THE ROLE OF RETINOIC ACID IN ANTERIOR-POSTERIOR PATTERNING OF THE MOUSE EMBRYO

by

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A Thesis Submitted in Conformity with the Requirements for the Degree of Master of Science
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ABSTRACT

Previous studies have suggested that retinoic acid (RA) is a signalling molecule important for the posteriorization of the developing vertebrate embryo. However, the evidence provided by these experiments has not been complete. To further investigate RA's role in anterior-posterior (A-P) patterning during early mouse embryogenesis, the complete removal of endogenous RA in the developing embryo was attempted by ubiquitously expressing an enzyme that can metabolize RA, called P450RAI. In this study various embryonic stem (ES) cell lines transgenic for a construct which would allow ubiquitous expression of P450RAI were generated, but they did not express the P450RAI transgene at a detectable level. Therefore, this prevented the successful execution of the project. Some likely problems have been considered and appropriate modifications proposed to allow for the successful future execution of this project, which will help elucidate how RA is implicated in A-P patterning during early vertebrate development.
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CHAPTER 1

INTRODUCTION
INTRODUCTION

General Introduction

Vitamin A, the precursor of retinoic acid (RA), has a remarkably wide range of biological activities. It is required for vision, reproduction, hematopoiesis, cell growth and differentiation, and is essential for various embryological processes (reviewed in Sporn et al., 1994; Zile, 1998).

RA was first identified because of its role as a teratogen. By the mid 1930s, it had been recognized that vitamin A deficiency (VAD) during mammalian embryonic development caused fetal death and severe developmental defects (Hale, 1937; Mason, 1935). Later studies showed that VAD during fetal development resulted in a series of congenital malformations of many systems, that included the eyes, urogenital tract, cardiovascular, diaphragm, and lungs (Morriss-Kay and Sokolova, 1996; Wilson et al., 1953). The third generation VAD mice from mothers continually maintained on VAD diets, showed a variety of deformities that included some of those mentioned above, as well as umbilical hernia, edema, thymus and liver defects, facial clefts, underdeveloped palatal shelves, hypoplastic mandible, and forelimb defects (Morriss-Kay and Sokolova, 1996).

It was initially realized by Cohlan in 1953 that excessive vitamin A during mammalian embryonic development also caused malformations. In this study pregnant rats were treated with excess vitamin A, resulting in a decrease in the size of the litters and congenital malformations. Since then many studies have been done to address the teratogenic effect of excess retinoids (vitamin A and its biologically active derivatives) on development. These studies have shown that the primary areas that RA affects are the limb, craniofacial structures, heart, central nervous system, brain, skull and eyes (Zile, 1998).

Thus, the observation that teratogenic abnormalities occurred when there is either a decrease or increase in the level of retinoids suggests that they have a crucial
role during normal embryonic development. This introduction will focus on what is known about RA's role in anterior-posterior (A-P) patterning during early embryogenesis, and how its role as a potential posteriorizing factor can be more precisely elucidated in the mouse.

**RA Biosynthesis**

Vitamin A (retinol) must be metabolized into RA in order for it to become an active signalling ligand. Retinol is first converted to retinal, then to RA by various alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) enzyme family members, respectively. The ADH enzyme family can metabolize retinol as well as ethanol. However some family members prefer retinol as a substrate to ethanol. The ADH family member ADH-IV has a high efficiency for the oxidation of retinol to retinal and it is the best-known candidate to perform this reaction during early mouse development. This enzyme is inefficient with ethanol as its substrate. Other members of the ADH family, such as, ADH-I is 100-fold less effective and ADH-III is inactive in retinol oxidation. ADH-I is most efficient for ethanol oxidation and ADH-III is a glutathione-dependent formaldehyde dehydrogenase (Duester, 1998). Similarly, in the ALDH family there are members that prefer retinal as a substrate to acetaldehyde. The ALDH family member ALDH-I can function to oxidize retinal to RA during development. Other members of the ALDH family, ALDH-II and ALDH-III, can not perform retinal oxidation. (Duester, 1998). RALDH2 and V1, two novel retinal dehydrogenase, can also specifically metabolize retinal to RA during mouse embryogenesis (Duester, 1998; McCaffery et al., 1993; Zhao et al., 1996). ALDH-II is most efficient in performing the oxidation of acetaldehyde. Thus, in the ADH and ALDH families, the forms most effective in retinol metabolism differ from the forms that are most effective for ethanol metabolism (Duester, 1998).
RA Signalling

The ligand RA exists in two isomeric forms that are biologically active: all-trans-RA and 9-cis-RA (van der Saag, 1996). RA can signal through multiple nuclear receptors, namely the retinoic acid receptor (RAR) and the retinoid X receptor (RXR). There are three types of RARs, α, β, and γ, each having multiple isoforms (α1, α2, β1, to β4, γ1, and γ2). There are also three types of RXRs, α, β, and γ and their isoforms (α1, α2, β1, β2, γ1 and γ2) (Chambon, 1996). These isoforms are a result of differential usage of two promoters and alternative splicing. RARs can use all-trans-RA or 9-cis-RA as their ligand but RXR recognizes only 9-cis-RA (Figure 1-1). Either all-trans-RA or 9-cis-RA ligand can penetrate the cell membrane, enter the nucleus and bind to the nuclear heterodimeric receptors RAR-RXR. This complex binds to response elements called the retinoic acid response elements (RAREs) in the target gene to trigger a transcriptional response (Figure 1-1). 9-cis-RA can also bind homodimers of RXRs, which can then bind to retinoid X response elements (RXREs), again leading to a transcriptional response in the target gene (Figure 1-1, Chambon, 1996; Conlon, 1995; Sporn et al., 1994). The RXR receptor can bind other types of receptors other than RARs (Chambon, 1996). This is a simplified explanation of RA signalling. There are multiple proteins that can bind retinoids which may modulate RA signalling, for example retinol binding protein (RBP), cellular retinol binding protein (CRBPI) and the cytoplasmic RA binding proteins (CRABPI and CRABPII) (Morriess-Kay and Sokolova, 1996). There are also nuclear coregulators that modify transcriptional response of the target gene (Chambon, 1996).

RA Is Implicated In Anterior-Posterior (A-P) Patterning

Gastrulation and A-P Axis Initiation

Gastrulation is a process by which an embryo with two germ layers is converted into one with three, the ectoderm, mesoderm and endoderm. This is the
Figure 1-1. RA signalling.

RA can signal through various nuclear receptors, which include the retinoic acid receptor (RAR) and the retinoid X receptor (RXR). The heterodimeric receptor RAR-RXR can bind either the all-trans-RA or 9-cis-RA ligand. This complex can bind to a retinoic acid response element (RARE) in the target gene, which then leads to its transcriptional activation. RXR homodimers can only bind the 9-cis-RA ligand. This complex then binds to a retinoid X response element (RXRE), again resulting in the transcriptional activation of the target gene.
first sign of embryonic pattern. The mouse gastrulates at about embryonic day (E) 6.5, with the formation of the primitive streak, which defines the A-P axis, marking the future posterior side of the embryo. At the streak, the ectoderm (epiblast) cells make an epithelial to mesenchymal transition, and migrate between the epiblast and endoderm to become mesoderm and definitive endoderm germ layers (Beddington and Robertson, 1999; Tam and Behringer, 1997). After gastrulation the A-P axis of the mouse embryo begins to be subdivided into different morphological structures through the expression of various developmental genes.

The family of nuclear transcription factors known as HOM-C (Drosophila)/Hox (vertebrate) homeotic genes, are thought to specify regional identity along the body axis. These genes were first discovered in Drosophila, and they contain a conserved homeobox that encodes a DNA binding homeodomain within the protein (Marshall et al., 1996). In vertebrates, four copies of the Hox complexes exist. It is thought they have been replicated from the original HOM-C complex, to Hoxa, Hoxb, Hoxc and Hoxd (Figure 1-2). Each of the Drosophila genes has more than one vertebrate homologue therefore each homologous group is called a paralog group (Means and Gudas, 1995). These Hox genes tend to be expressed in a colinear manner, from the hindbrain posteriorly. The more 3' genes are expressed in more anterior domains while the more 5' genes are limited to more posterior domains (Figure 1-2 and 1-3, Hogan et al., 1994; Marshall et al., 1996; Means and Gudas, 1995). The hindbrain is divided into eight regions called rhombomeres. Some of the 3' Hox genes have shared expression domains that extend into the hindbrain region. The limits of the anterior expression of the most 3' Hox genes correspond to the specific rhombomere boundaries (Figure 1-3, Marshall et al., 1996). Hox gene mutagenesis studies have shown that they can affect rhombomere segmentation and identity (reviewed in Means and Gudas, 1995).
Figure 1-2. **Organization of Drosophila HOM-C and mouse Hox homeotic Complexes.**

The *Drosophila* HOM-C genes include, *labial (lab)*, *proboscipedia (pb)*, *Deformed (Dfd)*, *Sex Combs Reduced (Scr)*, *Antennapedia (Antp)*, *Ultrabithorax (Ubx)*, and *Abdominal (Abd)*. In the mouse there are four separate complexes *Hoxa*, *Hoxb*, *Hoxc*, and *Hoxd*, which have been duplicated from the original *HOM-C* complex. The mouse *Hox* complexes are placed beneath its *Drosophila* homologue. There is a high degree of sequence identity between the vertical alignment of the *Drosophila* and mouse genes. These homologous genes within a vertical column are called a paralog group (for example *a1, b1* and *d1*) of which there are 13. These genes are expressed in a colinear manner in the anterior-posterior body axis. Low levels of RA can activate expression of more 3' genes while higher levels will activate more 5' genes.
Figure 1-3. 3'tHox gene expression in the hindbrain region.

Summary of 3' Hox gene expression in the hindbrain of E9.5 mouse embryos. The hindbrain is divided into eight regions called rhombomeres (r1-r8). Filled boxes represent the approximate limits of expression of the indicated Hox genes. There are similar anterior boundaries of expression for members of the same paralog group except for Hoxa2 and Hoxb2. Moving 3' along the Hox cluster, the anterior boundary of the Hox paralog shifts more anteriorly by two rhombomeric units.
Anterior

Posterior

Hindbrain Spinal cord

r1 r2 r3 r4 r5 r6 r7 r8

Hoxb1

Hoxa2

Hoxb2

Hoxa3

Hoxb3

Hoxd3

Hoxd4

Hoxb4

Hoxa4

Hoxb5

3'

5'
The paraxial mesoderm also becomes patterned along the A-P axis. The notochord is an epithelial rod that extends from the base of the head into the tail. On either side of the notochord are thick bands of mesodermal cells, referred to as paraxial mesoderm (Gilbert, 1997). Somites are blocks of segmented paraxial mesoderm on either side of the neural tube and they develop in a rostral to caudal direction, that is, the most anterior somite is older than the more posterior somites. These somites will give rise to the vertebrae, bone, muscle, cartilage and dermis. Somites are transient structures, but they are important in organizing the segmental pattern of the vertebrate embryo (Gilbert, 1997). The vertebral column consists of vertebrae with different A-P identities including four occipital vertebrae at the base of the skull, seven cervical vertebrae (C1-C7), thirteen thoracic vertebrae (T1-T13), six lumbar vertebrae (L1-L6), three or four vertebrae fused to form the sacral bone (S1-S4), and the caudal vertebrae. The occipital vertebrae are most anterior and the caudal vertebrae are most posterior. The expression of different Hox genes determines the type of vertebra along the A-P axis (Gilbert, 1997).

The vertebrate heart is derived from lateral plate mesoderm. These cells migrate and fuse to a ventral midline position where they become a contracting tube (Gilbert, 1997). This heart tube also has an A-P polarity (Stainier and Fishman, 1992). The linear heart tube has different structures along its A-P axis. The most anterior structure is the outflow tract followed by the right ventricle, left ventricle and atrium which is the most posterior structure (Gilbert, 1997).

**Treatment of Embryos with Excess RA**

Many embryologists became very interested in RA, since it had been identified as a potent teratogen. Over the past years many experiments have been done where developing embryos have been exposed to exogenous RA, to try and further refine its specific role. For the most part, these types of experiments resulted
in a posteriorizing phenotype, suggesting that RA signalling may be involved in A-P patterning of the embryo.

Many studies have been done that showed that treatment of embryos with excess RA results in anterior truncations. Treating mouse or rat embryos with high doses of RA caused posteriorization of the embryo and induced dose-dependent truncation of the head (Avantaggiato et al., 1996; Cunningham et al., 1994; Simeone et al., 1995). Similar results are seen when Xenopus or zebrafish embryos are treated with high levels of RA (Durston et al., 1989; Ruiz i Altaba and Jessell, 1991; Zhang et al., 1996).

There are several lines of evidence suggesting that RA is involved in A-P patterning of the hindbrain via regulation of Hox genes. Multiple studies have been done which show rhombomere alterations in embryos treated with excess RA. Exposure of mouse embryos to exogenous RA resulted in a shorter preotic hindbrain and the anterior expansion of expression of some of the 3' Hox genes, which included Hoxb2, Hoxb1, Hoxb4, and Hoxb5 within the prospective hindbrain (Conlon and Rossant, 1992; Marshall et al., 1992; Morriss-Kay et al., 1991). Moreover, it has been shown that the 3' Hox genes are more sensitive to RA than are the 5' Hox genes. Low doses of RA activate primarily 3' Hox genes, whereas higher concentrations will induce more 5' Hox genes (Figure 1-2). RAREs have been found in the some of the 3' Hox genes: Hoxa1, Hoxb1, Hoxa4, Hoxb4 and Hoxd4 suggesting a direct interaction between the genes and RA (Marshall et al., 1996). Other genes that were misexpressed in the presence of excess RA included RARβ, which was expressed more anteriorly, and Krox-20, whose expression was reduced. Therefore, the analysis of various Hox genes and other hindbrain markers in these mouse embryos treated with RA, suggested that more anterior rhombomeres were transformed into a more posterior identity (Conlon and Rossant, 1992; Marshall et al., 1992; Morriss-Kay et al., 1991). In addition, the study done by Marshall, et al. (1992), showed that
neurons that exit the transformed rhombomeres and some of the branchial arches were also transformed to a more posterior phenotype. Therefore, the ectopic expression of the 3' Hox genes that resulted upon RA treatment in the mouse implied that a transformation of the anterior hindbrain to a posterior identity had occurred. As in the mouse, when *Xenopus*, zebrafish or chick embryos were treated with excess RA, it also caused hindbrain posteriorization with associated ectopic Hox gene expression (Dekker et al., 1992; Durston et al., 1989; Hill et al., 1995; Holder and Hill, 1991; Papalopulu et al., 1991; Sundin and Eichele, 1992).

Another interesting homeobox containing gene that RA has been shown to affect is *Otx2*. The mouse *Otx2* gene is a homologue of the *Drosophila* head-specific homeobox gene, *orthodenticle* (Conlon, 1995). This gene is expressed throughout the early mouse embryo. At approximately E7.5, *Otx2* expression becomes restricted to the anterior region of the embryo, which forms the forebrain and midbrain and at this time both *Hoxa1* and *Hoxb1* are turned on in regions that become the hindbrain and spinal cord. Therefore, at this stage *Otx2* expression is complementary to the expression of *Hoxa1* and *Hoxb1* (Conlon, 1995). Studies have shown that treatment of embryos with RA resulted in the restriction of *Otx2* expression in a more anterior domain, while there was an induction of expression of *Hoxa1* and *Hoxb1* more rostrally (Conlon, 1995; Simeone et al., 1995). These changes in gene expression upon RA treatment correlate with the hindbrain expansion described by Simeone et al. (1995). This is further evidence supporting the idea that RA may be acting as a posteriorizing factor by inhibiting anterior specific genes, such as *Otx2*, and promoting the expression of more posterior genes like *Hoxa1* and *Hoxb1* and others.

Treatment of embryos with excess RA has also been shown to cause posteriorizing phenotypes in paraxial mesoderm derivatives, the somites and vertebrae. Treatment of mouse embryos with RA caused somites to be displaced more anteriorly. Normally the first somite is level with the fourth branchial arch,
but upon RA administration, the first somite was level with the second branchial arch (Morriss-Kay et al., 1991). Embryos exposed to excess RA has also caused homeotic transformations of the vertebrae. These embryos exhibited vertebrae transformations from an anterior to a more posterior identity (Kessel and Gruss, 1991; Means and Gudas, 1995). Some of the posterior transformations that occurred included anterior cervical vertebrae to more posterior cervical vertebrae, the last two cervical vertebrae to rib-bearing thoracic vertebrae, anterior thoracic vertebrae to more posterior thoracic vertebrae, thoracic to non-rib-bearing lumbar vertebrae, unfused lumbar to fused sacral vertebrae, and fused sacral to unfused caudal vertebrae (Kessel and Gruss, 1991; Means and Gudas, 1995). These transformations occurred along with ectopic anterior prevertebral expression domains of Hox genes like Hoxa7 Hoxc8 and Hoxa3 (Kessel and Gruss, 1991). The above homeotic transformations that occurred upon RA treatment are similar to the phenotypes that resulted from the Hox gene mutational analyses (Means and Gudas, 1995). This suggests that RA may be involved in vertebra development via Hox gene regulation.

There is experimental evidence that suggests that RA is also involved in A-P patterning of the heart. Headfold stage mouse embryos cultured in the presence of excess RA, developed various heart abnormalities, one of which included the posteriorization of the heart tube, characterized by an expansion of more posterior structures and a reduction or loss of more anterior structures (Chazaud et al., 1999). Similar heart tube posteriorization phenotypes have resulted when zebrafish or chicken embryos were treated with RA (Osmond et al., 1991; Stainier and Fishman, 1992; Yutzey et al., 1994). These results suggest that RA can induce posteriorization of the heart tube by promoting truncations or reductions of anterior structures and enlarging posterior structures. Thus, RA may be acting as a posteriorizing factor during heart tube development.
Excess RA treatment of developing embryos has also been shown to induce a displacement of the otic vesicle to a more anterior location. Mouse embryos treated with RA caused an anterior shift in the position of the otic vesicle. Instead of being level with the second branchial arch, they were level with the first branchial arch (Morriss-Kay et al., 1991; Sulik et al., 1988). Similarly, when Xenopus and zebrafish embryos were exposed to RA, the otic vesicles were also positioned more anteriorly (Durston et al., 1989; Holder and Hill, 1991; Papalopulu et al., 1991; Ruiz i Altaba and Jessell, 1991). It has been proposed that the possible reason for the abnormal rostral position of the otic vesicle in RA treated embryos may be due to anterior hindbrain loss (Morriss-Kay et al., 1991; Papalopulu et al., 1991; Sulik et al., 1988). It has been proposed that the otic placod, ectodermal precursor of the otic vesicle, may be induced by a signal from the mid-hindbrain. Therefore, the other possible explanation for the abnormal position of the otic vesicle is that in the RA treated embryos there may be a change in the regional inductive properties of the hindbrain (Ruiz i Altaba and Jessell, 1991).

**Vitamin A Deficient Embryos**

It has been demonstrated that the addition of exogenous RA to developing embryos caused various posteriorization phenotypes. The caveat of these experiments is that it is possible that the posteriorizing phenotypes manifested may be an artefact, due to the addition of large amounts of RA to the embryo. Therefore, a better functional study is the removal of endogenous levels of RA signalling in the developing embryo. This has been attempted by generating vitamin A deficient (VAD) embryos by feeding the mothers a VAD diet. This approach would better determine if RA is truly a posteriorizing factor. Some of the phenotypes that are observed in various types of VAD embryos do suggest that RA may have an endogenous role in posteriorizing the embryo axis.
VAD studies on various embryos have provided further evidence of RA's role in A-P patterning of the hindbrain. The best-characterized system for obtaining VAD embryos is in birds. Quail embryos when developed in the absence of vitamin A exhibit various defects in the central nervous system (CNS), one of which indicates that RA has a role in hindbrain patterning (Maden et al., 1996). In these VAD embryos the posterior hindbrain fails to develop. This was confirmed by the analysis of the expression of various Hox genes and other genes that are specifically expressed in different rhombomeres (Maden et al., 1996; Maden et al., 1998). In this VAD situation the expression of the Hox genes was either absent or their boundaries of expression regressed posteriorly, again suggesting that RA regulates the expression of these Hox genes. The loss of this posterior hindbrain region was due to apoptosis, along with the misexpression of genes responsible for A-P patterning of the embryo axis (Maden et al., 1996; Maden et al., 1998; Maden et al., 1997).

VAD rats fed insufficient amounts of all-trans-RA also showed hindbrain abnormalities (White et al., 1998b). They exhibited a loss of posterior cranial nerves, which arise from the posterior hindbrain and there was also a loss of postotic branchial arches. Similar results were observed in a study done by Dickman et al. (1997). VAD rat embryos also exhibited multiple otic vesicles (White et al., 1998b). The first otic vesicle was positioned normally while the second was located posterior to the first. Otic placodes are thought to be induced by secreted factors from r 5/6. Therefore in the absence of RA, the adjacent postotic neurepithelium (posterior hindbrain) may have a more anterior identity, inducing ectopic otic vesicles dorsolateral to the posterior hindbrain (White et al., 1998b). This is the opposite phenotype to that observed with excess RA treatment, which caused otic vesicles to be positioned more anteriorly. The possible reason why complete posterior hindbrain loss is not seen in these VAD rats is that they must be maintained on low levels of RA so they can become pregnant (White et al., 1998b).
Vitamin A deficiency during embryonic development also affects A-P patterning of the heart. When headfold stage mouse embryos were cultured in the presence of a synthetic antagonist of all three RA receptors (RAR α, β, and γ) which inhibited RA signalling, it resulted in the anteriorization of the heart tube. This was characterized by a reduction of posterior structures and an enlargement of anterior structures (Chazaud et al., 1999). Similarly, anteriorization of the heart tube was seen in VAD quails (Heine et al., 1985; Kostetskii et al., 1999). These results are opposite to the posteriorizing phenotype of the heart tube seen with excess RA. In the mouse, RA is present in the posterior part of the heart tube along with the RA synthesizing enzyme RALDH2 (Moss et al., 1998). This supports the idea that the antagonist may impede RA signalling that is required for posterior heart development, causing anteriorization while excess RA would promote posterior heart development, leading to posteriorization (Chazaud et al., 1999).

Embryos developing under VAD conditions has resulted in the posterior truncations of the hindbrain and the anteriorization of the heart tube, while embryos exposed to excess RA caused the posteriorization of these same structures. These results are further evidence supporting RA’s role as a posteriorizing factor. Although these VAD embryo experiments are informative, the caveat is that these embryos (specifically rat and mouse) may not be completely VAD because the females need to be maintained on minimal levels of vitamin A to allow for pregnancy to occur. So the full spectrum of phenotypes may not be manifested.

**Endogenous RA Signalling**

One of the requirements for RA to be an endogenous regulator of A-P pattern is that it be present and active in the embryo in the appropriate domains. To detect endogenous RA signalling in the mouse embryo, transgenic mice were generated which contained a RARE upstream of a basal promoter (hsp) and the lacZ reporter (Rossant et al., 1991). The advantage of using the RAREhsplacZ transgene rather than
just looking for the presence of RA is that the expression of this transgene in vivo is suggestive of where RA is actively signalling in the embryo. The expression of this transgene detects the presence of activated retinoid receptors and RA-mediated gene activation during development. In the RAREhsp lacZ transgenic mouse, RA activity is not detected at E6.5 at the beginning of gastrulation, but is detected at E7.5 in the posterior half of the embryo. At E8.5 and E9.5, the transgene is expressed in the posterior trunk except for the very posterior tail bud, and there is an absence of expression anterior to the hindbrain-spinal cord boundary except for a region marking the optic eminence (Rossant et al., 1991). Similar results were obtained from other studies (Ang et al., 1996; Balkan et al., 1992; Wagner et al., 1992). These results indicate that RA signalling initiates during primitive streak formation.

Coincident with the expression domain of the RAREhsp lacZ transgene is the expression domain of Raldh2, which expresses an enzyme required for the synthesis of RA (Niederreither et al., 1997). Other genes that have a similar expression pattern to the RAREhsp lacZ transgene are Hoxa1 and Hoxb1, while Otx2 is expressed in a complementary domain (Conlon, 1995). These results suggest that RA is present in regions consistent with its repressive and inductive roles on Otx2 and Hoxb1 and Hoxa1, respectively. Therefore the expression pattern of the RAREhsp lacZ transgene supports a role for RA in early A-P patterning of the embryonic axis.

Exogenous RA treatment of various embryos has resulted in posteriorization phenotypes in the hindbrain, paraxial mesoderm, and heart tube. In the opposite situation, the removal of RA signalling, by generating VAD embryos induced posterior truncations in the hindbrain and caused anteriorization of the heart tube. Active endogenous RA is found in specific regions with defined boundaries in the embryo, which implies it may have a role in setting up A-P boundaries. Thus from all these results, the current hypothesis is that embryonic RA may be acting as a posteriorizing factor. Endogenous embryonic RA may be involved in repressing
anterior characteristics by inhibiting anterior specific genes, like Otx2 (and others) and promoting posterior characteristics by positively regulating more posterior genes like 3'Hox genes (and others) (Conlon, 1995).

**Functional Analysis of Retinoid Receptors**

To further elucidate RA's importance in development mice have been generated with targeted deletions for the various retinoid receptors, which are expressed during development in specific spatiotemporal patterns (Morris-Kay and Sokolova, 1996). The loss of a single retinoid receptor, for the most part, resulted in minor or no physical abnormalities, except for the RXRα null mutant, which had chorioallantoic placental defects, heart and ocular defects. RARα or RARγ null mutants also showed some posterior to anterior homeotic transformations and malformations of the vertebrae (Kastner et al., 1995; Sapin et al., 1997). In general, the complete inactivation of a retinoid receptor was not as severe as might have been expected, suggesting functional redundancy amongst the receptors.

Compound RAR and/or RXR null mutations exhibited phenotypes that were more severe than single null mutants. The various combinations of these double mutants exhibited almost all of the VAD phenotypes. These included abnormalities in the nervous system, eyes, face, heart, neural crest, urogenital tract, respiratory tract and limb. These double mutants also had phenotypes that were not present in VAD embryos, for example, exencephaly, specific skeletal, ocular and glandular abnormalities (Kastner et al., 1995). These results suggest that the VAD embryos previously generated were not completely deficient. The other possible reason for the difference in phenotypes is that these receptors may be involved in other developmental processes, which are not mediated by RA. It has also been determined that the severity of some of the phenotypes seen in single RXR null mutants increased with the removal of specific RAR alleles. This indicated a convergence between RXR-and RAR-dependent signalling pathways. These
phenotypes are also seen in VAD mice. Therefore, these synergistic effects suggest that RAR/RXR heterodimers act as the functional units in transducing the RA signal in vivo (Kastner et al., 1995; Kastner et al., 1997). These results indicated that RA is important during development and its effects are mediated via the retinoid receptors.

Null mutants which provided evidence of RA's role in A-P patterning were the RARγ and (to a lesser extent) RARα, single mutants and some of the double mutants involving RARα and RARγ/β or RXRα and RARα/γ. These mutants showed homeotic transformations along the axial skeleton and abnormalities in vertebrae formation (Kastner et al., 1995; Kastner et al., 1997). RARγ null mutants (all isoforms disrupted) exhibited an anteriorization of the cervical and thoracic vertebrae; that is, more posterior vertebrae were transformed to a more anterior identity (Lohnes et al., 1993). RARα null mutants (all isoforms disrupted) also showed homeotic transformations of the cervical vertebrae, but at a low frequency (Lohnes et al., 1994). Similar homeotic defects were also manifested in double mutants RARα1γ, RARα1α2+/- and RARαγ. These abnormalities increased in penetrance and expressivity, in a graded manner upon removal of RARα alleles from the RARγ null background. RARβ2 also showed a high frequency of anterior transformations of the cervical vertebrae, suggesting that RARβ2 is also involved in A-P patterning of the axis (Kastner et al., 1995; Lohnes et al., 1994). RXRα+/-/RARα+/- and RXRα+/-/RARγ+/- null mutants also exhibited some homeotic transformations of the cervical vertebrae (anteriorization) (Kastner et al., 1997). Almost all these transformations are confined to the cervical vertebrae and for the most part, are an anteriorization phenotype, which fits with RA's proposed role as a posteriorizing factor. Similar phenotypes are also seen in Hox mutants (loss-of-function). This suggests that the transformation seen in the receptor mutants may be a result of altering Hox gene expression (Kastner et al., 1995; Lohnes et al., 1994).
general, even with double receptor null mutants, the phenotypes were not as dramatic as expected. Since actively signalling RA is present at E7.5, additional earlier phenotypes were also expected.

The likely explanation for the milder than expected phenotypes in single and double mutants is functional redundancy amongst the different forms of the receptors. It is possible that some of RAR isoforms and RXR isoforms are functionally interchangeable for the transcriptional control of RA target genes. Thus, there still may be residual RA signalling in the double null mutants which prevents the full spectrum of phenotypes from being observed (Kastner et al., 1995). Therefore, it is difficult to prevent all RA signalling by targeting the receptors to try and address its role as a posteriorizing factor.

Objective

Treatment of embryos with excess RA, VAD embryos and retinoid receptor null mutant experiments have provided evidence, supporting RA's role as a posteriorizing factor, but have not determined its absolute requirement for early embryogenesis. Therefore, to more definitively determine RA's role in A-P patterning at the early stages of embryogenesis, the aim of my project was to remove all endogenous RA in the developing embryo by ubiquitously expressing a cytochrome P450 enzyme, called P450RAI. This enzyme has been shown to metabolize RA (Abu-Abed et al., 1998; Fujii et al., 1997; Hollemann et al., 1998; White et al., 1997; White et al., 1996).

The Cytochrome P450 Superfamily

The cytochrome P450 gene superfamily encodes many enzymes that catalyze a variety of chemical reactions and can use many different substrates (Porter and Coon, 1991). Over 1000 P450 genes have been characterized and this superfamily has been divided into over 70 families. They are found in microorganisms, plants and animals (Peterson and Graham, 1998). P450 is localized mostly in the
membranes of endoplasmic reticulum and mitochondria and it is most abundantly found in the liver (Porter and Coon, 1991). The mechanisms of regulation of these P450 genes are also diverse (Coon et al., 1992; Porter and Coon, 1991). There is regulation at the level of transcription, processing and mRNA stabilization, translation and enzyme stabilization (Coon et al., 1992).

For the most part P450 enzymes are monoxygenases. The reaction they catalyze involves the reductive activation of molecular oxygen followed by the insertion of one oxygen atom into the substrate (Guengerich, 1992; Porter and Coon, 1991). Substrates for cytochrome P450 include many xenobiotics (foreign substances), like drugs (including antibiotics), carcinogens, antioxidants, anesthetics, dyes, pesticides, odorants, flavorants, alcohols, organic solvents and unusual substances found in plants and microorganisms which are foreign to animals. Some of the physiologically occurring substrates are steroids, eicosanoids, fatty acids, lipid hydroperoxides, retinoids, acetone and acetal. Some of the P450s are fairly specific in their choice of substrates (Coon et al., 1992; Porter and Coon, 1991). In mammals, P450s have several different functions. They have a role in steroidogenesis and in detoxification of foreign substances by forming more polar compounds that can easily be excreted. In some cases however, this reaction can result in the formation of a more powerful carcinogen. They are also involved in the arachidonic acid cascade, producing prostaglandins and eicosanoic acids (Peterson and Graham, 1998; Porter and Coon, 1991).

Although different P450s may have a unique active site and method of substrate recognition, they all seem to have a similar structural core. In general, these enzymes contain four β sheets and approximately thirteen α helices (Peterson and Graham, 1998). Some of the common structural motifs found in P450s include the heme-binding region, transmembrane region, substrate-binding site and a
proline rich region, which links the transmembrane and catalytic domains (Chen and Kemper, 1996; Peterson and Graham, 1998).

**Characterization of P450RAI**

*P450RAI* is a novel member of the cytochrome P450 family. It was first isolated in the zebrafish by using differential display PCR on fin tissue regenerating in the presence or absence of RA. Since zebrafish fins regenerate through a RA-sensitive process, this approach identified genes regulated by RA. It was determined for the first time that a cytochrome P450RAI could specifically metabolize RA into more polar metabolites (White et al., 1996).

The mouse homologue was then isolated by Fujii et al. (1997), by applying a subtraction procedure on P19 cells, a pluripotent embryonic carcinoma (EC) cell line, which were cultured in the presence or absence of RA. These cells can differentiate to neural cell types when induced with all-trans-RA (Fujii et al., 1997). A number of genes were isolated whose expression RA induces and one of these clones was *P450RAI*. Upon analysis of this clone it was determined that the P450RAI enzyme could specifically metabolize (*in vitro*) biologically active retinoids, all-trans-RA, 9-cis-RA and 13-cis-RA, into 5,8-epoxy-all-trans-RA. Overexpression of *P450RAI* in cell culture caused the cells to become hyposensitive to all-trans-RA. P19 cells overexpressing *P450RAI* remain undifferentiated at a concentration of RA that would normally cause differentiation. Overexpression of *P450RAI* in HeLa cells, which also contained the RARβ expression vector, prevented RARβ expression at a concentration of RA that would normally activate it (Fujii et al., 1997). Therefore, these analyses suggest P450RAI metabolizes RA into a biologically inactive form. Thus, *P450RAI* in the mouse embryo may be involved in regulating intracellular levels of RA and setting up the differential distribution of active RA in the embryo (Fujii et al., 1997).
The mouse homologue was later independently cloned again by two other groups (Abu-Abed et al., 1998; Ray et al., 1997). The human and Xenopus homologues have also been cloned (Hollemann et al., 1998; White et al., 1997). These were all cloned by screening for genes which are induced by RA, with the exception of the Xenopus homologue, which was cloned from a cDNA library prepared from activin-induced animal caps (Hollemann et al., 1998). The conservation of the amino acid sequence across the different species is high. The mouse P450RAI is 93% identical to the human and 68% identical to the zebrafish (Abu-Abed et al., 1998; White et al., 1997) and Xenopus homologues (Hollemann et al., 1998). There was less than 30% amino acid identity between the P450RAIs and other known cytochromes, suggesting they comprise a new class of cytochromes called CYP26 (White et al., 1997). P450RAI contains structural motifs that are common to all cytochrome P450 family members. It contains the characteristic heme-binding domain at the C-terminus and the first 35 amino acids encode a highly hydrophobic region, which is probably the transmembrane domain. P450RAI also contains a characteristic proline-rich domain and substrate-binding region (Fujii et al., 1997; Ray et al., 1997; White et al., 1997; White et al., 1996). These further studies also showed that the human, Xenopus and mouse (as previously shown) forms of P450RAI can also metabolize RA (Abu-Abed et al., 1998; Hollemann et al., 1998; White et al., 1997). Therefore, it appears that P450RAI from different species is an enzyme that specifically catabolizes RA.

Amongst the various studies that showed P450RAI could metabolize RA, there is a discrepancy regarding the identity of the metabolic by-products. To analyse if the P450RAI enzyme from the various species could metabolize RA, its cDNA was transfected into cells. The cells were incubated with RA and the metabolites were analysed by HPLC. (Abu-Abed et al., 1998; Fujii et al., 1997; White et al., 1997; White et al., 1996). The initial study done with mouse P450RAI, by Fujii
et al. (1997), showed that the metabolic product was 5, 8-epoxy-all-trans-RA. However, other studies showed that zebrafish, mouse and human P450RAI metabolized RA into, 4-oxo-RA, 4-OH-RA and 18-OH-RA (Abu-Abed et al., 1998; White et al., 1997; White et al., 1996). These discrepancies were attributed to technical differences in the extraction and characterization of the metabolites (Abu-Abed et al., 1998). There is some evidence that suggests that 4-oxo-RA and 4-OH-RA may be biologically active (Pijnappel et al., 1993; White et al., 1996), while 5,8-epoxy-all-trans-RA was determined to be inactive in the analysis done by Fujii et al. (1997). Therefore, there is controversy in the literature concerning the activity of these different metabolic by-products generated by P450RAI (Fujii et al., 1997; Hollemann et al., 1998; White et al., 1996). There is also controversy in the literature regarding P450RAI's substrate specificity. Some studies have shown that P450RAI can metabolize all-trans-RA, 9-cis-RA and 13-cis-RA, while other studies suggested it is specific for only all-trans-RA (Abu-Abed et al., 1998; Fujii et al., 1997; Marikar et al., 1998; Sonneveld et al., 1998; White et al., 1997; White et al., 1996).

In addition to identifying P450RAI as an enzyme that metabolizes RA, studies have helped to further characterize this novel cytochrome. The expression of mouse P450RAI has been characterized in different cell lines. Undifferentiated ES cells do not express detectable levels of P450RAI. RA- induced neural differentiation of ES cells in vitro results in an upregulation of P450RAI mRNA, but ES cells undergoing non-neural differentiation only transiently expressed it (Ray et al., 1997). Studies have also demonstrated that RA-inducible RA metabolism correlates with P450RAI expression, therefore it is concluded that the P450RAI enzyme may be functioning in a RA feedback loop system (Abu-Abed et al., 1998; White et al., 1997; White et al., 1996). The retinoid receptors are involved in regulating P450RAI expression. In wild type F9 cells, which is a murine embryonal carcinoma cell line, and F9 cells lacking RARγ, RARα and/or RXRα, there is a direct relationship between the level
of RA metabolic activity and RA-induced $P450RAI$ mRNA. This suggested that the RARγ and RARα receptors mediate the effects of RA on the expression of the $P450RAI$ gene (Abu-Abed et al., 1998). Other studies have also implicated the retinoid receptor in regulating $P450RAI$ expression (Marikar et al., 1998; Sonneveld et al., 1998).

At a clinical level RA has been used to treat patients with acute promyelocytic leukemia (APL), because it leads to terminal differentiation of APL cells. Most patients go into remission after RA treatment, but later develop resistance to RA. It was proposed that $P450RAI$ might be involved in the development of this resistance (White et al., 1997). $P450RAI$ maps to human chromosome 10q23-q24 and mouse chromosome 19C2-3. Several disease loci have been described in the 10q23-q24 region including infantile onset spinocerebellar atrophy, split hand-split foot (SHSF-3) and several tumor suppressor gene loci. However, it is still not known if mutations in $P450RAI$ play a role in these diseases (White et al., 1998a).

Conclusion

The identification of $P450RAI$ as an enzyme that can specifically metabolize RA has allowed me to develop a new strategy to further elucidate RA’s role in development. I have developed a vector to ubiquitously express $P450RAI$ in the embryo in the presence of a RA-responsive reporter. The reporter should provide a read-out of a reduction in RA activity and the resulting phenotypes will help determine if RA is truly acting as a posteriorizing factor in early embryonic A-P axis development.
CHAPTER 2

MATERIALS AND METHODS
Materials and Methods

Whole-mount in situ Hybridization

Whole-mount in situ hybridization was performed as described by Conlon and Rossant (1992). The probe used to detect endogenous P450RAI expression was an 800 bp cDNA (3'end) of a mouse expressed sequence tag (EST) of the P450RAI gene (accession number: AA239785). The antisense and sense probes were produced with T3 RNA polymerase and T7 RNA polymerase, respectively.

Vector Construction

A blunted fragment containing the β-actin-neo-pA was inserted into the Sma I site of the pRAREhsplacZ vector from Rossant et al. (1991), to generate the RAREhsplacZβ-neo vector (Ralph Zirngibl, unpublished).

To allow overexpression of P450RAI, the full-length cDNA (obtained from Martin Petkovich, Queen’s University) was subcloned into the eukaryotic vector, pCAGGS (Niwa et al., 1991). A c-myc tag was added to the 3'end of the full-length cDNA, and loxP sites were placed flanking the cDNA. The full length (1725 bp) cDNA was inserted at the Eco RI and Xho I sites of pBluescript SK, mP450RAISK (obtained from Martin Petkovich, Queen’s University). The c-myc epitope tag was added at the 3'end of the full length P450RAI cDNA by PCR. The endogenous stop codon and 3' UTR was removed, and the c-myc was added in frame with the rest of the gene. In addition a Xho I site was added at the very 3' end for subcloning. As a control, the 3' UTR was removed without adding the c-myc tag. The PCR primers were as follows: 5' CAGCTTCATTCCATTGGAGG 3' (nucleotides 1378-1399 of mP450RAISK), which is upstream of an unique Afl II site, and 5'CCTACTTCCAGGGAGATATCGAAACAAAAACTCATCTCAGAAGGAGGATCTG
AATTGACTCGAGCGG 3', which contains 20 bp of homology to the 3' end of P450RAI plus the c-myc tag followed by a stop codon and the Xho I site, or 5' ACTTCCAGGGAGATATCTGACTCGAGTTTC 3', which removes the 3' UTR and adds a Xho I site. These PCR products were digested with Afl II and Xho I and then subcloned into mP450RAISK, generating mP450RAI 3'mycSK and mP450RAI-3'UTRSK. These vectors were then digested with Xba I and Xho I, to release the P450RAI3'myc/-3'UTR cDNAs, which were then subcloned into the Avr II and Sal I site of the pBS2loxBAvrII vector (obtained from Andras Nagy). These two vectors were then digested with Xho I and Bam HI, to release the P450RAI3'myc/-3'UTR cDNAs now flanked with loxP sites. These fragments were then subcloned into the Xho I and Bgl II sites of the pCAGGS vector, generating the following expression vectors: pCAGGSloxPP450RAI3'myc and pCAGGSloxPP450RAI-3'UTR. The presence of the c-myc tag and loxP sites was verified by sequencing (Thermo Sequenase Radiolabeled Terminator Cycle Sequencing, Amersham).

**Generation of Transgenic ES Cell Lines**

The RAREhsplacZβ-neo vector (20 µg) was linearized with Sca I and introduced into ES cells by electroporation (Wurst and Joyner, 1993). Genomic DNA was isolated from neomycin resistant colonies and digested with Sph I, electrophoresed through 1% agarose gels and transferred to nylon membranes. Southern blots were hybridized with a 32P-labeled cDNA encoding the 5'end of the lacZ gene. Hybridization was performed at 42°C in 70% Church-Gilbert medium (10% BSA, 0.5M Na2HPO4, pH 7.2, 0.001M EDTA, 7% SDS) and 30% formamide containing 200 µg denatured salmon sperm DNA and radiolabeled probe (1x10^6 c.p.m. /ml). Filters were washed in 2x SSC at room temperature for 20 min, and 0.1x SSC containing 0.5% SDS at 65°C for 20-40 min. The membranes were exposed
overnight to a Molecular Dynamics Phosphor screen and analysed using the ImageQuant 5.0 program (Molecular Dynamics).

ES cells that were transgenic for RAREhsplacZβ-neo were co-electroporated with either pCAGGSloxPP450RAI3'myc or pCAGGSloxPP450RAI-3'UTR (30 μg) digested with Sca I and Sfi I, (linearizes and removes the plasmid backbone), and circular PGK-puro-PA vector (5 μg) for selection. For the first 24 hours after electroporation, the cells were cultured in ES cell media (Wurst and Joyner, 1993). After 24 hours, G418 was added to the media to maintain selection on the RAREhsplacZβ-neo transgene. After 48 hours, puromycin (1.5 μg/ml) was added to the media to select for the PGK-puro-PA co-electroporated with pCAGGSloxPP450RAI3'myc or pCAGGSloxPP450RAI-3'UTR. After 3 days with puromycin selection most of the cells died, and selection was removed. Colonies were picked 6-7 days after selection was removed. Genomic DNA was isolated from these colonies and subjected to Southern blot analysis, as previously described. The blots were probed with a 415 bp 3' fragment (nucleotides 1101-1516) of the P450RAI cDNA, or with a 379 bp fragment (nucleotides 1-379) of the CMV-enhancer from the pCAGGS vector.

β-Galactosidase Staining of Cells and Embryos

Embryos and ES cells were fixed in 0.2% gluteraldehyde, 5 mM EGTA, 2 mM MgCl₂, 100mM sodium phosphate, pH 7.3 at room temperature for 15 min. and 5 min., respectively. They were then washed in 2mM MgCl₂ 0.01% deoxycholate, 0.02% Nonidet-P40, and 100mM sodium phosphate, pH 7.3 and incubated in 2.12 mg ml⁻¹ potassium ferrocyanide, 1.64 mg ml⁻¹ potassium ferricyanide, 1 mg ml⁻¹ X-
gal and 100mM sodium phosphate, pH7.3 overnight at 37°C. Embryos were then washed as above, and preserved in 3.7% formaldehyde in PBS.

*In vitro* Response of RAREhspacZβ-neo transgene to RA.

ES cells transgenic for RAREhspacZβ-neo alone, and cells that were also transgenic for CMV-chickβ-actin-P450RAI were passaged onto gelatinized plates at the same density and allowed to grow for 24 hours in ES cell media (Wurst and Joyner, 1993). They were then treated with all-trans-RA (Sigma) ranging in concentration from 10^{-10}-10^{-6} M, diluted in 96% ethanol or treated with ethanol alone, as the control. After 12 or 24 hours of treatment the cells were rinsed with PBS and stained for β-galactosidase expression, as previously described.

Expression Analysis of CMV-chickβ-actin-P450RAI Transgene.

Northern blot analysis of P450RAI expression in ES cells transgenic for CMV-chickβ-actin-P450RAI was performed following the NorthernMax-Gly Blotting Kit (Ambion). Briefly, total RNA was isolated from ES cells using Trizol Reagent. RNA (15 µg) was electrophoresed, transferred to a nylon membrane, and hybridized with a 32P-labeled P450RAI cDNA (nucleotides 1101-1516). Hybridization was performed at 42°C in Prehybridization/Hybridization Solution. The filter was washed in Low Stringency Wash Solution #1 at room temperature for 2x5 min., and then in High Stringency Wash Solution #2 at 42°C for 2x15 min. As a control for equal sample loading, the Northern blot was stripped and re-hybridized with a cDNA encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Reverse transcription of RNA was performed using SuperScript II RNase H-Reverse Transcriptase (GIBCO BRL). Total RNA was purified as previously described, and treated with DNase I FPLC Pure (Pharmacia Biotech). First strand
cDNA synthesis was performed in a total volume of 20 µl containing 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 6 mM MgCl₂, 1 mM DTT, 1 mM of each dNTP, 1 µg total RNA, 300 ng oligo (dT), 200 ng random hexamers and 200 U SuperScript II. Reverse transcription was carried out at 65°C for 5 min., followed by incubation at 42°C for 2 hours. The enzyme was then inactivated at 72°C for 15 min. 1 µl of the cDNA was used in a 50 µl PCR reaction to give a final concentration of 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 0.001% gelatin, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 2.5 U Taq DNA polymerase (Tsg Bio Science Inc.) and 45 ng of each gene specific primer. PCR amplification was performed by incubation at 95°C for 2 min. followed by 30 cycles of incubation at 94°C for 30 s, 62°C for 30 s and 72°C for 40 s. Primers used were specific to the chickβ-actin promoter (5' TGACTGACCGCGTTACTCCC 3') and P450RAI cDNA (5' TGCGCACCGACGCGGGCCAGT 3'). These primers spanned an intron and the expected sizes are 0.55 Kb for cDNA and 2.9 Kb for genomic DNA. Primers specific for the hypoxanthine guanine phosphoribosyl transferase (HPRT) gene were also used (5' CCTGCTGGATTACATTAAAGCACTG 3') and (5' GTCAAGGGCATATCCAACAACAAAC 3'). The expected size is 0.3 Kb.

To detect protein expression by Western blotting, 1 ml of 1% Tx-100 lysis buffer (10% glycerol, 2 mM EDTA, 138 mM NaCl, 1% triton (Tx-100), 20 mM Tris pH 8, and protease inhibitors) was added to a 10 cm confluent dish of ES cells transgenic for RAREhsplacZβ-neo and CMV-chickβ-actin-P450RAI3'myc. The lysates were clarified by centrifugation (14000 rpm for 15 min. at 4°C). 40 µl of this lysate was denatured and loaded on a SDS-PAGE, and transferred onto a PVDF membrane. The membranes were washed in 1X TBS (750 mM NaCl, 100 mM Tris base, pH 7.5), and then blocked for 1 hour at room temperature in 5% milk-powder,
1% BSA and 0.05% Tween-20. They were then probed with the primary antibody, c-Myc mouse monoclonal IgG (9E10, Santa Cruz Biotechnology), diluted 1:1000, for 2 hours at room temperature. The membranes were then washed in 1X TBS-T (0.05%) and probed with the secondary anti-body, goat anti-mouse IgG (H+L)-HRP conjugate (Bio-Rad Laboratories), diluted 1:10000, for 1 hour at room temperature. The membranes were then washed in 1XTBS-T (0.2%), followed by ECL (PIERCE) for detection.

In vivo Analysis

To assess the expression pattern of the RAREhsplacZβ-neo transgene in vivo, transgenic ES cells were aggregated with two tetraploid ICR embryos (Nagy et al., 1993). Lois Schwartz performed this procedure. These tetraploid cells will only contribute to the trophoblast and primitive endoderm, such that the chimeric embryos will be totally ES cell derived (Nagy et al., 1993). Two ICR diploid blastomeres at the two-cell stage were electrofused to produce tetraploid embryos. The aggregates were then transferred into uteri of pseudopregnant ICR mothers. I dissected the resulting embryos at early to mid-gestational stages, and assessed them by morphology and β-galactosidase staining. To analyse the response of the RAREhsplacZβ-neo transgene to RA in vivo, embryos were generated as above. They were dissected at E 7.5 in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose, antibiotics, 10% FCS and 25 mM HEPES pH7.4, being careful not to damage the ectoplacental cone (EPC) and removing Reichert’s membrane. These embryos were then cultured in vitro in the presence of 10⁻⁶ M RA or ethanol, as the control, for 24 hours. The culturing conditions were as follows: four-five embryos were placed in one of the 24-wells of an Ultra Low Cluster dish (Costar) with 1 ml culturing
media (50% rat serum in DMEM with high glucose and antibiotics) containing $10^6$ M RA or ethanol, in a 37°C, 5%CO$_2$ incubator for 24 hours (Crockcroft, 1990). After 24 hours of culture the embryos were stained for β-galactosidase expression (as previously described).

To assess the affect of ubiquitously expressing the P450RAI gene in the embryo, the tetraploid aggregation technique was used (Nagy et al., 1993), with ES cells transgenic for RAREhsplacZβ-neo and CMV-chickβ-actin-P450RAI (performed by Lois Schwartz). I analysed the phenotype of the resulting embryos by gross morphology and by β-galactosidase staining.
CHAPTER 3

RESULTS
Results

Expression pattern of endogenous $P450RAI$ gene is complementary to the $RAREhsplacZ$ transgene in the mouse embryo

A sensitive RA-responsive transgene was previously generated, which consists of three RA response elements (RARE) upstream of the basal promoter $hsp68$, and the $E. coli lacZ$ gene (Rossant et al., 1991). It is expressed in specific domains of E7.5, E8.5, E9.5 (Figure 2-1 b,d,f) and later stage embryos. This expression pattern suggests where RA is actively signalling in the embryo (Rossant et al., 1991). Since studies have shown that $P450RAI$ metabolizes RA (Abu-Abed et al., 1998; Fujii et al., 1997; White et al., 1997; White et al., 1996), I predicted that its expression pattern would be complementary to regions in the embryo where the $RAREhsplacZ$ transgene is expressed. To test this hypothesis, endogenous $P450RAI$ expression was visualized by whole-mount in situ hybridization using an anti-sense RNA probe made from the mouse $P450RAI$ EST. At E7.5, expression is found in the anterior region of all three embryonic germ layers. In the extra-embryonic region only the endoderm is positive (Figure 2-1 a). When compared to $RAREhsplacZ$ expression (Figure 2-1 b) in an embryo of the same stage, there is a very striking complementary expression pattern. At late E8.5, $P450RAI$ is highly expressed in the posterior region: caudal neural plate, tail bud mesoderm and hindgut endoderm (Figure 2-1 c, this study, Fujii et al., 1997). The complementarity is again very evident in this posterior region, compared to the $RAREhsplacZ$ expression (Figure 2-1 d). At late E8.5, $P450RAI$ is also expressed in the anterior region, including prospective rhombomere 2, foregut epithelium and the first branchial arch epithelium (Figure 2-1 c, this study, Fujii et al., 1997). These are also regions in which $RAREhsplacZ$ is not
Figure 2-1. Complementary expression pattern of endogenous $P450RAI$ versus the $RAREhsplacZ$ transgene.

(a,c,e) Endogenous $P450RAI$ expression visualized by whole-mount in situ hybridization. (b,d,f) β-galactosidase whole mount staining of $RAREhsplacZ$ transgenic embryos. (a,b) Early E7.5 (head-fold-stage) embryos. $P450RAI$ expression is present in the anterior region of all three embryonic germ layers, and in the extraembryonic region, only the endoderm is positive (a). The $RAREhsplacZ$ expression is present in the complementary posterior region (b). (c,d) Late E8.5 embryos. $P450RAI$ is highly expressed in the posterior region: caudal neural plate, tail bud mesoderm and hindgut endoderm. It is also expressed in the anterior region: prospective rhombomere 2, foregut epithelium and first branchial arch (c). These are regions where the $RAREhsplacZ$ transgene is not expressed (d). (e,f) E9.5 embryos. $P450RAI$ is expressed in two regions: the caudal neural plate (hindgut and tail bud mesoderm), and the neural crest cells for cranial ganglia, which are regions where $RAREhsplacZ$ is not expressed.
expressed (Figure 2-1 d). At E9.5, P450RAI is expressed again in two main regions. In the posterior region, it is expressed in the caudal neural plate, hindgut and tail bud mesoderm (Figure 2-1 e, this study, Fujii et al., 1997). Again the complementary expression pattern of P450RAI with respect to RAREhsplacZ expression (Figure 2-1 f) in this posterior region is well maintained. In the anterior region, P450RAI is expressed in neural crest cells for cranial ganglia, a region where RAREhsplacZ is not expressed (Figure 2-1 f).

Therefore by comparing the RAREhsplacZ transgene expression to the endogenous P450RAI expression, it is very evident that there is a striking, though not completely complementary expression pattern in the early embryo. This interesting expression pattern of P450RAI fits with its role as an enzyme that metabolizes RA and it is not present in regions were RA is actively signalling.

**Strategy for the overexpression of P450RAI in the mouse embryo**

To allow overexpression of the P450RAI enzyme, which should lead to the depletion of RA in the embryo, its full length cDNA was cloned into the pCAGGS expression vector (Niwa et al., 1991). More specifically, the P450RAI cDNA was placed downstream of the CMV-IE enhancer and the chicken β-actin promoter (Figure 2-2 a,b), a strong, ubiquitous promoter. The cDNA was also followed by rabbit β-globin gene sequences, including a polyadenylation signal for proper processing. The c-myc epitope tag was placed at the C-terminus of the P450RAI transgene to assess correct protein expression (Figure 2-2 a). loxP sites were placed flanking the P450RAI cDNA to allow for future excision using the Cre recombinase system, as a rescue experiment. The control construct is the same as in Figure 2-2 a,
Figure 2-2. Structure of the overexpression vector.

The full-length cDNA of the P450RAI gene was placed downstream of the CMV-IE enhancer and the chicken β-actin promoter, which is a strong promoter that allows ubiquitous expression. An epitope tag, c-myc, was placed at the C-terminus, to monitor correct protein expression (a). The control was the same construct minus the c-myc tag (b). loxP sites were placed flanking the P450RAI cDNA to allow for future excision using the Cre recombinase system, as a rescue experiment.
minus the c-myc tag (Figure 2-2 b). For simplicity, this construct is named CMV-chickβ–actin-P450RAI.

The overexpression strategy involved making ES cells transgenic for both the RAREhsplacZβ-neo and CMV-chickβ–actin-P450RAI (Figure 2-3). The RAREhsplacZβ-neomycin (neo) construct was electroporated into wild type (WT) ES cells, and neomycin resistant colonies were selected. I tested these cells for RAREhsplacZ expression and induction by RA in vitro and in vivo. Those cells containing the RAREhsplacZβ-neo transgene were then co-electroporated with the overexpression vector, CMV-chickβ–actin-P450RAI, as described above, along with the puromycin containing vector, PGK-puro-PGK-PA, for selection. Puromycin resistant colonies were selected. These final cells should be transgenic for both RAREhsplacZβ-neo and CMV-chickβ–actin-P450RAI. These cells were used to carry out both in vitro and in vivo analysis. The advantage of this strategy is that the RAREhsplacZβ-neo transgene can be used as a tool to monitor the absence of RA signalling in vitro and in vivo, which can then be compared with the phenotypes seen in vivo.

ES cells transgenic for RAREhsplacZβ-neo are responsive to RA in vitro and in vivo

The presence of the RAREhsplacZβ-neo transgene in individual ES clones was confirmed by Southern blot analysis (Figure 2-4). B1, F1, F2, G2, H3, D4, C5 and D5 are examples of positive transgenic cell lines, indicated by the 5.5 Kb band. The higher molecular weight bands in the positive control are due to incomplete digestion of the plasmid.
Figure 2-3. Overexpression Strategy.

The RAREhsplacZ β-actin neomycin (neo) construct (blue) was electroporated into WT ES cells. Neomycin resistant colonies (blue) were selected. Cells containing the RAREhsplacZ β-neo transgene were co-electroporated with the overexpression vector (yellow), which has the full length P450RAI cDNA driven by the CMV-enhancer-chicken-β-actin promoter (as previously described), and the puromycin (puro) containing vector for selection. Puromycin resistant colonies (blue and yellow) were selected for. These cells contained both the CMV-chickβ-actin-P450RAI and the RAREhsplacZ β-neo transgenes.
OVEREXPRESSION STRATEGY

WT ES Cells

select for neomycin resistance

CMV Chick β-actin loxP P450RAI myc loxP

PGK puro PGK-PA

select for puromycin resistance

P450RAI transgene

+ RAREhsplacZ transgene

in vitro and in vivo analysis
Figure 2-4. ES cells transgenic for RAREhsplacZβ-neo.

Genomic DNA was isolated from neomycin resistant ES cell colonies that had been electroporated with the RAREhsplacZβ-neo construct. These samples were then digested with SphI and subjected to Southern blot analysis. (TOP) The RAREhsplacZβ-neo construct. The 5’ end of the lacZ gene was used as a probe (shown in red). (BOTTOM) Southern blot analysis. The 5.5 Kb band indicates the presence of the RAREhsplacZβ-neo transgene. B1, F1, F2, G2, H3, D4, C5 and D5 are examples of positive transgenic cell lines. The positive control (+) is the RAREhsplacZβ-neo plasmid digested with SphI and probed as above. Because the positive control gave a very intense signal, it was exposed for a shorter time compared to the cell line samples.
The image shows a diagram of a genetic marker with restriction sites labeled by Sph I enzymes. The probe is indicated, and the marker size is marked as 5.5 kb. Below the diagram is a gel analysis showing bands for different cell lines labeled B1, F1, F2, G2, H3, D4, C5, D6, and a positive control '+' with a 5.5 Kb marker for RAREhaploZ Tg.
The RAREhsplacZβ-neo transgenic ES cell lines were cultured in the presence of 10^{-6} M RA (dissolved in ethanol) or ethanol alone, as the control, and then stained for β-galactosidase expression to determine their responsiveness to RA (Figure 2-5). F2, G2, D4, and H3 are examples of cell lines that were responsive to RA. There was little or no staining in the presence of ethanol at 12 and 24 hours, but in the presence of RA there was a significant increase in β-galactosidase expression. This suggests that the RAREhsplacZβ-neo transgene was responsive to RA in vitro.

To determine if these RAREhsplacZβ-neo transgenic cell lines could also give the expected expression pattern in vivo, the tetraploid aggregation technique was used to generate totally ES cell derived embryos (Nagy et al., 1993). Implementing this technique using ES cells transgenic for RAREhsplacZβ-neo, that were responsive to RA in vitro, two cell lines (F2 and G2) out of four lines tested, were identified which gave the expected in vivo expression pattern. Figure 2-6 a) shows the expression pattern of the F2 cell line at E8.5 and E9.5 (G2 cell line, data not shown), which is almost the same as the expression pattern in the previously derived RAREhsplacZ transgenic mice (Rossant et al., 1991). The other two RAREhsplacZβ-neo transgenic lines tested displayed morphological abnormalities, most likely due to positional effects or lower levels of transgene expression.

RAREhsplacZβ-neo transgenic embryos were generated using the tetraploid aggregation technique and tested to determine if the transgene was responsive to RA throughout the embryo, as shown in Rossant et al. (1991). These transgenic embryos were dissected at E7.5 and cultured in vitro in the presence of RA or ethanol (control) for 24 hours, and then stained for β-galactosidase expression (Figure 2-6 b). The embryos treated with ethanol (+EtOH) showed normal β-galactosidase
Figure 2-5. ES cells transgenic for RAREhsplacZβ-neo are responsive to RA in vitro.

To determine if the RAREhsplacZβ-neo transgene was responsive to RA in these ES cells, they were treated with 10^{-6} M RA or ethanol, as the control, for 12 and 24 hours and then stained for β-galactosidase expression. F2, G2, D4, and H3 are examples of cell lines that were responsive to RA. At 12 and 24 hours there was little or no staining in the presence of ethanol, but in the presence of RA, there was a significant increase in β-galactosidase expression (blue staining).
Figure 2-6. ES cells transgenic for RAREhsplacZβ-neo give the expected expression pattern in vivo.

a, b) The tetraploid aggregation technique was used to generate totally ES cell derived embryos using RAREhsplacZβ-neo ES cells, that were responsive to RA in vitro (F2 line). a) Embryos were dissected at E8.5 and E9.5 and stained for β-galactosidase expression. The F2 line gave the expected expression pattern in vivo.

b) To show that this RAREhsplacZβ-neo transgene is responsive to RA throughout the embryo, the transgenic embryos were dissected at E7.5 and cultured in vitro in the presence of 10⁻⁶M RA or ethanol, as the control, for 24 hours and then stained for β-galactosidase expression. The embryo treated with ethanol (+EtOH) showed normal β-galactosidase expression, while the embryo treated with RA (+RA) showed an expansion of staining posteriorly and anteriorly.
expression, while the embryos treated with RA (+RA) showed an expansion of staining posteriorly and anteriorly. Therefore there is an expansion of expression of the \textit{RAREhsplacZ\beta-neo} transgene in the embryos treated with RA, with respect to the ethanol treated ones. There is not a complete anterior expansion of expression, which is most likely due to the fact that this transgene is less sensitive than the previously generated \textit{RAREhsplacZ} transgenic mice (Rossant \textit{et al.}, 1991). Since there is an expansion of expression of the \textit{RAREhsplacZ\beta-neo} transgene in embryos treated with RA, this suggests that it is responsive to RA in areas where it is normally not expressed.

**Generation of ES cells transgenic for \textit{RAREhsplacZ\beta-neo} and \textit{CMV-chick\beta-actin-P450RAI}**

Once it had been confirmed that the \textit{RAREhsplacZ\beta-neo} transgenic ES cell line F2 gave the correct expression pattern \textit{in vivo} and was responsive to RA \textit{in vitro} and \textit{in vivo}, the F2 cell line was co-electroporated with the overexpression construct, CMV-chick\beta-actin-P450RAI (with or without the c-myc tag) and the PGK-puro-PGK-PA vector. Puromycin resistant colonies were subjected to Southern blot analysis (Figure 2-7 a, b), to determine if the \textit{CMV-chick\beta-actin-P450RAI} transgene had also integrated into the genome. 2\% of the puromycin resistant cell lines were positive for the full \textit{CMV-chick\beta-actin-P450RAI} transgene. Figure 2-7 a) shows the result of a Southern blot of genomic DNA digested with \textit{Hind} III and probed with the \textit{P450RAI} cDNA. The following cell lines were positive for the 3' end of the overexpression construct (indicated by the presence of the 2 Kb band): -myc D7, -myc E7, -myc F7, -myc H7, -myc A8, -myc C8, +myc A1, +myc B1, +myc C1 and +myc E1. Figure 2-7 b) shows the result of a Southern blot of genomic DNA from various cell lines that
Figure 2-7. ES cells transgenic for CMV-chickβ-actin-P450RAI.

(TOP) The overexpression vector containing the full length P450RAI cDNA. ES cells transgenic for RAREhsplacZβ-neo were co-electroporated with this overexpression construct and the PGK-Puro-PA vector, for selection. Genomic DNA was isolated from puromycin resistant ES cell colonies and subjected to Southern blot analysis. -myc and +myc refers to cell lines that were electroporated with the construct without the myc tag or with the myc tag at the C-terminus, respectively. F2 is the negative control, the parent line transgenic for only RAREhsplacZβ-neo. The positive control (+) is the overexpression plasmid. (a) Genomic DNA from different cell lines and controls was digested with HindIII and probed with a 415 bp 3' end fragment (nucleotides 1101-1516) of the P450RAI cDNA (purple box). The 2 Kb band confirms the presence of the P450RAI transgene (3'end of the overexpression construct), and the 6 Kb band corresponds to the endogenous P450RAI locus. (b) Genomic DNA from various cell lines that were positive for the presence of the P450RAI transgene, along with controls were digested with SalI and HindIII. A 379 bp fragment (nucleotides 1-379) of the CMV-enhancer from the pCAGGS vector was used as a probe (red box). The 1.8 Kb band confirms the presence of the 5'end of the construct, which includes the following regions of the construct: CMV-IE, chickβ-actin promoter, intron and a loxP site. Cell lines +myc A1, -myc H7, and -myc C8 contain the full CMV-chickβ-actin-P450RAI transgene. Because the positive controls in a) and b) gave a very intense signal they were exposed for a shorter time with respect to the other cell line samples.
were positive in Figure 2-7 a), digested with Sal I and Hind III and probed with part of the CMV-enhancer. Cell lines +myc A1, -myc H7, and -myc C8 are positive for the presence of the 5' end of the CMV-chickβ-actin-P450RAI transgene (1.8 Kb band). Therefore these three cell lines contain the full CMV-chickβ-actin-P450RAI transgene. The larger molecular weight bands seen in Figure 2-7 b) are likely due to incomplete digestion, multiple integration or tandem repeats of the CMV-chickβ-actin-P450RAI transgene.

**Analysis of CMV-chickβ-actin-P450RAI transgene expression in ES cells**

A Northern blot analysis was performed to determine the expression of the CMV-chickβ-actin-P450RAI transgene at the transcriptional level (Figure 2-8). The Northern blot was probed with a P450RAI cDNA. The P450RAI gene encodes a 1.9 Kb mRNA (Ray et al., 1997). As a control WT ES cells were treated with RA (ES+RA), which induces endogenous P450RAI expression, represented by the 1.9 Kb band (Figure 2-8). Cell line +myc A1, -myc H7 and -myc C8 contain the full CMV-chickβ-actin-P450RAI transgene, while +myc E1 and -myc E7 contain only part of the CMV-chickβ-actin-P450RAI transgene. P450RAI mRNA was not expressed in any of these double transgenic cell lines. This Northern was stripped and re-probed with GAPDH. The presence of the GAPDH band confirms that the RNA was not degraded and that the concentration in each sample was consistent. These results showed that the CMV-chickβ-actin-P450RAI transgene is not expressed transcriptionally at the level of Northern detection in the cell lines tested.

A more sensitive assay, RT-PCR, was used to detect mRNA expression and to confirm the Northern blot results (Figure 2-9 a). Primers specific for the chickβ-actin promoter and the P450RAI cDNA were used to amplify CMV-chickβ-actin-P450RAI
Figure 2-8. Analysis of transcript levels of P450RAI in ES cells transgenic for CMV-chickβ-actin-P450RAI.

Total RNA was prepared from cell lines that were transgenic for both RAREhsplacZβ-neo and CMV-chickβ-actin-P450RAI. Expression of P450RAI was assessed by Northern blot analysis. 15 μg of RNA was electrophoresed, transferred to a nylon membrane and probed with a 415 bp DNA fragment of the P450RAI cDNA (nucleotides 1101-1516). F2 is the negative control, which is the parent line transgenic for only RAREhsplacZβ-neo. As a positive control, WT ES cells were treated with 10^-6M RA for 24 hours (ES+RA), which induces endogenous P450RAI expression (1.9 Kb mRNA). As a control for RA treatment, ES cells were treated in parallel with ethanol (ES+EtOH). None of the double transgenic cell lines showed P450RAI expression. Size markers are the location of ribosomal bands. The blot was reprobed with a cDNA encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as a control for RNA concentration and integrity.
Figure 2-9. Reverse transcriptase (RT)-PCR analysis of transgenic P450RAI expression in ES cells.

(a) From 1 µg of total RNA, first-strand cDNA was synthesized with (+) or without (-) RT. Primers specific to the chickβ-actin promoter and P450RAI cDNA (spanning an intron) were used to amplify the CMV-chickβ-actin-P450RAI transgene (expected size 0.55 Kb) (Figure 2-2). Hypoxanthine guanine phosphoribosyl transferase (HPRT) primers were also used to amplify the endogenous HPRT gene as a control for RNA concentration and integrity. +myc E1, -myc C8 and +myc A1 are cell lines transgenic for both RAREhspZβ-neo and CMV-chickβ-actin-P450RAI. F2 is the negative control, since it only contains the RAREhspZβ-neo transgene. (H2O) contains water alone. The positive control is the circular plasmid of CMV-chickβ-actin-P450RAI (expected size 2.9 Kb). The HPRT primers gave the correct size fragment in all samples, while the primers specific for the CMV-chickβ-actin-P450RAI transgene gave no 0.55 Kb product. (b) Samples in (a) were then subjected to Southern blot analysis and probed with the 5’end of the P450RAI cDNA (Eco RI-Acc I fragment 546 bp), which spanned the RT-PCR product. The 0.55 Kb expected band was not present in any of the samples. The positive control (+) had the correct 2.9 Kb band and also a ~6 Kb, which is most likely due to linear amplification of the plasmid, since it was not linearized. This positive control gave a very intense signal so it was exposed for a shorter time with respect to the other cell line samples.
transgenic cDNA from cell lines -myc C8, +myc A1 (contain the full CMV-chickβ-actin-P450RAI transgene) and +myc E1 (contains only part of the CMV-chickβ-actin-P450RAI transgene). The expected size of the PCR product is 0.55 Kb, which is not visible on the agarose gel containing ethidium bromide, for any of the cell lines. The positive control is the circular plasmid of CMV-chickβ-actin-P450RAI. The expected size of the PCR product from this plasmid is 2.9 Kb, because the primers span an intron. This band was not visible on the agarose gel because the PCR reaction was not very efficient with such a large product. HPRT primers were also used on these samples. These primers gave the correct size fragment in all the samples, indicating the RNA had not degraded and that the concentration between samples was consistent. One possibility is that the P450RAI transgene is expressed at such a low level that the RT-PCR product could not be detected on the agarose gel. The samples in Figure 2-9 a), were subjected to Southern blot analysis and probed with P450RAI cDNA to address this possibility (Figure 2-9 b). The 0.55 Kb product was not present in any of the samples, confirming that the P450RAI transgene is not expressed. The positive control had the correct 2.9 Kb product and also a ~6 Kb product, which is likely due to linear amplification of the plasmid.

In parallel with the Northern blot and RT-PCR analysis, Western blot analysis was performed on CMV-chickβ-actin-P450RAI transgenic cell lines, to determine protein expression of the P450RAI transgene (Figure 2-10). Protein lysates were prepared from +myc A1 (contains the full CMV-chickβ-actin-P450RAI transgene) and +myc E1 (contains only part of the CMV-chickβ-actin-P450RAI transgene). The positive control is the grb-4 cDNA containing a c-myc tag, transfected into COS-1 cells, from which protein was isolated (donated by Venus Lai). This protein product
Figure 2-10. Analysis of protein expression of P450RAI in ES cells transgenic for CMV-chickβ-actin-P450RAI.

Protein lysates were prepared from ES cell lines that were transgenic for RAREhsplacZβ-neo and CMV-chickβ-actin-P450RAI that was c-myc tagged, +myc A1 and +myc E1. F2 is the parent line containing the RAREhsplacZβ-neo transgene (negative control). The positive control is Grb-4 protein tagged with c-myc (expected size 50kDa). These samples were Western blotted with a monoclonal antibody to c-myc. The positive control gave the correct size band, while the doubly transgenic lines were negative.
is 50 kDa. The predicted translation product of the P450RAI transgene is 56.1 kDa (Ray et al., 1997), plus the c-myc tag, gives a total estimated size of about 58 kDa. The above samples were subjected to Western blot analysis using a monoclonal antibody to c-myc. The positive control gave the correct size translation product, while the transgenic lines were negative, suggesting that the P450RAI transgene is not expressed. Therefore the Western blot, Northern blot and RT-PCR analysis suggest that the P450RAI transgene was not detectably expressed at the protein or transcriptional level in the cell lines generated.

ES cells transgenic for RAREhsp lacZβ-neo and CMV-chickβ-actin-P450RAI treated with RA show a downregulation in β-galactosidase expression

While the analysis of the expression of the P450RAI transgene was being carried out in ES cells transgenic for RAREhsp lacZβ-neo and CMV-chickβ-actin-P450RAI, they were also tested for their in vitro response to RA (Figure 2-11). The following cell lines were treated with varying concentrations of RA, and cultured for 24 hours, and then stained for β-galactosidase expression: -myc C8 (contains the full CMV-chickβ-actin-P450RAI transgene), +myc E1, -myc E7 (contain part of the CMV-chickβ-actin-P450RAI transgene) and F2 which is transgenic for only the RAREhsp lacZβ-neo. It is estimated that the endogenous level of RA in the mouse is approximately 10⁻⁸ M (Fujii et al., 1997). When treated with 10⁻⁹ and 10⁻⁸ M RA, there was an obvious decrease in the expression of the RAREhsp lacZβ-neo in the double transgenic lines compared with the F2 line. As the RA concentration was increased to 10⁻⁷ and 10⁻⁶ M, the reduction in RAREhsp lacZβ-neo was not as evident.

In vivo analysis: embryos transgenic for RAREhsp lacZβ-neo and CMV-chickβ-actin-P450RAI were generated
Figure 2-11. *In vitro* response to RA.

Cell lines -myc C8, +myc E1 and -myc E7 were treated with varying concentrations of RA or ethanol (EtOH), as a control, for 24 hours and then stained for β-galactosidase expression. F2 is the control, which contains only the RAREhsplacZβ-neo transgene. -myc C8, +myc E1 and -myc E7 are transgenic for both RAREhsplacZβ-neo and CMV-chickβ-actin-P450RAI. All three of these cell lines do not express detectable levels of this transgene, but they do show a downregulation of RAREhsplacZ transgene expression at $10^{-9}$ and $10^{-8}$ M RA.
While the analysis of the CMV-chickβ-actin-P450RAI expression was being carried out, the in vivo analysis was also being performed. Using ES cells transgenic for RAREhsplacZβ-neo and CMV-chickβ-actin-P450RAI, totally ES cell derived embryos were generated with the tetraploid aggregation technique (Nagy et al., 1993). The cell lines that gave the most dramatic decrease in β-galactosidase expression when treated with RA in vitro (Figure 2-11) were aggregated with the tetraploid embryos. The cell lines used were –myc C8 (contains the full CMV-chickβ-actin-P450RAI transgene), +myc E1 (contains a partial form of the CMV-chickβ-actin-P450RAI transgene), and the F2 parent line (RAREhsplacZβ-neo). These embryos were dissected at E8.5 and E9.5, and then stained for β-galactosidase expression to determine if RA signalling was absent. They were also analysed for any morphological abnormalities. A total of eleven E8.5 and sixteen E9.5 embryos were analysed from the –myc C8 and +myc E1 cell lines and at these developmental stages showed normal RAREhsplacZ expression, and no abnormal morphological phenotype (data not shown). This suggests that the P450RAI transgene did not have any detectable developmental affects on the embryo.
CHAPTER 4

DISCUSSION AND FUTURE WORK
DISCUSSION AND FUTURE WORK

Past studies have not been able to definitively determine RA's role in A-P patterning of the developing vertebrate embryo. Therefore, to gain a deeper understanding of RA's physiological role in determining the A-P polarity of the body axis, I attempted to remove all endogenous RA signalling in the developing embryo by ubiquitously expressing P450RAI, which is an enzyme that has been shown to specifically metabolize RA (Abu-Abed et al., 1998; Fujii et al., 1997; Hollemann et al., 1998; White et al., 1997; White et al., 1996). The strategy undertaken to allow ubiquitous expression of P450RAI involved generating ES cells transgenic for RAREhsplacZβ-neo and CMV-chickβ-actin-P450RAI. The RAREhsplacZβ-neo reporter was used to assess RA signalling. These double transgenic ES cells were then subjected to an in vitro and in vivo analysis.

The endogenous expression pattern of P450RAI in the early mouse embryo was complementary to the expression pattern of the RAREhsplacZ transgene. This result agreed with the biochemical data which suggested that P450RAI metabolized RA (Abu-Abed et al., 1998; Fujii et al., 1997; White et al., 1997; White et al., 1996). Therefore, these data supported the idea that overexpression of P450RAI throughout the embryo would result in endogenous RA metabolism.

ES cell lines transgenic for RAREhsplacZβ-neo were derived and two of these lines were found to be responsive to RA in vitro and in vivo. One of these cell lines was then made transgenic for CMV-chickβ-actin-P450RAI. Three independent lines, transgenic for both RAREhsplacZβ-neo and CMV-chickβ-actin-P450RAI were generated. These double transgenic lines did not show detectable levels of expression of the P450RAI transgene at the protein or transcriptional level. Although these double transgenic lines did not show expression of the P450RAI transgene, when they were treated with RA they showed an in vitro downregulation
in β-galactosidase expression, which is the expected result if P450RAI was being expressed. This downregulation was likely an artefact due to a clonal effect, that is, the different RAREhspacZβ-neo clones derived from the parent line (F2), after the electroporation of the CMV-chickβ-actin-P450RAI construct, may show different intrinsic levels of β-galactosidase expression in response to RA. In vivo analysis was also carried out by generating embryos transgenic for RAREhspacZβ-neo and CMV-chickβ-actin-P450RAI. These embryos had a normal phenotype and showed normal β-galactosidase expression. Thus, in vitro and in vivo analysis of these double transgenic ES cells suggested that the P450RAI transgene was not being expressed at a detectable level in the lines analysed.

Possible Problems

There are several possible reasons why the P450RAI transgene is not being expressed. The absence of transgene expression can be attributed to its site of integration into the genome. The transgene may have integrated into a region of the genome that prevents its expression. Since I only had three independent lines, this is a likely possibility. Another possibility is that there may be a problem with the construct, which is not allowing expression of the P450RAI cDNA insert. After the various cloning steps during the construction of the CMV-chickβ-actin-P450RAI vector, the sequence of the ligation sites between the insert and the vectors were analysed and they appeared correct. There is a possibility that during the cloning process, changes in the vector may have been introduced which were not detected by the sequencing done and subsequently interfered with the expression of the insert. Another possible explanation for the lack of expression of the P450RAI transgene is that overexpression of this enzyme may be toxic to ES cells, so only those cells that were not expressing P450RAI or expressing it at a very low level survived, while those cells that did express P450RAI died.
Future Work

Determining the Problem

Further experiments can be done to address if the possible problems mentioned above are the cause of the lack of expression of the P450RAI transgene from the CMV-chickβ-actin-P450RAI construct I generated. An experiment that can be carried out is in vitro transcription and translation (TNT™ T7-Coupled Reticulocyte Lysate System-Promega) of the CMV-chickβ-actin-P450RAI construct to determine if the P450RAI transgene can be properly transcribed and translated. Transient transfection of the myc tagged version of the construct into ES cells followed by anti-myc Western blot analysis can also be performed to assess if P450RAI can be properly expressed in ES cells and determine its potential toxic effect to the cells. If the result of the in vitro transcription and translation is negative, it would suggest that there is something wrong with the construct, which is preventing P450RAI's expression. If the in vitro transcription and translation experiment is positive and the transient transfection experiment is negative it would indicate that the expression of P450RAI is toxic to the ES cells. If these two experiments result in the expression of the P450RAI transgene, it would mean that the construct is functioning properly and it would imply that the site of integration of the P450RAI transgene in the stable cell lines might have prevented its expression. If expression of P450RAI is seen in the above in vitro transcription and translation, and transient transfection experiments, a further functional test can be done. It would involve repeating the transient transfection with the CMV-chickβ-actin-P450RAI construct into ES cells transgenic for RAREhsplacZβ-neo followed by treatment with RA and then staining for β-galactosidase expression. If a decrease in blue staining is observed it would suggest P450RAI is metabolizing RA.

A further test experiment that can be done is to generate transient transgenic embryos by injecting circular CMV-chickβ-actin-P450RAI DNA plasmid into the
pronuclei of fertilized mouse eggs carrying the RAREhspacZ transgene by standard procedure (Hogan et al., 1994). If the transgenic embryos generated exhibited a phenotype, a decrease in β-galactosidase expression and expressed P450RAI, it would suggest that the construct is allowing proper expression.

**Modifications to the Strategy for Future Experiments**

Several changes can be attempted which may help in the successful execution of the original strategy outlined if the construct is not expressed properly. If the CMV-chickβ-actin-P450RAI construct does not allow expression of P450RAI even in an in vitro transcription and translation system, subcloning P450RAI cDNA into a different expression vector with a different promoter that has been shown to give ubiquitous expression in the mouse embryo may solve the problem.

If overexpression of P450RAI is toxic to ES cells, different promoters, which are ubiquitous but have a lower level of expression than the chickβ-actin promoter, can be used to drive the expression of P450RAI. These constructs can be transiently transfected into ES cells and assayed for apoptosis, to assess the level of P450RAI expression tolerated by ES cells. However, these promoters must be tested in vivo to assess whether they can still completely deplete RA in the embryo.

Another possible way to overcome the toxic effect of overexpressing P450RAI in ES cells is to use an inducible system, which would allow control of the initiation and duration of P450RAI expression. The idea is to use an inducible system such that the P450RAI transgene would still be downstream of a ubiquitous promoter but it would be silent in the ES cells and then induced during development in the P450RAI transgenic embryos generated by tetraploid aggregation. An example of such a system includes the tetracycline regulated inducible system (Tet-off System-CLONTECH). In this system the P450RAI cDNA would be subcloned downstream of a tetracycline regulated promoter (Tet-off System-CLONTECH); the presence of tetracycline or a tetracycline derivative, doxycycline, in the culturing media, would
prevent \(P450RAI\) expression in the ES cells, but upon the removal of tetracycline or doxycycline, \(P450RAI\) would be expressed ubiquitously. \(P450RAI\) transgenic cells would be aggregated with two tetraploid embryos and cultured in the presence of tetracycline/doxycycline until transferred into foster mothers, to ensure that the \(P450RAI\) transgene is not being expressed. The foster mothers are free of tetracycline/doxycycline therefore this would allow \(P450RAI\) to be expressed early in development in the transgenic embryos. Another possibility is to turn on the \(P450RAI\) transgene later in development. This can be done as stated above with the exception that the foster mothers would be administered tetracycline/doxycycline prior to embryo transfer and then taken off of tetracycline/doxycycline after the transferred embryos had developed to the desired gestational stages (Furth et al., 1994).

Another strategy that can be used to circumvent the possible toxic effect of overexpressing \(P450RAI\) in ES cells is to conditionally express it by using the Cre recombinase/loxp site specific recombination system which will also control the timing of expression of \(P450RAI\) in the embryo (Lobe et al., 1999). This would involve placing a reporter gene followed by a stop signal (three polyadenylation signals) to ensure transcriptional stop, flanked by loxp sites, just downstream of a ubiquitous promoter, but upstream of the \(P450RAI\) cDNA. This construct would prevent expression of \(P450RAI\) (Lobe et al., 1999). In order to get expression of \(P450RAI\) from this construct; transgenic mice would first be generated from ES cells containing this transgene. These transgenic mice would then be crossed to another transgenic mouse line that expressed Cre recombinase at an early stage of mouse development (Lewandoski et al., 1997; Meyers et al., 1998). In the resulting double transgenic lines, expression of Cre would allow excision of the stop signal and thus allow the ubiquitous expression of the \(P450RAI\) transgene (Lobe et al., 1999). Thus,
in this experiment P450RAI would not be expressed in the ES cells but would be expressed early in embryogenesis.

It is possible that the site of integration of the P450RAI transgene into the genome, in the transgenic lines I generated, may have prevented its expression. I may not have analysed enough different cell lines, therefore to further determine if site of integration is the problem I would generate more cell lines transgenic for both RAREhspZβ-neo and CMV-chickβ-actin-P450RAI and then test for expression of the P450RAI transgene. Another way to resolve this problem is to target the construct, into the hypoxanthine phosphoribosyltransferase (HPRT) locus. This would allow integration of the transgene into a chosen location in the genome, which has been shown to allow proper expression of an inserted transgene (Bronson et al., 1996). Therefore, this strategy would ensure that the site of integration of the P450RAI transgene would not interfere with its expression.

With each of the above modifications mentioned the resulting overexpression construct of P450RAI would be tested for its expression and function by in vitro transcription and translation, and transient transfection experiments, prior to its electroporation into ES cells. If the results are positive from these test experiments, the rest of the strategy that would be followed is the same as stated previously. In general, the overexpression strategy is still valid but what prevented its successful execution was the initial technical problem involving the lack of expression of the P450RAI transgene from the overexpression construct.

Recent Advances in the Field

Recent studies have contributed to the understanding of RA's role in A-P patterning in early embryogenesis. Retinaldehyde dehydrogenase type 2 (RALDH2) was identified as an enzyme that functions in the synthesis of RA in the early embryo. It has a high substrate specificity for retinal, that is, it oxidizes retinal into RA (Wang et al., 1996; Zhao et al., 1996). The endogenous expression pattern of the
Raldh2 gene in early mouse development is similar to the expression pattern of the RAREhsplacZ transgene. This suggests that this enzyme may be responsible for RA synthesis in the embryo (Niederreither et al., 1997; Rossant et al., 1991; Zhao et al., 1996). To determine Raldh2's importance in RA synthesis and to further determine RA's role in embryonic development, a study was done by Niederreither et al. (1999), which generated mice that were deficient for the Raldh2 gene, via the gene targeting technique. Raldh2-/- embryos die at midgestation without undergoing axial rotation. At E9.5 and E10.5 these embryos showed severe posterior region shortening which remained open ventrally and no limb buds formed. The anterior region of these mutant embryos was less affected. Some of the abnormalities included truncation of the frontonasal region, misformed branchial arches, medial cleft and open anterior neuropore. The whole trunk of Raldh2-/- embryos had the appearance of the posterior-most regions of age-matched control embryos (Niederreither et al., 1999).

Raldh2-/- embryos also exhibited heart defects. Their hearts consisted of a single medial dilated ventricle-like cavity. Analysis of heart specific markers suggested there was an absence of heart looping and anteriorization of the heart tube (Chazaud, C., personal communication). These embryos also had reduced otic vesicles and they were abnormally distant from the hindbrain neurepithelium. Structural and marker analysis also indicated abnormal mesoderm formation (Niederreither et al., 1999).

The study done by Niederreither et al. (1999) also showed that the defects in Raldh2-/- mutants were due to a lack of RA synthesis, as shown by the absence of expression of the RAREhsplacZ transgene in the Raldh2-/- background. This suggests that RALDH2 is the primary enzyme that catalyzes the conversion of retinal to RA (Eichele, 1999). Raldh2-/- mutant defects also included altered expression of a RA-target homeobox gene, Hoxa1 that was reduced in the posterior region. Otx2 expression appeared normal in Raldh2-/- embryos. Therefore, they concluded RA
might not be required to repress Otx2 expression (Niederreither et al., 1999). In addition, to confirm that the defects in Raldh2^-/- mice were a result of the absence of RA, they demonstrated an almost complete rescue of the mutant phenotype upon maternal administration of RA. Overall, this study finally confirms that local embryonic RA synthesis (by RALDH2) is essential for early mouse development (Niederreither et al., 1999).

Past studies have proposed that RA may be a posteriorizing factor, that is, it can promote posterior characters in the developing embryo and that a lack of RA would lead to an anteriorization of the embryo. Due to the lack of expanded Otx2 expression in Raldh2^-/- embryos, Niederreither et al. (1999), suggested that RA is not involved in anterior patterning. However, more extensive analysis of these Raldh2^-/- embryos needs to be performed by testing additional markers to determine if RA can affect anterior patterning by causing an anteriorization phenotype in its absence. It would be interesting to look at specific, anterior, midbrain-hindbrain boundary, hindbrain, hindbrain-spinal cord boundary markers to determine if there are abnormalities in anterior patterning which cause an anteriorization of the embryo. Examples of these markers include Emx1, En2, 3' Hox genes and RARβ. Although this study by Niederreither et al. (1999), has not fully determined RA's role in anterior patterning it has clearly shown that RA is implicated in posterior patterning.

Another recent study that has contributed to the understanding of RA's role in A-P patterning is the one done by Hollemann et al. (1998). In this study they cloned the Xenopus homologue of P450RAI or CYP26, called XCYP26 and found it is differentially expressed during Xenopus embryonic development. Overexpression of XCYP26 in the Xenopus embryo was carried out and it was determined it could rescue the developmental defects that occur upon RA treatment and it also restored the normal expression pattern of hindbrain and anterior neural markers that are
disrupted in RA treated embryos. These results suggest that XCYP26 inactivates RA without producing any new signalling activity (Hollemann et al., 1998). These results also resolve the controversy raised in past studies, regarding the metabolic activity of the by-products produced by P450RAI.

Hollemann et al. (1998), showed that ectopic XCYP26 expression caused a compression of the A-P body axis, that is, shortening of the trunk and tail structure and they also showed it exclusively affected the expression of hindbrain-specific molecular markers. The ectopic expression of XCYP26 caused the molecular identity (Krox-20 and Pax6) of individual caudal rhombomere segments to be shifted posteriorly by either one or two rhombomeric units, in a dose-dependent manner, while the rostral rhombomere identity was partially duplicated. The overall results obtained from overexpressing XCYP26 in the Xenopus embryo suggested a homeotic transformation of r4/r6 (low dose) or r5/r7 (high dose) to a r3/r5 identity. Thus, these results suggest that ectopic expression of XCYP26 anteriorizes the developing hindbrain and demonstrates a primary function of RA signalling in hindbrain development (Hollemann et al., 1998). This anteriorization of the hindbrain in Xenopus embryos overexpressing XCYP26, is the opposite to the effect of adding excess RA to mouse embryos which caused posteriorization of the rhombomeres (Conlon and Rossant, 1992; Marshall et al., 1992; Morriss-Kay et al., 1991). In addition, the position of anterior neural markers (Otx2, Sox3) and markers for the mid-hindbrain (En2) and hindbrain/spinal cord (Hoxb9) boundaries were also analysed in embryos overexpressing XCYP26. Their expressions were found to be, for the most part, normal with some exceptions, like the slight expansion in expression of Otx2 and En2 and the loss of Sox3 in the lens placode (Hollemann et al., 1998). Therefore, this study, as the study done by Niederreither et al. (1999), suggests RA is involved in posterior patterning. These two studies have definitely
provided some convincing data to support RA's role as a posteriorizing factor during embryonic development.

**Predictions**

If P450RAI had been successfully ubiquitously expressed in the developing mouse embryo as outlined in the strategy, the expected phenotypes would be an absence or downregulation in the expression of the RAREhsplacZ reporter transgene and a disruption in A-P patterning. The A-P patterning defects expected would be similar to those seen in Raldh2\textsuperscript{+} embryos (Niederreither et al., 1999), such as, posterior truncations of the embryo axis, anteriorization of the heart tube frontonasal truncations and abnormalities in branchial arches and otic vesicles. Another patterning defect expected in these mouse embryos ubiquitously expressing P450RAI, would be abnormalities in the developing hindbrain. I would expect an anteriorization of the hindbrain, that is, more posterior rhombomeres assuming a more anterior identity, as seen in Xenopus embryos overexpressing XCYP26 (Hollemann et al., 1998) and/or loss of the posterior hindbrain as seen in VAD quail embryos (Maden et al., 1996). Along with hindbrain anteriorization, I would expect to see an alteration of molecular markers specific for hindbrain development. I would expect to see a downregulation or regression posteriorly in expression of 3' Hox genes thought to be regulated by RA such as, Hoxb1, Hoxb2, Hoxb4, and Hoxb3 and also altered expression of other markers like Krox-20 and Fgf3. Although the results from overexpressing XCYP26 in Xenopus embryos suggested that the anterior region was not dramatically affected, except for a slight expansion of Otx2 and En2 expression (Hollemann et al., 1998), it is still possible that RA has a role in restricting the expression of anterior markers. RA may have a role in refining A-P boundaries; but whether RA has a role in anterior patterning in the mouse embryo still needs to be verified. To determine if RA affects anterior patterning, that
is, its absence causes anterior expansions, in mouse embryos overexpressing \textit{P450RAI}, the expression pattern of specific anterior markers would be analysed such as \textit{Otx2}, \textit{Wnt-7b} and \textit{Emx1}. To determine if overexpression of \textit{P450RAI} in the mouse embryo causes anteriorization of other regions of the embryo, the expression of other markers can be analysed, including midbrain-hindbrain markers, such as \textit{En2} and \textit{Pax2}, and hindbrain-spinal cord boundary markers like RAR\textbeta. Therefore, the phenotypes of mouse embryos ubiquitously expressing \textit{P450RAI} will hopefully provide more convincing data to support RA’s role as a posteriorizing factor during embryonic A-P patterning and help determine if in fact RA has a role in anterior patterning in vertebrates.

\textbf{Conclusion}

With the aim of determining RA’s role in A-P patterning, I attempted to generate embryos devoid of RA signalling by ubiquitously expressing an enzyme that could metabolize RA, \textit{P450RAI}. Due to technical problems the \textit{P450RAI} transgene was not expressed, thus the overexpression strategy could not be properly completed. Considering the possible problems and then making the appropriate adjustments to the strategy, should allow the successful execution of this project in the future. Recent studies have provided more evidence supporting RA’s role in posterior patterning, but whether it has a role in anterior patterning needs to be further elucidated. The ubiquitous expression of \textit{P450RAI} in the mouse embryo will provide important information because different \textit{P450RAI} transgenic lines will have different levels of \textit{P450RAI} expression. Thus, there will be a graded loss of RA activity, which can be easily monitored with the \textit{RAREhsplacZ} transgene and this graded loss would result in phenotypes with ranging degrees of severity. Therefore, the overexpression of \textit{P450RAI} during mouse embryogenesis along with the results from past studies will help clarify RA’s role during the early stages of mouse
morphogenesis with respect to A-P patterning and will help determine RA's exact role in establishing gene expression boundaries along the A-P axis.
REFERENCES
References


Hill, J., Clarke, J. D., Vargesson, N., Jowett, T., and Holder, N. (1995). Exogenous retinoic acid causes specific alterations in the development of the midbrain and


