mM GTP, 0.5 mM ATP and 0.5 mM UTP, 2 μM CTP, 50 units of RNase Inhibitor (Boehringer Mannheim, Lewes, Sussex), 2-3 μg DNA template, 50 μCi [α-^{32}P] CTP (New England Nuclear Inc., Boston, U.S.A.), and 1.5 μl (approximately 15 units) of the appropriate RNA-polymerase, in a total volume of 25 μl. These reagents were mixed and incubated at room temperature for 1 hour.

After transcription, the DNA template was removed by adding 50 units of RNase Inhibitor (Boehringer Mannheim, Lewes, Sussex), 10 μg of *E. coli* tRNA (SIGMA, Poole, Dorset) and 10 units of RNase-free DNase I (Boehringer Mannheim, Lewes, Sussex) incubating 10 minutes at 37°C. Finally, the reaction volume was increased to 115 μl with autoclaved double deionised water, and after adding 10 μl of 5 M LiCl, the probe was precipitated with 300 μl of ethanol at -70°C for 15 minutes.

After spinning in a microfuge for 5 minutes, the pellet was washed with ice-cold 70% (v/v) ethanol, air dried at room temperature for 5 minutes, resuspend in 50 μl of autoclaved double deionised water, and stored for up to 4 days at -70°C. At this point 0.5 μl of the probe were counted in 8 ml of Ecoscint (National Diagnostics, Atlanta, USA).

2.4.6 - Hybridization of northern blots

Northern blots were hybridized with RNA probes as described by Rowe *et al.* (1991).

The membranes, interleaved with sheets of nylon mesh, were placed in hybridization bottles with prehybridization mixture (5x SSC, 60% (v/v) deionised formamide, 20 mM sodium phosphate pH 6.0, 5x Denhardt's solution, 1% (w/v) SDS, to which were added 100 mg/ml of heat-denatured sheared herring sperm DNA, 100 μg/ml yeast total RNA (SIGMA, Poole, Dorset) and 10 μg/ml poly-(A)$^+$ RNA (SIGMA, Poole, Dorset), and incubated at 65°C in a Hybaid rotary hybridization oven, for at lest 2 hours.

Hybridization was carried out at 65°C overnight, in a mixture identical to the prehybridization mixture with extra 7% (w/v) dextran sulphate, and with ^{32}P-labelled RNA probe to a final concentration of approximately 10^6 cpm/ml of hybridization mixture. The probe was combined with the herring sperm DNA, yeast total RNA and the poly-(A)$^+$ RNA, and denatured by heating to 80 °C for 5 minutes before adding to the hybridization mixture.

After hybridization, the membranes were transferred from the hybridization bottles to sandwich boxes and washed as follows:

- 3x SSC, 1.0 % (w/v) SDS, 30 minutes at 65°C
- 2x SSC, 0.5 % (w/v) SDS; 30 minutes at 70°C
- 1x SSC, 0.5 % (w/v) SDS; 30 minutes at 75°C
- 0.1x SSC, 0.5 % (w/v) SDS; 30 minutes at 80°C
- 0.1x SSC, 0.1 % (w/v) SDS, 30 minutes at 80°C

Finally the membranes were wrapped in Saran wrap without allowing them to dry, and exposed to Fuji RX film at -70°C for an appropriate length of time.

2.4.7 - RNase protection assays

The method outlined here, was adapted from the protocol for the use of RNase ONE™ (Promega) recommended by the supplier, optimized for our system by running a series of mock experiments in which different amounts of probe, RNA, RNase, and the different temperatures of hybridization were tested.

Restriction fragments of around 200-300 nucleotides, encompassing the relevant regions of the cDNAs, were subcloned to allow their transcription in both the sense and the antisense orientations. Typically, at least 90% of the transcripts synthesized from short templates such as these were full length transcripts (Krieg, 1990), and so gel purification of the probes was not necessary. However, all the probes were checked before use, by running an aliquot on a sequencing gel.

Antisense ^{32}P-labelled riboprobes and control unlabelled sense RNA transcripts synthesized from the same templates, were prepared according to the protocol described in section 2.4.5, and were used immediately after transcription, to minimise degradation of the transcripts as a result of radioactive decay.

Typically, 25 μg of total RNA from each sample, were combined with approximately 2.5x 10^5 counts/minutes of probe. After making the total volume of the mixture to 25 μl with autoclaved double deionised water, the probe and RNA were coprecipitated by adding 5 μl of 3 M sodium acetate pH5.2, followed by 150 μl of ethanol, and incubating at -70°C for 30 minutes.

The same amount of probe was also precipitated with similar volumes of unlabelled sense transcripts, and with 25 μg of *E.coli* tRNA as a control. After washing with 70% (v/v) ethanol, the pellets were dried at room temperature for 5 minutes, and thoroughly resuspended in 30 μl of hybridization buffer (80% (v/v) deionised formamide, 40 mM PIPES pH

6.4, 0.4 M sodium acetate and 1 mM EDTA). The resuspended samples were then denatured at 85°C for 5 minutes, and quickly transferred to an oven at 45°C, where they were left to hybridize overnight.

The hybridized samples were cooled to room temperature, and digested by adding to each, 3-4 units of RNase ONE™ in 300 μl of RNase digestion buffer (10 mM Tris-HCl pH 7.5, 0,2 M sodium acetate and 5 mM EDTA), and incubating at 37°C for 1 hour. Digestion was stopped by adding 5 μl of 10 % (w/v) SDS and 20 μg of poly(A)$^+$ RNA, and the protected fragments were precipitated by adding 850 μl of absolute ethanol and incubating at -70°C for 30 minutes.

After centrifugation in a microfuge for 5 minutes, the pellets were washed with 70% (v/v) ethanol, and briefly air dried at room temperature. The pellets were then thoroughly resuspended in 4 μl of sequencing loading buffer, denatured at 70°C for 5 minutes, and loaded on to a sequencing gel, next to a DNA sequence ladder, for estimation of the sizes of the protected fragments.

2.5 - Primary cultures of dissociated cells from dorsal root ganglia (DRG) and sympathetic ganglia (SG).

The procedure for the preparation of the primary cultures of dissociated cells from dorsal root ganglia and sympathetic ganglia, is based on the method described by Ernsberger and Rhorer (1989).

Dorsal root ganglia and sympathetic ganglia, were dissected aseptically from the lumbo-sacral region of 7 and 10 day old chicken embryos, and collected in Ham's F14 medium, supplemented with 1.176 g/l of sodium bicarbonate (to give a final concentration of 0.2%), 1 mM glutamine and 100 units/ml of penicillin and streptomycin (Gibco/BRL, Paisley, Scotland). They were then gently spun down, resuspended in 2 ml of medium with 2.5 mg/ml trypsin (Gibco/BRL, Paisley, Scotland), and incubated at 37°C for 15 minutes. Digestion was stopped by adding foetal calf serum to a concentration of 10% (v/v), and the ganglia gently spun down and washed twice in medium with 10% (v/v) foetal calf serum. Cells were dissociated by pipeting up and down through a sterile micropette and filtered through a 220 μm cell sieve (SIGMA, Poole, Dorset), to remove debris and undissociated clumps of cells.

Finally, the cells were plated at a density of approximately 10,000-20,000 cells per chamber on 2.0x2.0x0.89 cm Nunc Lab-Tek® chamber slides (previously coated with 100 μg/ml poly-l-ornithine (SIGMA, Poole, Dorset)), and cultured for 2-3 days in medium with 10% (v/v) foetal calf serum, supplemented with 50 ng/ml NGF-2.5S (SIGMA, Poole, Dorset) to promote neurone survival.

2.6 - Protocols for in situ hybridization

This protocol for in situ hybridization, is based on the methods described by Hogan *et al.*, (1986), and optimized for hybridization of chick embryo tissues in our laboratory by Dr. Annie Rowe (1991).

2.6.1 - Tissue preparation

Chick embryos, staged according to Hamburger and Hamilton (1951), were collected in ice-cold PBS, dissected whenever necessary to allow access of the fixative to the tissues, and rinsed in PBS before fixation.

Most tissues were fixed overnight at 4°C, in freshly made 4% (w/v) paraformaldehyde in PBS. The fixative was removed by washing twice in PBS at 4°C for 30 minutes, and the tissues dehydrated through an ethanol series: first in 1:1 (v/v) ethanol/PBS for 15 minutes at 4°C; then in 70% (v/v) ethanol twice for 15 minutes at room temperature; 85% (v/v) ethanol for 30 minutes; 95% (v/v) ethanol for 30 minutes; twice in absolute ethanol for 30 minutes. Finally the tissues were cleared in Histoclear (National Diagnostics, Atlanta, USA) twice for 20 minutes.

Chick embryo eyes were dissected out and washed in ice-cold PBS, then fixed overnight in 94:5:1 (v/v) ethanol/water/glacial acetic acid. The next day they were washed three times in ice-cold 95% (v/v) ethanol for 20 minutes, and then twice for 20 minutes in absolute ethanol, allowing them to warm up to room temperature. Finally they were cleared in Histoclear twice for 20 minutes.

Fixed tissues were then gradually embedded in freshly melted, filtered paraffin wax (Fibrowax - BDH, Lutterworth, Leics.), starting with 1:1 Histoclear/wax for 20 minutes allowing it to warm up from room temperature to 60°C; then in 1:3 Histoclear/wax for 20 minutes at 60°C; finally twice in wax only for 20 minutes at 60°C. Samples were then

orientated in plastic casting dishes and allowed to cool down to room temperature. The blocks could then be stored for several months at 4°C with dessicant, until they were sectioned.

Slides with the dissociated dorsal root ganglia and sympathetic ganglia cultures (prepared as described in section 2.5), were simply rinsed twice in PBS at room temperature, and fixed in 2% (w/v) paraformaldehyde in PBS for 5 minutes at room temperature. Next, they were washed twice in PBS for 2 minutes and dehydrated through an ethanol series (75% (v/v) ethanol; 90% (v/v) ethanol; absolute ethanol, for 2 minutes each). Finally, they were dried at room temperature for 1 hour, and stored at -20°C with dessicant for up to two months.

2.6.2 - Preparation of slides and sections

Glass slides (Superfrost - BDH, Lutterworth, Leics.) were cleaned by washing with Teepol, and rinsed in deionised water. They were then washed in 10% (v/v) HCl for 20 minutes, rinsed in double deionised water, dried and finally baked at 180°C for at least 2 hours. They can be stored indefinitely at this point.

Prior to use, the slides were coated with TESPA (3-aminopropyl-etoxysilane) (SIGMA, Poole, Dorset), by dipping for 10 seconds in a freshly made solution of 2% (v/v) TESPA in acetone, and rinsed twice in acetone for 10 seconds, once in double deionised water, and finally drained and baked dry at 42°C for at least 1 hour. At this stage they were stored at room temperature and used within four days.

7 μm sections were cut in a standard microtome, and ribbons collected and stored in frames. The relevant sections were selected, and floated on the slides on a drop of double deionised water, allowing them to spread

by heating the slides briefly to 45°C on a hot plate. Once the sections were completely spread, the water was blotted off. The sections were then dried overnight at room temperature, and sealed on to the slides by baking them at 60°C for between 6 hours and overnight. At this stage, the slides with the sections can be stored at 4°C with dessicant, for up to four months.

2.6.3 - Probe preparation

For each probe, a subclone of the relevant region of the cDNA clone was made, typically including the region coding for the E domain and the 3' untranslated regions, and linearized templates were prepared as described in section 2.4.5. For each clone, two linearized templates were made, so that both strands could be transcribed separately. The antisense probe was used to detect mRNA, whilst the sense probe was used as a negative control on adjacent sections.

The procedure for making probes for in situ hybridization is essentially similar to the protocol for making riboprobes described in section 2.4.5, with some modifications for labelling with [α^{35}S] CTP. In particular, the concentration of DTT must be maintained at/or above 10 mM at all times, to prevent the degradation of the isotope.

For a 25 μl labelling reaction, 70 μCi of [α^{35}S] CTP was used. After transcription, DNase treatment and ethanol precipitation were carried out in the presence of 10 mM DTT, and the probe was finally resuspended in 50 μl of 50 mM DTT. 0.5 μl of the probe were counted in Ecoscint (National Diagnostics, Atlanta, USA).

After transcription, probes were alkali-hydrolysed, to generate fragments of an average size of 200-300 nucleotides, to improve tissue

penetration. The hydrolysis times were calculated using the formula (Hogan *et al.*, 1986):

$$X = (L_o - L_f) / K.L_o.L_f$$

with: X= hydrolysis time (minutes); L_o= original transcript length (kb); L_f= final transcript length (kb); K= 0.11

The probes, in 50 μl of 50 mM DTT, were hydrolysed by adding 50 μl of hydrolysis buffer (80 mM $NaHCO_3$, 120 mM Na_2CO_3, 10 mM DTT, pH 10.2) and incubating at 60°C for the required length of time. The reactions were then stopped by adding 50 μl of neutralising buffer (0.2 M sodium acetate, 1% (v/v) glacial acetic acid, 10 mM DTT). Finally, 15 μl of sodium acetate pH 5.2 were added, and the probe fragments were precipitated by adding 500 μl of absolute ethanol, and incubating at -70°C for 15 minutes. The hydrolysed probes were spun down in a microfuge for 5 minutes, washed with 10 mM DTT in 70% (v/v) ethanol, and resuspended in 50 mM DTT as before. 0.5 μl of each probe were counted in Ecoscint (National Diagnostics, Atlanta, USA). There was no significant difference from the value obtained before hydrolysis.

The probe was now ready for use, and was stored at -70°C for a maximum of 4 days.

2.6.4 - In situ hybridization procedure

All solutions used in the pretreatment and hybridization procedures were autoclaved prior to use, or, when that was not possible, made up with autoclaved (but not DEPC treated) double deionised water. The solutions used for hybridization, were also filtered through 0.45 μm filters. All the glassware and slide racks used in the pretreatment and

hybridization, were baked at 180°C for at least 2 hours to inactivate contaminating RNases.

2.6.4.1 - Pretreatment

For cell cultures:

Slides with cultures, were allowed to warm up gently to room temperature in the sealed box where they had been stored, and were washed for 20 minutes in 2x SSC at 70°C (continued below).

For wax sections:

Slides with sections, were allowed to warm up to room temperature in the sealed box where they had been stored, and were dewaxed in xilene for 15 minutes, rehydrated through an ethanol series (absolute, 95%, 90%, 80%, 60%, and 30% (v/v) ethanol, for 2 minutes each), washed twice for 2 minutes in double deionised water, and incubated once for 15 minutes in 1/49 HCl.

From this point, slides carrying cultured cells and sections were treated in the same way, as follows:

The slides were washed once in 2x SSC at room temperature for 5 minutes, followed by incubation with 5 μg/ml proteinase K (SIGMA, Poole, Dorset), in 100 mM Tris pH 7.5, 50 mM EDTA at 37°C for 10 minutes. They were then washed for 2 minutes at room temperature in 2 mg/ml glycine in PBS, to inactivate the enzyme, and rinsed twice for 1 minute in PBS. Next, the slides were incubated in freshly prepared 4% (w/v) paraformaldehyde in PBS for 20 minutes, and rinsed twice in PBS for 2 minutes. The slides were then acetilated in 1:400 (v/v) acetic anhydride in 0.1 M triethanolamine pH 8.0 for 10 minutes, followed by a 5 minutes wash in PBS, and rinsed in double deionised water for 2

minutes. Finally, they were dehydrated through an ethanol series (30%, 60%, 80%, 95% (v/v) and absolute ethanol, for 2 minutes each), and air dried under dust cover for at least 1 hour.

2.6.4.2 - Hybridization

The in situ hybridization mixture consists of: 1x salts (20x salts stock solution: 20 x Denhardt's solution, 400 mM Tris pH 8.0, 100 mM EDTA, 200 mM Phosphate buffer pH 6.8); 50% (v/v) formamide (freshly deionised); 10% (w/v) dextran sulphate; 50 mM DTT; 500 μg/ml yeast total RNA (SIGMA, Poole, Dorset); 50 μg/ml poly-(A)$^+$ RNA (SIGMA, Poole, Dorset) and probe to a concentration of 5x 10^4 - 1x 10^5 counts/minutes/μl of hybridization mixture.

Prior to use, the hybridization mix. with the probe, was vortexed, spun for 5 minutes to remove air bubbles, and heated to 80°C for 2 minutes, and rapidly quenched on ice. After denaturation, the hybridization mixture was spread directly on sections with the aid of a piece of Nescofilm, (approximately 50-100 μl of hybridization mixture per slide), and covered with coverslips avoiding trapping any air bubbles.

The slides were then placed horizontally on top of glass rods, inside the hybridization chambers (plastic boxes with dry 3MM (Whatman) paper sellotaped on the lid to prevent condensation, and 3MM paper soaked in 2x SSC and 50% (v/v) formamide on the bottom). The hybridization chambers were sealed and incubated at 55°C overnight (8-12 hours) in an oven.

2.6.4.3 - Post-hybridization washes

After hybridization, the chambers were opened and the slides rapidly transferred to a slide rack. The first wash was in 2x SSC, 50% (v/v)

formamide,10 mM DTT for 15 minutes at 55°C. During this time the rack was frequently shaken, to dislodge the coverslips from the slides. The slides were then transferred to another rack, and washed in fresh wash solution for 20 minutes. During this time, the waterbath was warmed to 65°C. Next, the slides were washed in fresh wash solution for 20 minutes at 65°C, rinsed twice for 15 minutes at 37°C in RNase buffer (500 mM NaCl; 10 mM Tris pH 8.0; 1 mM EDTA), and incubated in 40 μg/ml pancreatic RNase A (SIGMA, Poole, Dorset) in RNase buffer, for 30 minutes at 37°C. The slides were then rinsed once more in RNase buffer, for 15 minutes at 37°C, washed twice in wash solution (same as above) at 65°C for 20 minutes, followed by a rinse in 0.1x SSC; 10 mM DTT at 65°C for 20 minutes, and in 0.1x SSC for 5 minutes at room temperature. Finally, the slides were dehydrated through an ethanol series (300 mM ammonium acetate in 70% (v/v) ethanol, 95% (v/v) ethanol, and absolute ethanol, for 2 minutes each), and air dried under dust cover for at least 1 hour.

2.6.5 - Dipping, exposure, developing and staining

Before dipping, the hybridized slides were exposed to ordinary Fuji-RX film overnight, to get an idea of the intensity of the signal and background in each section.

Slides were then dipped in a dark room under a safelight, in Ilford K5 emulsion (Ilford, London) diluted 6:8 in 2% (v/v) glycerol, kept liquid at 42°C.

After dipping, excess emulsion was drained off, and the slides were placed horizontally on a tray, and left for at least 2 hours at room temperature under dust cover, to allow the emulsion to solidify and harden. They were then transferred to racks, stored at 4°C with dessicant in light tight boxes, and exposed for the appropriate length of time.

After 1-2 weeks, depending on the intensity of the signal, test slides were allowed to warm up to room temperature and developed (5 minutes in Kodak D19 developer; rinsed in water; 5 minutes in Kodak Unifix fixer). If the exposure was satisfactory, all the other slides were developed in the same way, rinsed in running water for 10 minutes and stained.

Sections were stained for 3 minutes in a 0.01% (w/v) solution of Malachite green, and excess stain was removed by washing under running water until a satisfactory result was obtained. Slides were then allowed to dry overnight at room temperature, and coverslips mounted with DPX (BDH, Lutterworth, Leics.).

Cell cultures were stained by dipping them for 12 minutes in a 5% (v/v) solution of Giemsa stock solution (Raymond Lamb, London), in 10 mM phosphate buffer pH 7.0. Excess stain was washed off under running water for approximately 30 seconds. The slides were then dried overnight at room temperature, and coverslips mounted with Aquamount (BDH, Lutterworth, Leics.), to allow viewing under phase contrast microscopy, and sealed with nail varnish.

CHAPTER III

DISTRIBUTION OF RXRγ IN EMBRYONIC AND ADULT CHICKEN TISSUES

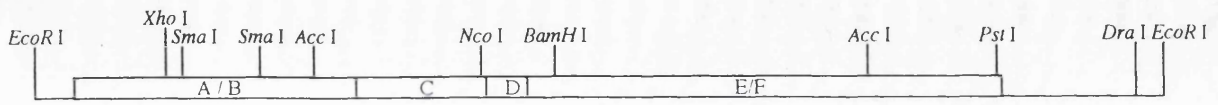
3.1 - Introduction

A cDNA clone of chicken RXRγ (pR2), was previously isolated in our laboratory (Rowe *et al.*, 1991), by low stringency screening of a 10 day whole embryo cDNA library constructed in λgt11 (Clontech), with a radiolabelled probe containing the sequences corresponding to the A, B and C domains of the human RARα cDNA clone, p63 (Petkovich *et al.*, 1987). Analysis of the nucleotide sequence of pR2, identified a putative coding region of 467 amino acids (fig. 3.1), whose predicted amino acid sequence showed homology to human RXRα (Mangelsdorf *et al.*, 1990) and to H-2RIIBP (Hamada *et al.*, 1989) both in the DNA- and ligand-binding domains. The subsequent publication of the sequences for the three mouse RXRs (Mangelsdorf *et al.*, 1991), identified pR2 as corresponding to the chicken homolog of mouse RXRγ (fig. 3.2).

Preliminary characterization of the expression pattern of the RXRγ gene in the chicken (Rowe *et al.*, 1991), revealed that at embryonic stages 16 to 18, transcripts of this gene were present along the pathways of migration of neural crest cells. At later stages (stages 24-27), RXRγ transcripts were abundant in the liver, and in some neural crest derivatives, including elements of the peripheral nervous system, but not in the mesectodermal derivatives of the cranial neural crest, such as the mesenchymal cells of the facial primordia.

The initial objective of the work presented in this thesis was to complete the analysis of the expression pattern of the chicken RXRγ gene, and to compare this with the expression pattern of the same gene in other

A



B

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gaattcCGGTGAGAGGAGACGGTCGTGTTTTGAAAGGCGAGAGATGAACTGAAGATTAAACATGTATGGGAATTA      75
                                                          M  Y  G  N  Y            5

TCCTCACTTCATTAAGTTTCCTGCGGGCTTCGGCAATTCCCCTGTTCATGCCAGCTCCACGTCTGTGAGCCCATC     150
  P  H  F  I  K  F  P  A  G  F  G  N  S  P  V  H  A  S  S  T  S  V  S  P  S     30

ATCCAGCCTGTCCGTGGGGAGCACGGTGGACGGACACCACAACTACCTCGAGGCCCCCACAAACGCCTCCCGGGC     225
  S  S  L  S  V  G  S  T  V  D  G  H  H  N  Y  L  E  A  P  T  N  A  S  R  A     55

GCTGCCATCCCCCATGAACACCATTGGGTCTCCAGTGAACGCGTTGGGCTCACCCTACAGGGTCATCGCATCCTC     300
  L  P  S  P  M  N  T  I  G  S  P  V  N  A  L  G  S  P  Y  R  V  I  A  S  S     80

CATTGGCTCGCATCCCGTTGCTCTGTCGTCCTCAGCCCCGGGCATGAATTTTGTGACCCACAGCCCACAGCCCAA     375
  I  G  S  H  P  V  A  L  S  S  S  A  P  G  M  N  F  Y  T  H  S  P  Q  P  N     105

TGTGCTCAACAATGTCAGCAGCTCGGAGGACATCAAGCCCTTGCCAGGTCTACCAGGGATTGGGAACATGAATTA     450
  V  L  N  N  L  S  S  S  E  D  I  K  P  L  P  G  L  P  G  I  G  N  M  N  Y     130

TCCATCCACAAGCCCAGGTTCTTTAGCCAAACACATCTGTGCCATCTGTGGGGACAGGTCTTCAGGGAAGCACTA     525
  P  S  T  S  P  G  S  L  A  K  H  I  C  A  I  C  G  D  R  S  S  G  K  H  Y     155

TGGGGTGTACAGCTGCGAGGGCTGCAAGGGCTTCTTTAAGAGGACCATCCGGAAAGACCTGATCTACACCTGCCG     600
  G  V  Y  S  C  E  G  C  K  G  F  F  K  R  T  I  R  K  D  L  I  Y  T  C  R     180

TGACAACAAGGACTGCCTCATCGACAAGCGCCAGCGCAACCGCTGCCAGTACTGCCGCTATCAGAAGTGCCTCGC     675
  D  N  K  D  C  L  I  D  K  R  Q  R  N  R  C  Q  Y  C  R  Y  Q  K  C  L  A     205

CATGGGGATGAAGAGGGAAGCCGTGCAGGAGGAGAGGCAGGGGAGCAGGGAGCGCAGTGAGAACGAAGCTGAATC     750
  M  G  M  K  R  E  A  V  Q  E  E  R  Q  G  S  R  E  R  S  E  N  E  A  E  S     230

CACGAGCGGCGGCAGCGAGGACATGCCTGTGGAGAGGATCCTGGAAGCCGAGCTGGCAGTCGAACCCAAGACGGA     825
  T  S  G  G  S  E  D  M  P  V  E  R  I  L  E  A  E  L  A  V  E  P  K  T  E     255

GGCATACAGCGACGTGAACACGGAGAGCTCAACCAATGACCCCGTCACCAACATCTGCCACGCTGCTGACAAGCA     900
  A  Y  S  D  V  N  T  E  S  S  T  N  D  P  V  T  N  I  C  H  A  A  D  K  Q     280

GCTCTTCACCCTCGTCGAGTGGGCCAAGCGCATCCCCCACTTCTCCGACCTGACCCTGGAGGACCAAGTCATCCT     975
  L  F  T  L  V  E  W  A  K  R  I  P  H  F  S  D  L  T  L  E  D  Q  V  I  L     305

CCTGCGGGCAGGCTGGAACGAGCTGCTCATCGCATCCTTCTCCCATCGCTCCGTGTCGGTGCAGGATGGCATCCT    1050
  L  R  A  G  W  N  E  L  L  I  A  S  F  S  H  R  S  V  S  V  Q  D  G  I  L     330

GCTGGCCACAGGCTTGCATGTGCACCGCAGCAGTGCTCACAGTGCAGGCGTGGGCTCCATCTTTGACAGAGTTTT    1125
  L  A  T  G  L  H  V  H  R  S  S  A  H  S  A  G  V  G  S  I  F  D  R  V  L     355

GACAGAGCTGGTGTCCAAAATGAAGGACATGCAGATGGATAAGTCGGAGCTGGGGTGCCTGCGAGCCATTGTCCT    1200
  T  E  L  V  S  K  M  K  D  M  Q  M  D  K  S  E  L  G  C  L  R  A  I  V  L     380

CTTCAACCCAGACGCCAAGGGCCTGTCCAGCCCCTCCGAAGTGGAGTCGCTGCGGGAAAAGGTCTACGCCACGCT    1275
  F  N  P  D  A  K  G  L  S  S  P  S  E  V  E  S  L  R  E  K  V  Y  A  T  L     405

GGAAGCCTACACGAAGCAGAAGTACCCCGAGCAGCCGGGGCGGTTTGCCAAACTCCTCCTGCGCCTGCCAGCACT    1350
  E  A  Y  T  K  Q  K  Y  P  E  Q  P  G  R  F  A  K  L  L  L  R  L  P  A  L     430

ACGGTCCATCGGGCTGAAGTGCCTGGAGCACCTCTTCTTCTTCAAGCTGATCGGGGACACCCCCATCGACACCTT    1425
  R  S  I  G  L  K  C  L  E  H  L  F  F  F  K  L  I  G  D  T  P  I  D  T  F     455

CCTTATGGAGATGCTGGAGACACCCCTGCAGGTCACTTGAGGGTCCGCTCCCCCCCGCCATATCCCCACACAGCT    1500
  L  M  E  M  L  E  T  P  L  Q  V  T  *                                        467

CCCACCCCTCCTCTCCGGGCTGCAAGAGCCGCCCTCCACCCTCTGCTCCTCTTTGGTGGGATTGTTGTGTCTTTG    1575

TACAGCTGTAAATCGTCCCCTCTAACCCTCCCGGTGGGTTGCGGACCCTCTGCCCAGCTAACCACACACCTCCCT    1650

GTACAGATAATTGCTTTAAATTATTTTTTCACTCTCAATAAAAGCCAACAACAACCGgaattc                 1713
```

Figure 3.1

Figure 3.1

(A) Restriction map of the insert of the cDNA clone pR2, of cRXRγ. The 5' and 3' untranslated regions are shown as solid lines. The coding region is boxed, showing the approximate position of the boundaries of the main protein domains (A-F).

(B) Nucleotide sequence and predicted amino acid sequence derived from the cDNA clone pR2. *Eco*RI linker sequences are shown in lower case. The putative DNA binding domain is boxed. The nucleotide and amino acid sequences are numbered on the column on the right.

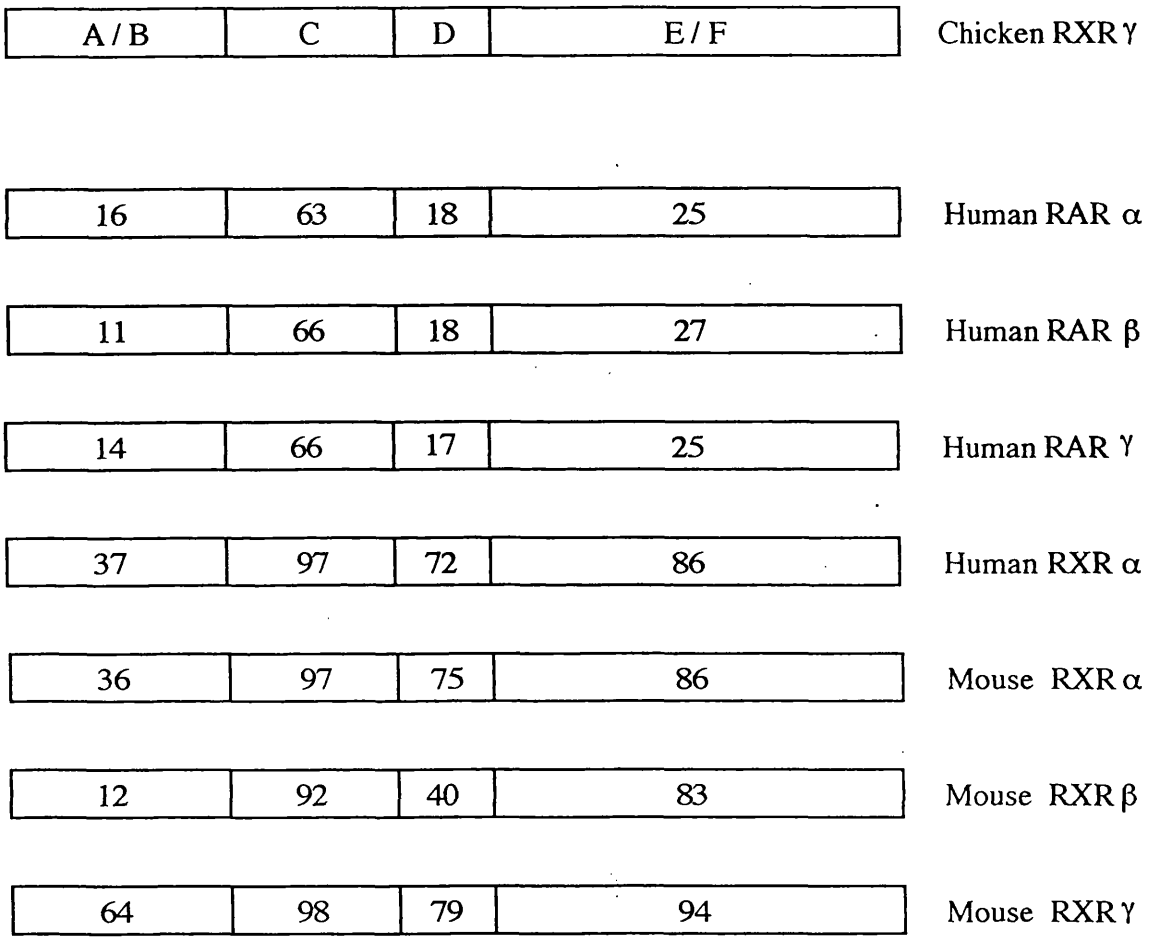


Figure 3.2

Comparison of the predicted amino acid sequence of chicken RXRγ (Rowe *et al.*, 1991), with those of human RARα (Petkovich et al., 1987), RARβ (Brand et al., 1988) and RARγ (Krust et al., 1989), human RXRα (Mangelsdorf et al., 1990) and mouse RXRα (Mangelsdorf et al., 1992), RXRβ (Hamada et al., 1989) and RXRγ (Mangelsdorf et al., 1992). In each domain, the percentage amino acid homology with the sequence of chicken RXR**γ** is indicated.

animals, as well as with the expression patterns of other related genes such as the other RXRs, the RARs, and the retinoid binding proteins.

This chapter describes the results of experiments designed to answer some of the questions that arose from the preliminary analysis of the distribution of chicken RXRγ transcripts described above. These are:

- Is the expression of the chicken RXRγ gene maintained in the same tissues throughout development and in the adult animal, or is its expression limited to embryonic structures;
- Is the chicken RXRγ gene expressed in other tissues apart from the liver and the peripheral nervous system, in particular, is this gene expressed in other non mesectodermal neural crest derivatives.

3.2 - Analysis of chicken RXRγ gene expression in embryonic and adult tissues by northern blot hybridization.

A *Bam*HI-*Eco*RI fragment of chicken RXRγ clone pR2 (nucleotides 786-1713), containing the sequences coding for the E-F domains and the 3' untranslated region, was subcloned into pBluescript SK+ producing the plasmid pR2-B/E (see map in appendix A). This was then used as a template for the synthesis of radiolabelled antisense RNA probes. This subclone was designed so that the probes produced did not contain sequences that are highly conserved amongst the members of the RXR family, namely, the sequences coding for the C and D domains, ensuring that the probe detected specifically RXRγ transcripts.

Hybridization of the pR2-B/E antisense riboprobe to northern blots of total RNA extracted from a variety of embryonic and adult chicken tissues, revealed a complex pattern of expression of RXRγ transcripts in the chicken (fig. 3.3). There are at least two major transcripts of this gene, each of them with different tissue distributions:

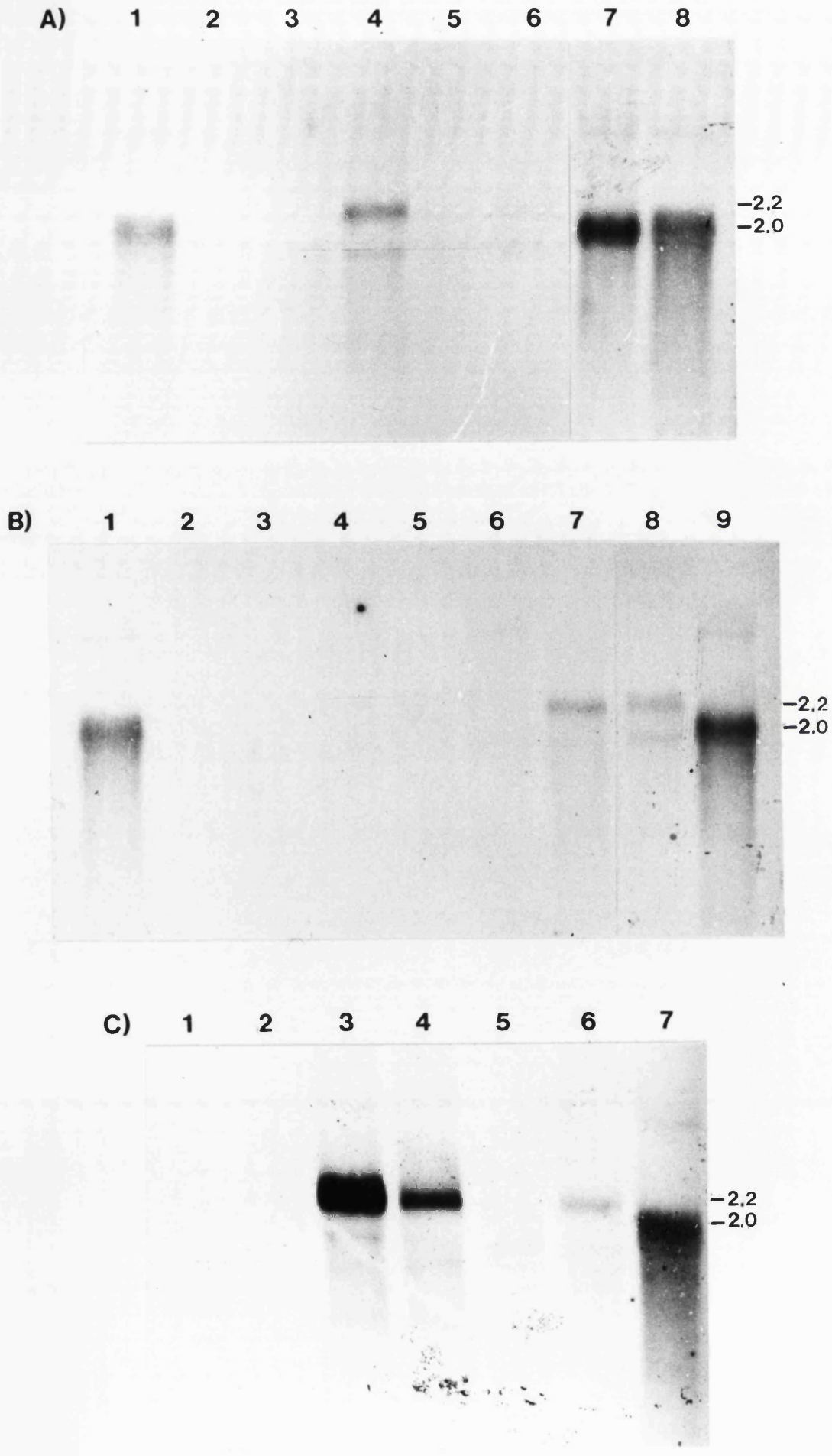


Fig. 3.3

Figure 3.3

Northern blot analysis of the expression of cRXRγ transcripts in embryonic and adult chicken tissues.

Northern blots of total RNA isolated from embryonic and adult chicken tissues, hybridized with a probe specific for cRXRγ transcripts (antisense riboprobe synthesized with T7 RNA polymerase, using *Bam*HI-linearised pR2-B/E as a template).

Tracks contain approximately 20μg of total RNA isolated from:

(A) 1 - adult liver; 2 - adult kidney; 3 - adult heart; 4 - adult brain; 5 - adult lung; 6 - adult duodenum; 7 - 10 day embryo liver; 8 - adult liver.

(B) 1 - adult liver; 2 - adult pancreas; 3 - adult oviduct; 4 - adult spleen; 5 - adult gizzard; 6 - adult skeletal muscle; 7 - adult skin; 8 - adult adrenal glands (approximately 10μg); 9 - adult liver.

(C) 1 - adult skin; 2 - adult spinal nerve (approximately 10μg); 3 - 7 day embryo sympathetic ganglia; 4 - 10 day embryo sympathetic ganglia; 5 - adult ovary; 6 - adult brain; 7 - adult liver.

The size of the transcripts is indicated in kilobases.

Notice the existence of at least two cRXRγ transcripts of different sizes, one of approximately 2.0kb, present only in embryonic and adult liver, and another of approximately 2.2kb, present in a variety of other tissues.

- a smaller transcript of approximatelly 2.0 kb was found exclusively in the liver, where it was expressed at fairly high levels, both in the embryo and in the adult (fig. 3.3, panel A tracks 7 and 8 respectively, panel B tracks 1 and 9, panel C track 7).

- a larger transcript of about 2.2 kb, was found in all the other tissues where the RXRγ gene was expressed. In the embryo, this larger RXRγ mRNA was very abundantly expressed in the sympathetic ganglia (fig. 3.3, panel C tracks 3 and 4), whilst in the adult, comparatively low levels of expression of this larger transcript were detected in a variety of tissues, such as the brain, adrenal glands, skin, and also in the duodenum and in the spinal nerves (fig. 3.3, panels A, B and C).

Northern blot analysis of poly $(A)^+$ RNA prepared from 10 day embryo tissues hybridized with the same probe (fig. 3.4), showed that the larger transcript, of approximately 2.2 kb, is the predominat RXRγ mRNA in 10 day whole embryo (fig. 3.4, tracks 1 and 2). This transcript is expressed at extremely high levels in the embryonic eye (fig. 3.4, track 4), and is also relatively abundant in embryonic dorsal root ganglia (fig. 3.4, track 3). Traces of a transcript of similar size were also detected in embryonic liver (fig. 3.4, track 5), which was confirmed as the only site of expression of the smaller transcript of chicken RXRγ. Several large mRNA species were detected in both tissues where the expression of RXRγ transcripts was most abundant, namely the eye and the liver, probably corresponding to unprocessed or partially processed chicken RXRγ mRNAs.

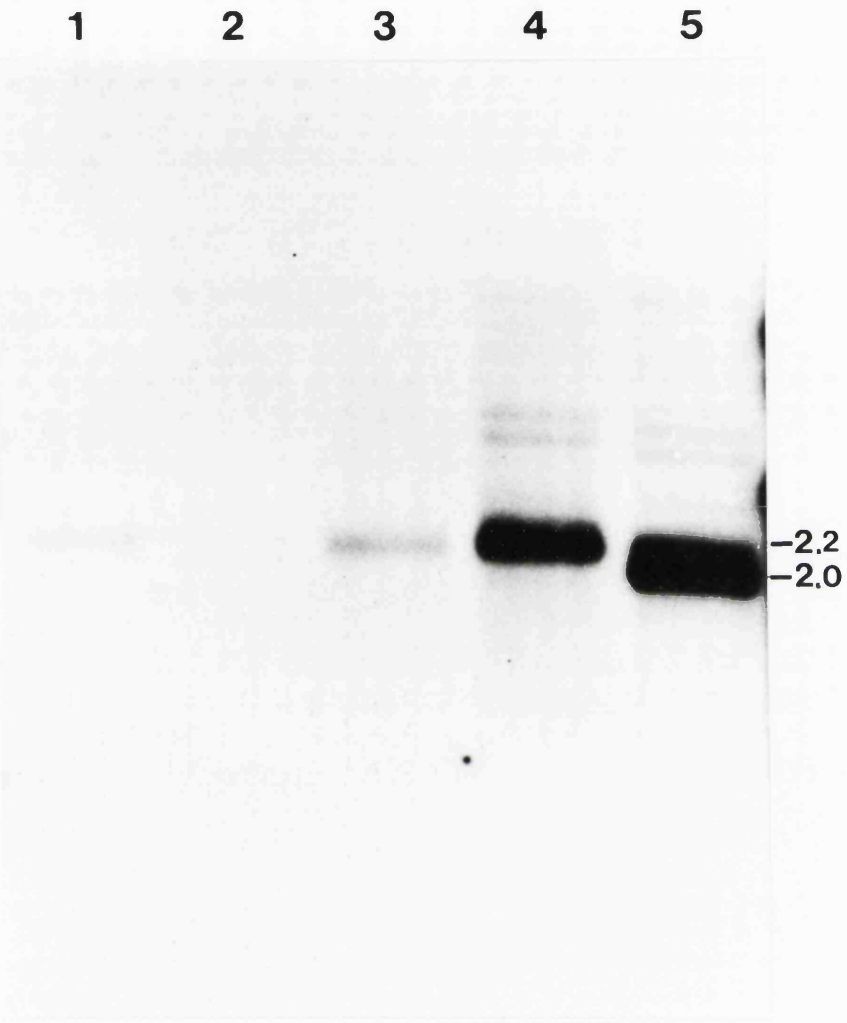


Fig. 3.4

Figure 3.4

Northern blot analysis of the expression of cRXRγ transcripts in embryonic chicken tissues.

Northern blot of poly(A)$^+$ RNA isolated from embryonic chicken tissues, hybridized with a probe specific for cRXRγ transcripts (antisense riboprobe synthesized with T7 RNA polymerase, using *Bam*HI-linearised pR2-B/E as a template).

Tracks contain approximately 2μg of poly(A)$^+$ RNA isolated from:

1 - 10 day embryo head; 2 - 10 day embryo body; 3 - 10 day embryo dorsal root ganglia (approximately 1μg); 4 - 10 day embryo eye; 5 - 10 day embryo liver.

The size of the transcripts is indicated in kilobases.

In this blot the existence of at least two cRXRγ transcripts of different sizes is more obvious. Notice the expression of traces of a larger transcript of cRXRγ in the liver, with a similar size to the transcript expressed in the other tissues (2.2kb). Also notice the detection of some very large bands, in tracks 4 and 5 (eye and liver poly(A)$^+$ RNA respectively), probably corresponding to unprocessed or partially processed transcripts of cRXRγ.

3.3 - Analysis of chicken RXRγ gene expression during development by in situ hybridization

In situ hybridization was carried out on sections of chicken embryos at various developmental stages, to determine in detail the cellular expression pattern of chicken RXRγ during embryogenesis. ^{35}S-labelled antisense RNA probes were synthesised from the same template as the probes used in the northern blot hybridization experiments (pR2-B/E), ensuring that both major transcripts of chicken RXRγ were detected. In all cases, adjacent sections were hybridized to a negative control probe, consisting of a radiolabelled sense RNA probe synthesised from the same template.

The results obtained in these in situ hybridization experiments with the chicken RXRγ probe, generally confirm the pattern of expression of this gene determined in the northern blots of embryonic and adult tissues, and complement the results previously reported by Rowe *et al.* (1991). They demonstrate that the developing eye and the liver, are the major sites of expression of RXRγ transcripts in the later stages of chick embryonic development. Lower levels of RXRγ transcripts are found in a subset of the structures derived from the neural crest, including the peripheral nervous system and the adrenal glands (fig. 3.5 and fig. 3.6).

Expression of the chicken RXRγ gene in each of these tissues is analysed in detail in the following sections of this chapter.

3.3.1 - RXRγ gene expression in the developing chick embryo eye.

The eye is one of the organs with the most complex and interesting embryological development, involving a series of complex inductive events between tissues from a variety of origins: the choroid and the

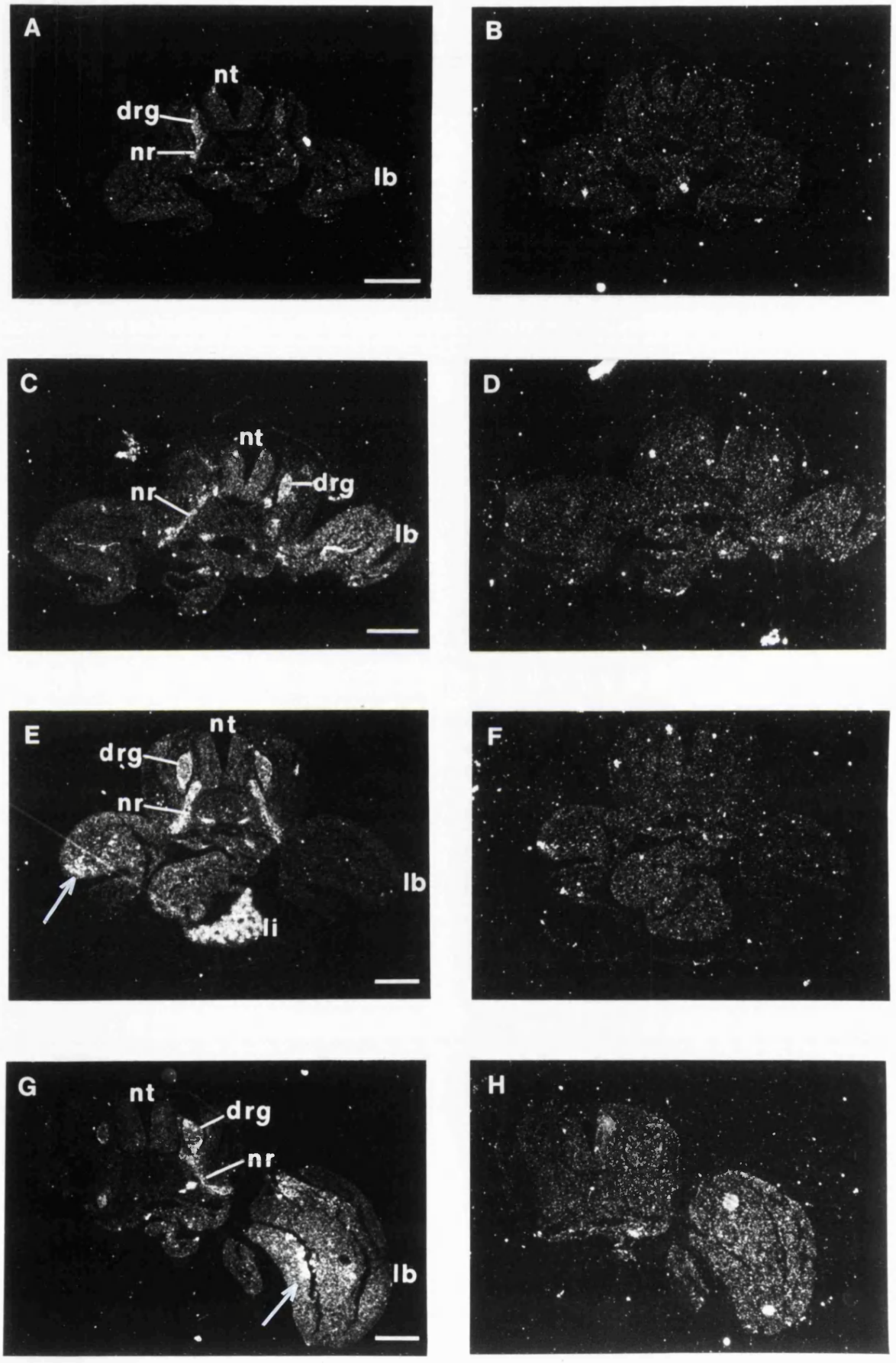


Fig. 3.5

Figure 3.5

In situ hybridization analysis of the distribution of cRXRγ transcripts in early chick embryos.

Panels A, C, E and G show transverse sections at the level of the wing buds of chick embryos at stages 18, 20, 22 and 24 respectively, hybridized with a probe specific for cRXRγ transcripts; panels B, D, F and H show the corresponding adjacent sections hybridized with the negative control probe. Notice the punctate hybridization observed in the limb buds in panels E and G (arrows). drg - dorsal root ganglia; li - liver; lb - limb bud; nt - neural tube; nr - nerves.

Sections are shown under dark-field illumination. Scale bar = 250 μm.

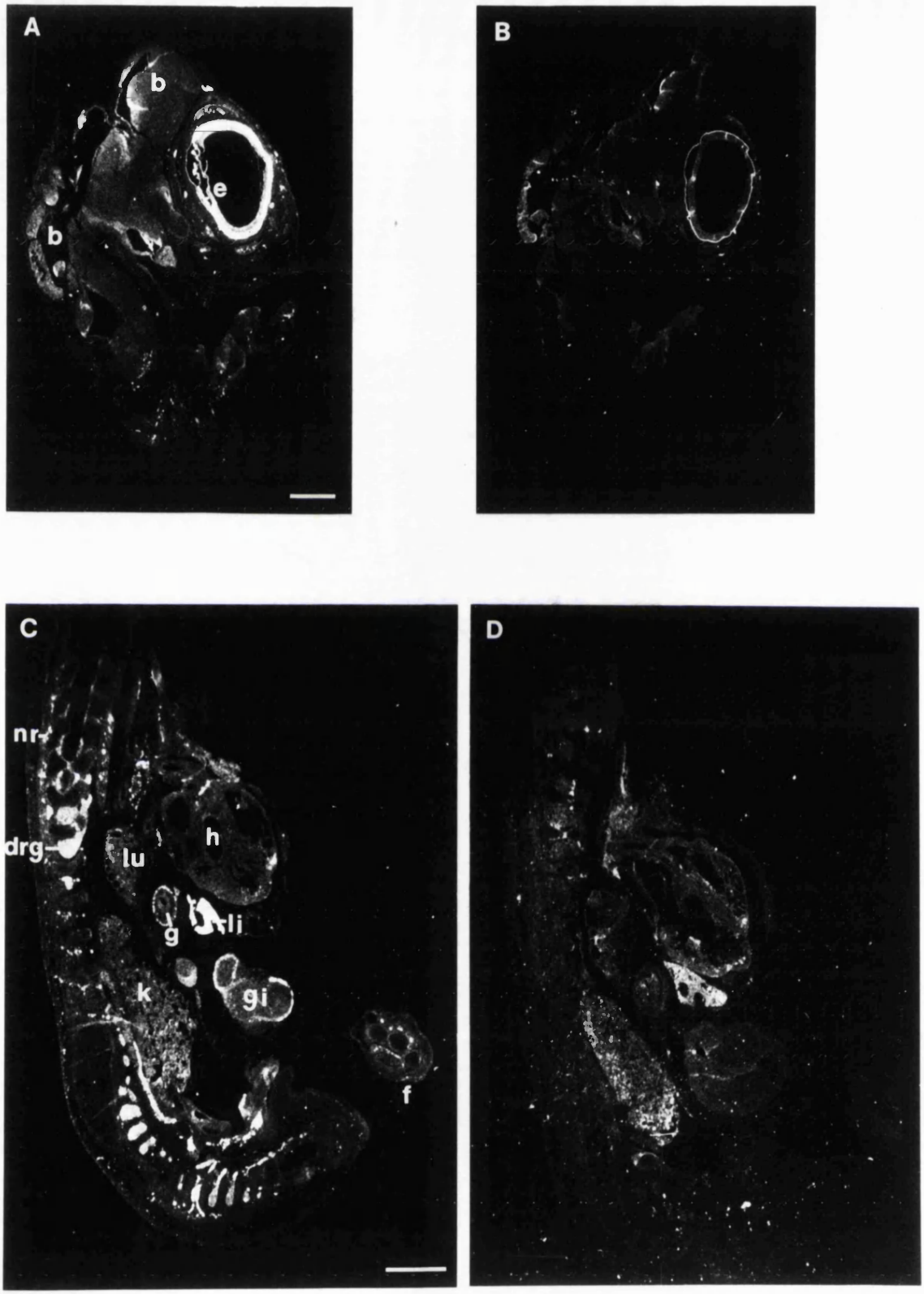


Fig. 3.6

Figure 3.6

In situ hybridization analysis of the distribution of cRXRγ transcripts in stage 31 (approximately 7 days of incubation) chick embryo.

Panel A shows a parasaggital section of the head of a stage 31 chick embryo, hybridized with a probe specific for cRXRγ transcripts; panel B shows an adjacent section, hybridized with the negative control probe.

Panel C shows a parasaggital section of the body of a stage 31 chick embryo, hybridized with a probe specific for cRXRγ transcripts; panel D shows an adjacent section, hybridized with the negative control probe.

b - brain; drg - dorsal root ganglia; e - eye; f - foot; g - gut; gi - gizzard; h - heart; k - kidney; li - liver; lu - lung; nr - nerves.

Sections are shown under dark-field illumination. Scale bar = 1 mm.

sclera are originated from mesencephalic neural crest cells, the lens is originated from the ectoderm of the developing head, the cornea and the iris have both an ectodermal and neural crest origin, and both layers of the retina, the neural and the pigmented retina, are originated from the neuroectoderm of the developing forebrain (Sadler, 1885; Browder *et al.*, 1991). A detailed description of the development of the eye is given in appendix B.

Expression of the chicken RXRγ gene in the tissues surrounding the developing eye is first detected around stages 12-14, in the neural crest cells originated from the midbrain, that surround the optic vesicles (Rowe, A. personal communication). Soon after, expression disappears in this region, more or less coinciding with the mesenchymal differentiation of these neural crest cells into the various connective tissue structures of the eye, as indicated above.

The definitive site of expression of RXRγ transcripts in the chicken eye is the neural retina, where they can be detected from approximately stage 24 onwards (figs. 3.7 and 3.8), well before any morphological stratification of the retina can be observed (see appendix B). At these early stages, expression of the RXRγ gene is more or less homogeneous along the external surface of the retinal epithelium (fig. 3.8, panels A to D), but becomes progressively restricted to the nuclear layers, as the various neuronal cell types differentiate, and take their respective positions (fig. 3.8, panels E to H). Around day 7 (approximately sages 30-31), sparse patches of expression corresponding to the nuclei of the ganglion cells, become separated from the bulk of the expression on the external surface of the retinal eipthelium. Shortly after, this inner region of expression starts to become separated into two bands, corresponding to the inner and outer nuclear layers (fig. 3.8, panels E and F). From this point onwards, there is a refinement of this expression pattern, with two

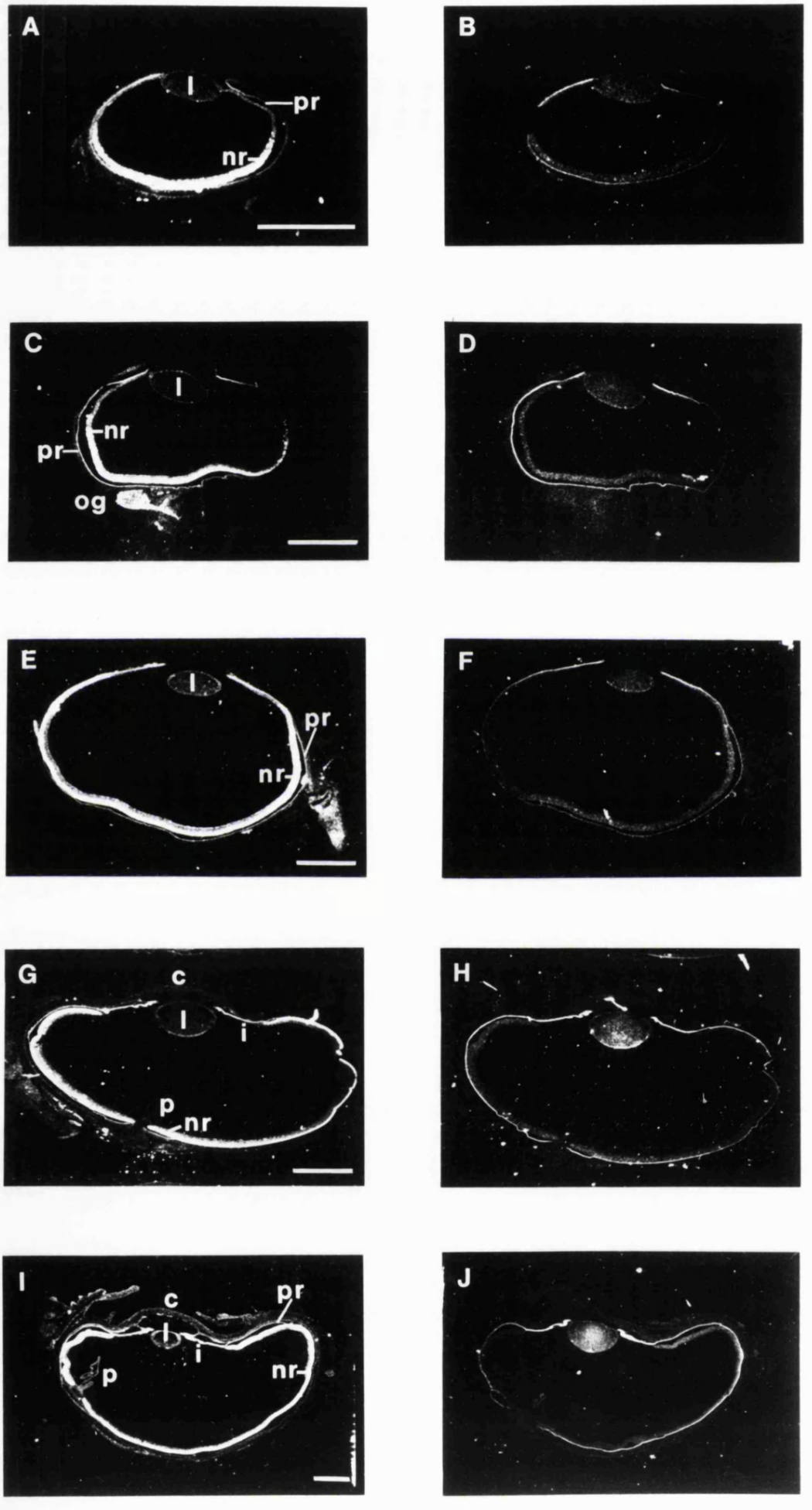


Fig. 3.7

Figure 3.7

Expression of cRXRγ transcripts in the developing chick embryo eye.

Panels A, C, E, G and I show saggital sections of the eyes of chick embryos at stages 27, 29, 32, 35 and 37 respectively, hybridized with a probe specific for cRXRγ transcripts; panels B, D, F, H and J show the corresponding adjacent sections, hybridized with the negative control probe. c - cornea; i - iris; l - lens; nr - neural retina; og - optic ganglion; p - pecten; pr - pigmented retina.

Sections are shown under dark-field illumination. Scale bar = 1 mm.

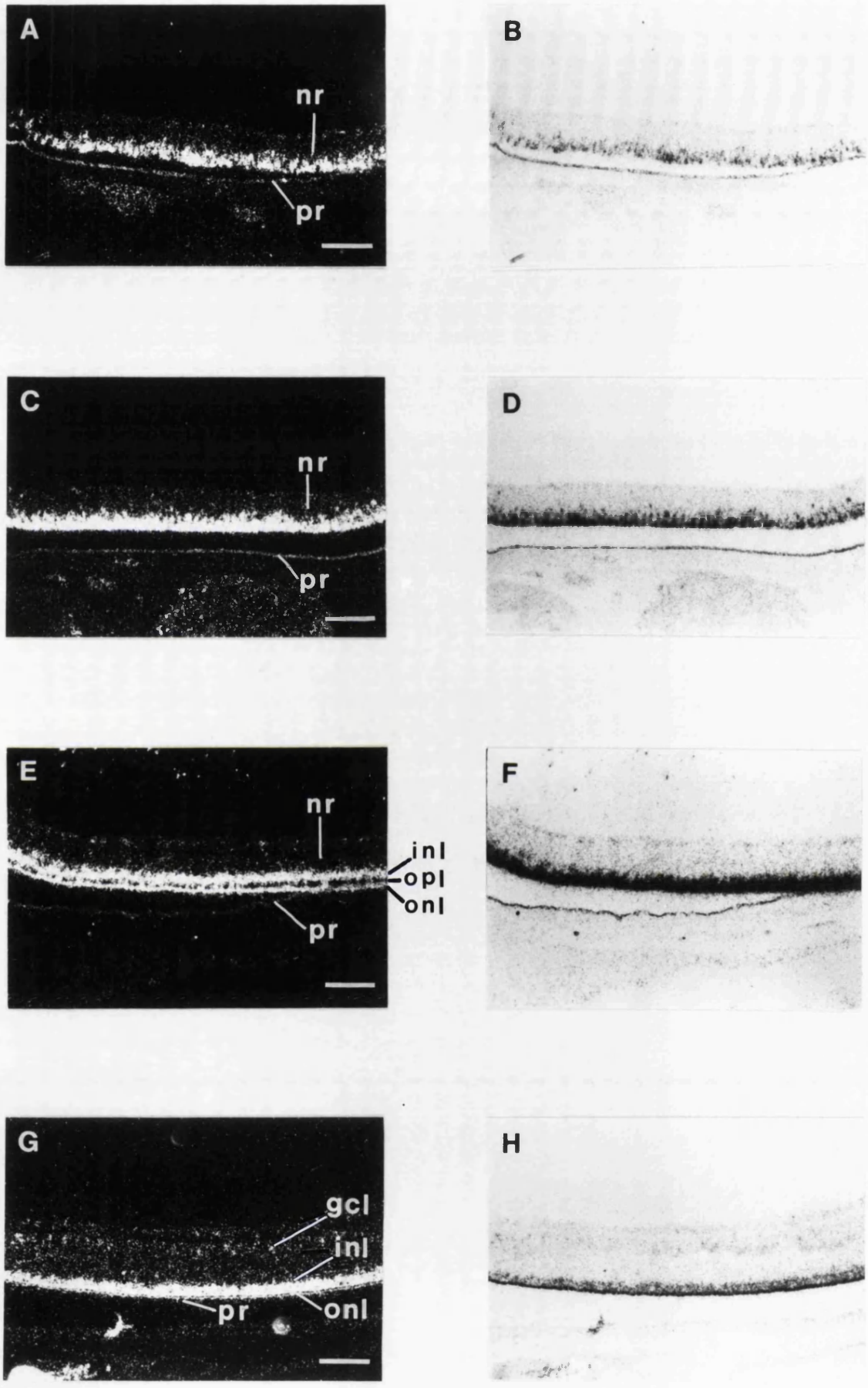


Fig. 3.8

Figure 3.8

Higher magnifications of the sections of chick embryo eye presented in the previous figure, showing in greater detail the way in which the expression of cRXRγ transcripts in the developing chick retina evolves as the differentiation and segregation of the different cell types of the neural retina takes place.

Panels A, C, E and G show dark-field views of the sections of the eyes of chick embryos at stages 27, 29, 32, and 37 respectively, hybridized with a probe specific for cRXRγ transcripts; panels B, D, F and H show a bright-field view of the same regions. Notice in panel E, the existence of a region where no hybridization of the probe is observed (opl.), between the two regions expressing the highest levels of chicken RXRγ transcripts (inl. and onl.). This pattern is not readily apparent in panel G due to over-exposure. nr - neural retina; pr - pigmented retina; gcl - ganglion cell layer; inl - inner nuclear layer; ipl - inner plexiform layer; onl - outer nuclear layer; opl - outer plexiform layer.

Scale bar = 100 μm.

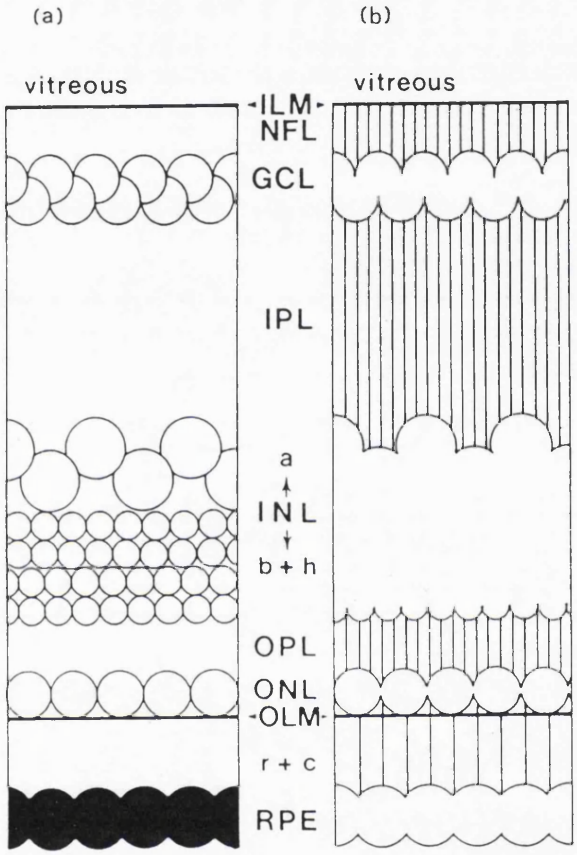


Figure 3.9

Schematic illustration of the cellular organisation of the mature avian neural retina. The location of the nuclear layers of the neural retina is shown in (a) with cell bodies being represented as circles. The anuclear layers are presented as vertical lines in (b) (not drawn to scale). ILM, inner limiting membrane; NFL, nerve fiber layer: GCL, ganglion cell layer; IPL, inner plexiform (synaptic) layer; a, amacrine cell bodies; b+h, bipolar and horizontal cell bodies; INL, inner nuclear layer; OPL, outer plexiform (synaptic) layer; ONL, outer nuclear layer; OLM, outer limiting membrane; r+c, rods and cones (photoreceptor processes); RPE, retinal pigmented epithelium (reproduced from Bee, 1982).

clear bands where no expression is observed, corresponding to the inner and outer plexiform layers. These bands become progressively better defined, until by day 10-11, the expression of the chicken RXRγ gene reflects exactly the stratified cellular pattern of the mature retina (fig. 3.8, panels G and H, and fig. 3.9).

3.3.2 - RXRγ gene expression in the developing chick embryo liver.

In vertebrates, the liver primordium starts as an outgrowth of the endoderm of the primitive gut, in the region which will later become the duodenum, the so called hepatic diverticulum or liver bud. This epithelial outgrowth, rapidly grows into the surrounding mesoderm, developing into highly branched cords of cells. In the most distal regions these cells will differentiate into the functional parenchymal cells of the liver, whilst the cords closest to the gut will become the lining of the biliary ducts (Sadler, 1885; Browder *et al.*, 1991).

The presence of RXRγ transcripts in the chicken embryo liver primordium is detected as early as stage 18 (fig. 3.10), in a pattern that suggests its expression in the endodermal cords of differentiating parenchymal liver cells, but not in the surrounding mesodermal tissue. As the liver develops, expression becomes more uniform, and is maintained like that throughout the rest of development up to adulthood.

3.3.3 - RXRγ gene expression in chicken neural crest cells and derivatives

As described in Chapter I, the neural crest is a population of pluripotent cells that originate from the margins of the neural plate at the

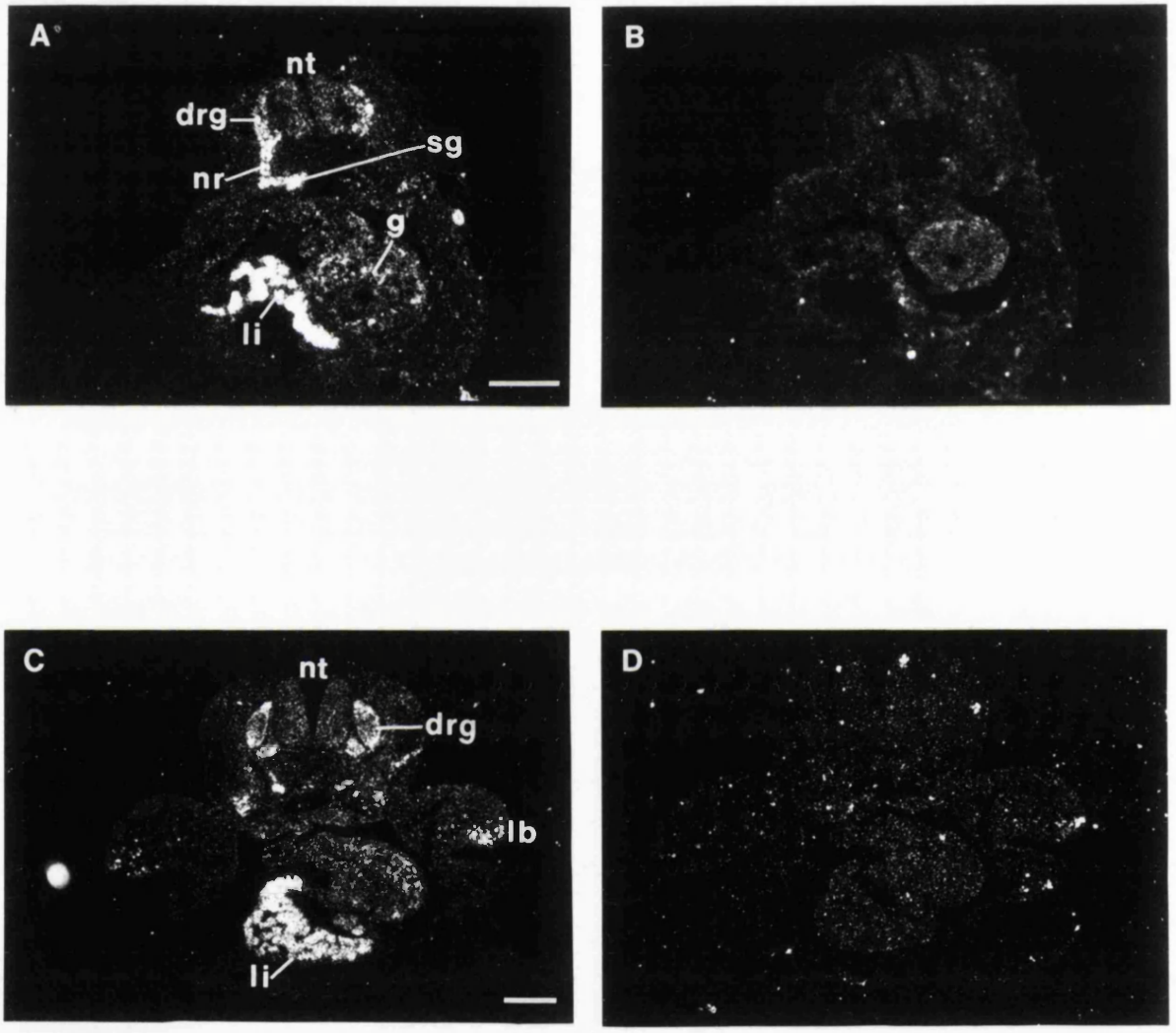


Fig. 3.10

Figure 3.10

Expression of cRXRγ transcripts in the developing chick embryo liver.

Panels A and C show transverse sections at the level of the trunk of chick embryos at stages 18 and 22 respectively, hybridized with a probe specific for cRXRγ transcripts; panels B and D show the corresponding adjacent sections, hybridized with the negative control probe. Notice the granular appearance of the expression of cRXRγ transcripts in the early chicken liver. drg - dorsal root ganglia; g - gut; li - liver; lb - limb bud; nr - nerves; nt - neural tube; sg - sympathetic ganglia.

Sections are shown under dark-field illumination. Scale bar = 1 mm.

time of neural tube closure, and that migrate extensively throughout the body of the embryo, before differentiating into a variety of cell types.

Rowe *et al.* (1991), showed that the earliest detectable expression of the chicken RXRγ gene is in migrating neural crest cells. Punctate expression is first seen as a stream of cells coming from the margins of the newly closed mid and hindbrain, progressively expanding caudally as the trunk neural tube closes (stages 12-14) (fig. 3.11).

Cranial neural crest cells contribute to the cranial nerves and ganglia, and give rise to melanocytes and to the mesenchymal cells of the facial primordia and pharyngeal arches. These will form the bones and connective tissue of the face and base of the skull, and contribute to the cardiac outflow tract and to the formation of the thymus (LeDouarin 1982). Neural crest cells from the trunk region give rise, amongst other structures, to the elements of the peripheral nervous system, to the chromaffin cells of the adrenal glands and to melanocytes.

Although chicken RXRγ seems to be expressed in the earlier stages in most cranial and trunk migrating neural crest cells, this expression is only retained in the subset of cells that do not undergo mesenchymal differentiation (Rowe *et al.*, 1991 and unpublished data), becoming restricted to the cells of the peripheral nervous system, to the adrenal glands and possibly to the melanocytes.

3.3.3.1 - RXRγ gene expression in the developing chick peripheral nervous system

By stage 17/22, expression of the chicken RXRγ gene is detected in the condensing masses of cells that will form the cranial ganglia and nerves (fig. 3.11), and the trunk dorsal root ganglia, sympathetic chain, and enteric plexuses, as well as the nerves that connect them to each other and to the spinal cord, such as the dorsal and ventral roots, the spinal nerves,

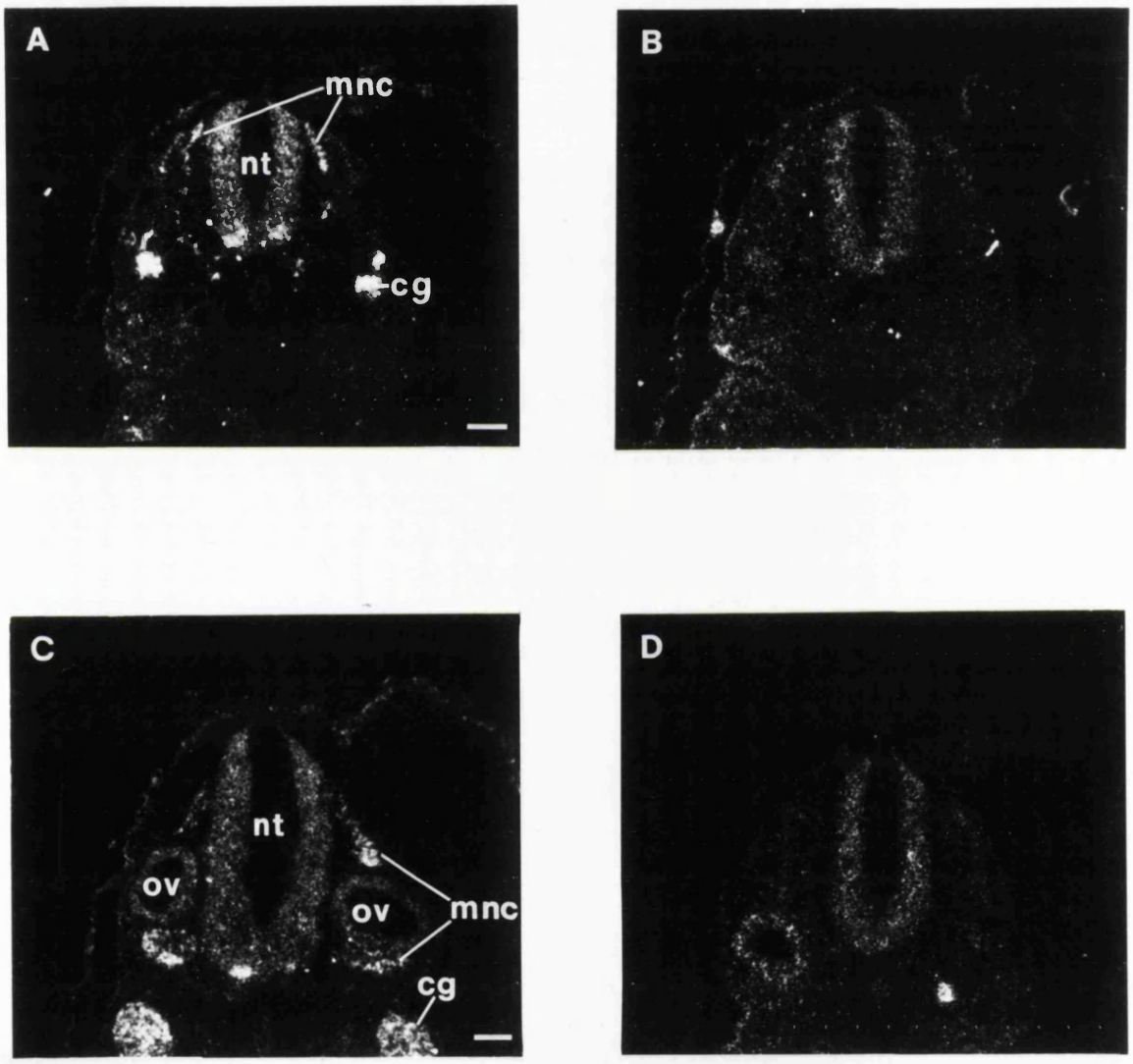


Fig. 3.11

Figure 3.11

Expression of cRXRγ transcripts in migrating neural crest cells.

Panels A and C show transverse sections at the level of the hindbrain of stage 17 chick embryos, hybridized with a probe specific for cRXRγ transcripts; panels B and D show the corresponding adjacent sections, hybridized with the negative control probe. cg - cranial ganglia; mnc - migrating neural crest cells; nt - neural tube; ov - otic vesicles.

Sections are shown under dark-field illumination. Scale bar = 100 μm.

and all the other nerve roots (fig. 3.12). From these stages onwards, high levels of expression of this gene are detected in all the elements of the peripheral nervous system, continuing into adult life. No expression is ever detected in the neural tube, in particular, no expression is detected in the regions containing the cell bodies of the neurons whose axons extend out and form part of the peripheral nerves, such as the motor neurons of the somatic nervous system and the preganglionic neurons of the autonomic nervous system, which have their cell bodies respectively in the ventral and intermediate horns of the neural tube.

The contribution of the neural crest to the peripheral nervous system includes both neuronal and glial cell types. The detection of chicken RXRγ gene expression along the length of the nerve roots, indicates that this gene must be expressed in the glial cells of the peripheral nervous system. In addition, the high levels of signal detected in sympathetic ganglia of 7 day old embryos, both on northern blots of total RNA (fig. 3.3) and on in situ hybridization, at a time when the cells present in these structures are almost exclusively neurons (aproximately 90-95%, H. Rhoehrer, personal communication), strongly suggests that the chicken RXRγ gene is also expressed in neuronal cells.

To determine whether the chicken RXRγ gene is indeed expressed in peripheral nervous system neurons, I performed a series of in situ hybridizations using the same probe, on dissociated dorsal root ganglia or dissociated sympathetic ganglia cultures, from 7 and 10 day old chicken embryos. Most of the cells present in both types of cultures seemed to express RXRγ transcripts, with the exception of a few cells that had the distictive morphology of fibroblasts. In the dorsal root ganglia cultures, strong signal was clearly detected in spindle shaped-glial cells (fig, 3.13, panels A and B). In these same cultures however, expression in the neuronal cells was very hard to judge, due to the pronounced spherical shape of their cell bodies. This causes these cells to be highly refractile,

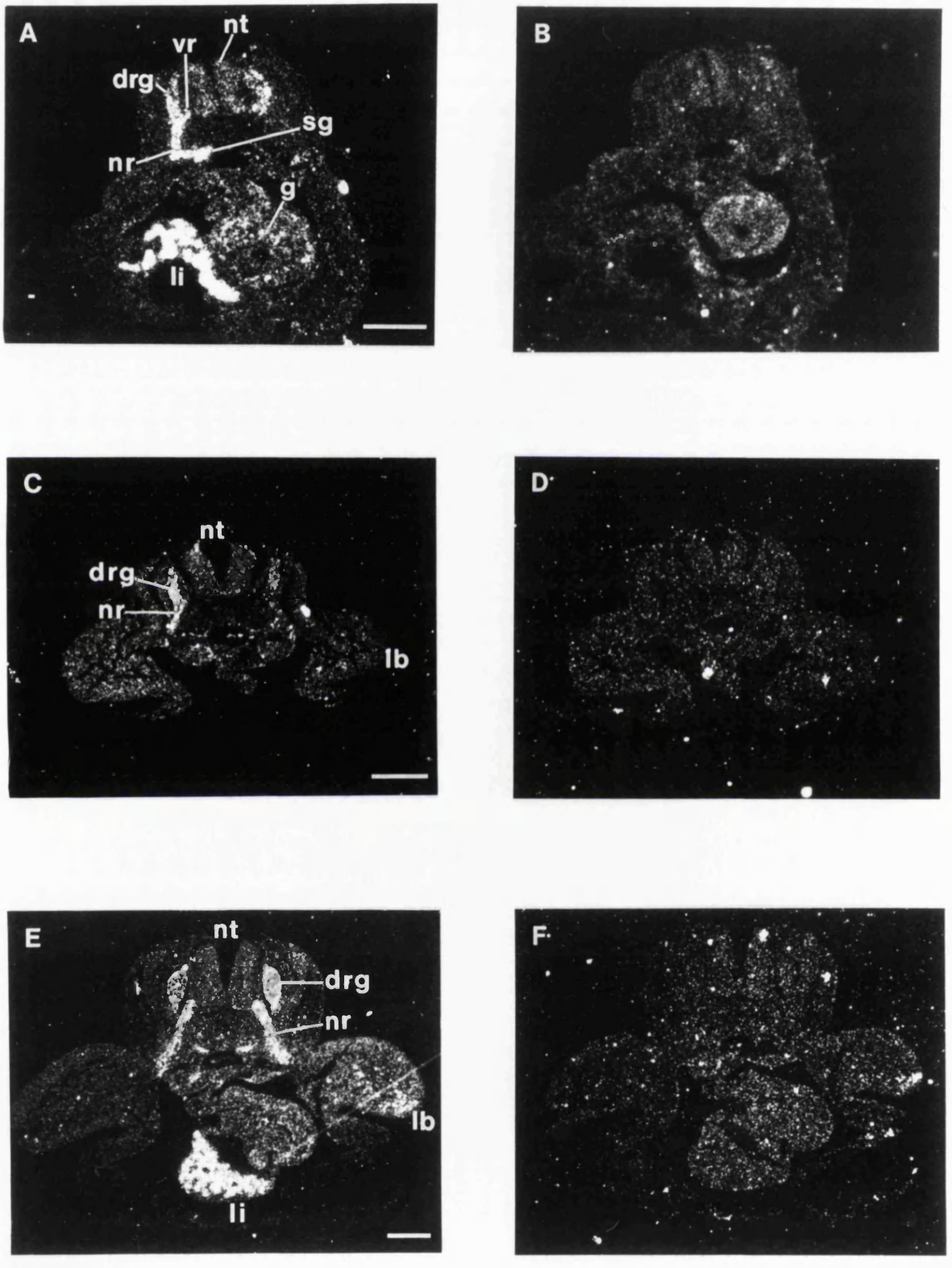


Fig. 3.12

Figure 3.12

Expression of cRXRγ transcripts in the developing peripheral nervous system of the chick embryo.

Panels A, C and E show transverse sections at the level of the wing buds of chick embryos at stages 18, 20 and 22 respectively, hybridized with a probe specific for cRXRγ transcripts; panels B, D and F show the corresponding adjacent sections, hybridized with the negative control probe. drg - dorsal root ganglia; g - gut; li - liver; lb - limb bud; nt - neural tube; sg - sympathetic ganglia; nr - nerves; vr - ventral root.
Sections are shown under dark-field illumination. Scale bar = 250 μm.

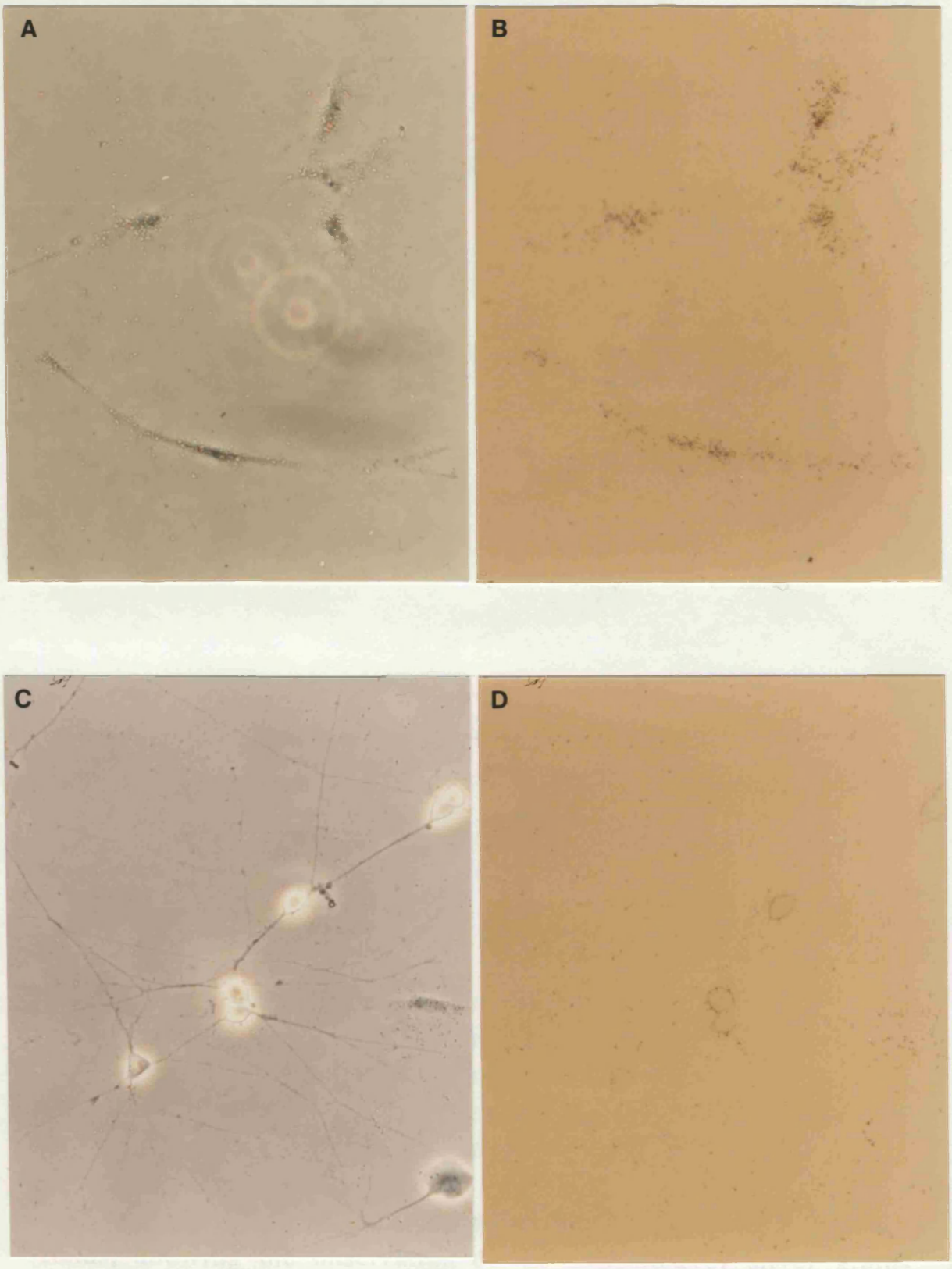


Figure 3.13

Figure 3.13

Expression of cRXRγ transcripts in stage 36 (approximately 10 days of incubation) chick dissociated dorsal root ganglion cultures.

Panels A and C show the phase contrast views of, respectively, a group of glial cells and a group of neurons, from a stage 36 chick dissociated dorsal root ganglion culture hybridized with a probe specific for cRXRγ transcripts.

Panels B and D show the corresponding bright field views of the same fields, allowing the observation of the deposited of silver grains corresponding to the hybridisation of the probe.

Magnification x360.

making it difficult to see the silver grains (fig, 3.13, panels C and D), and also produces an artefact on the photographic emulsion, an "edge effect" that generates the deposition of silver grains around these cells even in the negative controls. Sympathetic ganglia neurons however, are much flatter, and therefore in these cultures we were able to confirm that there is expression of chicken RXRγ in peripheral nervous system neurons (fig, 3.14).

3.3.3.2 - RXRγ gene expression in the developing chick adrenal glands

In mammals, the adrenal glands are divided into two regions with distinct functions which have separate embryonic origins: the cortex which is derived from mesodermal cells, and the medulla which contains the choromaffin cells of neural crest origin. Functionally, the chromaffin cells of the adrenal glands can be considered as forming the endocrine part of the sympathetic autonomic nervous system, and therefore it is not surprising that both groups of cells share the same embryonic origin.

In the chicken, the adrenals are not organised into cortex and medulla, but the chromaffin cells are simply arranged in clusters scattered between the cortical cells. This organisation correlates well with the type of signal that we detect in the in situs on sections of 7 and 10 day old embryos, where a region of patchy expression of RXRγ transcripts was seen immediately adjacent to the rostral tip of the definitive kidney (fig. 3.15).

3.3.3.3 - RXRγ gene expression in chick melanocytes

A small group of cranial and trunk neural crest cells, migrate laterally and superficially under the ectoderm, until they eventually enter the

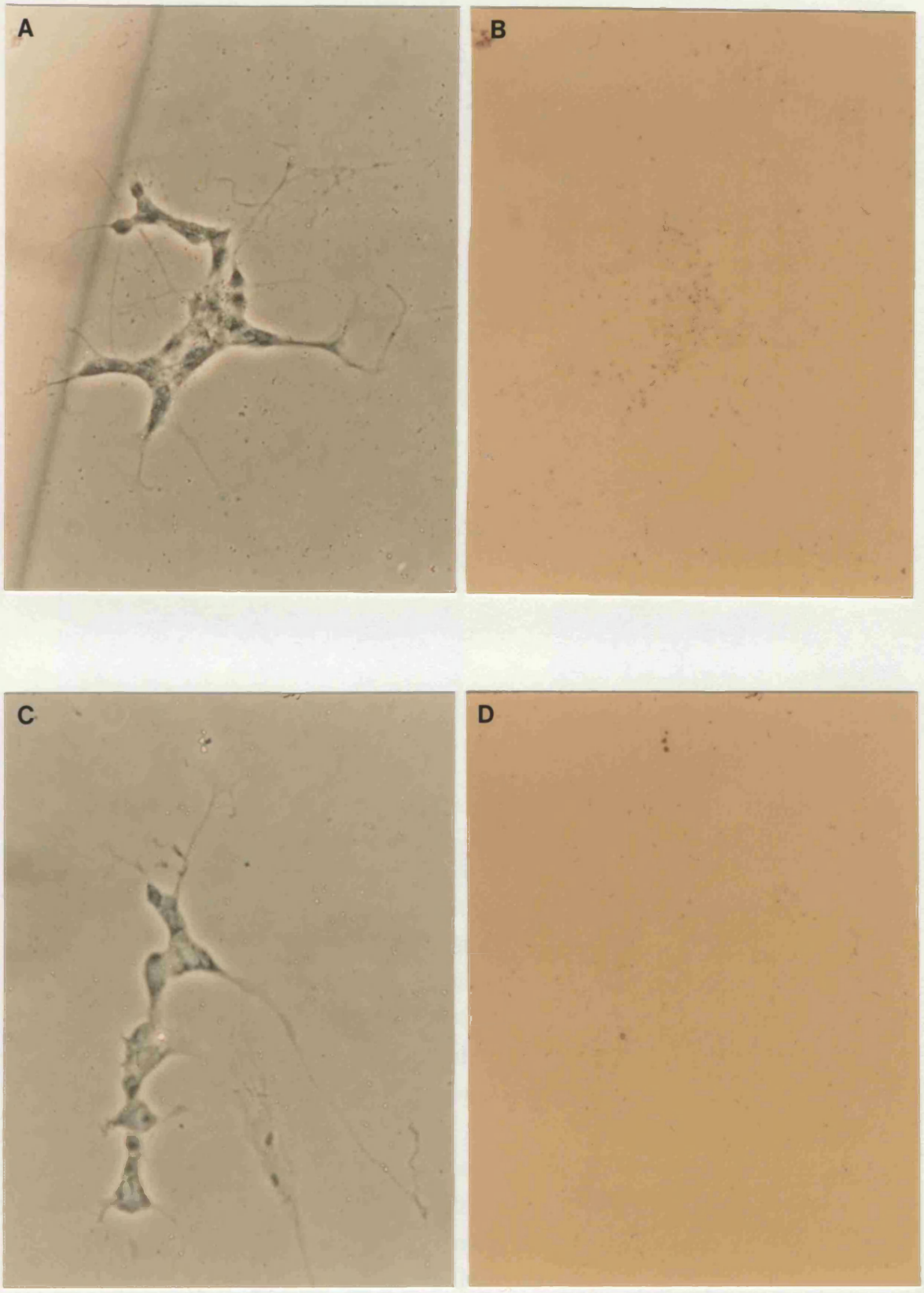


Figure 3.14

Figure 3.13

Expression of cRXRγ transcripts in stage 31 (approximately 7 days of incubation) chick dissociated sympathetic ganglion cultures.

Panel A shows the phase contrast view of a group of neurons, from a stage 31 chick dissociated sympathetic ganglion culture hybridized with a probe specific for cRXRγ transcripts. Panel B shows the corresponding bright field view of the same field, allowing the observation of the deposited of silver grains corresponding to the hybridisation of the probe.

Panel C shows the phase contrast view of another group of neurons, from a stage 31 chick dissociated sympathetic ganglion culture, hybridized with the negative control probe. Panel B shows the corresponding bright field view of the same field.

Magnification x360.

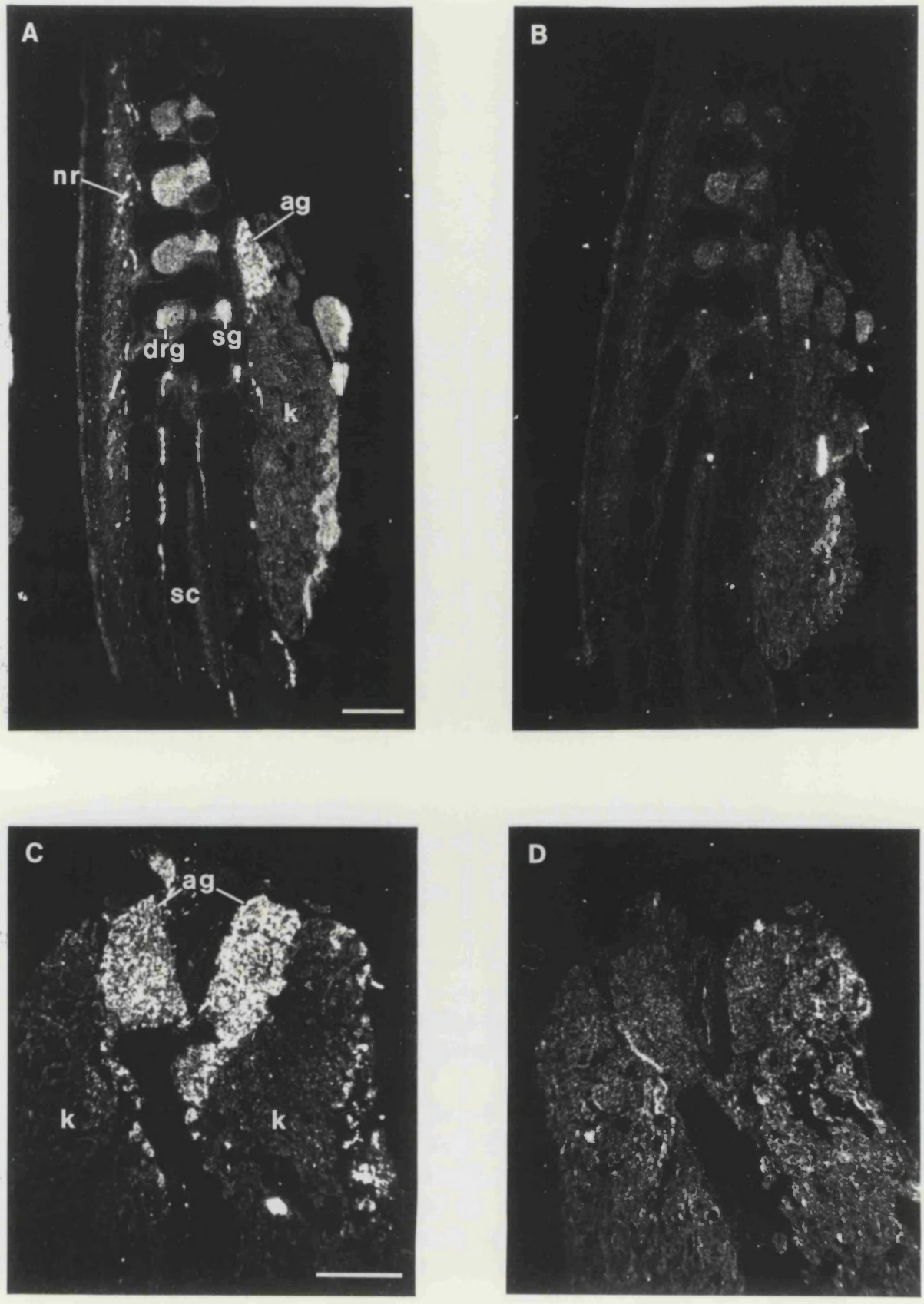


Fig. 3.15

Figure 3.15

Expression of cRXRγ transcripts in the periphereal nervous system and in the adrenal glands of stage 36 (approximately 10 days of incubation) chick embryos.

Panel A shows a parasaggital section of the body of a stage 36 chick embryo, hybridized with a probe specific for cRXRγ transcripts; panel B shows an adjacent section, hybridized with the negative control probe.

Panel C shows a frontal section of the kidneys of a stage 36 chick embryo, hybridized with a probe specific for cRXRγ transcripts; panel D shows an adjacent section, hybridized with the negative control probe.

ag - adrenal glands; drg - dorsal root ganglia; k - kidney; nr - nerves; sc - spinal chord; sg - sympathetic ganglia

Sections are shown under dark-field illumination. Scale bar = 500 μm.

dermis and epidermis and differentiate into the pigmented cells of the skin hairs and feathers, the melanocytes. Although the embryos used throughout this work were of an unpigmented strain of chickens (White Leghorn), the migration and early differentiation of the melanocytes in these animals does not seem to be affected: cells that produce melanin and are morphologically similar to normal melanocytes are seen in approximately normal numbers, in the skin and early feather papillae of white leghorn embryos at up to day 9 of incubation. After this point, the melanocytes of white leghorn embryos become progressively less dendritic and contain lower numbers of mature melanosomes than their normal counterparts, and seem to be unable to export melanosomes into the surrounding keratinocytes, until by approximately day 14-15 they degenerate and die (Jimbow *et al.*, 1974).

Rowe *et al.* (1991), detected the presence of chicken RXRγ transcripts on the dorsal surface of the wing buds of stage 24 embryos, in a region that coincides with the migratory pathway of the pre-melanocytic neural crest cells. This observation however, does not prove that there is expression of this gene in differentiated melanocytes, because, as in the case of the cranial neural crest cells that undergo mesectodermal differentiation, the expression of this gene could be downregulated as these cells start to differentiate.

At later stages however, it becomes very difficult to detect the expression of any gene in melanocytes by radioactive in situ hybridization. The melanin granules that accumulate inside these cells, are virtually indistinguishable from the silver grains of the photographic emulsion, both under light and dark field microscopy.

In sections of 7-10 day old embryos, a weak punctate "signal" was detected in the skin and feather papillae, but careful examination revealed

that it was present both in sections hybridized with the antisense and the sense (negative control) probes (fig. 3.16).

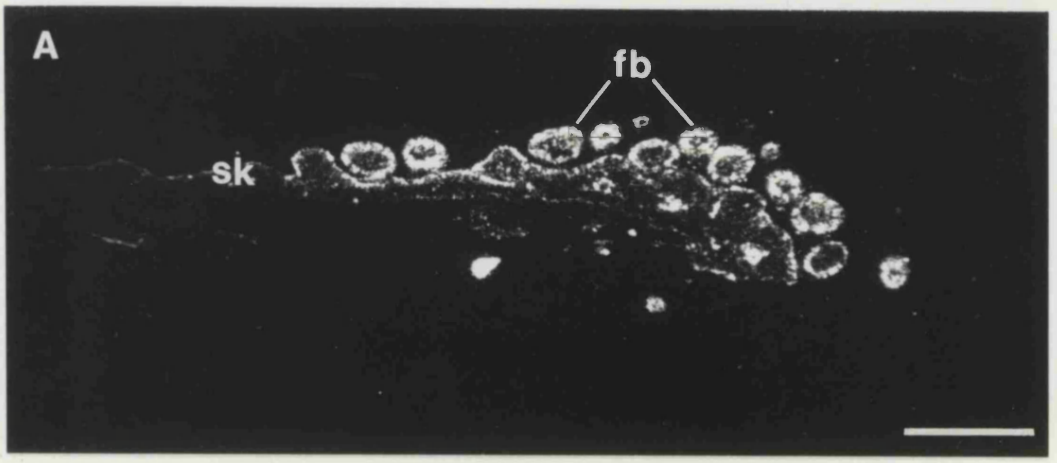
In an attempt to determine whether chicken RXRγ was genuinely expressed in differentiating melanocytes, we planned to perform a series of in situ hybridizations on 5-7 day chick embryo ectodermal cultures prepared by Dr. Michael Richardson. In these cultures, a small piece of ectoderm, usually from the wing bud, was isolated and cultured for 2 to 7 days in medium supplemented with foetal calf serum. During this time, some of the pre-melanocytic cells migrate away from the sheet of ectoderm, and continue differentiating, accumulating melanin and assuming their characteristic morphology. We expected to be able to get a clear result in the melanocytes present in these cultures before they started to produce melanin. However, we found that as soon as these cells could be identified by their morphology, they had already started to accumulate considerable amounts of melanin. Therefore, the question of whether or not there is real expression of the chicken RXRγ gene in melanocytes remains open.

3.3.4 - Other sites of RXRγ gene expression in the chick embryo

Expression of RXRγ transcripts was also detected in other regions of chicken embryos, that do no seem to have a direct correlation with any of the structures described above:

- Expression in the limb buds:

Intense punctate hybridization is detected in various regions of the mesenchyme of the wing buds of embryos at stages 20-24 (fig. 3.5, arrows in panels E and G). This signal is too intense and is in the wrong place (too deeply in the mesenchyme), to correspond to expression of chicken RXRγ in the migrating pre-melanocytic cells. It is strongly



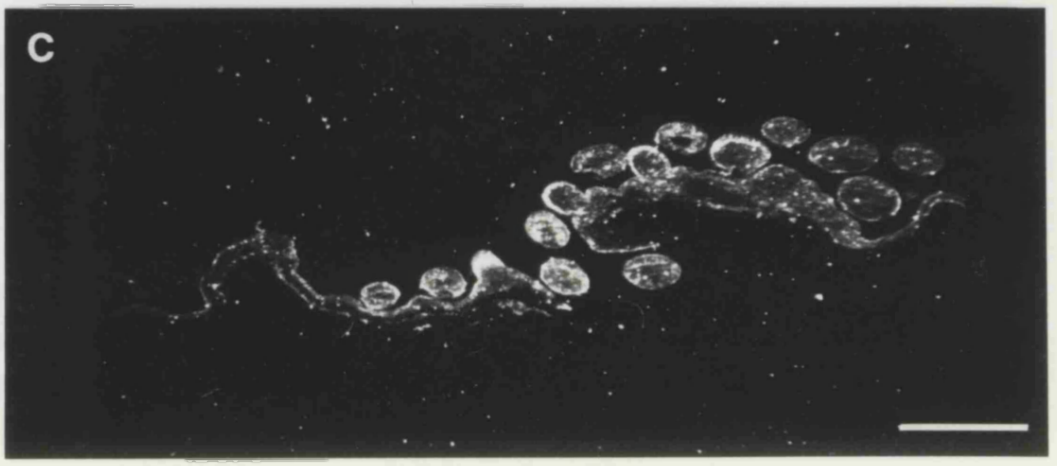


Fig. 3.16

Figure 3.16

Analysis of the expression of cRXRγ transcripts in the skin of stage 36 (approximately 10 days of incubation) chick embryos.

Panel A shows a section of stage 36 chick embryo skin, hybridized with a probe specific for cRXRγ transcripts; panel B shows the corresponding adjacent section, hybridized with the negative control probe.

Panel C shows an unhybridized section of stage 36 chick embryo skin.

sk - skin; fb - feather buds.

Sections are shown under dark-field illumination.Scale bar = 500 μm.

reminiscent of the punctate hybridization observed at later stages in the muscle of 7-10 day embryos that corresponds to the expression of this gene in the nerve roots of the peripheral nervous system (figs. 3.6 and 3.15). However, at the stages at which this punctate signal is detected in the limb buds, the peripheral nerves are just starting to extend into the proximal regions of the mesenchyme, where hybridization of the chicken RXRγ probe can also be observed (fig. 3.5, panels E and G).

In the preliminary characterization of the early expression pattern of the chicken RXRγ gene, carried out by Dr. A. Rowe, this type of punctate signal in the limb buds was not observed, either in sections or in wholemount in situs of embryos at these same stages. Therefore, it is possible that this punctate signal is simply an artefact, and does not correspond to real localised expression of this gene. Nevertheless, I would like to stress that a comparable "artefact" was never observed with the control probe of chicken RXRγ, or with the antisense or sense probes for chicken RXRα.

- Expression in the heart

In sections through the heart of 7-10 day old embryos, expression of the chicken RXRγ gene is detected in the atrioventricular valves (arrows in fig. 3.17). These structures are originated from the mesodermal endocardial cushion cells, and do not seem to have any contribution from the cranial neural crest cells, which as described in chapter I, give rise to part of the cardiac outflow tract, which as all the other mesectodermal neural crest derivatives, do not express chicken RXRγ transcripts.

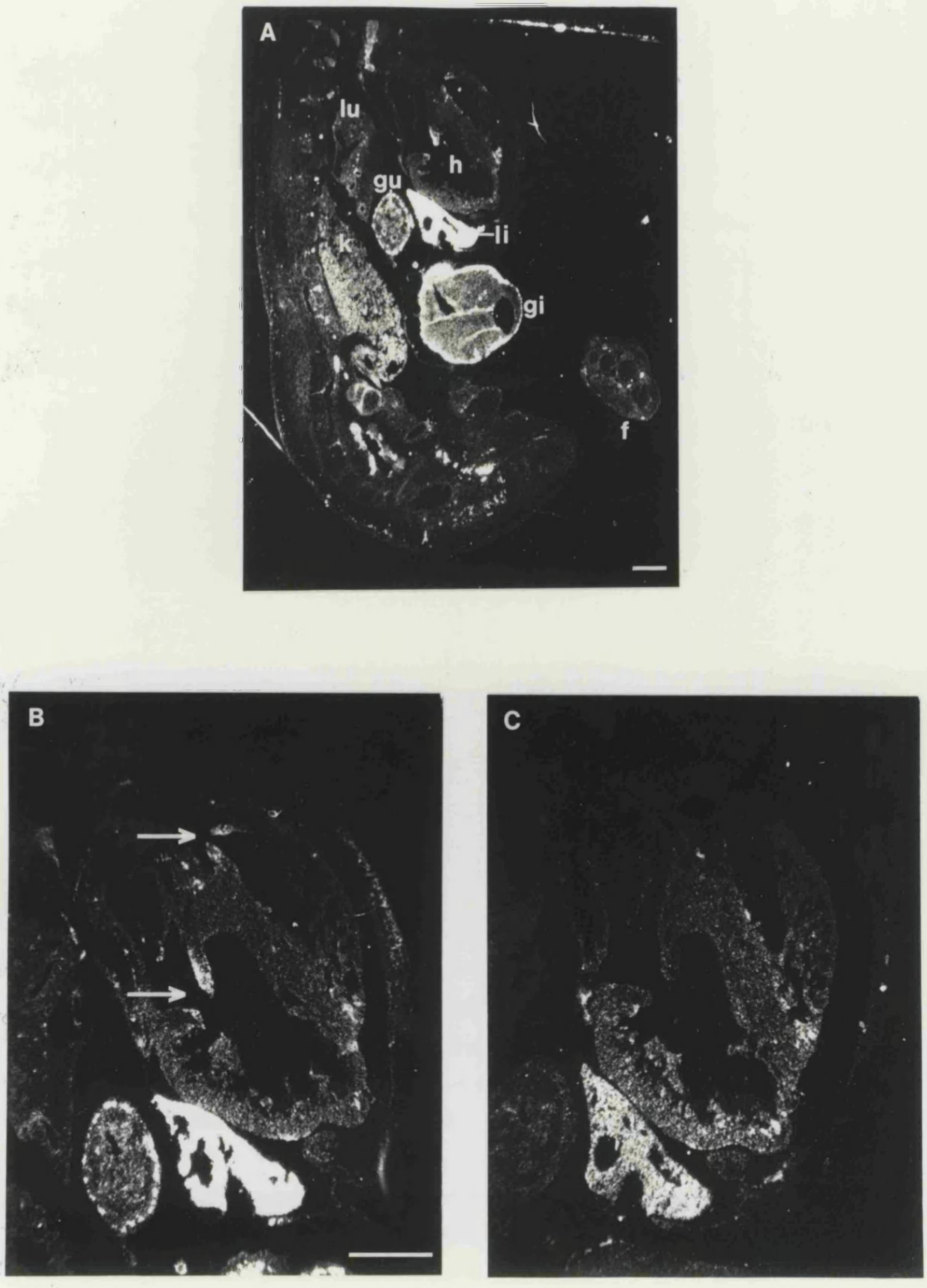


Fig. 3.17

Figure 3.17

Expression of cRXRγ transcripts in the heart valves of stage 31 (approximately 7 days of incubation) chick embryos.

Panel A shows a parasaggital section of the body of a stage 31 chick embryo, hybridized with a probe specific for cRXRγ transcripts.

Panel B shows a higher magnification view of the section presented in panel A, showing in greater detail the expression detected in the heart valves (arrows); panel C shows the corresponding adjacent section, hybridized with the negative control probe.

f - foot; gu - gut; gi - gizzard; h - heart; k - kidney; li - liver; lu - lung.

Sections are shown under dark-field illumination. Scale bar = 500 μm.

3.4 - Analysis and discussion of the results

The results presented in this chapter demonstrate that the pattern of expression of the chicken RXRγ gene in the adult is essentially similar to that observed in the embryo, and therefore, that the expression of this gene is maintained in the same tissues throughout development and differentiation, with the exception of the cranial neural crest derived mesectodermal structures.

Secondly, these results also demonstrate that the expression of the RXRγ gene in the chicken, is not limited to the liver and to the elements of the peripheral nervous system, but includes several other structures with distinct embryological origins, such as the adrenal glands and the eye.

The present work confirmed the earlier observations by Rowe *et al.* (1991), which identified the migrating cranial and trunk neural crest cells as the earliest site of expression of the RXRγ gene in the chicken embryo. As these cells differentiate and reach their destination, there is a progressive decrease in the proportion of cranial neural crest cells expressing high levels of this gene (Rowe *et al.*, unpublished results), and at later stages, no expression is observed in any of the mesectodermal derivatives of the cranial neural crest cells.

These results suggest that the chicken RXRγ gene is more or less widely expressed in early migrating cranial and trunk neural crest cells, and then becomes progressively restricted, as these cells become committed to their distinct differentiation pathways. The implications of this switch in the expression of the chicken RXRγ gene in the mesectodermal subset of cranial neural crest cells deserve to be carefully investigated, but at the moment we can only speculate about a possible

role of this gene in the regulation of neural crest cell commitment and differentiation.

As for the remaining (non mesectodermal) neural crest derivatives, the data presented in this chapter shows that there is expression of the chicken RXRγ gene in both the glial and neuronal cells of the nerves and ganglia, and in the chromaffin cells of the adrenal glands. However, I could not unequivocally determine the expression of this gene in other neural crest derivatives, such as the differentiated melanocytes. For this reason it is not possible to establish whether the expression of the chicken RXRγ gene is widespread in all non mesectodermal neural crest derivatives, or whether it is specifically associated with the peripheral nervous system cells (including the chromaffin cells), and corresponds to some specific functional characteristic common to this group of cells.

As described in the introduction, neural crest cells are one of the major targets of the teratogenicity of retinoids. However, these teratogenic effects seem to be restricted to the mesectodermal structures derived from cranial neural crest cells, precisely the ones where the expression of the RXRγ gene is turned off, and seem to be more of a consequence of the effects of RA in the patterning ot the regions of the neural tube from where the neural crest cells arise, than a direct effect on the differentiation of the neural crest cells themselves.

No clear teratogenic effects of retinoids have been observed in the non mesectodermal neural crest derived-structures where the RXRγ gene is expressed, such as the peripheral nervous system or the adrenal glands. Vitamin A deficiency has been associated with degeneration of the cranial and peripheral nerves, and of the grey and white matter of the spinal cord, but this was interpreted as an indirect result of the effects of the low levels of vitamin A on bone growth, although an active role in the

differentiation and maintenance of these structures could not be ruled out (Lohnes *et al.*, 1993).

In later embryos, the highest levels of expression of chicken RXRγ transcripts are detected in the liver and in the retina, suggesting a possible involvement of this receptor in vitamin A storage and metabolism.

However, on a more careful examination, there is not an obvious correlation between the cellular expression pattern the chicken RXRγ gene in the retina, and vitamin A metabolism in this tissue. The expression of the chicken RXRγ gene in the neural retina, is not strictly associated with those cells directly involved in the production of the visual pigments from retinol, namely the rods and cones. Also, there is no expression of chicken RXRγ transcripts in the cells of the pigmented retina, the cells that are involved in the absorption and translocation of retinol from the plasma to the photoreceptor cells. These results seem therefore to argue against a direct involvement of this receptor in the regulation of the production of visual pigments from retinol. It is possible however, that chicken RXRγ has some indirect role in the regulation of vitamin A metabolism in the eye.

Together with its importance in vitamin A metabolism, the retina is also a major site of production of retinoic acid, both in the embryo and in the adult. Strong β-galactosidase expression was detected in the retina of mice transgenic for a reporter construct containing the β-galactosidase gene under the control of the retinoic acid response element of the RARβ gene, at all stages of embryonic development (Rossant *et al.*, 1991). Similarly, rough estimation of the local levels of retinoic acid in cellular extracts of several embryonic mouse tissues, using a reporter cell line containing an analogous βRARE-lacZ reporter plasmid, placed the retina amongst the tissues with the highest endogenous content of RA, exceeded only by the spinal cord (McCaffery *et al.*, 1992). Furthermore, this study

also showed that RA had an asymetric distribution in the eye, with higher levels being found in the ventral half of the retina, suggesting that RA could play some role in the positional specification along the dorso/ventral axis of this organ (McCaffery *et al.*, 1992).

Several other retinoid receptors and binding proteins are also expressed in the mouse embryonic eye. These are, RARα (Dollé *et al.* 1990), RXRβ (Mangelsdorf *et al.*, 1992), CRBP and CRABP (Dollé *et al.*, 1990). However, none of these has such a restricted expression pattern, and is expressed at such high levels in the retina as the chicken RXRγ gene. It is important to stress however, that no expression of the RXRγ gene has been detected in the mouse retina., making it unlikely that RXRγ could mediate this putative patterning effect of RA during the development of the retina.

Chicken RXRγ could however be involved in the regulation of other events in the development and differentiation of the retinal cells. In this respect it is interesting to notice that the way in which the early expression of chicken RXRγ expands along the developing retina is closely reminiscent of the progression of the differentiation of the retinal neurons, which proceeds in a wave from the base to the margins of the optic cup (see fig. 3.7). It seems therefore possible, that RXRγ may play some role in the regulation of the differentiation of the retinal neurons.

Probably the more intriguing conclusion that emerged from these results is that the pattern of expression of the RXRγ gene in the chicken described in this chapter, is strikingly different from the expression pattern observed for this same gene in the rat and mouse (Mangelsdorf *et al.*, 1992; Georgiades and Brickell, unpublished results). As described in the introduction, in the rat and mouse embryos, RXRγ transcripts are predominantly expressed in two very localised regions of the developing

brain, in the somites and in the ventral horns of the spinal cord. In the adult rat, the RXRγ gene is predominantly expressed in the heart and in the muscle, and also at lower levels in the liver, brain, kidney and adrenal glands. Therefore, the only regions where this gene seems to be expressed in both animals, are the adult liver and the adult adrenal glands.

The fundamentally different expression patterns of the RXRγ gene observed in these two organisms are difficult to understand, and seem to preclude any possibility of RXRγ having a precisely conserved function across species.

Furthermore, the expression pattern of chicken RXRγ gene reported here, does not show any similarities with the expression patterns of any of the genes for other retinoid receptors or binding proteins, both in the chicken and in the mouse, apart from a very vague analogy to the expression pattern of the chicken and mouse CRABP genes. In mouse embryos, both CRABP I and II are expressed in the migrating neural crest cells, and CRABP II expression is maintained during development in the cranial- dorsal root- and spinal ganglia (Ruberte *et al.*, 1993); expression of CRABP transcripts in the mouse eye is also restricted to the neural retina, and seems to be associated with the differentiation of the ganglion cells (Dollé *et al.*, 1990). In the chicken embryo, CRABP expression is also found in migrating neural crest cells, and later, in their peripheral nervous system derivatives (Perez-Castro *et al.*, 1989; Maden *et al.*, 1989 and 1990)

However, the chick and mouse CRABP genes are also expressed in a range of other tissues where no expression of the chicken RXRγ gene is ever observed. This, together with the fact that the expression pattern of the RXRγ gene in the mouse is significantly different to that observed in the chicken, make it difficult to envisage a direct connection between the expression of these two genes.

Finally, in addition to the complexity of the distribution pattern of chicken RXRγ transcripts, the results presented in this chapter also revealed that the expression of this gene is further complicated by the existence of two different sized transcripts, each with specific tissue distributions.

This structurally complex pattern of expression of the RXRγ gene is also observed in the mouse, where two transcripts of different sizes with overlapping tissue distributions have been identified (Mangelsdorf *et al.*, 1992; Liu and Linney, 1993). The mouse RXRβ gene is also expressed as two transcripts, but in this case, both mRNAs have identical tissue distributions. Similar complex expression patterns, where several transcripts are expressed in a tissue specific fashion, are also common amongst the members of the RAR family.

We decided therefore, to isolate and characterise the structure of the two major transcripts of the chicken RXRγ gene. The results for this work are presented in the next chapter.

CHAPTER IV

CHARACTERISATION OF THE DIFFERENT CHICKEN RXRγ TRANSCRIPTS

4.1 - Introduction

In the previous chapter, I showed by northern blot hybridization, that the transcript of the chicken RXRγ gene present in embryonic and adult liver was smaller than that present in the other tissues expressing this gene (approximately 2.0 and 2.2 kb respectively). As described in the introduction, this type of complex expression pattern, with several transcripts of the same gene being expressed with a specific tissue distribution, is common amongst the members of the RAR, and RXR families. Each RAR gene is expressed as at least two major distinct mRNAs, that differ from each other only in their 5' untranslated regions and in the 5' end of the open reading frame, coding for protein isoforms that share the regions B-F but have different amino-terminal A domains.

Not much is known about the structure of the various trancripts of the RXR genes. Two distinct cDNA clones for human RXRβ have been identified so far, differing from each other only in the 5' untranslated regions and in the sequences coding for the A domain (Leid *et al.*, 1992; Fleischhauer *et al.*, 1992). Recently, a second cDNA clone for mouse RXRγ was isolated, that differs from the mouse RXRγ clone originally described, also only in the region 5' to the sequences coding for the B domain, but coding for a truncated version of the protein lacking the whole of the A domain (Liu and Linney, 1993). It seems therefore probable, that the two major transcripts of chicken RXRγ will have a structure similar to these, differing from each other in their 5' ends.

In this chapter I describe the isolation of the two major transcripts of the chicken RXRγ gene, and analyze the functional implications of these

differences, in the light of what is known about the structure and function of the different isoforms of the RARs, and of the mechanisms of action of the RXRs.

4.2 - Isolation of a cDNA clone corresponding to the liver specific transcript of the chicken RXRγ gene.

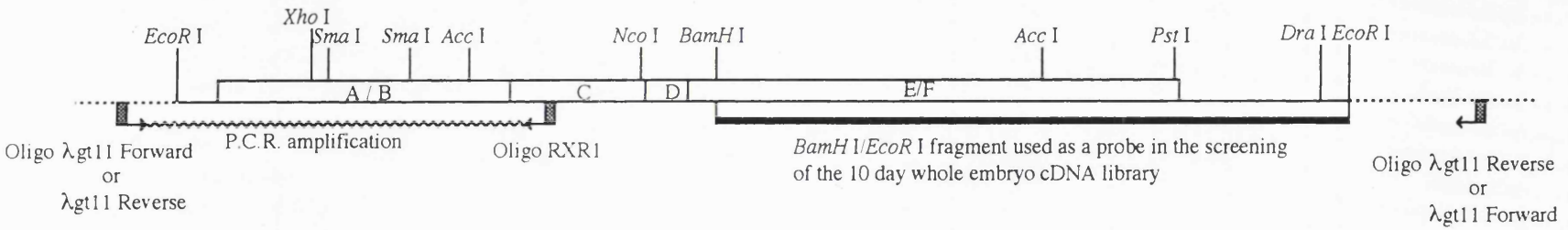
In an attempt to isolate the different cDNA clones corresponding to the various transcripts of chicken RXRγ, I screened a 10 day whole embryo cDNA library in λgt11 (Clontech), with a probe equivalent to that used in the northern blot hybridizations, which detects both major transcripts of this gene.

Membrane lifts were taken in duplicate as described in the methods section, from plates containing approximately 320 000 pfu, and hybridized with a radiolabelled DNA probe made from the insert of pR2-B/E (see appendix A), containing the sequences coding for the E-F domains and the 3' untranslated region of the cDNA clone pR2 (nucleotides 786-1713). The first round of screening resulted in the identification of a total of 26 presumptive positive clones.

Rapid screening of these presumptive positives for the presence of new RXRγ cDNA clones, was carried out by PCR amplification of the regions upstream of the DNA binding domain of chicken RXRγ, using a primer designed from the sequences present in positions 555-537 of pR2 (oligo-RXR1) and primers for sequences flanking the cloning site in the vector (λgt11 Forward or λgt11 Reverse) (fig. 4.1).

Of the 26 initial clones, 15 were specifically amplified using these primers. The size of the amplified products, allowed us to divide these positives into five different groups: three clones (clones number 12, 20 and 25) generated an amplified product of approximately 400 nucleotides; nine clones (clones number 2, 10, 13, 14, 15, 17, 19, 22, and

A) Screening strategy:



B) PCR products of the positive clones:

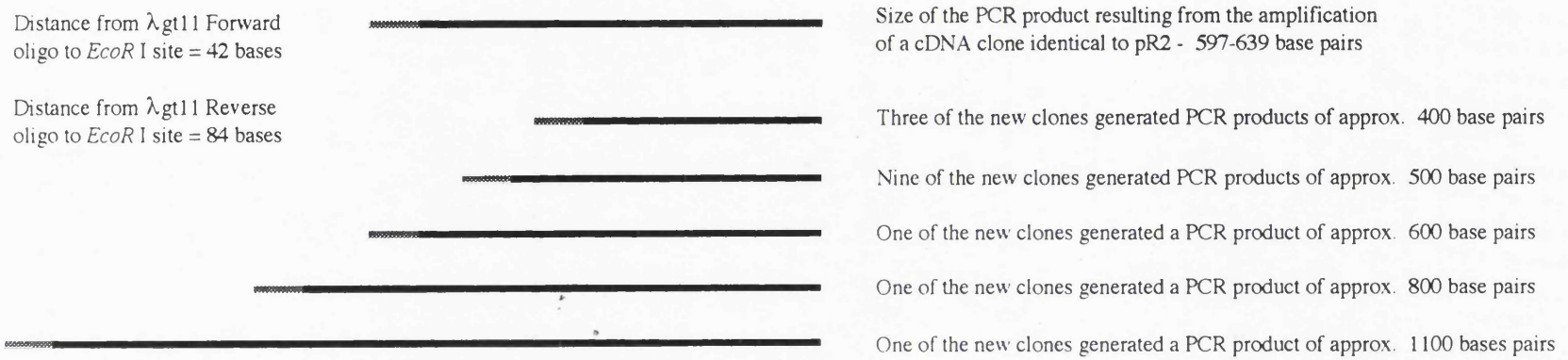


Figure 4.1

Figure 4.1

Strategy for the screening of the 10 day whole embryo cDNA library in λgt11, and rapid characterisation of the positive clones.

(A) A schematic representation of the screening strategy used, illustrated on a cDNA clone identical to pR2. Vector sequences are shown as dashed lines. Insert sequences are shown as solid lines. The coding region is boxed. The probe used in this screening is shown as a bold line. The position of the oligonucleotides used in the PCR amplification of the positive clones is shown.

(B) A schematic representation of the PCR products obtained from the amplification of the positive clones, using oligonucleotides RXR1 and λgt11Forvard or λgt11Reverse. The structure an amplified product generated from a cDNA clone identical to pR2 is shown for comparison. Amplified insert sequences are shown as solid lines. Amplified vector sequences are shown as shaded lines.

24) generated an amplified product of approximately 500 nucleotides; one clone (clone number 18) generated an amplified product of approximately 600 nucleotides, which is the expected size for a clone containing an insert of the same size as pR2; one clone (clone number 8) generated an amplified product of approximately 800 nucleotides; one clone (clone number 5) generated an amplified product of approximately 1100 nucleotides.

Partial restriction mapping and nucleotide sequence analysis of these PCR products, showed that clones number 5, 12 and 25 did not share any appreciable sequence identity with RXRγ, whilst the remaining were all equivalent, or shorter than pR2, with the exception of clone number 8. This clone was later isolated, and shown to contain the whole of the sequences of pR2, plus an additional stretch of 224 nucleotides at its 5' end (fig. 4.2). The presence of this longer clone of RXRγ in the library, suggests that pR2 probably corresponds to an incomplete cDNA of the chicken RXRγ mRNA.

All of the RXRγ cDNA clones isolated from the 10 day whole embryo cDNA library, seem to be derived from the same mRNA as pR2, judging from the sequences in their 5' ends. These results therefore suggest that pR2 corresponds to the major transcript of chicken RXRγ gene present in 10 day whole embryo RNA, that is, the larger RXRγ mRNA which is detected in the eye and in the peripheral nervous system. If this is true, the isolation from this library of the other transcript of chicken RXRγ, that is, of the smaller RXRγ mRNA expressed in the liver, would require the characterisation of a much larger number of RXRγ positive clones. For this reason we decided to acquire and screen a chicken liver cDNA library.

```
gaattccAGCCTCCGCCGTGAATTCGCAGGGAGCTCGGGTAGCTGAAGGCAGGAGCCGGCCCTGAGCTGCTGGGG     75
ACGGGAGACGCATCTCATCCCGGCCGAGCAGCTGTGCAAAGCGAGGATGGGGGCGAGGGAGGAGGCAGCTGCACC    150
GCAGCCCTCCGAGAGCCCCCGCAGAGCCGTGCCCCGAGCCGGGCGGGCGGCACCGCGCTCCGGGCAGCTTTGAAG    225
TGAGAGGAGACGGTCGTGTTTTGAAAGGCGAGAGATGAACTGAAGATTAAACATGTATGGGAATTATCCTCACTT    300
CATTAAGTTTCCTGCGGGCTTCGGCAATTCCCCTGTTCATGCCAGCTCCACGTCTGTGAGCCCATCATCCAGCCT    375
GTCCGTGGGGAGCACGGTGGACGGACACCACAACTACCTCGAGGCCCCCACAAACGCCTCCCGGG...           440
```

Figure 4.2

Partial nucleotide sequence derived from the cDNA clone ED8, of chicken RXRγ. The sequence shown, corresponds to the 5' untranslated region and the beginning of the coding region, down to the first *Sma*I site (shown in bold). The sequences downstream from this point, are identical to those found in pR2, and were not displayed.

The sequences present in pR2 are underlined. *Eco*RI linker sequences are shown in lower case. The nucleotide sequences are numbered on the column on the right.

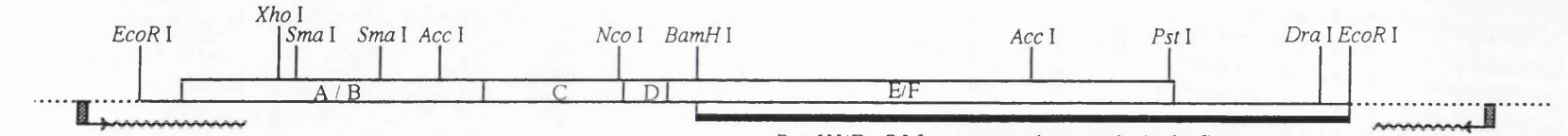
On the first screening of an adult chicken liver cDNA library in λ ZAP (Promega), approximately 450 000 pfu were plated, and duplicate lifts of these were hybridized with a radiolabelled DNA probe similar to the one used in the previous screening, corresponding to the sequences coding for the E-F domains and the 3' untranslated region of the cDNA clone pR2 (nucleotides 786-1713) (fig. 4.3-A). Five positive clones were isolated after two rounds of purification.

Following *in vivo* conversion of the recombinant λ ZAP clones to recombinant pBluescript clones, the first 250-300 nucleotides of either end of each positive were sequenced. Two of these clones, (pE2 and pE3) were shown to correspond to incomplete chicken RXRγ cDNAs, that contained sequences identical to those present in pR2 (fig. 4.3-B). One clone corresponded to a cDNA clone of RXRα (pE1), that was subsequently characterised and used for the study of the expression of this gene in the chicken (see Chapter 5). The sequences of the two remaining positive clones did not seem to share any significant sequence identity with RXRγ or any other sequence published.

On the second screening of this library a slightly different approach was used. Duplicate lifts corresponding to approximately 500 000 pfu were hybridized with a radiolabelled DNA probe, this time corresponding to a *Xho*I-*Acc*I fragment (nucleotides 199-425) coding for most of the B region present in pR2, thus ensuring that only longer cDNAs of RXRγ were isolated. Four positive clones were isolated, and as before the first 250-300 nucleotides of either end were sequenced, following *in vivo* conversion of the λ ZAP clones to pBluescript clones (fig. 4.4).

One of these positives (pP3) was again an RXRα cDNA clone, but shorter than pE1, the other RXRα clone isolated in the previous screening (see Chapter 5). The remaining three positive clones were

A) Screening strategy:



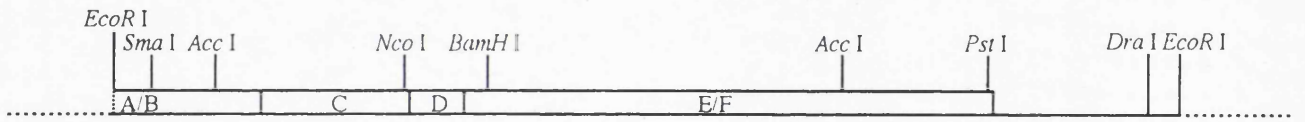
Sequence the first 250-300 bases of each end of the new cDNA clones with pBluescript T3 and T7 primers

BamH I/EcoR I fragment used as a probe in the first screening of the adult chicken liver cDNA library

Sequence the first 250-300 bases of each end of the new cDNA clones with pBluescript T3 and T7 primers

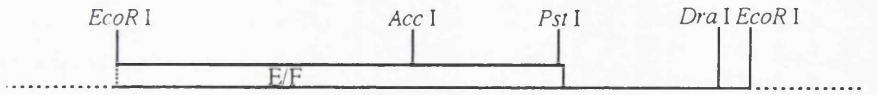
B) New RXRγ cDNA clones

pE2



Insert length:
approx. 1400 base pairs

pE3



Insert length:
approx. 850 base pairs

Figure 4.3

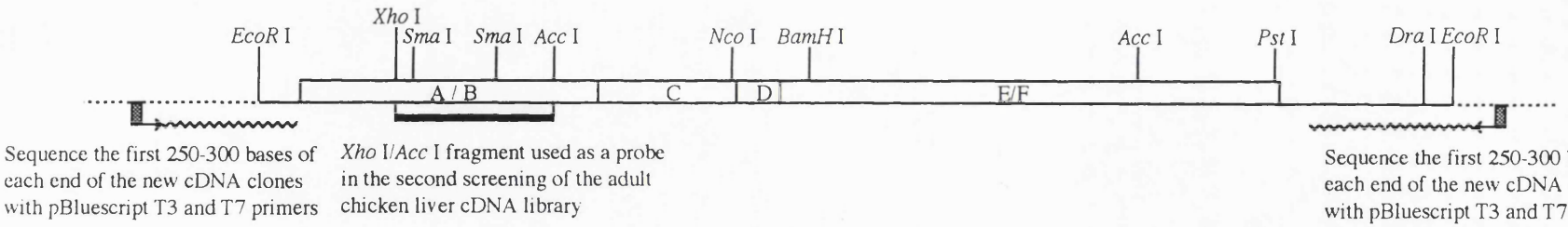
Figure 4.3

Strategy for the first screening of the adult chicken liver cDNA library in λ–ZAP.

(A) A schematic representation of the screening strategy used, illustrated on a cDNA clone identical to pR2. Vector sequences are shown as dashed lines. Insert sequences are shown as solid lines. The coding region is boxed. The probe used in this screening is shown as a bold line. The position of the oligonucleotides used in the preliminary sequencing and identification of the positive clones is shown.

(B) Restriction maps of the two positive clones that turned out to correspond to cRXRγ cDNAs (pE2 and pE3). Vector sequences are shown as dashed lines. Insert sequences are shown as solid lines. The coding region is boxed, showing the approximate position of the boundaries of the main protein domains (A-F).

A) Screening strategy:



B) New RXRγ cDNA clones

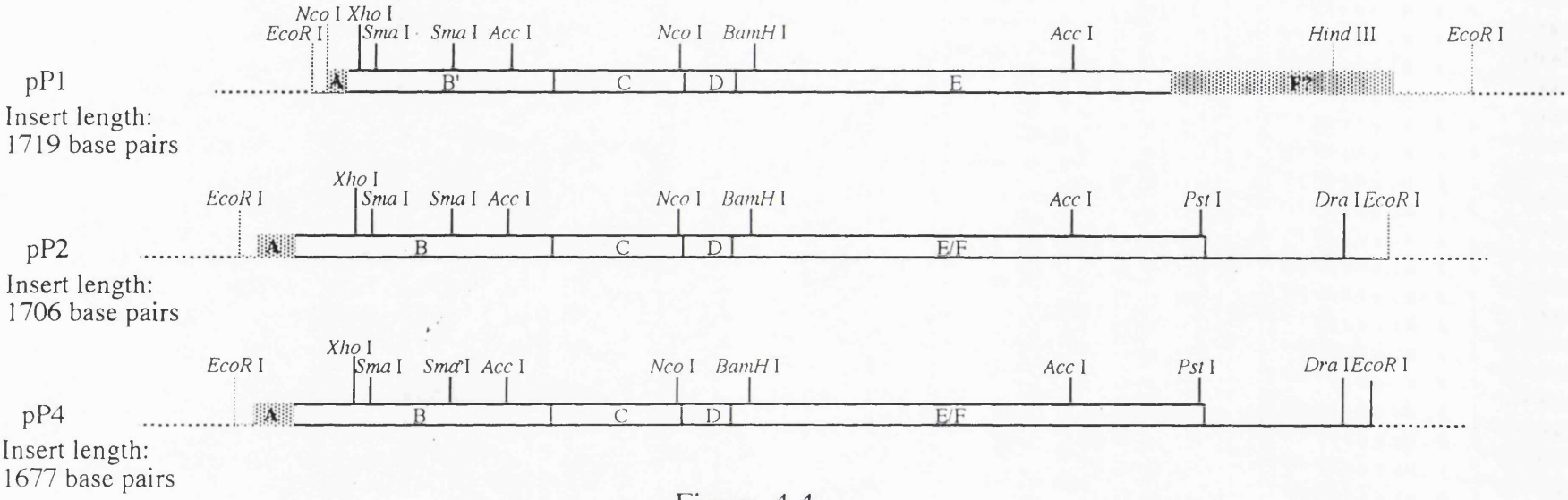


Figure 4.4

Figure 4.4

Strategy for the second screening of the adult chicken liver cDNA library in λ–ZAP.

(A) A schematic representation of the screening strategy used, illustrated on a cDNA clone identical to pR2. Vector sequences are shown as dashed lines. Insert sequences are shown as solid lines. The coding region is boxed. The probe used in this screening is shown as a bold line. The position of the oligonucleotides used in the preliminary sequencing and identification of the positive clones is shown.

(B) Restriction maps of the three positive clones that turned out to correspond to cRXRγ cDNAs (pP1, pP2 and pP4). Vector sequences are shown as dashed lines. Insert sequences are shown as solid lines. The coding region is boxed, showing the approximate position of the boundaries of the main protein domains (A-F). The regions that differ from those present in the cDNA clone pR2 are shown as shaded lines and boxes.

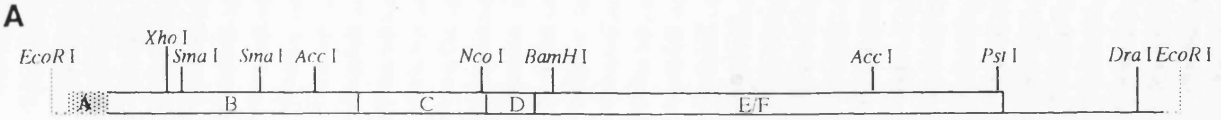
RXRγ cDNA clones, that shared the same nucleotide sequences as pR2 along most of their length, but differing from it at their 5' ends, and in one case also at the 3' end.

Two of these clones (pP2 and pP4), have a unique 83 nucleotide long region at their 5' ends, followed by sequences identical to those present in pR2, from position 84 to their 3' end (fig. 4.5). The divergent 5' end region present in these new RXRγ cDNA clones, contains several in-frame initiation codons (none of which conforms very well with Kozak's rules for the initiation of translation) (Kozak, 1991), therefore coding for a predicted protein product that differs from the one coded by pR2 only in the amino-terminal sequences. The longest of these two new clones (pP2) also contains 29 additional nucleotides at the end of its 3' untranslated region, that are not present in pR2 or pP4.

The other RXRγ clone isolated in this screening (pP1), differed from pR2, at both its 5' and 3' ends. At its 5' end, it contained a small unique stretch of 52 nucleotides. Its sequence was identical to those of pR2, pP2 and pP4, from position 53 to position 1277 (fig. 4.6). It then diverged again, containing an additional stretch of 442 nucleotides. These 5' and 3' divergent regions also contained respectively, a translation start site that conforms well with Kozak's rules for the initiation of translation (Kozak, 1991), and a stop codon in frame with the rest of the sequence, generating a predicted protein that differs from the one coded by pR2 both in the amino- and carboxy-terminal regions.

None of the chicken RXRγ cDNA clones isolated from the adult liver cDNA library, contained the same 5' end sequences found in pR2, supporting our initial hypothesis that the chicken RXRγ transcript corresponding to pR2, is the larger RXRγ mRNA that is expressed predominantly outside the liver.

A



B

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.....gaattcCAGAGAGACAGGACGTGGGTTGGAAATGCAGCCAGGCATGCAGGCTCC      54
                                  M  Q  P  G  M  Q  A  P          8
TTACTCCCTGGAGATGGGCTCCTTCCCTCACTTCTATTCCCCTGTTCATGCCAGCTCCACGTCTGTGAGCCCATC    129
 Y  S  L  E  M  G  S  F  P  H  F  Y  S  P  V  H  A  S  S  T  S  V  S  P  S     33
ATCCAGCCTGTCCGTGGGGAGCACGGTGGACGGACACCACAACTACCTCGAGGCCCCCACAAACGCCTCCCGGGC    204
 S  S  L  S  V  G  S  T  V  D  G  H  H  N  Y  L  E  A  P  T  N  A  S  R  A     58
GCTGCCATCCCCCATGAACACCATTGGGTCTCCAGTGAACGCGTTGGGCTCACCCTACAGGGTCATCGCATCCTC    279
 L  P  S  P  M  N  T  I  G  S  P  V  N  A  L  G  S  P  Y  R  V  I  A  S  S     83
CATTGGCTCGCATCCCGTTGCTCTGTCGTCCTCAGCCCCGGGCATGAATTTTGTGACCCACAGCCCACAGCCCAA    354
 I  G  S  H  P  V  A  L  S  S  S  A  P  G  M  N  F  Y  T  H  S  P  Q  P  N    108
TGTGCTCAACAATGTCAGCAGCTCGGAGGACATCAAGCCCTTGCCAGGTCTACCAGGGATTGGGAACATGAATTA    429
 V  L  N  N  L  S  S  S  E  D  I  K  P  L  P  G  L  P  G  I  G  N  M  N  Y    133
TCCATCCACAAGCCCAGGTTCTTTAGCCAAACACATCTGTGCCATCTGTGGGGACAGGTCTTCAGGGAAGCACTA    504
 P  S  T  S  P  G  S  L  A  K  H  I  C  A  I  C  G  D  R  S  S  G  K  H  Y    158
TGGGGTGTACAGCTGCGAGGGCTGCAAGGGCTTCTTTAAGAGGACCATCCGGAAAGACCTGATCTACACCTGCCG    579
 G  V  Y  S  C  E  G  C  K  G  F  F  K  R  T  I  R  K  D  L  I  Y  T  C  R    183
TGACAACAAGGACTGCCTCATCGACAAGCGCCAGCGCAACCGCTGCCAGTACTGCCGCTATCAGAAGTGCCTCGC    654
 D  N  K  D  C  L  I  D  K  R  Q  R  N  R  C  Q  Y  C  R  Y  Q  K  C  L  A    208
CATGGGGATCAAGAGGGAAGCCGTGCAGGAGGAGAGGCAGGGGAGCAGGGAGCGCAGTGAGAACGAAGCTGAATC    729
 M  G  M  K  R  E  A  V  Q  E  E  R  Q  G  S  R  E  R  S  E  N  E  A  E  S    233
CACGAGCGGCGGCAGCGAGGACATGCCTGTGGAGAGGATCCTGGAAGCCGAGCTGGCAGTCGAACCCAAGACGGA    804
 T  S  G  G  S  E  D  M  P  V  E  R  I  L  E  A  E  L  A  V  E  P  K  T  E    258
GGCATACAGCGACGTGAACACGGAGAGCTCAACCAATGACCCCGTCACCAACATCTGCCACGCTGCTGACAAGCA    879
 A  Y  S  D  V  N  T  E  S  S  T  N  D  P  V  T  N  I  C  H  A  A  D  K  Q    283
GCTCTTCACCCTCGTCGAGTGGGCCAAGCGCATCCCCCACTTCTCCGACCTGACCCTGGAGGACCAAGTCATCCT    954
 L  F  T  L  V  E  W  A  K  R  I  P  H  F  S  D  L  T  L  E  D  Q  V  I  L    308
CCTGCGGGCAGGCTGGAACGAGCTGCTCATCGCATCCTTCTCCCATCGCTCCGTGTCGGTGCAGGATGGCATCCT   1029
 L  R  A  G  W  N  E  L  L  I  A  S  F  S  H  R  S  V  S  V  Q  D  G  I  L    333
GCTGGCCACAGGCTTGCATGTGCACCGCAGCAGTGCTCACAGTGCAGGCGTGGGCTCCATCTTTGACAGAGTTTT   1104
 L  A  T  G  L  H  V  H  R  S  S  A  H  S  A  G  V  G  S  I  F  D  R  V  L    358
GACAGAGCTGGTGTCCAAAATGAAGGACATGCAGATGGATAAGTCGGAGCTGGGGTGCCTGCGAGCCATTGTCCT   1179
 T  E  L  V  S  K  M  K  D  M  Q  M  D  K  S  E  L  G  C  L  R  A  I  V  L    383
CTTCAACCCAGACGCCAAGGGCCTGTCCAGCCCCTCCGAAGTGGAGTCGCTGCGGGAAAAGGTCTACGCCACGCT   1254
 F  N  P  D  A  K  G  L  S  S  P  S  E  V  E  S  L  R  E  K  V  Y  A  T  L    408
GGAAGCCTACACGAAGCAGAAGTACCCCGAGCAGCCGGGGCGGTTTGCCAAACTCCTCCTGCGCCTGCCAGCACT   1329
 E  A  Y  T  K  Q  K  Y  P  E  Q  P  G  R  F  A  K  L  L  L  R  L  P  A  L    433
ACGGTCCATCGGGCTGAAGTGCCTGGAGCACCTCTTCTTCTTCAAGCTGATCGGGGACACCCCCATCGACACCTT   1404
 R  S  I  G  L  K  C  L  E  H  L  F  F  F  K  L  I  G  D  T  P  I  D  T  F    458
CCTTATGGAGATGCTGGAGACACCCCTGCAGGTCACTTGAGGGTCCGCTCCCCCCCGCCATATCCCCACACAGCT   1479
 L  M  E  M  L  E  T  P  L  Q  V  T  *                            470
CCCACCCCTCCTCTCCGGGCTGCAAGAGCCGCCCTCCACCCTCTGCTCCTCTTTGGTGGGATTGTTGTGTCTTTG   1554
TACAGCTGTAAATCGTCCCCTCTAACCCTCCCGGTGGGTTGCGGACCCTCTGCCCAGCTAACCACACACCTCCCT   1629
GTACAGATAATTGCTTTAAATTATTTTTTCACTCTCAATAAAAGCCAACAACAACCCAACAAAAAAAATAAGTAAT   1704
ATATGAGAGgaattc                                                  1719
```

Figure 4.5

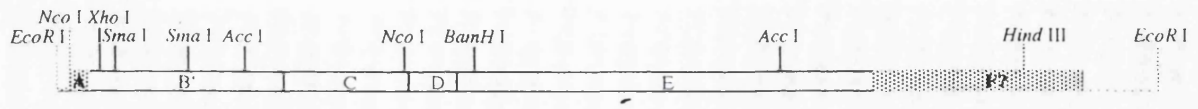
Figure 4.5

(A) Restriction map of the insert of the cDNA clone pP2, of cRXRγ. The 5' and 3' untranslated regions are shown as single lines. The coding region is boxed, showing the approximate position of the boundaries of the main protein domains (A-F). The regions that differ from those present in the cDNA clone pR2 are shown as shaded lines and boxes.

(B) Nucleotide sequence and predicted amino acid sequence derived from the cDNA clone pP2. *Eco*RI linker sequences are shown in lower case. The putative DNA binding domain is boxed. The sequences that differ from those present in the cDNA clone pR2 are underlined. The nucleotide and amino acid sequences are numbered on the column on the right.

The open reading frame displayed, starts at the first AUG codon, although it does not conform well with Kozak's rules for the initiation of translation (Kozak, 1991).

A



B

```
.....                                                                  
.....gaattcCTCTCTCCCGCCAGCGC                                      23
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GCTGCCATCCCCCATGAACACCATTGGGTCTCCAGTGAACGCGTTGGGCTCACCCTACAGGGTCATCGCATCCTC     173
  L  P  S  P  M  N  T  I  G  S  P  V  N  A  L  G  S  P  Y  R  V  I  A  S  S    50
CATTGGCTCGCATCCCGTTGCTCTGTCGTCCTCAGCCCCGGGCATGAATTTTGTGACCCACAGCCCACAGCCCAA     248
  I  G  S  H  P  V  A  L  S  S  S  A  P  G  M  N  F  Y  T  H  S  P  Q  P  N    75
TGTGCTCAACAATGTCAGCAGCTCGGAGGACATCAAGCCCTTGCCAGGTCTACCAGGGATTGGGAACATGAATTA     323
  V  L  N  N  L  S  S  S  E  D  I  K  P  L  P  G  L  P  G  I  G  N  M  N  Y   100
TCCATCCACAAGCCCAGGTTCTTTAGCCAAACACATCTGTGCCATCTGTGGGGACAGGTCTTCAGGGAAGCACTA     398
  P  S  T  S  P  G  S  L  A  K  H  I  C  A  I  C  G  D  R  S  S  G  K  H  Y   125
TGGGGTGTACAGCTGCGAGGGCTGCAAGGGCTTCTTTAAGAGGACCATCCGGAAAGACCTGATCTACACCTGCCG     473
  G  V  Y  S  C  E  G  C  K  G  F  F  K  R  T  I  R  K  D  L  I  Y  T  C  R   150
TGACAACAAGGACTGCCTCATCGACAAGCGCCAGCGCAACCGCTGCCAGTACTGCCGCTATCAGAAGTGCCTCGC     548
  D  N  K  D  C  L  I  D  K  R  Q  R  N  R  C  Q  Y  C  R  Y  Q  K  C  L  A   175
CATGGGGATCAAGAGGGAAGCCGTGCAGGAGGAGAGGCAGGGGAGCAGGGAGCGCAGTGAGAACGAAGCTGAATC     623
  M  G  M  K  R  E  A  V  Q  E  E  R  Q  G  S  R  E  R  S  E  N  E  A  E  S   200
CACGAGCGGCGGCAGCGAGGACATGCCTGTGGAGAGGATCCTGGAAGCCGAGCTGGCAGTCGAACCCAAGACGGA     698
  T  S  G  G  S  E  D  M  P  V  E  R  I  L  E  A  E  L  A  V  E  P  K  T  E   225
GGCATACAGCGACGTGAACACGGAGAGCTCAACCAATGACCCCGTCACCAACATCTGCCACGCTGCTGACAAGCA     773
  A  Y  S  D  V  N  T  E  S  S  T  N  D  P  V  T  N  I  C  H  A  A  D  K  Q   250
GCTCTTCACCCTCGTCGAGTGGGCCAAGCGCATCCCCCACTTCTCCGACCTGACCCTGGAGGACCAAGTCATCCT     848
  L  F  T  L  V  E  W  A  K  R  I  P  H  F  S  D  L  T  L  E  D  Q  V  I  L   275
CCTGCGGGCAGGCTGGAACGAGCTGCTCATCGCATCCTTCTCCCATCGCTCCGTGTCGGTGCAGGATGGCATCCT     923
  L  R  A  G  W  N  E  L  L  I  A  S  F  S  H  R  S  V  S  V  Q  D  G  I  L   300
GCTGGCCACAGGCTTGCATGTGCACCGCAGCAGTGCTCACAGTGCAGGCGTGGGCTCCATCTTTGACAGAGTTTT     998
  L  A  T  G  L  H  V  H  R  S  S  A  H  S  A  G  V  G  S  I  F  D  R  V  L   325
GACAGAGCTGGTGTCCAAAATGAAGGACATGCAGATGGATAAGTCGGAGCTGGGGTGCCTGCGAGCCATTGTCCT    1073
  T  E  L  V  S  K  M  K  D  M  Q  M  D  K  S  E  L  G  C  L  R  A  I  V  L   350
CTTCAACCCAGACGCCAAGGGCCTGTCCAGCCCCTCCGAAGTGGAGTCGCTGCGGGAAAAGGTCTACGCCACGCT    1148
  F  N  P  D  A  K  G  L  S  S  P  S  E  V  E  S  L  R  E  K  V  Y  A  T  L   375
GGAAGCCTACACGAAGCAGAAGTACCCCGAGCAGCCGGGGCGGTTTGCCAAACTCCTCCTGCGCCTGCCAGCACT    1223
  E  A  Y  T  K  Q  K  Y  P  E  Q  P  G  R  F  A  K  L  L  L  R  L  P  A  L   400
ACGGTCCATCGGGCTGAAGTGCCTGGAGCACCTCTTCTTCTTCAAGCTGATCGGGGACACGCAAGAAGATCTGTG    1298
  R  S  I  G  L  K  C  L  E  H  L  F  F  F  K  L  I  G  D  T  Q  E  D  L  C   425
CTCCTCCAACCCCTGCAAGAACAACGGCTGGTGTGAGATGAGAGGAAGCAATTTCACCTGCCTCTGCCCCAGGCC    1373
  S  S  N  P  C  K  N  N  G  W  C  E  M  R  G  S  N  F  T  C  L  C  P  R  P   450
CTATACCGGGGACACCTGCGGGGAAGTGGAGGACACGTGTGTGGACCACAACTGTGTCAGAGGAGACTGCCTCGT    1448
  Y  T  G  D  T  C  G  E  V  E  D  T  C  V  D  H  N  C  V  R  G  D  C  L  V   475
TACATTAACCAAACCTTACTATCAGTTGACATGCTGTAGCCATCCCTACAAGTTACCAGACTGCCAGTCAAGCTT    1523
  T  L  T  K  P  Y  Y  Q  L  T  C  C  S  H  P  Y  K  L  P  D  C  Q  S  S  F   500
CTTCACAGTGCCGGCCAAACCCATGAAAAAATGGAGGAATCTGTGTGCTTCACTGAATCAGATCAAAATTCACCT    1598
  F  T  V  P  A  K  P  M  K  K  W  R  N  L  C  A  S  L  N  Q  I  K  I  H  L   550
GCAAGTGCCCTGAACGTTACCGAGGGAGGTTTTGTGAGATTGAACCGGATGATTGTTATGAAGAGGACTCCTTTT    1673
  Q  V  P  *                                                               553
ATTACAGAGGAAGAGTGAACCAAGCACAGAGTGGCAGGACTTGCCTGCACTGgaattc                   1731
```

Figure 4.6

Figure 4.6

(A) Restriction map of the insert of the cDNA clone pP1, of cRXRγ. The 5' and 3' untranslated regions are shown as single lines. The coding region is boxed, showing the approximate position of the boundaries of the main protein domains (A-F). The regions that differ from those present in the cDNA clone pR2 are shown as shaded lines and boxes.

(B) Nucleotide sequence and predicted amino acid sequence derived from the cDNA clone pP1. *Eco*RI linker sequences are shown in lower case. The putative DNA binding domain is boxed. The sequences that differ from those present in the cDNA clone pR2 are underlined. The nucleotide and amino acid sequences are numbered on the column on the right.

The divergence point between the nucleotide sequences of pP2/pP4 and pR2, is situated in a position that could correspond to the boundary between the A and B domains, which would make the structure of these clones identical to that of the different isoforms of the RARs and RXRs described previously.

The structure of the protein product coded by pP1 is rather peculiar, when compared to the predicted protein sequences of the other cDNA clones of chicken RXRγ, containing a very small A and B regions and an enormous F region. This raises some doubts as to its authenticity, as a cDNA corresponding to a fully processed transcript of chicken RXRγ.

In order to establish whether these different cDNAs correspond to real mRNA isoforms, and to examine in detail their tissue distribution, ribonuclease protection assays were performed using probes derived from the divergent regions of the various cDNA clones of chicken RXRγ.

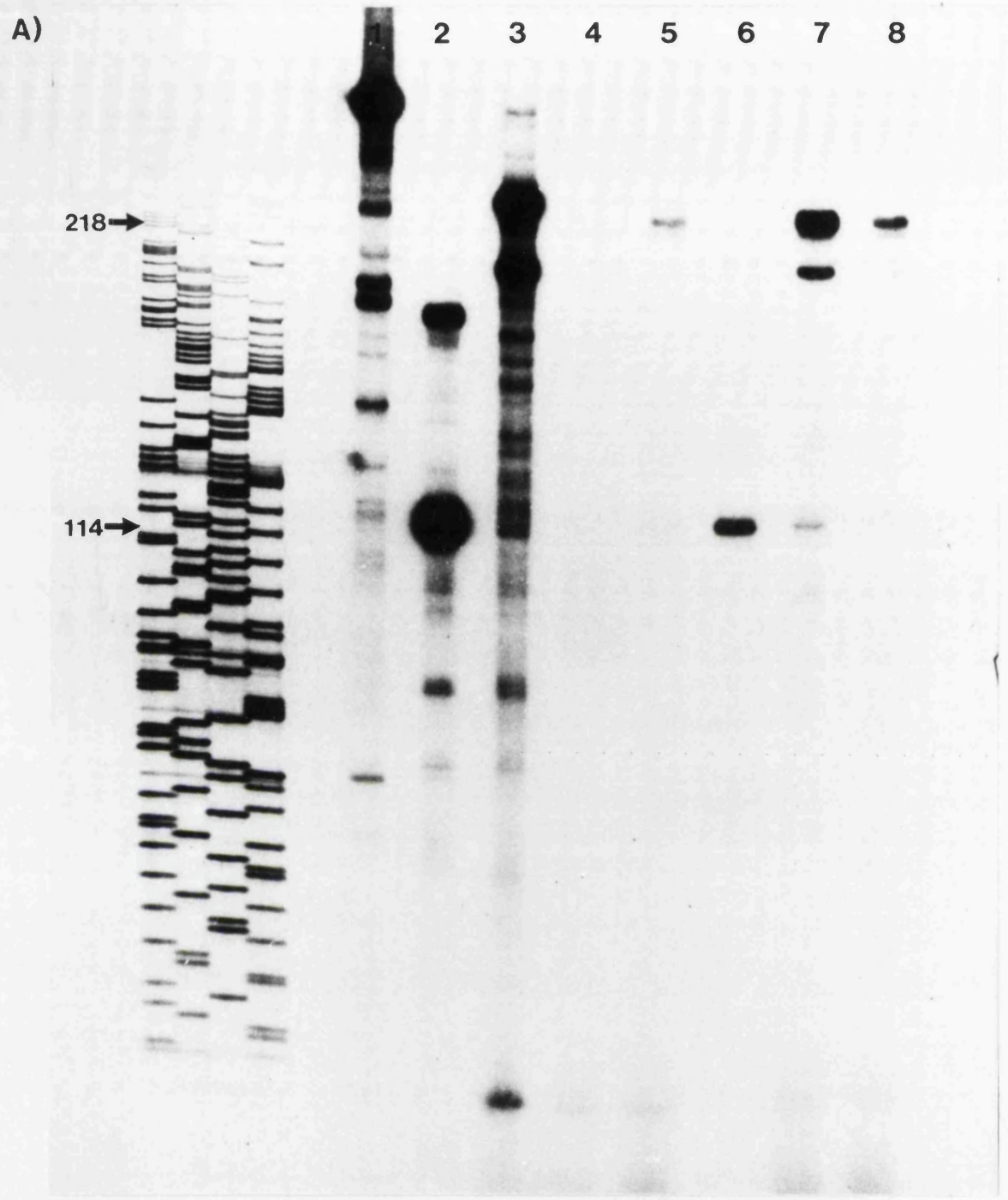
4.3 - RNase protection of probes derived from 5' regions of the different chicken RXRγ cDNA clones

From this point onwards, the transcripts corresponding to the different cDNA clones of chicken RXRγ, will be refered to as cRXRγ1 (mRNA corresponding to clone pR2) cRXRγ2 (mRNA corresponding to clones pP2 and pP4) and cRXRγ3 (mRNA corresponding to clone pP1).

Templates for probe synthesis were prepared by subcloning the 5' *Eco*RI-*Sma*I fragment of each of the cDNA clones of chicken RXRγ into pBluescript SK+, generating subclones pR2-E/S, pP2-E/S and pP1-E/S (see appendix A). Radiolabelled antisense RNA probes were prepared with T3 RNA polymerase, using *Eco*RI-linearised templates. Unlabelled sense RNA used as a control in the ribonuclease protection experiments, was also prepared from the same subclones, using T7 RNA polymerase and *Sma*I linearised templates.

Figure 4.7 shows the fragments of the pR2-E/S probe (cRXRγ1), that were protected from ribonuclease digestion by hybridization to total RNA extracted from a series of tissues from 10-day chick embryos. The undigested probe was 274 nucleotides long (fig. 4.7, track 1), of which 215 nucleotides correspond to the mRNA sequences, as shown by the protection of a fragment of approximately 218 nucleotides when the probe was hybridized to the control sense RNA synthesised from the same template (fig. 4.7, track 3) (the discrepancy in sizes corresponds to the protection of the *Eco*RI linker sequences). Surprisingly, a smaller protected fragment of 198 nucleotides was also present in this track, and therefore must represent artefactual cleavage of the probe, because no other partially matched RNA species were present in the reaction.

Total RNA from 10 day chick embryo liver, protected a 114 nucleotide fragment (fig. 4.7, track 6), whose size is identical to that of the fragment obtained by the hybridization of this probe to control sense RNA synthesised from pP2-E/S (fig. 4.7, track 2). No 215 nucleotide fragment was detectable, when the probe was hybridized to 10 day chick embryo liver, even when the autoradiography film was over-exposed (not shown), indicating that this tissue contains no, or very little cRXRγ1 mRNA. Total RNA from 10-day chick embryo eye (fig. 4.7, track 7) and 10-day chick embryo dorsal root ganglia (fig. 4.7, track 8), protected fragments of 215, 198 and 114 nucleotides. The smaller fragment is barely visible in figure 4.7, track 8, but was apparent upon longer exposure of the film (not shown). The presence of the 215 nucleotide protected fragment indicates that 10-day chick embryo eye and dorsal root ganglia, contain cRXRγ1 mRNA. The 198 nucleotide protected fragment is similar to that present in the control track 3, and as discussed above, probably corresponds to artefactual cleavage of the probe. The presence of a weak 114 nucleotide fragment in these tracks, apparently



B) pR2 E/S antisense probe:

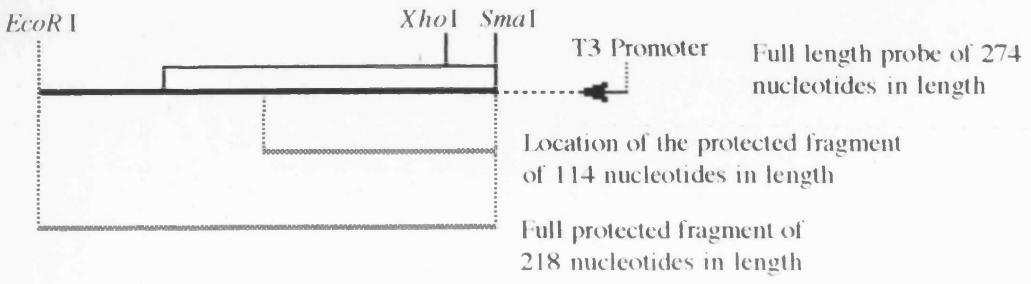


Fig. 4.7

Figure 4.7

Analysis of the structure of the chicken RXRγ mRNAs by ribonuclease protection of a probe corresponding to the 5' end of clone pR2.

(A) An antisense riboprobe was synthesized with T3 RNA polymerase, using *Eco*RI-linearised pR2-E/S as a template. Track 1 contains undigested probe. Tracks 2 and 3 contain probe fragments protected by hybridization to unlabelled RNA synthesized with T7 RNA polymerase, using *Sma* I-linearised pR2-E/S, or *Sma* I-linearised pP2-E/S respectively. Tracks 4-8 contain probe fragments protected by hybridization to 25μg of total RNA from: yeast (track 4); whole 10-day chick embryo (track 5); 10-day chick embryo liver (track 6); 10-day chick embryo eye (track 7) and 10-day chick embryo dorsal root ganglia (track 8). The DNA sequence ladder on the left, was synthesized using the oligonucleotide -40 as a primer and m13 as a template, and was used for the estimation of the sizes of the protected fragments. The sizes of the protected fragments are indicated in nucleotides.

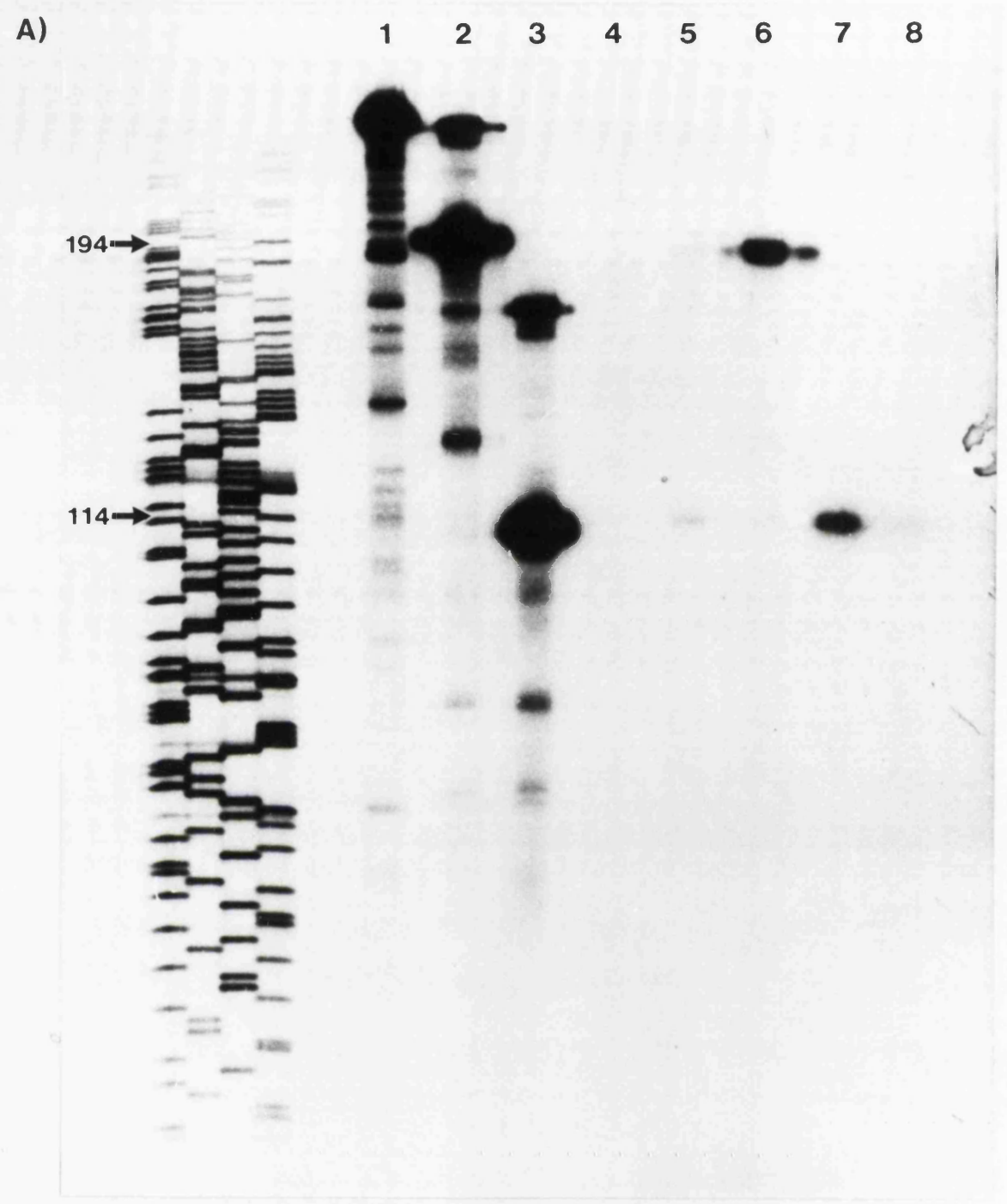
Notice the presence of a large partially protected fragment, approximately 198 bases long, in all samples where full protection of the probe has occurred, including in the control in track 3, where the probe was hybridized to cold sense RNA synthesised from the same template, suggesting that this protected fragment corresponds to an artefact.

(B) Structure of the probe used in this ribonuclease protection assay, showing the location and length of the protected fragments detected in this assay. Vector sequences are shown as a dashed line. Insert sequences are shown as a solid line. The coding region is boxed. Protected fragments are shown as shaded lines. The arrow indicates the direction of transcription.

suggests that 10-day chick embryo eye and 10-day chick embryo dorsal root ganglia may contain very small quantities of the same cRXRγ transcript present in the liver, but at much lower levels than cRXRγ1. As would be expected, whole 10 day chick embryo RNA (fig. 4.7, track 5) protected fragments of 218, 119 (artefactual) and 114 nucleotides, the latter being more readily apparent upon longer exposure of the film (not shown). None of these fragments was protected by yeast total RNA (fig. 4.7, track 4).

Figure 4.8 shows the fragments of the pP2-E/S probe (cRXRγ2), that were protected from ribonuclease digestion by hybridization to total RNA extracted from a series of 10-day chick embryo tissues. The undigested probe was 253 nucleotides long (fig. 4.8, track 1), of which 194 nucleotides correspond to the mRNA sequences, as shown by the protection of a fragment of approximately 197 nucleotides when the probe was hybridized to the control sense RNA synthesised from the same template (fig. 4.8, track 2) (the discrepancy in sizes corresponds to the protection of the *Eco*RI linker sequences).

Total RNA from 10 day chick embryo liver, protected a 194 nucleotide fragment (fig. 4.8, track 6), confirming that this tissue contains cRXRγ2 mRNA. No 114 nucleotide fragment was detectable in this track, even when the autoradiography film was over-exposed (not shown), confirming that the 10-day chick embryo liver contains no, or very little cRXRγ1 mRNA. Total RNA from 10-day chick embryo eye (fig. 4.8, track 7) and 10-day chick embryo dorsal root ganglia (fig. 4.8, track 8), protected a single 114 nucleotide fragment. This is barely visible in figure 4.8, track 8, but was more readily apparent upon longer exposure of the film (not shown). The size of this protected fragment is identical to that of the fragment obtained by the hybridization of this probe to control sense RNA synthesised from pR2-E/S (fig. 4.8, track 3)



B) pP2 E/S antisense probe:

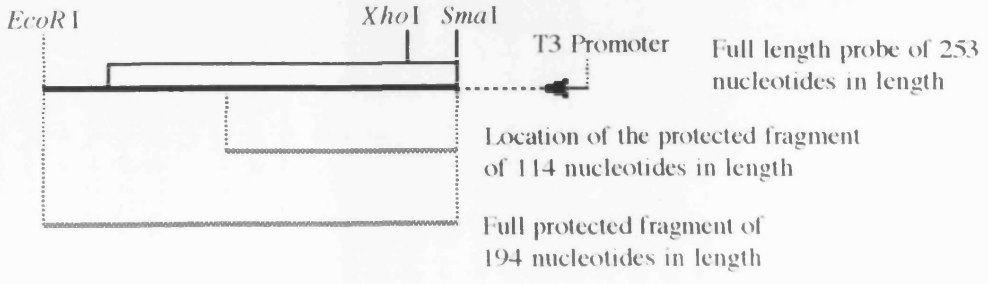


Fig. 4.8

Figure 4.8

Analysis of the structure of the chicken RXRγ mRNAs by ribonuclease protection of a probe corresponding to the 5' end of clone pP2.

(A) An antisense riboprobe was synthesized with T3 RNA polymerase, using *Eco*RI-linearised pP2-E/S as a template. Track 1 contains undigested probe. Tracks 2 and 3 contain probe fragments protected by hybridization to unlabelled RNA synthesized with T7 RNA polymerase, using *Sma* I-linearised pR2-E/S, or *Sma* I-linearised pP2-E/S respectively. Tracks 4-8 contain probe fragments protected by hybridization to 25μg of total RNA from: yeast (track 4); whole 10-day chick embryo (track 5); 10-day chick embryo liver (track 6); 10-day chick embryo eye (track 7) and 10-day chick embryo dorsal root ganglia (track 8). The DNA sequence ladder on the left, was synthesized using the oligonucleotide -40 as a primer and m13 as a template, and was used for the estimation of the sizes of the protected fragments. The sizes of the protected fragments are indicated in nucleotides.

Notice the absence of any partially protected fragments when the probe was hybridized to 10-day chick embryo liver RNA (track 6).

(B) Structure of the probe used in this ribonuclease protection assay, showing the location and length of the protected fragments detected in this assay. Vector sequences are shown as a dashed line. Insert sequences are shown as a solid line. The coding region is boxed. Protected fragments are shown as shaded lines. The arrow indicates the direction of transcription.

confirming that 10-day chick embryo eye and dorsal root ganglia contain cRXRγ1 mRNA. No 119 nucleotide fragment was detectable in tracks 7 and 8, even when the autoradiography film was over-exposed (not shown), indicating that 10-day chick embryo eye and dorsal root ganglia contain no, or very little cRXRγ2 mRNA. As would be expected, whole 10 day chick embryo RNA (fig. 4.8, track 5) protected fragments of 194 and 114 nucleotides. No smaller, artefactually protected fragments were observed in any of these tracks. None of these fragments were protected by yeast total RNA (fig. 4.8, track 4).

Ribonuclease protection assays with a probe derived from the 5' end sequences of pP1 (cRXRγ3), were also attempted, but did not generate interpretable results (data not shown). However, the fact that a small protected fragment of 38 nucleotides in length, corresponding to the common regions of cRXRγ3 with pR2-E/S and pP2-E/S, was never observed in any of tracks of both RNase protection assays described above, seems to support the doubts about the authenticity of this cDNA clone. Alternatively, it is possible that cRXRγ3 does correspond to a chicken RXRγ mRNA that is expressed at considerably lower levels than the other two transcripts, so that the small protected fragment produced by it in the ribonuclease protection experiments was not detected.

4.4 - Analysis of the genomic structure of the chicken RXRγ gene

In the previous sections of this chapter I showed that the chicken RXRγ gene gives rise to at least two major mRNAs, that differ from each other in their 5' end sequences, and that have the potential to code for two distinct protein products with different amino-terminal regions. The characterisation of the genomic organisation of this gene should allow us to determine whether the differences between these two chicken RXRγ

transcripts include exactly all the sequences coding for the A region, and are generated by alternative splicing of a single gene (as in the case of the RARs), or are originated by transcription of two divergent copies of the same gene.

A chicken RXRγ genomic clone, designated λRXR-Gen, was isolated by Dr. Nicholas Eager in our laboratory, by screening a chicken genomic library constructed in the vector λEMBL3, with a radiolabelled probe corresponding to the whole of the sequence of the cDNA clone pR2. Preliminary restriction analysis of this clone and Southern hybridization with radiolabelled DNA fragments of pR2 or with oligonucleotides derived from its sequence, was performed by Dr. Melanie Saville in our laboratory, revealing that λRXR-Gen only contained regions corresponding to the 5' end of this cDNA clone.

A *Pst*I fragment of λRXR-Gen of approximately 1 Kb in length, that hybridized to a probe made from the insert of pR2, was subcloned into pBluescript SK+ yielding clone pRGP1. This was sequenced by Dr. Nicholas Eager in our laboratory, revealing a region with nucleotide sequence identical to that present in position 1-110 of pR2, flanked at the 3' end by an almost perfect consensus splice donor site (fig. 4.9). Furthermore, the sequence upstream from this region was identical to the full length of the additional 5' sequence present in the cDNA clone ED8 isolated during the screening of the 10 day whole embryo cDNA library (see section 4.2). No obvious regulatory elements (such as a TATA box, or Sp1 sites or CCAAT box) could be detected in the region upstrean from the 5' end of the sequences identical to those present in the cDNA clone ED8.

Following the isolation of the new cDNA clones of chicken RXRγ (pP1, pP2 and pP4), a second *Pst*I fragment of λRXR-Gen of approximately 1.4 Kb in length, was found to hybridize to a probe made

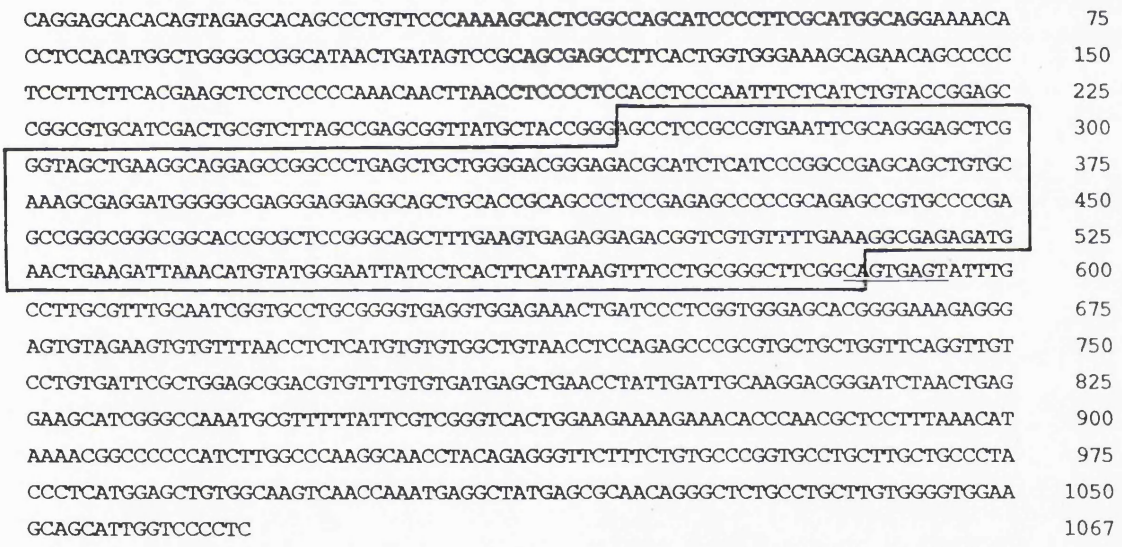


Figure 4.9

Nucleotide sequence of clone pRGP1 derived from the genomic clone λRXR-Gen, of chicken RXRγ. The sequences present in cDNA clone ED8 are boxed. The region corresponding to the splicing junction consensus sequences is underlined. The nucleotide sequences are numbered on the column on the right.

from the 5' region of pP2, and was subsequently subcloned into pBluescript SK+ yielding clone pRGP2. Sequencing of this clone revealed the presence of a region with nucleotide sequence identical to that present in position 1-83 of pP2, that was also bounded at the 3' end by a consensus sequence for an exon/intron boundary (fig. 4.10). No known regulatory elements could be identified in the region immediately upstream from this exon. Partial restriction mapping of λRXR-Gen, performed in collaboration with Dr. David Darling, showed that the two 5' exons of pR2 and pP2 are separated by approximately 9 Kb of genomic DNA (fig. 4.11).

The unique sequences of the 5' region of pP1 (nucleotides 1 to 58) did not seem to be present in λRXR-Gen, since no hybridization was detected in Southern blots of this genomic clone probed with a radiolabelled DNA probe made from this region of pP1. Due to the fact that none of the downstream RXRγ exons were present in λRXR-Gen, it was possible that the 5' sequences of pP1 were also located downtream of this genomic fragment. For this reason a second genomic clone of RXRγ (CRXR-Gen3), was isolated in collaboration with Miss Lobat Doostdar, from a chicken genomic library in the cosmid pWE15, screened with a radiolabelled probe made from the insert of pR2-B/E.

Preliminary mapping of CRXR-Gen3 was carried out by Southern hybridization with a series of radiolabelled oligonucleotide probes for the common and specific sequences of the three RXRγ mRNA isoforms. Although not enough data was collected to allow the construction of a complete map of CRXR-Gen3, this work showed that this genomic clone contained most of the sequences present in the pR2 and pP2 clones with the possible exception of the most 3' regions. Moreover, it was also found to hybridize to a radiolabelled oligonucleotide probe specific from the 5' sequences of clone pP1 (data not shown).

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GTTCCATCGGGAGCCAGGGTTAGAATCATAAAGGTTGTAAAAGACCTCTGTGATCACCTAGTCCTTGCGGCCAGC     375
CCTCCCCCCAGCATTCGAACACCTCGAAGGGACAGAAGCCCGAAGAGCAAACACAGGAAACCCCAGCCCTGGTCA     450
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CATTCACTGTGTGTGCATAGATGCATATACCTACACATACATGTGTGGGGTGGGAGAGAACTCCACTGAAAACTG    1050
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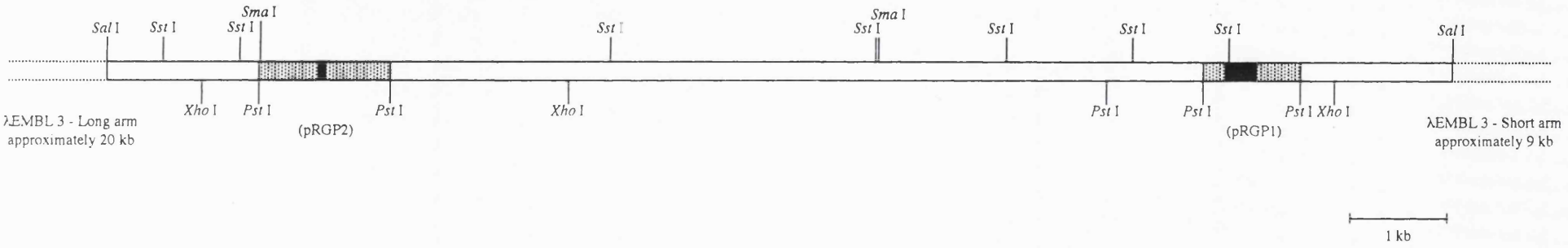
GATGGGTTGATGGTTGGTCTTGATGATCTTAATAGCCTTTTTCCAACCTTAATCATCCTATATCGACATCAAATT    1275
TGATTAGCATCTGATGATACATTATTGGTCTGATTCCTGTATATCAGGTAAACCAGTGCTGGAATTGATTTTCCT    1350
GGCAGTTAATTACGCTGCAG                                                            1370
```

Figure 4.10

Nucleotide sequence of clone pRGP2 derived from the genomic clone λRXR-Gen, of chicken RXRγ. The sequences present in cDNA clone pP2 are boxed. The region corresponding to the splicing junction consensus sequences is underlined. The nucleotide sequences are numbered on the column on the right.



Approximate size of the insert 13 kb

Figure 4.11

Figure 4.11

Restriction map of the insert of the genomic clone λRXR-Gen, showing the relative position of the alternative 5' exons (black boxes) found in both chicken RXRγ mRNAs (cRXRγ1 and cRXRγ2).

Insert sequences are shown as a solid lines. Vector sequences are shown as dashed lines. The sequences corresponding to the subclones pRGP1 and pRGP2 are shown as shaded boxes. The restriction sites shown above the insert sequences correspond to the complete restriction maps of those enzymes, whilst the restriction sites shown below the insert sequences correspond only to partial restriction maps of those enzymes.

4.5 - Analysis and discussion of the results

The protection of the full length of the probes corresponding to the 5' regions of pP2/pP4 and pR2, confirms that these cDNA clones represent *bona fide* chicken RXRγ mRNAs. Clone pR2 corresponds to the larger RXRγ mRNA, that is predominant in 10 day whole embryo RNA (cRXRγ1), and is expressed at very high levels in the eye and in neural crest derivatives, whilst clones pP2 and pP4, correspond to the smaller transcript of chicken RXRγ that is expressed in the liver (cRXRγ2).

The subsequent characterisation of the genomic structure of this gene showed that both unique regions of each of the major transcripts of chicken RXRγ are found as single exons, situated on the 5' end of the gene, flanked on their 3' ends by consensus splice donor sequences, confirming that the divergence point between these two chicken RXRγ mRNAs does correspond to the A/B domain boundary.

These results seem therefore to demonstrate, that the expression of the RXRγ gene resembles that of the RAR genes and of hRXRβ, which have previously been shown to give rise to more than one mRNA species with different or overlapping tissue distributions, that are generated by alternative promoter use and/or alternative splicing of the exons containing the 5' untranslated regions and the sequence coding for the A domains, onto a common downstream group of exons containing the sequences coding for the rest of the protein (see section 1.7.1.1 for details and references).

It is important to point out that due to the lack of another probe that could be used as an internal standard for the normalization of the amount of total RNA hybridized in each track, the results of the RNase protection assays can only be interpreted in quantitative terms within each sample, and therefore they do not allow us to draw any conclusions about the relative abundance of the different chicken RXRγ mRNAs in different

tissues. However, in qualitative terms these experiments clearly demonstrate the specificity of the tissue distribution of these two chicken RXRγ mRNAs.

Comparison of the structure of the two chicken RXRγ mRNAs described here, with that of the two RXRγ mRNAs identified in the mouse (Liu and Linney, 1993) reveals again some striking differences. As described earlier, the mouse RXRγ2 mRNA differs from the mouse RXRγ1 mRNA, only in the sequences 5' to the B domain, coding for a truncated protein isoform that lacks the A domain and probably also the amino-terminal half of the B domain. This truncated mouse RXRγ protein, is however still capable of activating the transcription of a reporter gene containing an RXR-response element in response to RA, albeit with a lower efficiency than the complete receptor. Both chicken RXRγ mRNAs decribed here, code full length protein isoforms with divergent amino-terminal A domains, not just truncated versions of the same protein.

The identification of the promoter regions of the different transcripts of the chicken RXRγ gene, requires further characterization of the upstream sequences of the pP2 and pR2 specific exons present in these genomic clones. Whether these transcripts are generated by alternative splicing of a single primary mRNA transcript, or whether the transcription of this gene involves the use of tissue specific promoters together with alternative splicing, as is the case with several of the RAR isoforms, remains to be proved. However, a *Pst*I-*Sma*I fragment containing the region immediately upstream of the 5' exon of pR2, was shown to have promoter activity, inducing the expression of a reporter gene (CAT) albeit weakly, in a recombinant eukaryotic expression vector (Eager and Brickell, unpublished results). This preliminary data seems to indicate

that the region upstream of the 5' exon of pR2, i. e., the region between the two different 5' exons of RXRγ, could have some promoter activity, suggesting that the transcription of these two mRNAs could involve the use of alternative promoters.

Careful analysis of the results of the northern hybridizations and RNase protection assays, also indicates that there may be several additional minor RXRγ mRNAS that diverge from pP2 and pR2 at their 5' ends:

- first, the hybridization of a northern blot including liver poly (A^+) RNA with an antisense riboprobe synthesized from pR2-B/E (fig. 3.4), showed that there are at least two different size transcripts of chicken RXRγ being expressed in the liver, suggesting that there must be other mRNA species that diverge from cRXRγ2 at a point downstream from the region corresponding to the pP2-E/S probe, since only full protection of this probe was observed when it was hybridized to RNA extracted from this tissue (fig. 4.8, track 6).

- secondly, the presence of a partially protected fragment of 114 nucleotides in both samples where full protection of the pR2 E/S probe was observed, namely 10-day chick embryo eye (fig. 4.7, track 7) and 10-day chick embryo dorsal root ganglia (fig. 4.7, track 8), seems to suggest that other transcripts of this gene are also expressed in these tissues. It is interesting to notice that this partially protected fragment has exactly the same size as the fragment produced with the same probe in the liver (fig. 4.7, track 6). This seems to suggest that small amounts of the smaller RXRγ mRNA present in the liver (cRXRγ2), are also being expressed in the tissues that express the larger RXRγ mRNA (cRXRγ1), however, we know that this is not the case, because no full protection of the cRXRγ2 specific sequences (i. e., of the pP2-E/S probe), was detected in these tissues (fig. 4.8, tracks 7 and 8), even when the autoradiograms

of the ribonuclease protection assays were over-exposed (not shown). These results therefore seem to indicate, that there must be other chicken RXRγ mRNA isoforms that are expressed at low levels in the same tissues that express cRXRγ1, and that probably differ from it at the same position as cRXRγ2.

Finally, the observation that the 5' specific sequences of pP1 are also present in one of the genomic clones of chicken RXRγ, indicates that this cDNA clone is not just an artefact originated during the construction of the cDNA library. Furthermore, analysis of the sequence of pP1 in its divergence point with the other cDNA clones of RXRγ also excludes the possibility that this clone represents an incompletely processed transcript, because no consensus splice acceptor sequences are present in this position.

It seems therefore likely, that this cDNA clone must correspond to a real chicken RXRγ mRNA present in the liver (cRXRγ3), therefore supporting the idea that there are other transcripts of this gene expressed in these same tissues, and also raising the possibility that there might be other chicken RXRγ mRNAs that diverge at their 3' ends. We have no direct evidence for the existence of such carboxy-terminal divergent RXRγ isoforms, although a simple way of testing this would have been to carry out a series of RNase protection assays with probes from the 3' ends of all the cDNA clones isolated.

CHAPTER V

ANALYSIS OF THE CHICKEN RXRα GENE EXPRESSION

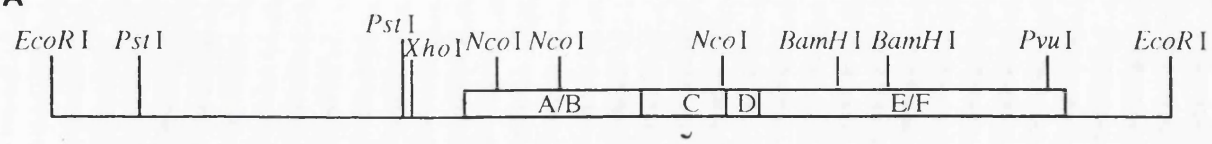
5.1 - Isolation and characterisation of two novel chicken RXRα cDNA clones

The cDNA clones pE1 and pP3 were isolated during the two successive screenings of the adult chicken liver cDNA library in λ–ZAP (Promega), with radiolabelled DNA probes derived from fragments of the chicken RXRγ cDNA clone pR2 (see section 4.2). Following *in vivo* conversion of the λ ZAP recombinant clones to pBluescript recombinant clones, partial sequencing and restriction mapping analysis of pE1 and pP3 showed that they did not correspond to chicken RXRγ mRNAs, although they shared high nucleotide sequence homology with pR2 in the region corresponding to the DNA binding domain, indicating that they probably corresponded to cDNA clones of another member of this family of nuclear receptors.

Clone pE1 contains a 2.6 Kb insert, that consists of a long 5' untranslated region of 959 nucleotides, followed by an open reading frame of 1401 nucleotides, with the potential to encode a protein of 467 amino acids (fig. 5.1). This open reading frame begins with an AUG codon that does not conform well with Kozak's rules for the initiation of translation (Kozak, 1991), however the other AUG codons that are present in the 5' untranslated regions, give rise only to small open reading frames.

Clone pP3 was only partially sequenced, but analysis of its restriction map and of the sequences of the first 250-300 nucleotides of both its 5' and 3' ends, showed that this clone was identical to the sequences present in pE1 from position 945 (right at the beginning of the putative open reading frame), and finishing 27 nucleotides short of its 3' end.

A



B

```
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                                                    M  D  T  K  H                  5
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T  P  S  L  H  P  S  I  G  P  G  I  G  S  S  L  G  S  P  G  Q  L  H  S  P      55
TCAGCACCCTGAGCTCACCCATCAATGGCATGGGGCCACCCTTCTCCGTCATCAGCTCCCCCATGGGCCCACACT    1200
I  S  T  L  S  S  P  I  N  G  M  G  P  P  F  S  V  I  S  S  P  M  G  P  H      80
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S  C  E  G  C  K  G  F  F  K  R  T  V  R  K  D  L  T  Y  T  C  R  D  N  K      180
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T  E  L  V  S  K  M  R  D  M  Q  M  D  K  T  E  L  G  C  L  R  A  I  V  L      380
TCAACCCCGACTCAAAAGGTCTCTCCAACCCGGCTGAAGTGGAGGCGTTACGGGAGAAGGTGTACGCATCGCTGG    2175
F  N  P  D  S  K  G  L  S  N  P  A  E  V  E  A  L  R  E  K  V  Y  A  S  L      405
AGGCGTATTGCAAGCACAAATACCCCGACCAGCCCCGGGAGGTTCGCCAAGCTCCTGCTCCGCCTGCCAGCCCTAC    2250
E  A  Y  C  K  H  K  Y  P  D  Q  P  G  R  F  A  K  L  L  L  R  L  P  A  L      430
GGTCCATCGGCCTCAAATGCCTGGAGCACCTCTTCTTCTTCAAGCTAATAGGCGACACGCCGATCGACACCTTCT    2325
R  S  I  G  L  K  C  L  E  H  L  F  F  F  K  L  I  G  D  T  P  I  D  T  F      455
TGATGGAAATGCTGGAAGCACCCCATCAAATGACTTAGAGAACTGCCGCTGGCTCAGTCCCTCACCCGCCAGGGC    2400
L  M  E  M  L  E  A  P  H  Q  M  T  *                                          467
CACGCGGGCTCCGATCCTCTGCTTCCTCCTCCTCAGCCCCAGCCCCCTTCCCTCTGCTCCCCAAATGCTGGAGGC    2475
ACCGTCCGAGCTTCTCCGCCTGGTCCCCGGGGCGAACCCGCACCTCCTTGCCGCCTCCTCACGTTGACCCTTCTG    2550
TCCGTTCGCTGTCACTGCTGCATCTAAAGACCTCTGATGGCTGTGgaattc                             2601
```

Figure 5.1

Figure 5.1

(A) Restriction map of the insert of the cDNA clone pE1; of cRXRα. The 5' and 3' untranslated regions are shown as solid lines. The coding region is boxed, showing the approximate position of the boundaries of the main protein domains (A-F).

(B) Nucleotide sequence and predicted amino acid sequence derived from the cDNA clone pE1. *Eco*RI linker sequences are shown in lower case. The putative DNA binding domain is boxed. The nucleotide and amino acid sequences are numbered on the column on the right.

The open reading frame displayed, starts at the first AUG codon in frame with the rest of the sequence, although it does not conform well with Kozak's rules for the initiation of translation (Kozak, 1991).

The predicted amino acid sequence of the putative coding region present in pE1 was compared with those of the other RXR genes, using the program PC-Gene (Intelligenetics Inc., California USA) (figs. 5.2 and 5.3). High levels of identity were observed between the predicted amino acid sequence of pE1 and those of human and mouse RXRα (Mangelsdorf *et al.*, 1990, 1992), and with the sequences of the DNA- and ligand-binding domains of mouse RXRβ (Hamada *et al.*, 1989; Mangelsdorf *et al.*, 1992) and of mouse- (Mangelsdorf *et al.*, 1992), rat- (Georgiades and Brickell, unpublished results), and chicken RXRγ (Rowe *et al.*, 1991). No significant amino acid sequence identity was shared with the RARs or any other member of the steroid/thyroid hormone receptor superfamily, outside of the highly conserved DNA-binding domain.

From these results we can conclude that pE1 (and pP3) corresponds to a cDNA clone of chicken RXRα.

5.2 - RXRα gene expression in embryonic and adult chicken tissues

A *Bam*HI-*Eco*RI fragment of the cDNA clone pE1 (nucleotides 1937-2590), containing the sequences coding for the last 140 amino acids of the coding region and the 3' untranslated region, was subcloned into pBluescript SK+, producing the plasmid pE1-B/E (appendix A). As pR2-B/E, this subclone was designed so that it contained sequences corresponding to part of the E and F domains, and none of the regions that are highly conserved amongst the members of the steroid thyroid hormone superfamily, ensuring that it could be used for the synthesis of radiolabelled antisense RNA probes specific for RXRα transcripts.

Preliminary analysis of the expression of the chicken RXRα gene, was carried out by probing two northern blots of total RNA extracted from a range of embryonic and adult chicken tissues, with a ^{32}P-labelled antisense riboprobe derived from pE1-B/E.

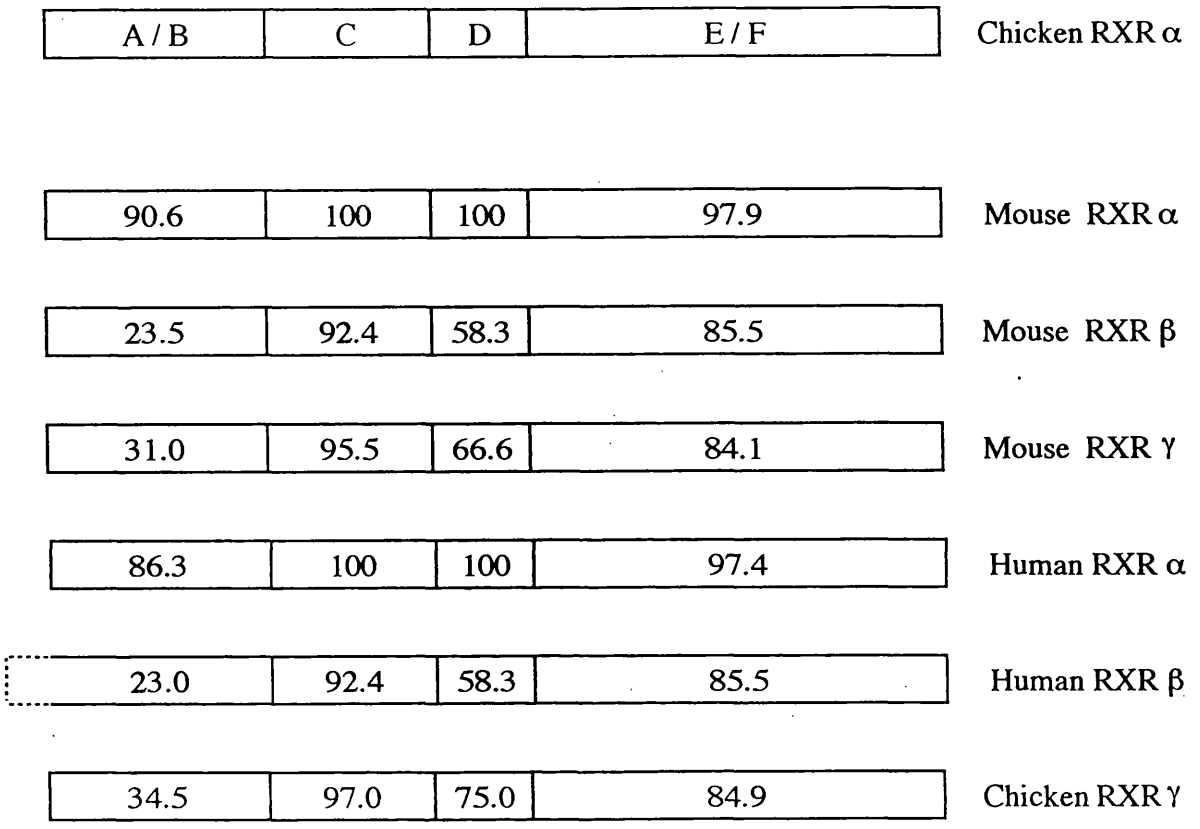

Figure 5.2

Comparison of the predicted amino acid sequence of chicken RXRα, with those of mouse RXRα (Mangelsdorf et al., 1992), RXRβ (Hamada et al., 1989) and RXRγ (Mangelsdorf et al., 1992), human RXRα (Mangelsdorf et al., 1990) and RXRβ (Leid et al., 1992), and chicken RXRγ (Rowe *et al.*, 1991). In each domain, the percentage amino acid homology with the sequence of chicken RXRα is indicated.

The dashed lines represent a region of 40 to 60 amino acids present in the amino-termini of both isoforms of hRXRβ, that has no appreciable homology with the predicted sequences of any of the other RXRs.

```
cRXRα    MDTKHFL--PLDFSNQVNSTSLNSPTSRGPMATPSLHPSIGPGIGSSLGSPGQLHSPIS-TLSS    61
mRXRα    .......--.....T....S......G..S..V......L......P.............-....    61
hRXRα    .......--.....T....--.T...G..S..A......L......P-------------....    56
mRXRβ                            MLGPDSRSPDS.SPNPLSQ..RP.SPPGPP.TPSA------    35
hRXRβ  (...)GGEQQT.EPEPGEAGRDGMGDSGRDSRSPDS.SPNPLPQ.VPPPSPPGPP.PPSTAP..GG    95
mRXRγ  MYGNYS..MKF.TG.GGSPGHTGST.MSPSVALP.GKPMD.HPSYTDTPVSA.RT.SA-----VGT    61
cRXRγ  MYGNYP..IKF.AG.G.SPVHA.ST.VSPSSSLSVG.TVDGHHNYLEAPTNASRA.P..MN-.IG.    65

cRXRα  ---------PINGMGPPFSVISSPMGPHSMSVPST-PSLGFGTSSPQLNSPIN----PVSSSEDIK   113
mRXRα  ---------.............................T.-.T.....G.......M.----....T....   113
hRXRα  ---------.............................T.-.T...S.G....S..M.----.........   108
mRXRβ  ----PPPPM.PPPL.S..P....S..SPGLPP.AP-.GFSGPV....I..TVSLPGGGSGPP..V.    96
hRXRβ  SGAPPPPPM.PPPL.S..P....S..SPGLPP.AP-.GFSGPV....I..TVSLPGGGSGPP..V.   160
mRXRγ  ---------.L.AL.S.YR..T.A...P.GALAAPPGINLVAPP.S...V-V.----S........   113
cRXRγ  ---------.V.AL.S.YR..A.SI.S.PVALS.SA.GMN.V.H...P.V-L.----N........   117

cRXRα  PP-LGLNGVLKVPAHPSGTMASFTKHICAICGDRSSGKHYGVYSCEGCKGFFKRTVRKDLTYTCRD   178
mRXRα  ..-..............N.........................................          178
hRXRα  ..-..............N.........................................          173
mRXRβ  ..V..VR.LHCP.--.P.GPGA-G.RL.............................I......S...   159
hRXRβ  ..V..VR.LHCP.--.P.GPGA-G.RL.............................I......S...   223
mRXRγ  .L-P..P.IGNM-NY..TSPG.LV...............................I....I.....   177
cRXRγ  .L-P..P.IGNM-NY..TSPG.LA...............................I....I.....   181

cRXRα  NKDCLIDKRQRNRCQYCRYQKCLAMGMKREAVQEERQRGKDRNENEVESTSSANEDMPVEKILEAE   244
mRXRα  ..........................................................          244
hRXRα  ..........................................................R.....   239
mRXRβ  ....TV..................T..............K-DGDGDGAGG.P.E...DR.....   224
hRXRβ  ....TV..................T..............K-DGDGEGAGG.P.E...DR.....   288
mRXRγ  ......................V..............SRE.A.S.A.CA..SH......R.....   243
cRXRγ  ....................................GSRE.S...A....GGS......R.....   247

cRXRα  LAVEPKTETYIEAN--MGLTPSSPNDPVTNICQAADKQLFTLVEWAKRIPHFSELPLDDQVILLRA   308
mRXRα  ..........V.------.N.....................................          308
hRXRα  ..........V.------.N.....................................          303
mRXRβ  ....Q.SDQGVEGPGAT.GGG..................................S.....      290
hRXRβ  ....Q.SDQGVEGPGGT.GSG..................................S.....      354
mRXRγ  ........S.GDM.------VEN.T........H.....................D.T.E.....   304
cRXRγ  ........A.SDV.------.E..T........H.....................D.T.E.....   308

cRXRα  GWNELLIASFSHRSIAVKDGILLATGLHVHRNSRHSAGVGAIFDRVLTELVSKMRDMQMDKTELGC   374
mRXRα  ..................................A.....................          374
hRXRα  ..................................A.....................          369
mRXRβ  ..............D.R..............A.....................R.....      356
hRXRβ  ..............D.R..............A.....................R.....      420
mRXRγ  ..............VS.Q.............S.A..R...S.............K......S....   370
cRXRγ  ..............VS.Q.............S.A......S.............K......S....   374

cRXRα  LRAIVLFNPDSKGLSNPAEVEALREKVYASLEAYCKHKYPDQPGRFAKLLLRLPALRSIGLKCLEH   440
mRXRα  ..........................................E.....................   440
hRXRα  ..........................................E.....................   435
mRXRβ  ....I.....A......G...I..........T...Q...E.Q.....................   422
hRXRβ  ....I.....A......S...V..........T...Q...E.Q.....................   486
mRXRγ  ..........A......S...T.......T....T.Q...E.....................   436
cRXRγ  ..........A....S.S...S.......T....T.Q...E.....................   440

cRXRα  LFFFKLIGDTPIDTFLMEMLEAPHQMT*                                      467
mRXRα  ........................A.                                       467
hRXRα  ........................                                         462
mRXRβ  ........................LA                                       449
hRXRβ  ........................LA                                       513
mRXRγ  ............S.......T.L.I.                                       463
cRXRγ  ..................T.L.V.                                         467
```

Figure 5.3

Figure 5.3

Multiple alignment of the predicted amino acid sequences of chicken RXRα, with those of mouse RXRα (Mangelsdorf et al., 1992), RXRβ (Hamada et al., 1989) and RXRγ (Mangelsdorf et al., 1992), human RXRα (Mangelsdorf et al., 1990) and RXRβ (Leid et al., 1992), and chicken RXRγ (Rowe *et al.*, 1991). Multiple sequence alignment was performed with the program "Clustal", of the PC/GENE sequence analysis package (Intelligenetics Inc., California USA).

The sequences corresponding to the DNA binding domains are boxed. "(...)" represents a region of 40 to 60 amino acids, present on the amino-termini of both isoforms of hRXRβ, that has no appreciable homology with the predicted sequences of any of the other RXRs. The character "." indicates that the amino acid present in that position is identical to that present in cRXRα. The character "-" indicates a gap in the alignment of the sequences. The amino acid sequences are numbered on the column on the right.

The results of these northern blot hybridizations show that the chicken RXRα gene is expressed as a single major transcript of approximately 5.1 kb, which is present in most of the tissues analysed (fig. 5.4). The highest levels of expression of the chicken RXRα gene are detected in 10 day embryo tissues (fig. 5.4, panel A tracks 2 and 3, panel B track 2), with lower levels of expression in some of the adult tissues, such as the eye, brain, duodenum, spleen, ovary and kidney (fig. 5.4, panel A tracks 5, 8, 9 and 10, panel B tracks 3 and 6, respectively), and traces of expression in most of the remaining adult tissues, which are apparent upon over-exposure of the hybridized blot (not shown).

A minor transcript of approximately 5.3 kb can also be seen in the tissues where there are higher levels of expression of the chicken RXRα gene, such as 10 day whole embryo, 10 day embryo liver and adult eye (fig. 5.4, panel A tracks 2, 3 and 5).

A more detailed analysis of the pattern of expression of the chicken RXRα gene during development, was carried out by in situ hybridization of an ^{35}S-labelled antisense RNA probe, synthesised from the same template as the probe used in the northern blot hybridizations (pE1-B/E), on sections of chicken embryos at various developmental stages. In all cases, adjacent sections were hybridized to an ^{35}S-labelled negative control sense probe, synthesised from the same template.

These experiments revealed that the chicken RXRα gene is expressed more or less ubiquitously in the chicken embryo at all the stages examined, with some variation of the intensity of the signal detected in different tissues (figs. 5.5 and 5.6).

At the earliest stage tested in this work (stage 18), the chicken RXRα gene is already ubiquitously expressed, with slightly higher levels of expression in the neural tube (fig. 5.5, panel A). At later stages, the

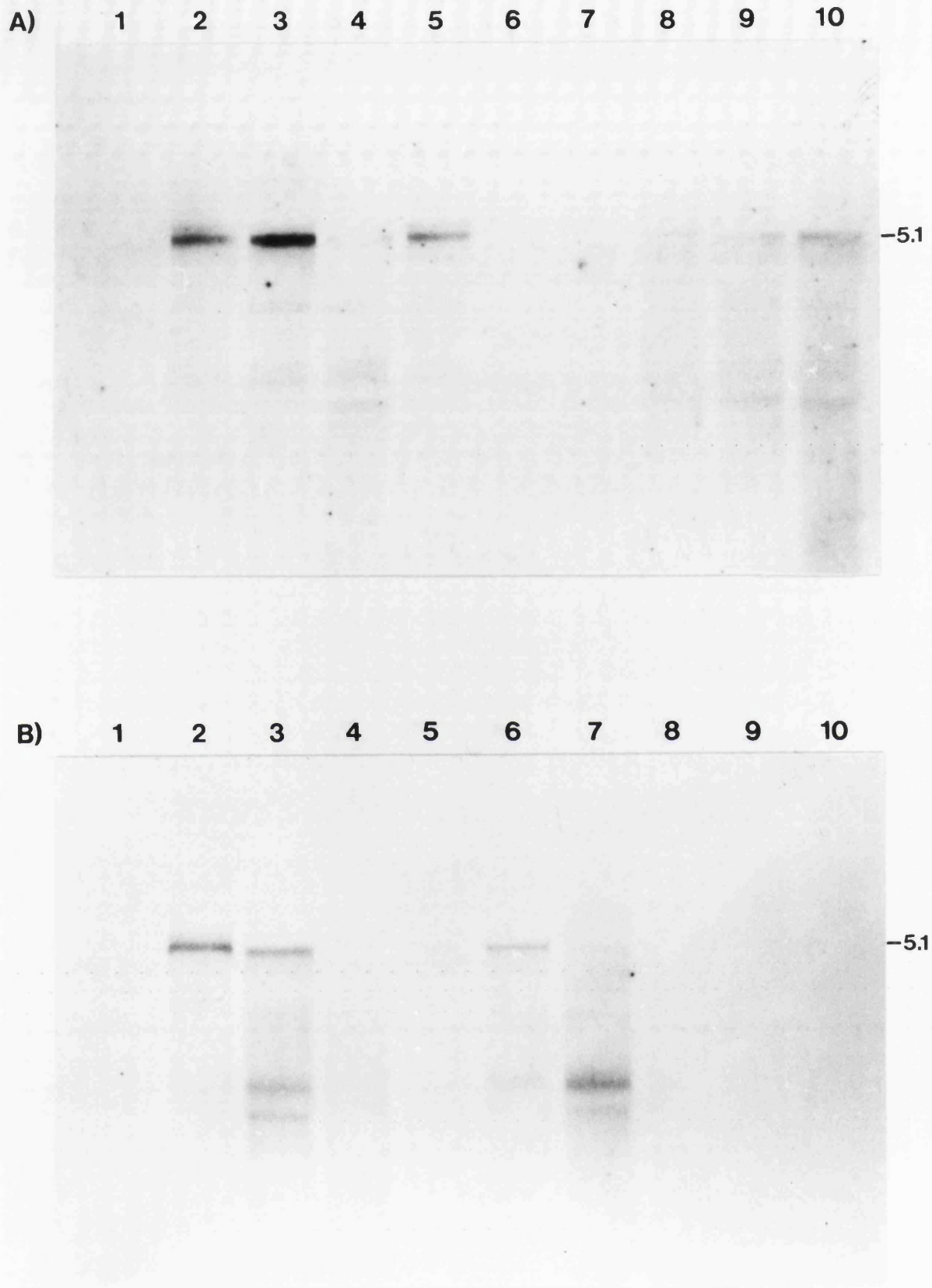

Fig. 5.4

Figure 5.4

Northern blot analysis of the expression of cRXRα transcripts in embryonic and adult chicken tissues.

Northern blots of total RNA isolated from embryonic and adult chicken tissues, hybridized with a probe specific for cRXRα transcripts (antisense riboprobe synthesized with T7 RNA polymerase, using *Bam*HI-linearised pE1-B/E as a template).

Tracks contain approximately 20μg of total RNA isolated from:

(A) 1 - RNA size marker (GIBCO-BRL, approximately 5μg); 2 - 10 day whole embryo; 3 - 10 day embryo liver; 4 - adult liver; 5 - adult eye; 6 - adult adrenal glands (approximately 10μg); 7 - 10 day embryo dorsal root ganglia (approximately 10μg); 8 - adult brain; 9 - adult duodenum; 10 - adult spleen.

(B) 1 - RNA size marker (GIBCO-BRL, approximately 5μg); 2 - 10 day whole embryo; 3 - adult ovary; 4 - adult skin; 5 - adult heart; 6 - adult kidney; 7 - adult skeletal muscle; 8 - adult gizzard; 9 - adult pancreas; 10 - adult lung.

The size of the transcript is indicated in kilobases.

Notice the existence of a minor transcript of the chicken RXRα gene just above the 5.1 kb RXRα mRNA, that is apparent in those tissues where expression of this gene is more abundant. It is also possible that there is another transcript of the chicken RXRα gene that comigrates with the chicken 18S rRNA, that is expressed in some tissues like the adult liver (panel A, track 4) adult ovary (panel B, track 3) and adult adult skeletal muscle (panel B, track 7).

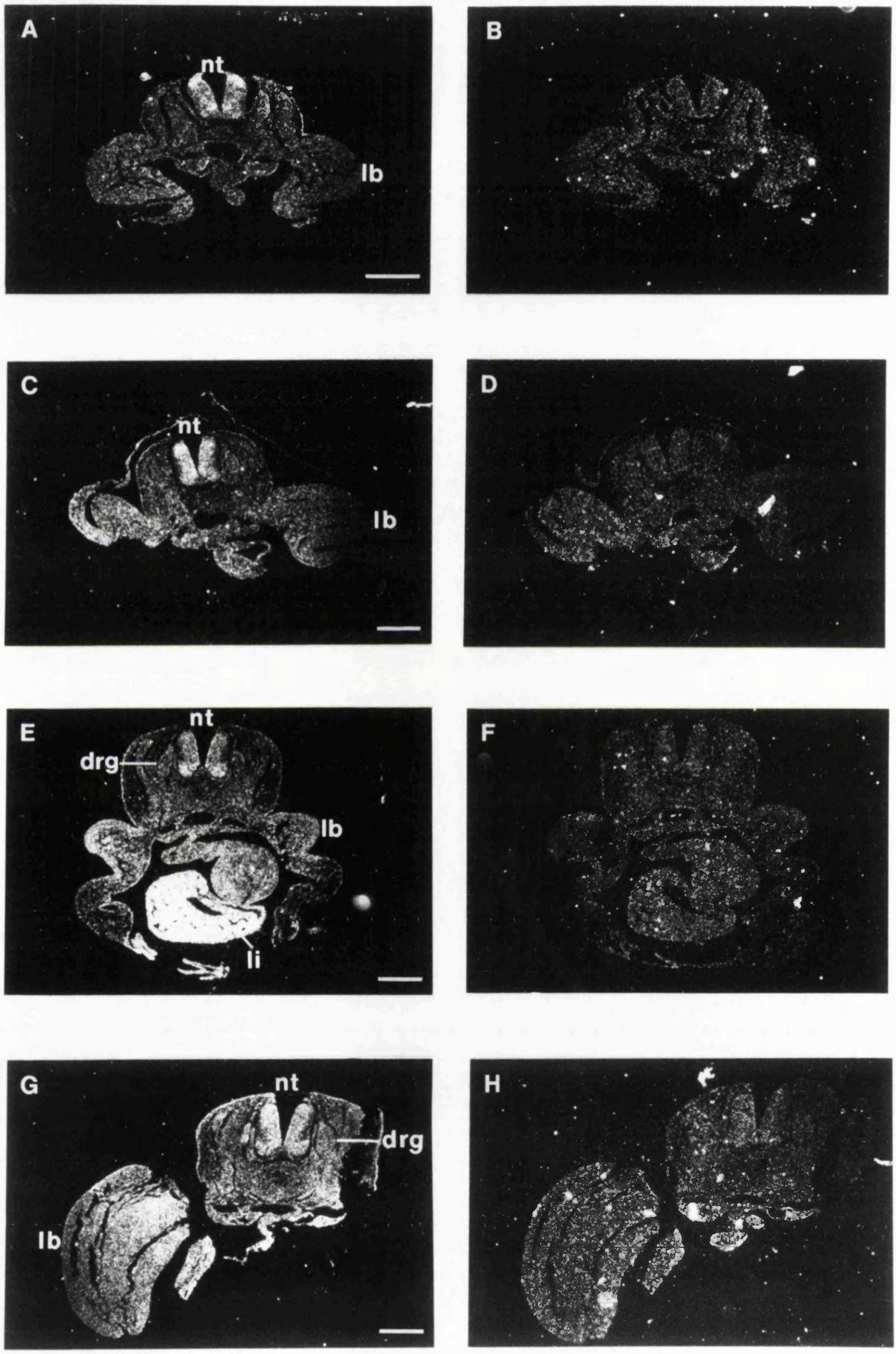

Fig. 5.5

Figure 5.5

In situ hybridization analysis of the distribution of cRXRα transcripts in early chick embryos.

Panels A, C, E and G show transverse sections at the level of the wing buds of chick embryos at stages 18, 20, 22 and 24 respectively, hybridized with a probe specific for cRXRα transcripts; panels B, D, F and H show the corresponding adjacent sections hybridized with the negative control probe. drg - dorsal root ganglia; li - liver; lb - limb bud; nt - neural tube; nr - nerves.

Sections are shown under dark-field illumination. Scale bar = 250 μm.

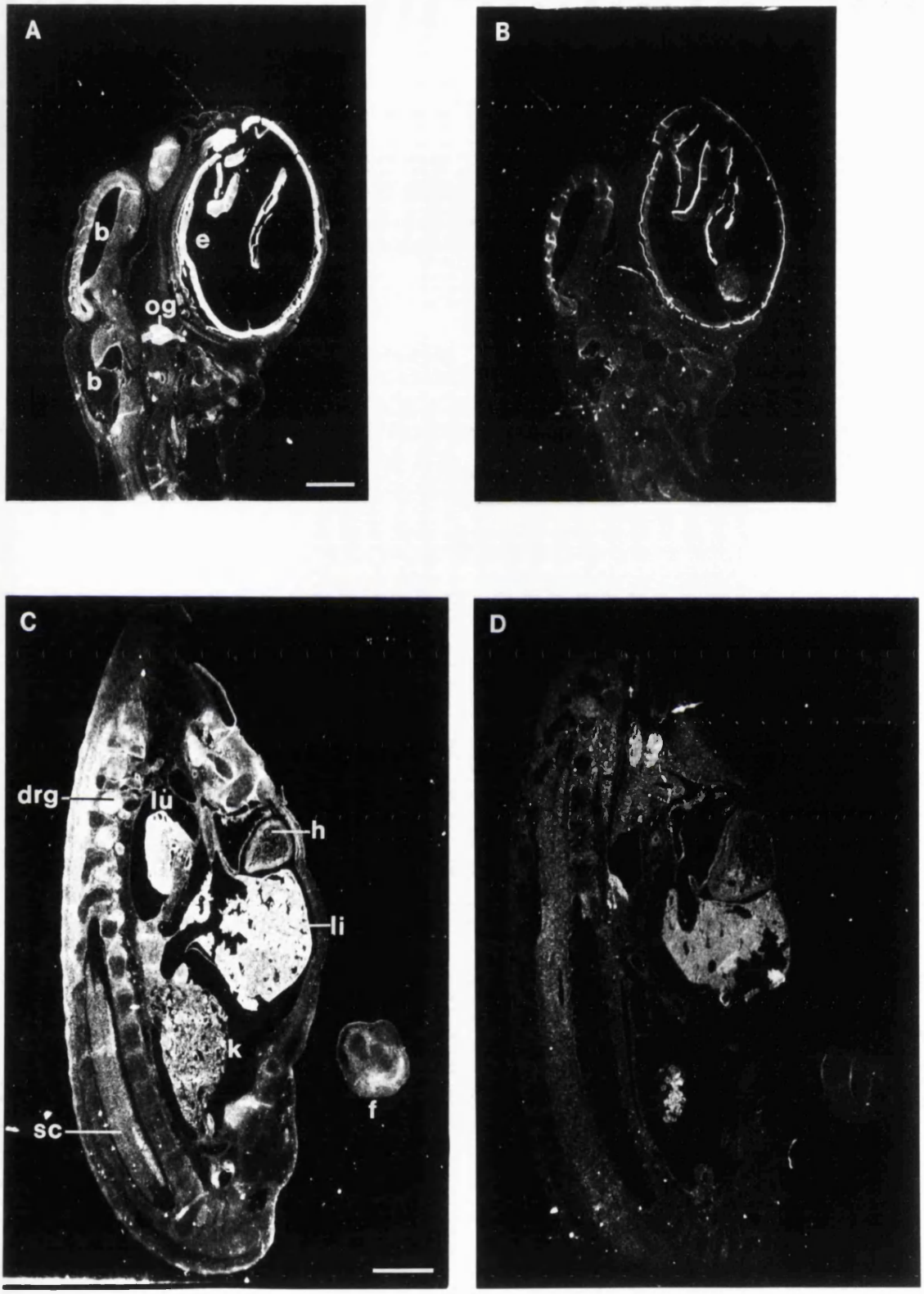

Fig. 5.6

Figure 5.6

In situ hybridization analysis of the distribution of cRXRα transcripts in stage 31 (approximately 7 days of incubation) chick embryo.

Panel A shows a parasaggital section of the head of a stage 31 chick embryo, hybridized with a probe specific for cRXRα transcripts; panel B shows an adjacent section, hybridized with the negative control probe.

Panel C shows a parasaggital section of the body of a stage 31 chick embryo, hybridized with a probe specific for cRXRα transcripts; panel D shows an adjacent section, hybridized with the negative control probe.

b - brain; drg - dorsal root ganglia; e - eye; f - foot; h - heart; k - kidney; li - liver; lu - lung; optic ganglion; sc - spinal cord.

Sections are shown under dark-field illumination. Scale bar = 1 mm.

neural tube remains one of the tissues with higher levels of expression of this gene, together with the liver, and the proximal mesenchyme of the limb buds (fig. 5.5, panels C, E and G).

In 7 day old embryos, the highest levels of expression of chicken RXRα are detected in the brain, in elements of the peripheral nervous system, in the eye, and in the viscera, with lower levels of expression in the remaining tissues (fig. 5.6, panels A and C).

5.3 - Analysis and discussion of the results

The results of the northern blot hybridizations with the RXRα specific probe, reveal an expression pattern for this gene in the adult chicken that is closely similar to that previously reported in the adult rat (Mangelsdorf *et al.*, 1992). In both cases, a single major RXRα mRNA of 5.1 in the chicken and 5.6 kb in the rat, was expressed in most of the tissues analysed, with higher levels of expression in visceral tissues (and in the eye, not analysed in the rat northern blots).

The in situ hybridization experiments show however significant differences in the pattern of expression of this gene in chicken and mouse embryos. The RXRα gene is abundantly expressed in most tissues of the developing chicken embryo, whilst in the embryo and neonatal mouse, this gene is predominantly expressed in the epithelia of the digestive system, the skin and the liver.

The early ubiquitous expression of the RXRα gene in the chicken, and the later more restricted expression pattern in the adult, do not give any clues about the possible fuctions of this receptor in this animal. The pattern of expression of the RXRα gene in the mouse, particularly in the embryo, seems to be strongly associated with tissues involved in vitamin A and lipid metabolism, such as the liver, kidneys, spleen and intestine.

The expression pattern of this gene in the chicken embryo seems however to be more closely related to the expression patterns of the RARα or RXRβ genes in the mouse, which are both widely expressed in the developing and adult animal.

Finally, the results of the northern hybridizations also seem to argue in favour of the existence of at least another different size transcript of this gene. It is impossible at this point to determine whether this corresponds to a real different isoform of the chicken RXRα gene, but a more careful examination of its expression should allow us to establish the structure of this different transcript, and how it compares with the general structure of the isoforms of the other RAR and RXR genes.

CHAPTER VI
CONCLUSIONS AND OUTLOOK

6.1 - General discussion

In this work I have shown that the expression of the chicken RXRγ gene is similar to that of most other members of the RXR and RAR families, which have been shown to give rise to several different mRNA species coding for protein isoforms differing only in the sequences corresponding to their amino-terminal A domains.

This complex pattern of expression seems therefore to be a common feature of the expression of most genes of both families of retinoid receptors. The probable evolution of these two types of receptors from a common ancestral nuclear receptor gene, must have played a part in the generation of the similarities between the structure of the isoforms of both RAR and RXR genes, however, the maintenance of the expression of all these different variants throughout the significantly divergent evolution of these two families, indicates that the existence of multiple RAR and RXR protein isoforms must play an extremely important function in the activity of these receptors.

As mentioned in the Introduction, the A and B domains of the RARs have been shown to modulate the extent of the activation of transcription of promoters containing retinoic acid response elements, in a promoter-context and cell-type specific fashion (Nagpal *et al.*, 1992a Mader *et al.*, 1993a and b). The existence of several tissue specific isoforms of the same RAR with different A domains, therefore provides an elegant way of changing the specificity of their activity, allowing the tissue specific regulation of the patterns of expression of distinct or overlapping sets of target genes. It has recently been shown that the A/B regions of the RXRs also have a cell- and promoter-context specific transactivation function,

and therefore that the different A regions of the various RXR isoforms, will play a similar function in the modulation of the activity of these proteins (Nagpal *et al.*, 1993Liu and Linney, 1993). Furthermore, in the case of the RXRs, the existence of multiple isoforms, will not only increase the diversity of the response induced by homodimers of these receptors, but also, of the heterodimers formed with the other Type II nuclear receptors, including RARs and the receptors for thyroid hormone, vitamin D and peroxisome-proliferator-activated receptors.

In conclusion, the existence of several tissue- and developmental stage-specific, amino-terminal isoforms of the RARs and of the RXRs, has an important effect in the amplification of the diversity of transcriptional responses mediated by these molecules, allowing them to play different functions in different tissues at various stages of development and differentiation, therefore providing a mechanism for the generation of the highly pleiotropic effects of retinoids in development and in the adult animal.

A striking feature of the expression of both the RXRγ and RXRα genes, are the differences in their expression patterns in the chicken and in the mouse or rat, which are in sharp contrast with the generally very conserved expression patterns of the RARs in different organisms. These differences are extremely puzzling, and seem to exclude any possibility of these receptors having an important, and therefore conserved role across species.

It has been proposed that there must be a considerable degree of functional redundancy between the different members of each of the retinoid receptor families. The main support for this argument so far, has been the equally surprising results obtained with transgenic mice in which the RAR genes have been disrupted by homologous recombination. No or very minor malformations are observed respectively in RARα or RARγ

null homozygous transgenic mice, in sharp contrast with the widespread expression patterns of these genes (Lohnes *et al.*, 1993 Lufkin *et al.*, 1993), but the severity of the effects is dramatically increased when these mice are crossed, and double RARα and RARγ null homozygous mice are generated (P. Chambon personal communication). These results were interpreted in terms of the existence of a broad functional overlap between the different members of this multigene family, so that they can substitute for each other's functions in most tissues. As the expression of more receptors of this family is disrupted, it becomes progressively more difficult to maintain normal control of retinoid-responsive gene expression, and more defects become evident.

Similarly, it is possible that there is a considerable degree of functional redundancy between the different members of the RXR family. The widely divergent patterns of expression of the RXR genes might therefore be explained in terms of the high level of functional overlap between these receptors, which would allow the rapid divergence of their distribution patterns during evolution, without major effects on the regulation of the expression of the target genes. On the other hand, the fact that such a divergence of the expression patterns is never observed within the RARs, could reflect a slightly lesser degree of functional redundancy of these receptors.

This explanation is however purely speculative, and it does not solve the paradox that although there is an apparent relaxation of the pressure to conserve the expression patterns of these genes throughout evolution, particularly between the RXR genes, there has not been an equivalent significant divergence of the sequences of the various receptors and isoforms across species.

These results seem to suggest that the functions of the various receptors within a family are very closely interconnected. In this respect it is interesting to note that the expression of both RXRα transcripts in the mouse, and of RXRγ transcripts in the chicken, seem to be at least superficially associated with retinoid metabolism. The mouse RXRα gene is highly expressed in the intestine and in the liver and kidneys (Mangelsdorf *et al.*, 1991 and 1992), whilst the chicken RXRγ gene, as shown in chapter 3, has its highest levels of expression in the liver and retina, all tissues with an important role in the uptake, storage, mobilisation or metabolism of vitamin A. Furthermore, the demonstration that the CRBP II gene contains an RXR response element that is specific for homodimers of these receptors (Mangelsdorf *et al.*, 1991; Yu *et al.*, 1991) further connects the expression of the RXRs with vitamin A metabolism.

The regulation of vitamin A metabolism has obvious implications in retinoic acid synthesis and activity, and the teratogenic effects of both these molecules have long been recognised to be closely associated, as demonstrated by the observation that retinoic acid administration could prevent or reverse most of the effects generated by vitamin A deficiency in fetuses (Dowling *et al.*1960). It is therefore possible that the RXR genes have a more or less general role in maintaining a close control on various aspects of the retinoid metabolism.

In a recent report, Thaller *et al.* (1993) proposed that 9c-RA could be the active retinoid in the avian wing bud. They demonstrated that exogenously applied 9c-RA is approximately 25 times more potent than at-RA, in the induction of digit duplications, and that the magnitude of this increase is very similar to the ratio of *in vivo* conversion of exogenously applied at-RA to its 9-cis isomer, measured in the tissues of the limb bud.

This correlation between the isomerisation equilibrium of the two forms of retinoic acid, and the increased efficiency of the generation of limb duplications by 9c-RA, raises the possibility that 9c-RA could be the molecule responsible for respecifying the positional information of the cells across the anterio/posterior axis of the limb bud, in the retinoic acid bead implant experiments. If retinoic acid is indeed the morphogen responsible for the patterning of the anterio/posterior axis of the normal developing limb buds, then these results also raise the possibility that its active isomer might be 9c-RA, with at-RA being simply a virtually inactive precursor.

This emphasises the importance of the characterisation of the expression patterns of the various members of the RXR gene family in the early limb buds, since these receptors respond specifically to 9c-RA, as opposed to the RARs which have similar activities in the presence of either 9c-RA or at-RA. Using reverse transcriptase-polymerase chain reaction analysis, Thaller *et al.* (1993) showed that chicken RXRγ mRNA could be detected in the wing buds of embryos at the appropriate stages of development. This led to the speculation that chicken RXRγ could be involved in the interpretation of the polarizing activity of exogenous or endogenous 9c-RA.

In the present work I have demonstrated that there is no widespread expression of the chicken RXRγ gene in early limb bud mesenchyme at stages 18 to 24, when limb patterning is occuring, and so it cannot be involved in mediating this proposed function of 9c-RA. The detection of chicken RXRγ sequences from limb bud tissues at these stages by reverse transcriptase-polymerase chain reaction, probably results from amplification of RXRγ mRNA present in neural crest cells that are beginning to migrate into the proximal regions of the limb bud, and that will later form the local elements of the peripheral nervous system.

The early expression of chicken RXRα in the limb buds, at the stages when the patterning events are occuring, make it a possible candidate for mediating the proposed effects of 9c-RA as a positional signalling molecule, although its distribution in the mesenchyme of the limb bud, and its low level ubiquitous expression throughout all stages of development, make it hard to conceive that it could play such an important role in a local positional patterning event.

6.2 - Future work

It was not possible to determine, by the methods used in this study, whether there is real expression of RXRγ mRNA in chicken melanocytes. This could probably be studied by whole mount in situ hybridization with an RXRγ specific probe, on 7-10 day chick embryo skin. This information would allow us to establish whether the expression of this gene is maintained in all neural crest derivatives that do not undergo mesectodermal differentiation, or whether it is strictly associated with their differentiation into peripheral nervous system cells.

Additionally, it would be interesting to confirm whether there is expression of the RXRγ gene, in the neurons of the cranial ganglia that are derived from the ectodermal placodes. This would determine whether the expression of the chicken RXRγ gene in the peripheral nervous system is specifically associated with its embryonic origin, or with the regulation of some functional characteristic of this group of cells.

It would also be interesting to explore the possible roles of chicken RXRγ in chick retinal cell differentiation. This could be done in retinal cultures, varying the amounts and types of retinoids in the culture medium, and possibly with the use of recombinant retroviral vectors to study the effects of the overexpression of this gene on the differentiation of these cultures.

As for the structural analysis of the expression of the chicken RXRγ gene, I have shown in the present work that there must be other chicken RXRγ mRNAs, differing from the ones characterised in this work in their 5' ends. Furthermore, it would be interesting to examine the existence of chicken RXRγ mRNAs with 3' divergent regions, which could be carried out by RNase protection of probes constructed from the 3' ends of the chicken RXRγ cDNA clones already isolated. Further

screening of the same, or of other chicken cDNA libraries, should allow us to isolate and study these different mRNA isoforms of the chicken RXRγ gene.

It was not possible to determine by RNase protection whether clone pP1, isolated during this work, corresponded to a bona fide mRNA of chicken RXRγ. Northern blot hybridizations with probes specific for the unique sequences of this clone, or reverse transcriptase/PCR using primers spanning the divergence point between the sequences of pP1 and those of the other cDNA clones of chicken RXRγ, should allow us to finally answer this question.

A large part of the genomic characterisation of the chicken RXRγ gene remains to be done. The clone CRXR-Gen3 seems to contain the full length of the sequences present in the RXRγ cDNA clones. The construction of a full genomic map of the chicken RXRγ gene, would allow us to determine whether this gene has a distinctive genomic structure in the chicken, similar to the one reported in the mouse (Liu and Linney, 1993).

The characterisation of the genomic structure of chicken RXRγ would set the ground for the study of the nature and the structure of the promoter region(s) of this gene, and of the mechanisms of regulation of the tissue specific expression of the different mRNA isoforms of chicken RXRγ.

In functional terms, it would be of extreme interest to compare the transactivation activities of each of the isoforms of chicken RXRγ. This study could be carried out in a culture system, by co-expressing each of these chicken RXRγ isoforms, alone or in the presence of one of their heterodimerisation partners, with a range of reporter constructs containing natural or artificial RA, VitD and TH responsive promoters.

This work should contribute to the understanding of how each of the unique regions of these different isoforms of the RXRs, modulate the response of these receptors in different cell types, different promoters, different response elements, and possibly it would also provide some clues as to the possible functions of RXRγ in the various tissues where it is expressed.

In the present work I have carried out only a very preliminary characterisation of the expression of the chicken RXRα gene, and most of this study still remains to be done.

The results presented in this work, seem to suggest the existence of at least twodifferentsize mRNAs of chicken RXRα. Therefore it would be interesting to examine in detail the structure of the transcripts of this gene expressed in different tissues.

The study of the genomic organisation of chicken RXRα has not yet been initiated. The comparison of the genomic structure of the chicken RXRα and RXRγ genes with each other, and with genomic structures of the same genes reported in other organisms, and with those of other members of the superfamily, would allow us to examine the evolution of this group of proteins.

Finally, it would be extremely important to study the expression of the other chicken RXR gene, RXRβ. During this work I have tried to isolate a cDNA clone of this gene, by differential screening of a chicken cDNA library with probes for the conserved sequences of all the RXRs, and probes specific for RXRα and RXRγ. This approach was not successfull, however, it is imperative that this work is carried out in the near future, particularly due to the important implications that this gene could have, on the modulation of the effects of 9c-RA in chick limb development.

In conclusion, although the present study contributed some new data about the expression of the RXRα and RXRγ genes in the chicken, it has by no means completed their characterization, instead, it has opened the path to a wide range of studies, that will eventually allow us to have a better understanding of the mechanisms of function, and of the physiological roles of this important group of nuclear retinoid receptors.

POSTSCRIPT

In a recent development, several groups have independently reported the isolation of several members of a new family of vertebrate genes, closely similar to the *Drosophila* segment polarity gene *hedgehog*.

In a series of elegant experiments, Riddle and collaborators (1993) showed that the product of the chicken *sonic hedgehog* gene, appears to function as the long sought putative morphogen signal produced by the cells of the polarising region, responsible for the anterio-posterior patterning of the developing limb buds. In a different report published simultaneously, Echelard and collaborators (1993), showed that the same gene product may play a role in the induction and patterning of the ventral region of the central nervous system.

These findings, forced a rapid (and sometimes painful) reevaluation of the proposed roles of retinoids as master regulators in development and differentiation. Indeed, some of the ideas discussed in this thesis, in particular the implications of the expression patterns of chicken RXRγ and chicken RXRα on the interpretation of a putative retinoid signal across the limb bud, were rendered virtualy meaningless. They have not however in my opinion, diminished the interest of the study of the roles of retinoids and of their receptors and binding proteins in development and in the adult animal; retinoids and their receptors are present at the correct times and in the correct places to play an important role in these patterning processes. In this respect it is interesting to notice that the local application of retinoic acid to the anterior margin of developing limb buds, induced the expression of the *sonic hedgehog* gene in the surrounding tissues.

The race is now on for the discovery of the true roles of retinoids in development and differentiation.

APPENDIX A

Restriction maps of some of the subclones constructed and used during this work. Insert sequences are shown as solid lines. Vector sequences are shown as dashed lines. Only the polylinker of the vector (pBluescript SK+) is displayed, showing the position of the T3 and T7 promoters, for orientation purposes. Sequences belonging to the coding regions of the cDNA clones are shown as boxes. Clones are not shown to scale.

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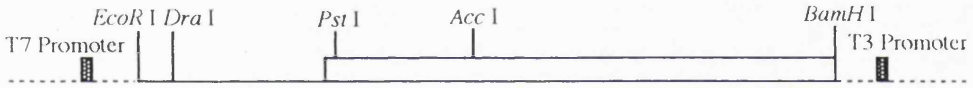
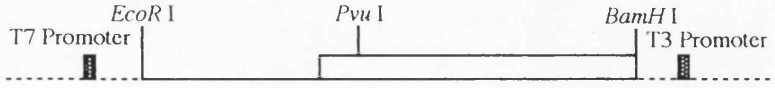
pR2 B/E



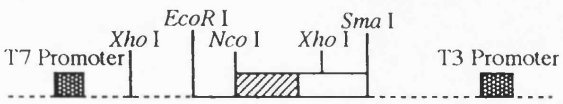
pE1 B/E



pR2 E/S

pP2 E/S

pP1 E/S



APPENDIX B

The embryonic development of the avian eye.

The developing eyes first appear as two lateral projections of the forebrain, the optic vesicles. These vesicles come in contact with the overlying ectoderm, subsequently inducing its differentiation, in to a thickened epithelium, the lens placode, that invaginates and eventually separates from the ectoderm, giving rise to a vesicle that will later become the lens. As the lens placode invaginates, the underlying neural epithelium invaginates with it, forming the double layered optic cup, which remains attached to the brain via a thinning process, the optic stalk, that will later provide the route though which the retinal neurons will send their axons back to the visual centers in the brain. The cells of the outer layer of the optic cup differentiate, forming the retinal pigmented epithelium, whilst the cell of the inner layer of the cup will give rise to the sensory layer of the eye, the neural retina, that will also produce the extracellular matrix component that fills the eye chamber, the vitreous humour.

During these early stages of eye development, a large number of mesencephalic neural crest cells, accumulate around the developing optic cup. Most of these cells will condense around the pigmented retina, and differentiate into the outer connective tissue layers of the eye, the choroid and sclera, that protect it and maintain its shape, and in the particular case of the avian eye they will also give rise to the scleral ossicles surrounding the cornea.

Some of these neural crest cells will also contribute to the formation of other structures in the eye such as the cornea and the iris. Both these structures have a composite origin:

- the outer epithelium of the cornea is derived from the layer of ectoderm that covers the lens vesicle after its complete separation, whilst the inner epithelium, the endothelium, is entirely originated from neural crest cells. The intermediate layer of connective tissue, the stroma, has again a composite origin; it is formed in two phases, with the original layer of extracellular matrix, the primary stroma, being secreted by the cells of the basal layer of the outer epithelium, which is later colonised and expanded by fibroblasts derived from neural crest cells, the secondary stroma

- the iris is formed by an extension of the retinal pigmented epithelium at the edge of the optic cup, that forms an inner double layered epithelium that is later covered by a spongy layer of mesenchyme and muscles, the iris stroma. The fibroblasts which constitute the mesenchyme of the iris stroma are derived from neural crest cells, whilst the origin of the muscles that control the aperture of the iris is still controversial, and it is not clear whether they are derived from epithelial or stromal cells.

The ciliary body, the muscle that controls the shape of the lens and is therefore responsible for focusing of the image in the back of the eye, is also of neural crest origin.

The neural layer of the retina is initially a pseudostratified columnar epithelium of actively dividing and apparently undifferentiated cells. Differentiation of the stratification characteristic of the retina, proceeds in an ordered fashion from the center to the periphery, and with the cell types nearer to the inside surface differentiating before the ones with progressively more external positions: first the ganglion cells differentiate and segregate to the inner surface of the retina; next the amacrine and horizontal cells differentiate followed by the bipolar cells and the Müller glial cells (the major non neuronal cell type present in the retina), forming the inner nuclear layer; finally the outer photoreceptor cells (rods and cones) differentiate, forming the outer nuclear layer.

These three cell layers containing the bodies of the various retinal cell types, are separated by the so called inner and outer plexiform layers where the processes from the different neuronal cells present in adjacent strata meet and establish the appropriate synapses. Finally there is an additional innermost layer of nerve processes, the nerve fiber layer, that converge to the optic disc where they exit into the optic stalk and finally make their way to the visual centers in the brain.

Due to the way by which the retina develops from the optic cup, the orientation of the retinal neuroepithelium is exactly opposite to the one that would be expected in a photoreceptor organ, with the sensory cells facing the outer regions, away from the light, whilst the basal lamina and the cells that process and transmit the information to the brain are present in the inner suface.

Finally the avian retina has a unique structure, the pecten, which is a highly vascularised pleated fold of the retina, that projects from the back of the eye, near to the exit point of the retinal neurons, and which is thought to be involved in the nutrition of the otherwise poorly vascularised avian retina.

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