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CELLULAR RETINOIC ACID BINDING PROTEIN (CRABP) mRNA EXPRESSION IN SPLOTCH MUTANT MOUSE EMBRYOS

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ABSTRACT

The *splotch* (*sp*) mutation has been identified as a mutation in the paired box gene, *Pax-3*. Heterozygous mice carrying this mutation are phenotypically normal, with the exception of a white spot on their bellies. Homozygous embryos do not live to birth, and suffer from a wide range of developmental defects, all of which result from delayed neural tube closure, or inadequate neural crest cell migration. Most notably, homozygotes have an increased rate of spina bifida with or without exencephaly. Retinoic acid (RA), which has been shown to be very important in the development of a number of systems, was shown to cause a selective mortality of the homozygous splotch embryos when administered maternally at day 9 p.c. (Moase and Trasler, 1987). Since cellular retinoic acid binding protein (CRABP) is localized to the tissues which are affected by both the *splotch* gene, and retinoic acid teratogenesis, its expression patterns in the developing *splotch* embryo were examined. It was found that the distribution of CRABP mRNA is normal, but its expression levels are excessive in *splotch* homozygous day 9 mouse embryos.

RESUME

L'anomalie *splotch* (*Sp*) est une alteration du gène *Pax-3*. Les souris hétérozygotes ne souffrent que par une tache blanche sur leurs poitrines. Les homozygotes, par contre, meurent avant naissance, et souffrent de nombreux défauts. Tous les défauts qui sont causés par cette anomalie sont un résultat direct de la faillite de la fermeture du tube neurale, ou de la faillite de la migration par les cellules neuronales de crête. En particulier, les homozygotes souffrent d'un taux élevé de défauts de tube neurale. L'acide retinoïc (RA) a été impliqué dans plusieurs processus dévélopmentale. RA cause la mortalité specifique des homozygotes *splotch* avec une dose maternelle à jour 9 de dévélopment (Moase and Trasler, 1987). Parce-que la protéine reliure d'acide retinoïc cellulare (CRABP) est associé avec les mêmes tissues qui sont affectées par l'anomalie *splotch* et par l'acide retinoïc, j'ai etudié sa distribution dans les embryons *splotch*. J'ai trouvé que la distribution de CRABP est normale, mais le taux d'éxpression est trop élevé dans les embryons homozygotes *splotch* de jour 9 de dévélopment.

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PREFACE

Part of the research presented in this thesis was presented at the 1994 meeting of the Teratology Society and published in Abstract:

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INTRODUCTION

One of the first and possibly one of the most important processes to occur in the development of vertebrates is neurulation: the formation of the neural tube which will develop into the brain and spinal cord. Many factors are involved in ensuring the neural folds rise, converge and fuse as they should. Two factors which prevent proper closure are retinoic acid and the *splotch* gene; both cause a dose dependent delay in closure of the neural tube. Homozygous *splotch* embryos have two copies of the mutant gene, and are pushed beyond the threshold of normal closure time to develop a neural tube defect (NTD; 89% of homozygous *splotch delayed* embryos have a neural tube defect; Moase and Trasler, 1989). *Splotch* heterozygotes, with only one copy of the gene, have a transient delay in neural tube closure, but develop a NTD only rarely. Retinoic acid also causes delayed closure of the neural tube by inducing an abnormally shaped neural groove (Kapron-Bras and Trasler, 1988a). The mechanisms by which *splotch* and RA delay closure are not known. Studying models in which neurulation is deviant leads to understanding the normal process.

In gene-teratogen interaction studies using RA and *splotch* mice, Kapron-Bras and Trasler (1985) found that RA treatment on day 9 of development reduced the frequency of spina bifida in *spl*+ X *spl*+ offspring. When Moase and Trasler (1987) repeated the experiment using *splotch delayed* mice and genetic markers, they found that the RA treatment was inducing selective mortality of the homozygotes. They found an increased rate of spina bifida in the heterozygotes coupled with the deaths of the homozygotes resulted in an overall reduction in the percent of the offspring suffering from neural tube defects. The increased sensitivity of the heterozygotes to RA results from an additional delay in the closure of the neural tube and not from a difference in the way that heterozygotes handle the teratogen.

The selective mortality of the homozygotes suggests that these embryos are handling the teratogen differently than their heterozygous and wildtype littermates. This would suggest that there is some RA associated protein downstream of *Pax-3*. The tissues affected by the *splotch* gene are tissues associated with cellular retinoic acid binding protein (CRABP) expression. In this study, I examine the possibility that aberrant CRABP expression is associated with the *splotch* mutation. This study is interesting for two reasons. Firstly, the *splotch* mouse model is of particular interest since it has been shown that it is homologous to Waardenburg syndrome I in humans (Foy *et al.*, 1990; Asher *et al.*, 1991). Secondly, retinoic acid has been shown to be associated with many developmental processes, and has also been shown to be an effective drug in the treatment of skin diseases (Larson *et al.*, 1992) and acute promyolocytic leukemia (Brand *et al.*, 1988, 1990). In understanding the role of retinoic acid in neurulation, we further our understanding of RA homeostasis, and how it can work as a drug.

LITTERATURE REVIEW

NEURULATION

Normal Neurulation

Gastrulation stage of development is responsible for transforming the embryo from a two layered ball to a three layered entity. This process ends with the initiation of neurulation. Neurulation can be divided into primary and secondary neurulation. Primary neurulation is the creation of the neural tube between the forebrain and caudal neuropore. Secondary neurulation is the formation of the caudal portion of the neural tube through the condensation of cord cells in the tail bud mesenchyme (Morriss-Kay *et al.*, 1994; Papalopulu and Kintner, 1994).

Primary neurulation begins with the formation of the notochord, a long rod shaped structure along the primitive streak, and the neural plate, a shield shaped structure consisting of elongated epithelial cells in the future fore- and midbrain region. The neural plate develops a neural groove down the midline which forms an attachment with the notochord (Morriss-Kay *et al.*, 1994). The apical end of the cells at the edges of the neural plate begin to constrict, forming bottle cells. This apical constriction, along with the flattening of adjacent epithelial cells is associated with the elevation of the edges of the neural plate: the creation of neural folds. The neural folds are convex, and initially present in the rostral region of the embryo, but as neurulation progresses, the neural plate extends caudally, followed by the neural folds. By looking at an embryo undergoing neurulation, a wide range of developmental stages can be seen: a flat neural plate caudally, and more advanced stages of neurulation rostrally. As the neural folds continue to rise, they change from convex to V-shaped, to concave (Morriss-Kay *et al.*, 1994), and finally the neural tube closure occurs with the fusion of the folds. Closure begins at the hindbrain/spinal cord junction,

and proceeds caudally the length of the neural tube, and rostrally toward the second site of neural tube closure. The second site of closure is at the forebrain/midbrain junction, and again, closure proceeds bidirectionally. The neuropore which is created by the first and second closure sites will result in exencephaly when left unclosed. Seven sulci and gyri form in the hindbrain region as it closes, forming the rhombomeres (Morriss-Kay *et al.,* 1994). A third closure site is at the very rostral tip of the neural tube, which zips caudally. These closure sites are quite rigid within a mouse strain; however, the location and timing of these closure sites varies between strains (Juriloff *et al.,* 1991).

Neural crest cells form as the neural tube closes. A small group of cells called the neural crest cells migrate away from the dorsal neuroepithilium, through the space between the neural tube and the overlying ectoderm. They give rise to a large number of derivatives throughout the body, including neuronal cells of the sensory and autonomic ganglia, nerve support structures, mesoectodermal structures, certain endocrine cells, and melanocytes (Moase and Trasler, 1989).

Neural Tube Defects

Neural tube defects arise as a result of the neural tube failing to close. The timing of neural tube closure can be described as having a threshold distribution (Fraser, 1976). The threshold is reached when the neural tube is not closed by a given stage of development (Kapron-Bras and Trasler, 1988a). Beyond this stage, the open neural tube will develop into spina bifida or exencephaly. The timing of the closure is determined by both environmental and genetic factors (Copp, 1994; Papalopulu and Kintner, 1994). Two such factors which will be discussed in this paper are retinoic acid and the *splotch* mutation.

PAX GENES

The Paired Box

Many genes which are important in embryonic development contain a DNA binding motif which allows them to affect the transcription of many other genes. Several types of structurally distinct DNA binding domains have been described, including helix-loop-helix, zinc fingers, homeodomain, pou domain, and paired domain (Kessel and Gruss, 1990). The paired box DNA binding motif was originally described in the *Drosophila melanogaster* pair rule gene *paired* (*prd*), and the two segment polarity genes *gooseberry-distal* and *gooseberry-proximal* (*gsb-p*, *gsb-d*; Bopp *et al.* 1986). In addition to the highly conserved paired box, the *paired* gene contains a paired type homeobox (Treisman *et al.*, 1991). Also identified are two other *Drosophila* genes which contain a paired box but not a paired type homeobox: *Pox meso* and *Pox neuro* (Bopp *et al.*, 1989). *Paired box*, or *Pax* genes have since been described in the zebrafish, nematode, frog, turtle, chicken, quail, mouse, and human, (Gruss and Walther, 1992; Chalepakis *et al.*, 1993; Gutjahr *et al.*, 1994). The *Pax* genes are so highly conserved throughout evolution, that vertebrate *Pax* genes can substitute for their *Drosophila* counterpart in transgenic *Drosophila* embryos (Pierpont and Erickson, 1993).

Nine murine *Pax* genes have been described to date, named *Pax-1* through *Pax-9*. These *Pax* genes contain three conserved motifs: first the paired box, followed by a short octapeptide motif which is not found in the *Drosophila* paired type genes, and then a paired type homeobox. While the paired box is intact in all nine *Pax* genes, the octapeptide motif and homeobox are not. These differences among the *Pax* genes has lead to their subgrouping into four classes based on their intron/exon structure and the motifs they contain (Walther *et al.*, 1991). *Pax-1* which contains an octapeptide but no homeobox is the only gene of its type. *Pax-2* and *Pax-8* both contain the octapeptide, and both have a similarly truncated homeodomain. Also included with them is *Pax-5*

which may show the same shortened homeobox, but has not yet been fully described. In the third group are *Pax-3* and *Pax-7*, which have all three motifs in their entirety. Lastly, group four consists of *Pax-4* and *Pax-6*, both of which lack the octapeptide motif (Deutsch and Gruss, 1991; Walther *et al.*, 1991). *Pax-9* has only recently been discovered (Stapleton *et al.*, 1993), and has not yet been well enough described to be classified. In light of the similarities and differences among these genes, it is likely that the evolution of *Pax* genes occurred via duplication and subsequent divergence (Gruss and Walther, 1992).

Czerny *et al.* (1993) elucidated the DNA consensus recognition sequence for Pax proteins using the murine *Pax-5* binding protein (BSAP). He found it to consist of two nonpalindromic half sites, and suggested the following model for paired domain-DNA binding: a paired domain binds with 2 successive major grooves in a bipartite manner, that is the amino terminal binding site specifically recognizes the 3' half site, and the carboxy terminal, the 5' half site. The consensus sequence he described has optimal binding capabilities: all *Pax* genes bind to it. Specificity of a particular *Pax* gene occurs with alterations in either the 3' or the 5' half site.

The exact function of the *Pax* genes in mice is not known; however, there is evidence that they are involved in cell differentiation. Maulbecker and Gruss (1993) overexpressed *Pax* genes in cell lines known for their lack of ability to spontaneously transform. They found that the paired domain was able to promote oncogenesis both *in vitro*, and, when these cells were injected into nude mice, *in vivo*. The spatially and temporally specific expression patterns of the *Pax* genes in mouse embryogenesis are also indicative of their involvement in cell differentiation. Whereas *Hox* genes are expressed in the developing neural tube with longitudinally discrete patterns (Kessel and Gruss, 1990), *Pax* genes are expressed with transversely discrete patterns (Deutsch and Gruss, 1991). *Pax* genes which contain homeoboxes are expressed in mitotically active cells in the neural tube, and those without the paired type homeobox are expressed slightly later, in post-mitotic differentiating neural tube cells (Tremblay

and Gruss, 1994). The fact that *Pax* genes are expressed as a cell's fate is being determined implicates them in this process. The spatial pattern of expression was shown to be crucial for normal development of the neural tube by Goulding *et al.* (1993a). *Pax-3* is normally expressed in the dorsal region of the neural tube, and *Pax-6*, in the lateral regions of the neural tube. Removal of the notochord, or addition of supernumerary notocords causes aberrations in the expression patterns of *Pax-3* and *Pax-6* in undifferentiated cells of the neural tube. This leads to eventual ventralization of the neural tube, in the case of supernumerary notochords, or dorsalization, in the case of the removal of the neural tube. Goulding suggests that *Pax-3* and *Pax-6* expression patterns are established early in neural tube development, and are responsible for appropriate cellular differentiation. The ability of *Pax-3* to be rapidly induced by nerve growth factor (NGF) and brain derived growth factor (BDNF) in cerebellar cells (Kioussi and Gruss, 1994) is another indication that *Pax* genes act in the chain of molecular events which cause neuronal differentiation.

Although all of the *Pax* genes, with the exception of *Pax-1* and *Pax-9*, are expressed in the developing nervous system, their expression is not restricted to it. *Pax* genes have also been found in the prevertebrae, developing thymus gland, limbbud, kidney, excretory system, eye, and nose (Dressler *et al.*, 1990; Plachov *et al.*, 1990; Deutsch and Gruss, 1991; Jostes *et al.*, 1991; Love and Tuan, 1993; Rothenpieler and Dressler, 1993).

Pax-3

The *Pax-3* gene contains all three elements characteristic of the *Pax* genes, that is, the paired box, the octapeptide motif and the homeobox, and is encoded on chromosome 1 (Epstein *et al.*, 1991a). Expression of *Pax-3* is first detected in the tips of the rising neural folds on day 8 of development. The tips of the neural folds fuse, and form the roof plate of the neural tube and the neural crest cells, both of which continue to express *Pax-3*. By day 10 post coitum, *Pax-3* is localized to those cells of the alar and roof plates of the developing

neural tube, hindbrain, midbrain, craniofacial mesectoderm, somites (Goulding *et al.*, 1991), neural crest cells and their derivatives (Walter and Goodfellow, 1992), and limb buds (Bober *et al.*, 1994). *Pax-3* is not expressed in adult tissue (Goulding *et al.*, 1991).

The role of *Pax-3* in development is not known, although there is evidence that it is involved in determining cell fate. During the process of gastrulaltion, primitive ectoderm is induced by endodermal signals to form mesoderm. It is the mesoderm which then induces nearby primitive ectoderm to become neuroectoderm. Pruitt (1992) showed that one of the genes to be induced by mesoderm is *Pax-3*, and he suggests that this *Pax-3* induction by mesoderm may be the mechanism by which neuroectoderm is induced (Pruitt, 1992; Williams and Ordahl, 1994). Furthermore, *Pax-3* is expressed very early in cells which are involved in the generation of dorsoventral patterning in the neural tube (Goulding *et al.*,1993a; Tremblay and Gruss, 1994).

Splotch

Six *splotch* alleles have been described to date. The *splotch* (*Sp*) and *splotch delayed* (Sp^{d}) mutations both arose spontaneously in inbred strains. Four others, *splotch retarded* (Sp^{r}), Sp^{1H}, Sp^{2H} and Sp^{4H} were created by X irradiation (Goulding *et al.*, 1993b; Goulding and Paquette, 1994). The original *splotch* gene has a point mutation which yields a truncated *Pax-3* protein (Epstein *et al.*, 1993). Heterozygous *splotch* mice have a lack of pigmentation on their bellies and sometimes back, feet and tail, but are otherwise normal. The homozygotes, however, die at d 13 -15 p.c. (Goulding and Paquette, 1994). When the *splotch* mice are outcrossed onto a different genetic background, the homozygotes survive longer, dying at or near birth (Moase and Trasler, 1987). Before their deaths, homozygous *splotch* embryos have numerous defects, including spina bifida with or without exencephaly (Dempsey, 1981), reduced or absent spinal ganglia (Moase and Trasler, 1989), defective basal lamina and extracellular matrix in the area of the neuroepithilium (O'Shea and Liu, 1987a;

Yang, 1989; Trasler and Morriss-Kay, 1991), persistent truncus arteriosus (Franz, 1989), defective ensheathment of motor nerves (Franz, 1990), and limb abnormalities (Franz, 1993). All of the defects seen in the *splotch* mice result from defective neural tube closure and defective neural crest cell migration. The mutations in Sp^{1H}, Sp^{2H} and Sp^{4H} all result in similar phenotypes for the heterozygotes and homozygotes (Epstein *et al.*, 1991b; Goulding *et al.*, 1993). *Splotch delayed* animals have a mutation in the paired box of the *Pax-3* gene which results in reduced transcript levels in the homozygote (Vogan *et al.*, 1993). This mutation is less severe, and homozygotes die at birth, with neural tube defects and small dorsal root ganglia. The Sp^{r} mutation is a deletion of the entire *Pax-3* gene and of other neighbouring genes, including the genes encoding villin, desmin, inhibin A, and intestinal alkaline phosphatase (Epstein *et al.*, 1991b). This is a more severe mutation, and heterozygotes have retarded growth in addition to white spotting. Homozygotes are preimplantation lethal. The phenotypes of all these mice imply that *Pax-3* is crucial for normal neural tube development and neural crest cell migration.

N-CAM expression is associated with neural plate induction, neural tube closure, and migration and differentiation of neural crest cells. Since it is these processes which are aberrant in *splotch* mice, a look at N-CAM expression in developing *splotch* embryos was warranted. Moase and Trasler (1991) had found altered N-CAM species in *splotch* mutants. Neale and Trasler (1994) investigated further, and showed that in fact sialylation occurs earlier in development in *splotch* embryos than in wildtype controls. Sialylation of N-CAM is associated with a decrease in adhesion, and early sialylation of N-CAM in *splotch* embryos may mean decreased cell-cell adhesion. Present in the *splotch* embryos, but less so in wildtype, are gap junctional vesicles which are associated with a loss of gap junctions, and therefore a loss of cell-cell communication (Wilson and Finta, 1979; Yang and Trasler, 1989). An increase in the amounts of chondroitin and heparin sulfate proteoglycans in the *splotch* embryo has also been documented (Trasler and Morriss-Kay, 1991). These

three lines of evidence suggest altered cell-cell communication and adhesion during neurulation, a time during which cell recognition and interaction is crucial. Neale and Trasler suggest that these factors together can account for the abnormalities observed in *splotch* mutants.

Waardenburg Syndrome

PAX3, previously named *HUP2*, is the human homologue of *Pax-3*, and has been documented to be disrupted in several patients with Waardenburg syndrome (WS; Asher *et al.*, 1991). Three types of WS have been described. WSI is characterized by dystopia canthorum (lateral displacement of inner corners of the eyes), cochlear deafness, and pigmentation deficiencies (heterochromia irides, white forelock and eyelashes, premature graying, hypopigmented skin lesions). Waardenburg syndrome type II is similar to WSI, with the addition of megacolon, and without dystopia canthorum. Waardenburg syndrome type III is also similar to WSI, with the addition of limb abnormalities and megacolon (Waardenburg, 1951; Hageman and Delleman, 1977; Klein, 1983). A very small number of cases have been described of WS families associated with spina bifida or meningomyolocele (Hol *et al.*, 1995). Although the phenotype is varied, all defects seem to arise from defects in cranial neural crest cells. The phenotypic heterogeneity in WS not seen in *splotch* likely results from individual genetic background, genetic modification and environmental influences (Hageman and Delleman, 1977).

Waardenburg syndrome is not a life threatening disease, nor is it prevalent in society (incidence $1{:}4 \times 10^4$; Foy *et al.*, 1990). WS is interesting for study because understanding its etiology is a step towards understanding the development of the nervous system. The understanding of the nervous system is a goal mankind has sought to achieve for many centuries, and which will no doubt help in alleviating the suffering caused by diseases such as Alzheimer's, Parkinson's, and Huntington's, and may help in increasing our ability to recover from spinal cord injury.

RETINOIC ACID

Vitamin A

The term Vitamin A refers to all molecules able to reproduce the biologic effects of retinol. The two main sources of vitamin A are provitamin A carotenoids found in vegetables, and in retinyl esters found in animal tissue, both of which are hydrolyzed into retinol in the intestinal lumen. Retinol then reacts with the fatty acids to form esters, which are incorporated into chylomicrons (CM). The CM then enter general circulation where they are transformed into chylomicron remnants (CMR). Once the CMR reach the liver, they are converted back into retinol. Retinol is then either stored in the stellate cells, or bound to retinol binding protein (RBP) and sent back into general circulation, and transported to the cells where it is metabolized (Blomhoff *et al.*, 1990).

Mice reared on a vitamin A deficient diet suffer from keratinizing squamous epithelium, atrophy of glandular organs, eye lesions, testes degeneration, emaciation, and they eventually die (De Luca, 1991; Lufkin *et al.*, 1993). Retinol supports all biological functions of vitamin A, and animals suffering from hypovitaminosis A can be completely rescued by administration of retinol. All-*trans* retinoic acid (RA), a metabolite of retinol, can rescue all phenotypes with the exception of those lesions associated with vision and reproduction (Williams and Napoli, 1985; Lohnes *et al.*, 1992). Two forms of retinoic acid exist endogenously, all-*trans* retinoic acid and 9-*cis* retinoic acid. All references to retinoic acid in this thesis refer to the all-*trans* form unless otherwise stated.

Retinoic Acid in Development

It has been clear for decades that vitamin A must be present within a well defined concentration range for normal development. Too much or too little retinoic acid will result in a wide range of developmental abnormalities. Offspring

of vitamin A deficient female mice have abnormalities of the eye, genitourinary tract, kidney, heart and lung, and suffer from growth retardation and emaciation. These animals die though they show no obviously lethal lesions (Langman and Welsh, 1967; Lohnes *et al.,* 1992). Offspring of dams treated with an excess vitamin A also have abnormalities, including craniofacial, cardiovascular, thymic, central nervous system (CNS), urogenital and limb malformations, exencephaly, and spina bifida, depending on the dosage and the stage of development at which it is given. This condition, hypervitaminosis A, has been well documented both in rats (Kochhar, 1967; Morriss, 1972) and humans (Lammer *et al.,* 1985; Rosa *et al.,* 1986).

Ho (1994) showed that RA could disrupt the developmental patterns in the brain of *Xenopus*. Anterior structures were most sensitive to RA, and could be abolished with low levels of the teratogen. Higher levels were required to eliminate more posterior structures such as the hindbrain and midbrain. This has also been shown to be true for mice (Riuz i Albata and Jessel, 1991; Lee *et al.,* 1995) RA is therefore involved in antero-posterior pattern formation of the developing nervous system. Riuz i Albata and Jessel (1991) suggest that the ground state of neural tissue is anterior, and retinoic acid acts to induce neural cells to differentiate into hindbrain. Digit pattern formation can also be disrupted by the application of RA. When RA was applied to the wing bud in chicks, duplication of the digit pattern was observed (Tickle *et al.,* 1982). RA is produced in the limbbud (Thaller and Eichele, 1988), and induces the cells in the posterior margin of the limb bud to differentiate into the zone of polarizing activity (ZPA), which in turn induces digit pattern formation (Wanek *et al.,* 1991). The ability of RA to affect the differentiation of cells has been seen in other models as well. Retinoic acid has been shown to induce the differentiation of teratocarcinoma cells, keratinocytes, melanoma cells, human blastoma cells, and human promyelocytic leukemia cells (Wang and Gudas, 1985; William and Napoli, 1985; Williams *et al.,* 1987; LaRosa and Gudas, 1988; Castaigne *et al.,* 1990).

Mode of Action

The mechanism by which RA has its pleiotropic effect has yet to be fully understood. Three major components of the pathway have been described: the nuclear receptors (RARs and RXRs), and the retinoic acid response elements (RARE) and the cellular binding proteins (CRBP and CRABP).

Retinoic Acid Receptors (RARs)

Retinoic acid receptors (RARs) belong to a nuclear receptor multigene family which includes receptors for steroid hormones, thyroid hormones and vitamin D_3 (Evans, 1988; Green and Chambon, 1988; Beato, 1989). Binding of a hormone to a receptor in this family induces an allosteric change in the receptor which allows the receptor-hormone complex to bind DNA. DNA binding can then induce up or down regulation of many genes (Chiocca *et al.*, 1988; Hu and Gudas, 1990; Nicholson *et al.*, 1990; Rowe *et al.*, 1991).

Three RAR subtypes have been cloned: RARα, RARβ, and RARγ. Homologues for each have been found in amphibians (Ragsdale, 1989), chicken (Smith and Eichele, 1991), mouse (Giguère *et al.*, 1990a; Leroy *et al.*, 1991a), rat (Rees *et al.*, 1989; Haq *et al.*, 1991; Mattei *et al.*, 1991) and human (Petkovich, 1987; Giguère, 1987; Benbrook 1988; Brand, 1988, 1990; Zelent *et al.*, 1989; Mattei *et al.*, 1991). Each receptor belonging to this multigene family has two zinc fingers which confer DNA binding properties, a ligand binding region, and a hypervariable domain which is responsible for dimerization (Green and Chambon, 1987; Kastner *et al.*, 1990; De Luca, 1991). Interspecies conservation of any given member of the RAR subfamily is much higher than the conservation of all three receptors within a given species. This suggests that each isoform has its own function (Krust *et al.*, 1988; Zelent *et al.*, 1989). This hypothesis is supported by the distribution and expression patterns of the RARs. RARα is ubiquitous in the developing embryo, whereas RARβ and RARγ have spatially and temporally confined distribution (de Thé *et al.*, 1989; Dollé *et al.*,

1989, 1990; Rees *et al.*, 1989; Ruberte *et al.*, 1990, 1991, 1992; Rowe *et al.*, 1992; Schofield *et al.*, 1992). It is interesting to note that RARβ and RARγ have mutually exclusive distributions in the late stages of gestation. In the developing neural tube, for example, RARβ is confined to the closed regions, and RARγ to the open regions (de Luca, 1991).

Null mutant mice have been created to study the specific roles genes play in development. In the case of RARα, null mutants of the primary isoform, RARα-1, appear normal. When the two most abundant RARα isoforms, RARα-1 and -2 are knocked out, the mice die early postnatally (Lufkin *et al.*, 1993). In humans, acute promyelocytic leukemia (APL) has been linked to a translocation between chromosomes 15 and 17 (t(15:17)(q21-q11-22); de Thé *et al.*, 1990a). The gene for RARα has been mapped to the translocation site, so that its disruption is associated with this form of cancer. Researchers have found that RA treatment of APL patients causes differentiation of the blast cells, resulting in complete remission (Castaigne *et al.*, 1990). De Thé (1990) suggests the mutant RARα competes with normal receptor for the DNA binding sites and blocks expression of RA target genes involved in the differentiation of granulocytic cells. This raises a curious question: how does excess RA overcome this deficiency?

The existence of the three different subtypes of RARs, the fact that each subtype has several isoforms (Leroy, *et al.*, 1991a,b; Mendelson *et al.*, 1991; Smith and Eichele, 1991), and the distinct distribution of each all contribute to RA's ability to have a pleiotropic effect. Also contributing to this wide range of effects is the ability of the RARs to heterodimerize with each other, and with other hormone receptors (Glass *et al.*, 1989; Lohnes *et al.*, 1992).

Retinoic "X" Receptors (RXRs)

The RXRs belong to the same receptor multigene family as the RARs. There are also three subtypes to the RXRs: RXRα, RXRβ, and RXRγ. Again, all three subtypes are conserved across species (Mangelsdorf *et al.*, 1990). Beato

(1989) suggests that the existence of a *Drosophila* RXR homologue means the RXR subfamily is more ancestral than that of the RARs. When discovered, the ligand for this receptor family was unknown, hence the name Retinoid "X" Receptor. Since then, however, the ligand was shown to be 9-*cis* retinoic acid (Lohnes *et al.*, 1992). RXRs will bind all-*trans* RA, but only when it is present in much higher concentrations. Whether RXRs have a role distinct from the RARs is not yet known.

The RXR isoforms, like those of the RARs, have distinct distribution patterns (Mangelsdorf *et al.*, 1990, 1992). RXRs are able to dimerize with RARs, as well as with other receptors in the nuclear receptor multigene family (Mangelsdorf *et al.*, 1992). The retinoic acid nuclear receptor system is very complicated. There are many receptor subtypes, many isoforms of each, leading to a great number of heterodimers, and resulting in the pleiotropic effect RA has on a developing embryo.

Retinoic Acid Response Elements (RAREs) and Retinoid "X" Response Elements (RXREs)

The retinoic acid response element (RARE) is the motif present in the promoter region of an RA-responsive gene to which RAR dimers bind (Vivanco Ruiz *et al.*, 1991). Rossant *et al.* (1991) attached a RARE to a marker gene, and found that the RARE was able to direct spatially and temporally specific domains of transcription during mouse embryogenesis. Many genes are known to have a RARE, including osteocalcin, RARβ-2 (both mouse and human), mCP-H (mouse complement factor H), hADH-3 (human alcohol dehydrogenase), laminin B1, and rPEPCK (rat phosphoenolpyruvate carboxykinase; De Luca, 1991). The gene for rCRB II (rat cellular retinol binding protein II) has been shown to have a retinoid "X" response element (RXRE; De Luca, 1991). RXREs are activated by RXRs, but are inhibited by RARs (Mangelsdorf *et al.*, 1990). Many more genes likely have an RARE or RXRE in their promoter.

RAREs and RXREs consist of two groups of bases, called half sites. Specificity of a response element is conferred by the bases which make up the half sites, the directional arrangement of the half sites (palindrome, inverted palindrome, or direct repeat) and the spacing between the half sites (De Luca, 1991). The variability of these response elements allows for the high specificity of hormonal responses and cross talk between hormonal pathways.

The gene early retinoic acid-1 (ERA-1; LaRosa and Gudas, 1988) mRNA is elevated by a continuous presence of RA, is not dependent on protein synthesis, and is inhibited when mRNA production is blocked. Although Era-1 has not yet been found to contain a RARE, it is likely that it, or some such gene is activated in response to the presence of RA. Its gene product is then responsible for activating genes such as fibronectin, laminin, and collagen III and IV which are activated in the presence of RA, but which don't necessarily contain RAREs (Wang *et al.*, 1985; Chiocca *et al.*, 1988; LaRosa and Gudas, 1988; Nicholson *et al.*, 1990).

Cellular Retinoic Acid Binding Protein (CRABP)

The cellular retinoic binding proteins (CRtBP) and fatty acid binding proteins (FABP) make up a family of low molecular weight cytoplasmic binding proteins. The FABPs include heart fatty acid binding protein (H-FABP), intestinal fatty acid binding protein (I-FABP), adipocite binding proteins (AP), gastrotropin, and the P2 protein of peripheral nerve myelin (Balling, 1991; Bass, 1993). The CRtBPs include cellular retinol binding protein (CRBP), and cellular retinoic acid binding protein (CRABP). The retinoid and fatty acid binding proteins solubilize their otherwise hydrophobic ligands, thus stabilizing them in the cytosol (Rizo *et al.*, 1994).

Two forms of cellular retinoic acid binding protein have been detected, CRABP I (Giguère *et al.*, 1987) and CRABP II (Giguère *et al.*, 1990b; Astrom *et al.*, 1991). Studies have shown that their distributions in the developing embryo differ (Lyn and Giguère, 1994; Maden, 1994). Where CRABP I is expressed in

those tissues specifically associated with RA sensitivity (Dollé *et al.*, 1990; Ruberte *et al.*, 1991), CRABP II is expressed uniformly at low levels throughout the embryo (Ruberte *et al.*, 1992; Lyn and Giguère, 1994; Maden, 1994). These differing distribution patterns suggest the two CRABPs have distinct functions in the embryo. The focus of this study is on CRABP I, and the term CRABP refers to CRABP I unless otherwise stated.

Once inside the cell, retinol is bound by CRBP which catalyzes its metabolism into retinyl esters and retinoic acid (Bass, 1993). CRABP binds, solubilizes and stabilizes RA; it's function beyond that is unknown. Two quite different theories exist: the first is that CRABP transports RA to the nucleus, the second theory is that CRABP limits the amount of free RA by assisting in its metabolism. Support for both of these is plentiful.

The theory of cellular retinoic acid binding protein acting as a transport mechanism is supported by several lines of evidence. In 1986, Takase found that H_3[RA] bound to CRABP could specifically bind nuclei, and that this binding was saturable, and could be competed out with cold RA. Although his assay was done *in vitro*, the results strongly suggest a role for CRABP in transferring RA to the nucleus. He also found that CRABP had specific DNA binding properties. Since CRABP is not found in the nucleus, it should not bind chromatin specifically. There may be some evolutionary significance to this finding; however, it is also possible that these findings are incorrect, and that leads us to question his first set of results: does CRABP-RA specifically bind the nucleus? Since retinoic acid has been found to be a useful drug, many analogues have been isolated and their properties established. Willhite (1992) found in his study of 26 such analogues, that those which do not cause teratogenic activity in embryos also fail to bind CRABP. Stoner and Gudas (1989) found that several mutant teratocarcinoma stem cell lines which lack detectable H_3[RA] binding to CRABP had lost their ability to differentiate in response to RA. Also, Perez-Castro (1993) found that expression of CRABP in the eye and pancreas, where it is not normally expressed, was sufficient to

cause tumors. Lastly, Napoli (1991) showed that the presence of CRABP slows but does not arrest the metabolism of RA. The above all suggest that CRABP has a role in mediating the biological activity of RA, possibly by transporting it to the nucleus.

Equally as much evidence supports the opposing theory, that CRABP serves to metabolize RA, thereby protecting the nucleus from its effects. Bass (1993) found that there exist RA analogues which don't bind CRABP but can still induce differentiation, and some cells which lack CRABP but which can still differentiate in response to RA. Boylan and Gudas (1991) overexpressed CRABP vectors in teratocarcinoma cell lines, and found that elevated levels of CRABP reduced the cells response to RA. Studies have also shown that the RA-CRABP complex is a substrate for RA metabolizing enzymes. The complex has a 27 fold higher affinity for catabolizing enzymes than does free RA, and excess CRABP increases the rate of RA metabolism (Fiorella and Napoli, 1991). All of this suggests that CRABP is involved in the metabolism of RA.

CRABP has been found to be associated with tissues which are susceptible to RA teratogenesis. One way of understanding the biological effects of RA in development is to study the expression and distribution patterns of its binding proteins and receptors. Many studies have examined the distribution of CRABP during embryonic development. CRABP staining first appears in the neuroepithilium at the presumptive hindbrain level in day 8.5 (4 - 6 somites) mouse embryo (Lyn and Giguère, 1994; Maden *et al.*, 1992). By day 9.5, it becomes apparent that staining in the hindbrain is restricted to rhombomeres 4, 5 and 6, less intensely rhombomere 2, and the neural crest cells which are migrating out from these regions (Maden *et al.*, 1988, 1989a, 1990, 1991, 1992; Ruberte *et al.*, 1991) . As development continues, CRABP staining from the hindbrain gradually progresses caudally along the spinal cord (Momoi *et al.*, 1990a,b). CRABP expression also progresses rostrally into the midbrain roof, and appears in craniofacial mesenchyme, developing limb buds, and in those tissues which receive the migrating neural crest cells, including

heart bulge, branchial arches, and dorsal root ganglia (Kwarta *et al.,* 1985; Maden, 1986, 1988, 1989a,b, 1990, 1991, 1992; Eriksson *et al.,* 1987; Dollé *et al.,* 1989; Perez-Castro *et al.,* 1989; Denker *et al.,* 1990, 1991; Ruberte *et al.,* 1991, 1992, 1993; Gustafson *et al.,* 1993). The association of CRABP with these RA tissues can be interpreted in two ways. One, CRABP acts as a shuttle, and transports RA, including exogenous RA, to the nucleus, hence mediating RA's teratogenic effect. The second possibility is that CRABP sequesters excess free endogenous RA, but cannot protect the nuclei from an exogenous dosage.

METHODS

PROBE PREPARATION

Subclone

This protocol was derived with the help of Franz Omann from methods described in Ausubel *et al.* (1989) and in Sambrook *et al.* (1989).

Preparation of Vector and Insert

The 756 base pair CRABP cDNA was removed from the pGEM vector by restriction enzyme digest with EcoRI (pGEM vector kindly supplied by Dr. Vincent Giguère, formerly at Hospital for Sick Children, Toronto, and now in Department of Basic Cancer Research, McGill). Insert was isolated by paper purification. 10 µg of the digest were run on a 1% agarose gel, which was then stained with ethidium bromide. Slices were cut in the gel just above the desired band (of 756 base pairs), and small strips of S&S paper (Schleider & Schuell paper, NA 45-#23400) were inserted into the slits. The gel was then run backward for 45 minutes, until the bands could be seen on the paper under an ultraviolet lamp. The bands were removed from the paper by rinsing in a low salt solution (50 mM Tris.Cl pH8.0, 0.15 M NaCl, 10 mM EDTA pH 8.0) to remove agarose, then by washing in high salt solution (50 mM Tris.Cl pH8.0, 1 M NaCl, 10 mM EDTA pH 8.0) to extract the DNA. The isolated bands were then run on another 1% agarose gel to verify that the desired DNA bands had been isolated. pBluescript vector was incubated with the restriction enzymes EcoRI and shrimp alkaline phosphatase to linearize the plasmid, and dephosphorylate its ends, respectively. To ensure that the restriction enzyme digest had gone to completion, the reaction mixture was run on a second 1% agarose gel.

Ligation and Transformation

The CRABP was inserted into the Bluescript vector using T4 DNA ligase. Two reactions were set up, with insert to vector ratios of approximately 1:1 and 1:5. The reaction was left for 4 hours, then added to freshly prepared (by calcium chloride method) competent *E.coli* (DH5) cells. Cells were heat-shocked at 42 °C for 90 seconds, then incubated at 37 °C for 45 minutes. Cells were then plated overnight on ampicillin-X-galactosidase plates. Ten white colonies were selected the following morning and were assayed for plasmid DNA.

Plasmid DNA Miniprep

500 µl of an overnight culture were centrifuged, and the cells recovered and digested with lysosyme for 1 hour at 60 °C. Cellular debris was removed by centrifugation, and the plasmid DNA in the supernatant was recovered by alcohol precipitation overnight at -20 °C. Each of the plasmid preps was digested with EcoRI, to ensure the presence of the insert, and with ApaLI and BglI to confirm the orientation of the insert.

Large Scale Plasmid Preparation

500 ml of broth + 50 µl/ml ampicillin were inoculated with 500 µl of overnight culture of the desired bacteria, and were incubated at 37 °C at 250 rpm overnight. The cells were collected by centrifugation, the supernatant discarded, and the pellet resuspended in 2 ml of 50mM glucose/25mM Tris-pH8.0/10mM EDTA. Freshly prepared lysosyme (25 mg/ml; 0.5 ml) were added, and left at room temperature for 10 minutes. Freshly prepared 0.2 N NaOH - 1% SDS (0.5 ml) were added, mixed by inversion, and left on ice for 10 minutes. Potassium acetate solution (0.75 M CH2COOK, 5% v/v Formic acid; 3.75 ml) was added, and again left on ice for 10 minutes. The solution was pelleted, and the supernatant was filtered through sterile gauze. Isopropanol (0.6 volumes) was added and left at room temperature for 5 minutes. The plasmid DNA was then precipitated at -20 °C for at least an hour, then centrifuged. The pellet was

air dried and washed with 70% EtOH. The plasmid DNA was recovered by centrifugation, and resuspended in TE buffer (10mM Tris, 1mM EDTA).

The plasmid was purified using the cesium chloride gradient method. Plasmid DNA was mixed with 6.35 g of CsCl for a final volume of 15 ml in TE. The plasmid mixture was transferred to 5/8" x 3" Beckman quick seal polyalomer centrifuge tube. Ethidium bromide was then added, and mineral oil was layered on top. The tubes were balanced, sealed, and centrifuged at 50 000 rpm, at 4 °C for 24 hours. Two DNA bands were then visible in the mixture under UV light, and the lower plasmid band was removed using a syringe. The plasmid was cleaned using phenol-chloroform extraction, ethanol precipitation method, and resuspended in TE.

Labeling

CRABP-Bluescript vector was linearized with BamHI, and incubated with the T3 polymerase and digoxygenin labeling NTP mixture to make the antisense CRABP RNA probe. A sense CRABP RNA probe was made by digesting the same plasmid with ApaI, and incubating it with the T7 polymerase and digoxygenin labeling NTP mixture. To verify that probe was being produced, the reaction mixtures were run on a 1% agarose gel and the purified probes were blotted onto Schleider and Schuell nylon transfer membrane paper (S&S Nytran, pore size 0.45 μm), crosslinked using a UV statalinker and assayed using Boehringer Mannheim's Colourimetric assay (Boehringer Mannheim, 1993a). To assay the binding capabilities of the probes, RNA isolated from a day 11 embryo (using the acid guanidinium-phenol-chloroform method of RNA isolation described below) was blotted onto membrane paper, and crosslinked. Each time probe was made, it was tested by hybridization, according to Boehringer Mannheim's Northern hybridization and Colourimetric detection protocols (Boehringer Mannheim, 1993a,b).

PREPARATION OF EMBRYOS

Day 9 embryos were dissected from maternal tissue, and the somite stage and neural tube closure stage of each embryo was recorded. Each embryo was placed in a separate tube bearing a unique identification number. The amniotic sac was dissected from each embryo, also given a unique identification number, and then frozen at -70 °C. Embryos were genotyped by running PCR analyses on the amniotic membranes as described below. Embryos which were to be used in the Northern blot analysis were placed directly in 0.5 ml Trizol, and were frozen at -70 °C. Closed neural tubes of those embryos to be used for the *in situ* hybridization were opened by the insertion of a fine tungsten wire into the hindbrain region. The embryos themselves were originally left overnight in 4% paraformaldehyde then transferred to 70% ethanol for storage. With this method, however, embryos degraded quickly. The embryos remained in much better condition when the pretreatment described below was used.

WHOLE MOUNT *IN SITU* HYBRIDIZATION

The following is derived from the *in situ* hybridization protocol received from Gillian M. Morriss-Kay's lab (derived from Wilkinson *et al.*, 1992) and the protocol described by Boehringer Mannheim (1993b).

Pretreatment

Fix

The embryos, once dissected, were placed on ice until the entire dissection was complete. Each embryo was rinsed with 1 ml of freshly prepared cold 4% paraformaldehyde. The paraformaldehyde was replaced, and the embryos were left shaking for 4 hours on ice. The embryos were washed 3 x 10

minutes each in 1 ml of PBS containing 0.1% Tween 20 (PBT), then incubated for 1 hour at 4 °C shaking gently.

Storage

The embryos were then rinsed through a graded series of alcohol: 50% PBT: 50% methanol, 25% PBT: 75% methanol, then 2 x 100% methanol. The embryos were stored at this stage in 100% methanol at -20 °C for up to 1 month.

Bleaching

Embryos were bleached in 0.75 ml of a 5:1 solution of methanol and 30% hydrogen peroxide for 2 hours at room temperature. The embryos were rinsed 3 x in 0.5 ml methanol, and put through the reverse graded series of alcohol: 25% PBT: 75% methanol, 50% PBT: 50% methanol, then 2 x PBT (0.5 ml each solution). Embryos were hybridized immediately.

Hybridization

Embryos were shaken gently for all steps. Embryos were prehybridized for 1 hour at 55 °C in hybridization solution (50% deionized formamide, 5 x SSC pH 4.5, 10 µg/ml *E. coli* tRNA, 1% SDS, 50 µg/ml heparin). Probe (2 µl/ml) was added to the prehybridization solution, and the embryos were left to hybridize overnight at 55 °C.

Post Washes

Embryos were washed for 30 minutes at 55 °C with solution 1 (50% deionized formamide, 5X SSC, 1% SDS), for 10 minutes at 55 °C with 50% solution 1: 50% solution 2 (0.5 M NaCl, 0.01 M Tris.HCl pH 7.5, 0.1% Tween-20), for 3 x 5 minutes at room temperature with solution 2. The embryos were incubated for 30 minutes at 37 °C in solution 2 plus RNase A (final concentration 1 mg/ml), then washed for 5 minutes at room temperature with solution 2, for 5

minutes at room temperature with solution 3 (50% deionized formamide, 2X SSC). Embryos were incubated for 2 x 30 minutes at 55 °C in solution 3. The embryos were then treated for 3 x 5 minutes at room temperature with TBST (1.4 M NaCl, 27 mM KCl, 250 mM Tris.Hcl pH 7.5, 1% Tween-20), blocked for 1 hour at room temperature using Boeringer Mannheim's "buffer 2" (1% Blocking Reagent for nucleic acid hybridization dissolved in 100 mM maleic acid, 150 mM NaCl pH 7.5), and left over night at 4 °C in anti-DIG antibody (from Boehringer Mannheim: 1µl/ml in TBST). Unbound antibody was removed by washing with TBST for 5 x 1 hour at room temperature.

Colourimetric Assay

The embryos were washed for 3 x 10 minutes at room temperature with NTMT (100 mM NaCl, 10 mM Tris.HCl-pH 9.5, 50 mM MgCl, 0.1% Tween-20, 2 mM levamisole). The colour reaction was done in almost complete darkness, using 4.5 µl NBT, 3.5 µl X-Phosphate per 1 ml of NTMT at room temperature for 20 minutes. Excess colour was washed from the embryos by rinsing several times in PBS (at least 2 x 10 minutes).

Preparation of Slides

Prestained embryos were left in PBS until wax embedding. Embryos were incubated for 1 hour in 70%, 90%, 95% and 2 x 100% EtOH (in DepC water) on ice, in 2 x xylene at room temperature, and then in xylene:paraffin wax (1:1) and 2 x in paraffin at 60 °C (Pelton). The embryos in paraffin were poured into metal molds, and allowed to harden at room temperature overnight. The wax embedded embryos were mounted on blocks of wood, and sliced transversely into 5 µm thick sections. The sections were mounted onto glass slides by leaving them on a hotplate until dry. The wax was removed by dipping the slides in xylene. The embryos were counterstained using 0.05% eosin, and coverslipped.

POLYMERASE CHAIN REACTION (PCR)

DNA was extracted from the amniotic sacs by protein kinase K digestion, two phenol-chloroform extractions and ethanol precipitation. Doug Epstein designed oligonucleotides from unique sequences for *splotch* and wild type DNA for the PCR analysis. The PCR reaction was carried out using a DNA Thermal Cycler for 40 amplification cycles under the following conditions: 1 minute denaturation at 94 °C, 1 minute annealing at 65 °C, and 1.5 minutes polymerization at 72 °C. PCR products were then run on a 1% agarose gel for analysis.

NORTHERN HYBRIDIZATION

Total RNA Preparation

Preparation

Total RNA preparations were originally done using the acid guanidium-phenol-chloroform method for RNA isolation (Ausubel *et al.*, 1989); however, we switched to the Trizol method (Chomczynski, 1993; Simms et al., 1993; BRL, 1994) as we found it to be more efficient. Embryos were placed in 1 ml of the Trizol solution immediately after dissection. Glycogen (5 μg) was added to each embryo, and the samples were vortexed until each embryo was completely dissolved. The samples were stored at -70 °C for a maximum of 1 month.

Isolation

RNA was isolated first by incubating the samples for 5 minutes at room temperature. Chloroform (0.2 ml) was added, and the tubes were shaken vigorously for 15 seconds, then left to rest 2-3 minutes. Samples were then

centrifuged at 12 000 x g for 15 minutes. The aqueous phase which contained the RNA was transferred to a fresh tube.

Precipitation

After centrifugation, about 0.5 ml of aqueous phase was recovered without disturbing the interphase. Isopropyl alcohol (0.5 ml) was added, and the sample inverted several times. RNA was then precipitated by storing the samples at -20 °C for 30 minutes. Samples were then spun at 12 000 x g for 10 minutes. RNA is in the gel-like pellet. The supernatant was carefully removed with a pipettor. The pellet was respun, and remaining supernatant removed.

Wash

Ethanol (1 ml of 75 %) was added to each pellet, and respun at 7 500 x g for 5 minutes. The pellets were stored for up to 1 year at -20 °C. Again the supernatant was carefully removed, the pellets respun for approximately 10 seconds, and the remaining liquid removed. Pellets were then air dried for 10 minutes at room temperature.

Hybridization

This method for Northern hybridization is derived from both the suggested protocol for using with DIG (Boehringer Mannheim, 1993a), and the Northern hybridization protocol described in Ausubel (1989). The RNA pellet was dissolved in the following RNA loading mix: 5.0 µl DepC H_2O, 2.0 µl PO_4 buffer (10 mM $NaH_2PO_4 \cdot 2H_2O$ pH 7.0), 3.5 µl formaldehyde and 10.0 µl deionized formamide. The mix was then incubated for 15 minutes at 55 °C, or longer, until the pellet was no longer visible. Occasionally throughout this incubation, the mixture was vortexed and then briefly centrifuged. Loading buffer (3.0 µl of 50% glycerol, 1 mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol) and 1.0 µl ethidium bromide (1 mg/ml) were added to each sample before running the gel.

The RNA samples were run on a gel (1.2% agarose, 2.2 M formaldehyde) either at 100 - 150 volts for 3 - 4 hours, or at 30 - 40 volts overnight. The gel was then photographed under ultraviolet light to record the distance traveled by both 18S and 28S rRNA bands.

The RNA was transferred from the gel to a nitrocellulose membrane (S&S Nytran, pore size .45 μm) using the capillary transfer method. The transfer was deemed successful by the visualization of the ethidium bromide stained RNA on the membrane under ultraviolet light.

The membrane was probed using Boehringer Mannheim's Northern hybridization protocol and Colourimetric assay (Boehringer Mannheim,1993a).

RESULTS

The CRABP I cDNA was originally obtained in a pGEM-3 plasmid, and was subcloned into the pBluescript vector. The new plasmid was used to make both the antisense probe using the T3 promoter, and the sense probe using the T7 promoter. The binding efficiency of the CRABP sense and antisense probes were assayed by blotting and crosslinking total RNA from day 11 mouse embryos onto a membrane and hybridizing with each probe. The antisense probe showed dark staining, whereas the sense probe showed only background levels of staining. Each time probe was made, its concentration was determined by Boehringer Mannheim's suggested method of dot blotting then comparing to controls of known concentrations.

I attempted two Northern hybridizations, however no bands were seen in either attempt. The rest of the Northern hybridizations were done by Sean Sefsik, a summer student, under my supervision. He completed over a dozen Northerns, and was finally able to get only very faint bands, from which nothing could be determined.

A total of 84 pregnant mice were dissected, yielding 613 embryos of approximately d 9 - 9.5 p.c (Table A). Of the 84 dissections, the embryos from 17 were saved for RNA isolation and Northern hybridization. The rest of the embryos were fixed, and stored in alcohol for the *in situ* hybridizations. A total of 234 embryos were used in 22 *in situ* hybridizations: 198 of the embryos were stained with the antisense probe, and 36 were stained with the sense probe for control (Table B). The hybridizations included 58 +/+ and 48 *Sp*/*Sp* embryos (the rest were *Sp*/+).

Embryos were put through the procedure in separate vials, which were marked with only an identifying number. All the embryos were processed at the same time under as identical conditions as possible.

TABLE A: Summary of all dissections

Litter number	Dissection date	Type of cross	Number of embryos	PCR	Embryos for RNA	*in situ*
1*	93/08/25	*Sp*/+ X *Sp*/+	6	√		
2*	93/09/13	*Sp*/+ X *Sp*/+	3	√		√
3*	93/09/20	*Sp*/+ X *Sp*/+	1	√		
4*	93/09/22	*Sp*/+ X *Sp*/+	9	√		
5*	93/10/07	*Sp*/+ X *Sp*/+	3	√		
6*	93/10/13	*Sp*/+ X *Sp*/+	8	√	√	
7*	93/10/18	*Sp*/+ X *Sp*/+	2	√		
8*	93/10/18	*Sp*/+ X *Sp*/+	7	√		
9*	93/11/02	*Sp*/+ X *Sp*/+	7	√		
10*	93/11/17	+/+ X +/+	10	√		
11*	93/11/26	*Sp*/+ X *Sp*/+	6	√		√
12*	93/11/27	*Sp*/+ X *Sp*/+	10	√		√
13	94/01/10	*Sp*/+ X *Sp*/+	7	√		√
14	94/02/09	*Sp*/+ X *Sp*/+	5	√		√
15	94/02/09	*Sp*/+ X *Sp*/+	6	√		√
16	94/02/09	*Sp*/+ X *Sp*/+	8	√		√
17	94/02/24	*Sp*/+ X *Sp*/+	8	√		√
18	94/02/24	*Sp*/+ X *Sp*/+	5	√		√
19	94/02/24	*Sp*/+ X *Sp*/+	10	√		√
20	94/02/24	*Sp*/+ X *Sp*/+	6	√		√
21	94/02/25	*Sp*/+ X *Sp*/+	4	√		√
22	94/02/25	*Sp*/+ X *Sp*/+	6	√		√
23	94/02/25	+/+ X +/+	7	√		√
24	94/03/02	+/+ X +/+	11		√	

* Litters collected for Honours project.

TABLE A (continued): Summary of all dissections.

Litter number	Dissection date	Type of cross	Number of embryos	PCR	Embryos for RNA	*in situ*
25	94/05/06	*Sp*/+ X *Sp*/+	9	√		√
26	94/05/12	*Sp*/+ X *Sp*/+	11	√		√
27	94/06/08	*Sp*/+ X *Sp*/+	7	√		√
28	94/07/20	*Sp*/+ X *Sp*/+	12	√		
29	94/07/21	*Sp*/+ X *Sp*/+	9			
30	94/07/27	*Sp*/+ X *Sp*/+	8	√		√
31	93/08/03	*Sp*/+ X *Sp*/+	7			√
32	94/08/11	*Sp*/+ X *Sp*/+	11			
33	94/08/12	*Sp*/+ X *Sp*/+	8			
34	94/08/12	+/+ X +/+	10		√	
35	94/09/30	*Sp*/+ X *Sp*/+	10	√		
36	94/09/30	*Sp*/+ X *Sp*/+	8	√		
37	94/09/30	*Sp*/+ X *Sp*/+	7	√		
38	94/09/30	*Sp*/+ X *Sp*/+	7	√		
39	94/10/06	*Sp*/+ X *Sp*/+	10	√		
40	94/10/06	*Sp*/+ X *Sp*/+	6	√		
41	94/10/06	*Sp*/+ X *Sp*/+	11	√		
42	94/10/06	*Sp*/+ X *Sp*/+	8	√		
43	94/10/07	*Sp*/+ X *Sp*/+	9	√		√
44	94/11/11	*Sp*/+ X *Sp*/+	8	√		
45	94/12/?	*Sp*/+ X *Sp*/+	7			
46	94/12/04	*Sp*/+ X *Sp*/+	8	√		√
47	94/12/08	+/+ X +/+	8		√	
48	94/12/08	+/+ X +/+	8		√	

TABLE A (continued): Summary of all dissections.

Litter number	Dissection date	Type of cross	Number of embryos	PCR	Embryos for RNA	*in situ*
49	94/12/08	+/+ X +/+	9		√	
50	94/12/10	+/+ X +/+	8		√	
51	94/12/11	+/+ X +/+	8		√	
52	94/12/11	+/+ X +/+	6		√	
53	94/12/11	+/+ X +/+	6		√	
54	94/12/11	*Sp*/+ X *Sp*/+	8		√	
55	95/01/23	*Sp*/+ X *Sp*/+	8	√		√
56	95/01/26	*Sp*/+ X *Sp*/+	5			
H	95/04/07	*Sp*/+ X *Sp*/+	4			
61	95/04/05	*Sp*/+ X *Sp*/+	8	√		
62	95/04/13	*Sp*/+ X *Sp*/+	8	√		√
63	95/04/20	*Sp*/+ X *Sp*/+	7			
64	95/04/20	*Sp*/+ X *Sp*/+	10	√		√
65	95/04/20	*Sp*/+ X *Sp*/+	4			
66	95/04/21	*Sp*/+ X *Sp*/+	8			
67	95/04/21	*Sp*/+ X *Sp*/+	5			
68	95/06/05	*Sp*/+ X *Sp*/+	6	√		
69	95/06/06	*Sp*/+ X *Sp*/+	10			
70	95/06/06	*Sp*/+ X *Sp*/+	7			
71	95/06/14	*Sp*/+ X *Sp*/+	3			
72	95/06/14	*Sp*/+ X *Sp*/+	9	√		
73	95/06/14	*Sp*/+ X *Sp*/+	4			
74	95/06/15	*Sp*/+ X *Sp*/+	7	√		
75	95/07/03	*Sp*/+ X *Sp*/+	7	√		

TABLE A (continued): Summary of all dissections.

Litter number	Dissection date	Type of cross	Number of embryos	PCR	Embryos for RNA	*in situ*
76	95/07/03	*Sp*/+ X *Sp*/+	5	√		
77	95/07/03	*Sp*/+ X *Sp*/+	7	√		
78	95/07/04	*Sp*/+ X *Sp*/+	8	√		
79	95/07/05	*Sp*/+ X *Sp*/+	7	√		
80	95/07/05	*Sp*/+ X *Sp*/+	9	√	√	
81	95/07/05	*Sp*/+ X *Sp*/+	6	√	√	
82	95/07/05	*Sp*/+ X *Sp*/+	9	√	√	
83	95/07/05	*Sp*/+ X *Sp*/+	5	√	√	
84	95/07/05	*Sp*/+ X *Sp*/+	7	√	√	
85	95/07/05	*Sp*/+ X *Sp*/+	10	√	√	
86	95/07/06	*Sp*/+ X *Sp*/+	6	√		
87	95/07/06	*Sp*/+ X *Sp*/+	7	√		

Total Number Disections:84 Total Number of Embryos Collected: 613

TABLE B: Summary of *in situ* Hybridizations.

Date of *in situ*	Litters numbers used	Total number of embryos in *in situ* *	Number of +/+ : *Sp/Sp* embryos in *in situ***	Number of embryos success-fully stained	+/+ : *Sp/Sp* where *Sp/Sp* have **lighter** staining than +/+****	+/+ : Sp/Sp where *Sp/Sp* have **darker** staining than +/+****
93/11	1	6 (1)	2 : 1	5	0 : 0	2 : 1
93/11	2	3 (1)	0 : 3	3	---	---
94/02/22	16	8 (1)	2 : 1	0	0 : 0	0 : 0
94/03/10	20	6 (1)	?	0	0 : 0	0 : 0
94/03/21	22	6 (1)	1 : 2	0	0 : 0	0 : 0
94/04/12	17	8 (1)	4 : 0	0	---	0 : 0
94/04/26	19	8 (1)	3 : 1	0	0 : 0	0 : 0
94/05/02	18, 21	9 (1)	2 : 1	0	0 : 0	0 : 0
94//05/24	25	9 (4)	3 : 0	5	---	0 : 0
94/05/29	26	11 (5)	2 : 6	3	0 : 0	1 : 1
94/06/08	13, 14, 15	13 (2)	6 : 2	3	0 : 0	1 : 2
94/08/04	27, 30, 31	10 (1)	?	2	---	---
94/11/07	41, 42, 43	7 (1)	3 : 3	0	0 : 0	0 : 0
94/11/30	35, 36, 37, 41, 44	9 (2)	4 : 4	4	0 : 0	2 : 1

* Number of control embryos in brackets.

** Numbers do not necessarily total number of embryos in *in situ* since heterozygotes not included.

*** Numbers do not necessarily total number of embryos succesfully stained since controls not included.

TABLE B (Continued): Summary of *in situ* Hybridizations.

Date of *in situ*	Litters numbers used	Total number of embryos in *in situ* *	Number of +/+ : *Sp/Sp* embryos in *in situ***	Number of embryos success-fully stained	+/+ : *Sp/Sp* where *Sp/Sp* have **lighter** staining than +/+****	+/+ : Sp/Sp where *Sp/Sp* have **darker** staining than +/+****
95/12/04	46	8 (2)	?	0	---	---
95/02/01	55	7 (1)	?	0	---	---
95/05/09	64	10 (2)	3 : 2	9	0 : 0	3 : 2
95/05/25	62	8 (1)	4 : 1	7	0 : 0	1 : 1
95/06/22	70, 71, 72, 74	15 (2)	5 : 5	13	0 : 0	5 : 5
95/06/27	70, 71, 73, 74	8 (2)	1 : 3	6	0 : 0	1 : 1
95/07/13	86, 77, 79, 82, 84, 86, 87	14 (2)	6 : 6	12	0 : 0	4 : 5
95/07/14	69, 70, 73, 75, 78, 79, 81, 82, 87	15 (1)	7 : 7	15	0 : 0	3 : 7
Totals	---	198 (36)	58 : 48	87	0 : 0	23 : 26

* Number of control embryos in brackets.

** Numbers do not necessarily total number of embryos in *in situ* since heterozygotes not included.

*** Numbers do not necessarily total number of embryos successfully stained since controls not included.

Of the 234 embryos in the *in situ* experiments, only 87 embryos stained successfully. The embryos which did not stain successfully suffered either from being handled too roughly, from desiccation, or from fungal contamination. These embryos appeared mutilated, flat, or "fluffy", respectively. By day three of the *in situ* hybridization procedure, the outermost tissue of the embryos contaminated by fungus had lost its integrity and was covered with lint. The mutilated and contaminated embryos remained white, or had very faint pink staining. The flat embryos varied in their staining intensity, depending on when during the procedure they had become desiccated. Although several flat embryos had positive staining, no detail was discernible as the organs and tissues were compacted.

The *in situ* hybridizations yielded 87 well stained intact embryos, including 23 +/+ and 26 *Sp/Sp* embryos stained with the antisense probe (the rest include heterozygotes stained with antisense probe, and sense controls). In every case, the homozygous *splotch* embryos had a normal CRABP staining pattern which was darker than the staining pattern of the wildtype controls of the same somite stage. The staining pattern of the heterozygotes was indistinguishable from that of the wildtype embryos. Although the difference in intensity of staining between the +/+ and the *Sp/Sp* embryos was variable, it was always clear that the Sp/Sp embryos were darker. It appeared as though the difference in staining between the genotypes was greater for younger embryos. This conclusion cannot be drawn for sure, though, since incubation times and temperatures and quantities of exact solutions could not be exactly replicated between *in situ* procedures.

Figures 1 through 4 all compare the staining in the wildtype and homozygous *splotch* embryos. Figure 1 shows two embryos of the 8-9 somite stage. In both the *Sp/Sp* and +/+ embryo, staining is visible in the neural folds in the head region, and faint staining in the posterior neuropore. Staining in the *splotch* embryo is much darker. Figure 2 compares two embryos of 18 somites. Again, the distribution of staining in both embryos is normal, and staining in the *splotch* embryo is darker than the wildtype. Figure 3 shows two embryos of the

21 somite stage. Both embryos have positive staining in the frononasal mesenchyme, the hindbrain, the branchial arches, the otic vesicle, the somites, the limb buds, and the posterior neuropore. Again, the *splotch* embryo has darker staining. Figure 4 shows four embryos of the 23-24 somite stage. The staining pattern is similar to the embryos of the 21 somite stage shown in figure 3, and again the homozygote is darker than its wildtype counterpart. Also shown in this figure are the 2 control embryos stained with the sense probe. They show only background staining.

Embryos outside the somite stages shown in figures 1 through 4 were also used in the *in situ* hybridization experiments. Embryos below the 8-9 somite stage were very difficult to treat. They almost always got lost, mangled or contaminated. Data was therefore not collected for these low somite stages. Higher somite stages were also used. *Splotch* embryos of 30-33 somites continued to show the staining patterns seen in the homozygote embryo shown in figure 4. Wildtypes beyond the 25th somite stage were not examined.

FIGURE 1: Dorsal view of day 9 embryos (8-9 somites) stained with antisense probe, showing greater CRABP I expression in the *Sp/Sp* (B) than in +/+ (A). Positive staining is in the neural folds (nf) and posterior neuropore (pnp).

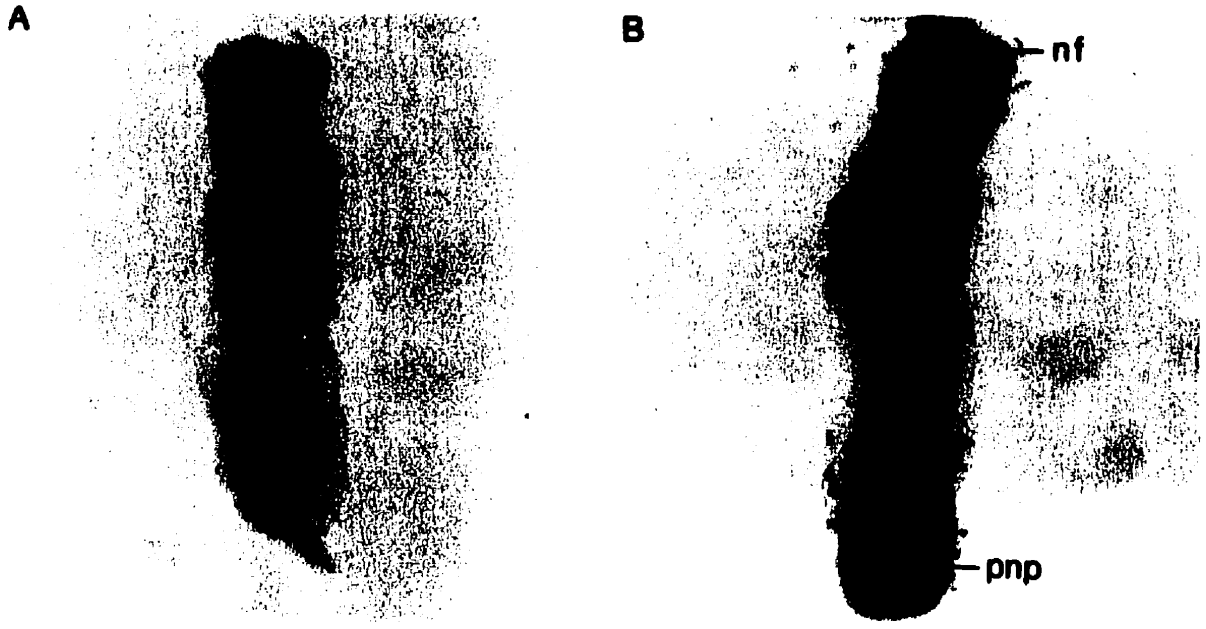

A
B
nf
pnp

FIGURE 2: Lateral view of day 9.5 embryos (18 somites) stained with antisense probe. Positive staining is in the midbrain (mb), hindbrain (hb), branchial arches (a followed by designating number), frontonasal mass (fn), heart bulge (h), and otic vesicle (ov). Note that CRABP I staining is similar in localization, but darker in the *Sp/Sp* (B) than the +/+ (A) embryo.

A

B

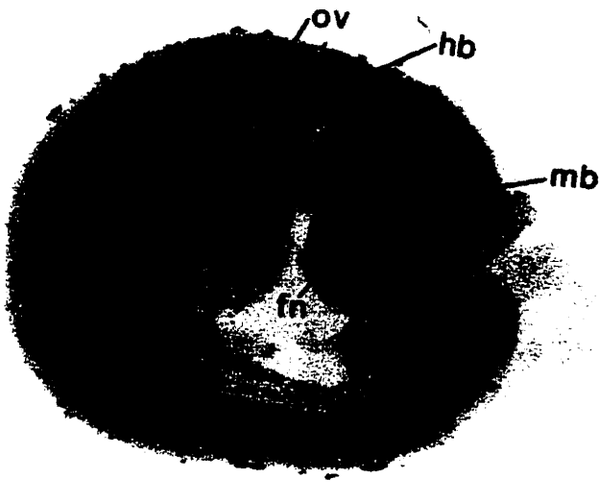
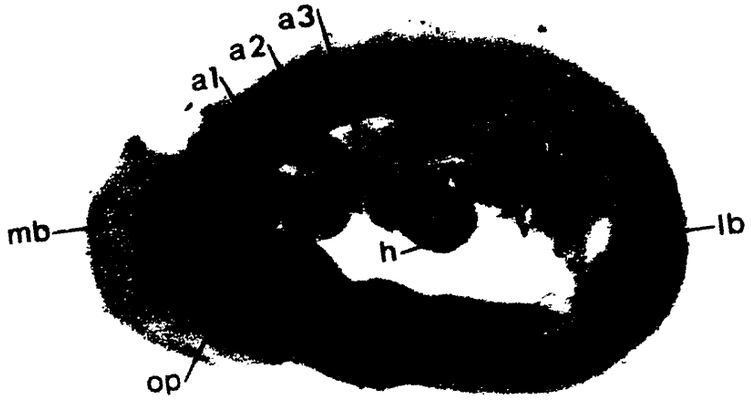


FIGURE 3: Lateral view of day 9.5 embryos (21 somites) stained with antisense probe. Positive staining is in the midbrain (mb), hindbrain (hb), branchial arches (a followed by designating number), frontonasal mass (fn), limbbud (lb), heart bulge (h) and otic vesicle (ov). The optic vesicle (op) is unstained. Note that CRABP I staining is similar in localization, but darker in the *Sp/Sp* (B) than the +/+ (A).

A



B

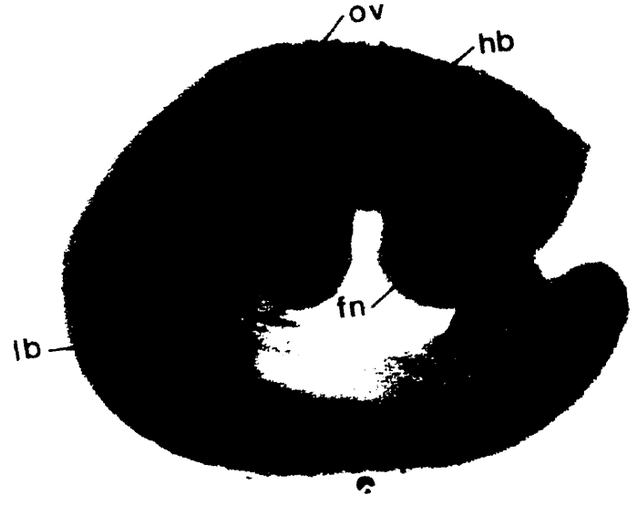
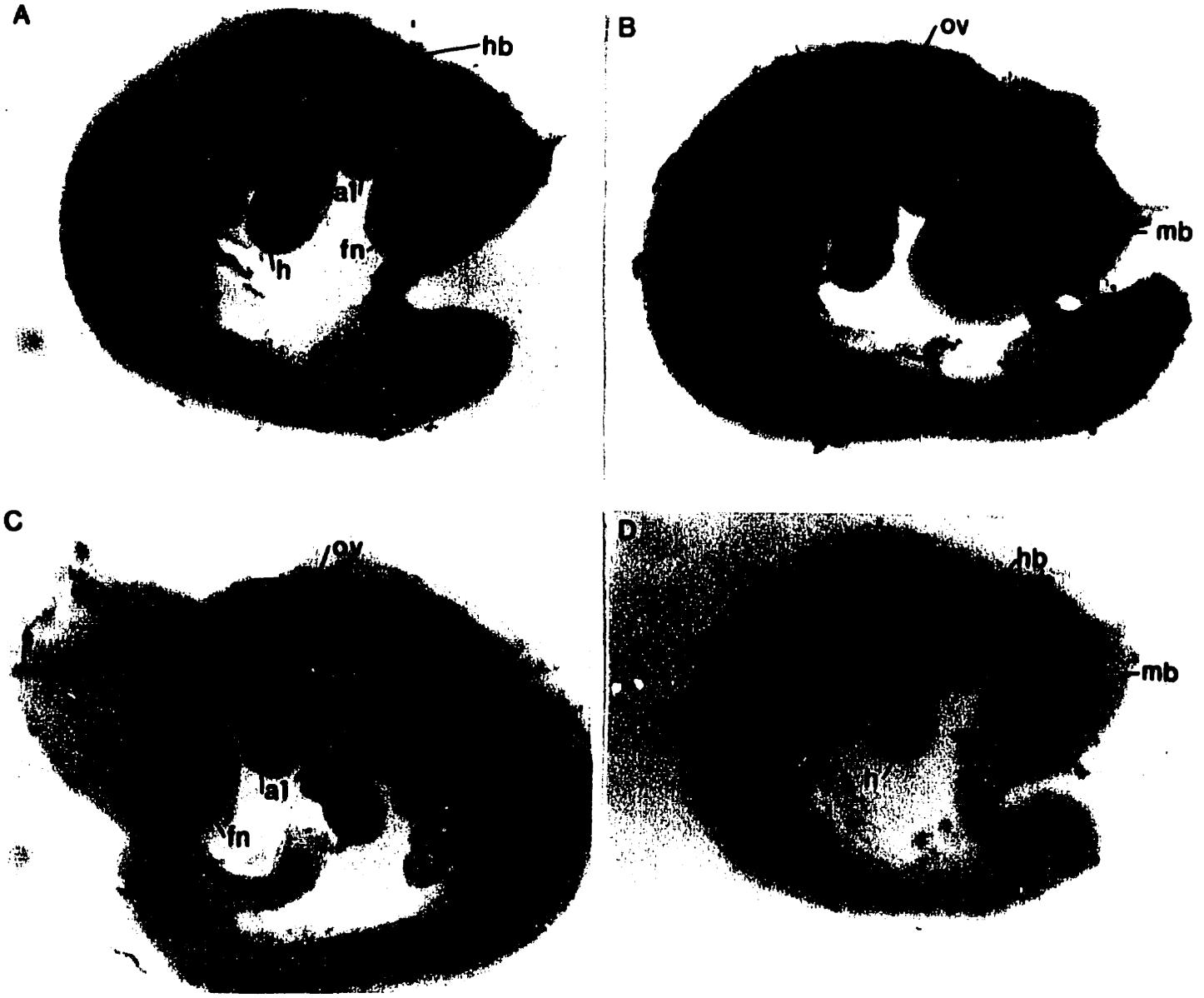


FIGURE 4: Lateral view of day 9.5 embryos (23-24 somites) stained with antisense probe (A, B) and sense probe (C, D). Positive staining is in the embryos treated with antisense in the midbrain (mb), hindbrain (hb), branchial arches (a followed by designating number), frontonasal mass (fn), limbbud (lb), heart bulge (h) and otic vesicle (ov). The optic vesicle (op) is unstained. Note that CRABP I staining is similar in localization, but darker in the *Sp/Sp* (B) than the +/+ (A) embryo. Note also that the staining with sense probe (C, D) is only general background and does not show the specificity of the antisense probe.


A
hb
al
fn
h
B
ov
mb
C
ov
al
fn
D
hb
mb
h

DISCUSSION

This research was originally undertaken as an honours project. Preliminary results indicated that CRABP I distribution was normal in *splotch* homozygotes; however, their CRABP expression levels were higher than in their wildtype littermates of the same developmental stage. For my masters project, I continued this research and confirmed these results. In *splotch* mice, CRABP is expressed only in those tissues normally associated with CRABP expression; however, these tissues have abnormally high CRABP expression levels.

In order to determine the distribution and expression pattern of CRABP, whole mount *in situ* hybridizations were done on day 9 mouse embryos. A full-length (756 bp) CRABP cDNA was used to create an antisense RNA probe to recognize and bind the mRNA for the CRABP protein. The control probe was made from the sense cDNA since it is comparable in length and G≡C content, and therefore in its non-specific binding properties.

Several groups have done experiments using DIG-labeled RNA probes, and have found that the SP6 promoter does not work well with the DIG system. The CRABP cDNA used in this project came from Dr. V. Giguère in a pGEM vector which contains the SP6 and T7 promoters. The CRABP cDNA was subcloned into pBluescript, which has T3 and T7 promoters, both of which were supposed to work well with the DIG system; however, the T3 promoter was also found to work less well than the T7. The concentrations of the sense and the antisense probes were estimated using Boehringer Mannheim's suggested dot blot system. This was done so that similar quantities of antisense and sense probe could be used in order to ensure that their degree of hybridization could be compared.

Once the CRABP was subcloned into the pBluescript vector, the orientation of the insert was assayed by creating a restriction enzyme map of the new construct. Antisense RNA was created by T3 polymerase reaction with the

vector digested at a unique site between the 5' end of the CRABP cDNA and the T7 promoter. This digestion ensured that no sense probe was also being made. The control probe was made in the same manner (with the vector digested at the unique restriction enzyme site between the 3' end of the cDNA and the T3 promoter). A binding assay was done with the probe which demonstrated that the control bound nonspecifically and yielded only background staining, whereas the antisense probe bound specifically and yielded a darker dot in the dot blot analysis. An initial *in situ* hybridization on three *Sp/Sp* embryos of approximately 30 somites also showed that the embryo treated with sense probe showed only non specific staining, whereas the two embryos treated with antisense probe had very dark, and very specific positive staining.

Northern blot analysis was done using the sense and antisense probes. Very careful and very stringent conditions were used, only to yield very faint bands. The Northern analysis was important to this project, not only to support the evidence found in the *in situ* hybridizations, but also to demonstrate the specificity of the probe. If only one band of the correct size is detected on a Northern, then the probe is specific to that type of RNA. If more than one band is detected, then the probe is recognizing other messages. Unfortunately, since the Northerns were unsuccessful, we were unable to demonstrate the specificity of our probe in this manner. I do, however, feel confident in assuming that the probe is specific to CRABP I since a full length probe and stringent washing conditions were used, and since we received this CRABP I cDNA from a lab which has found it to be CRABP I specific in their experiments (Lyn and Giguère, 1994).

The preliminary results obtained from the two litters stained for my honours project indicated that the pattern of CRABP mRNA expression in homozygous *splotch* embryos is the same as the wildtype pattern previous described (Dollé *et al.*, 1989; Maden *et al.*, 1989a, 1990, 1991, 1992; Perez-Castro *et al.*, 1989; Vaessen *et al.*, 1989, 1990; Ruberte *et al.*, 1991, 1992, 1993; Lyn and Giguère, 1994). Although the distribution of CRABP mRNA was

normal in the *splotch* embryos, the level of expression was higher than that of their wildtype littermates. This suggests that CRABP is present in the correct tissues but is being overexpressed by these tissues.

For my masters, I continued this work to confirm these initial findings. Further *in situ* hybridizations were performed, modifying the procedure to yield more intact, and better stained embryos. All embryos which had a closed anterior neuropore were "poked" during dissection: a fine tungsten wire was inserted into the dorsal aspect of the neural tube in the hindbrain region. This caused the neural tube to pop open along the back of the head, which ensured the circulation of the probe during hybridization. The fix time was reduced from overnight to four hours, and the embryos were pretreated with PBT (phosphate buffered saline with Tween 20) which helped to both permeabilize and stabilize the tissues of the embryos throughout the *in situ* procedure. Methods were improved by storing the embryos in 100% methanol, rather than 70% ethanol, and by bleaching the embryos in a hydrogen peroxide and methanol solution prior the *in situ* process. This step also helped in maintaining the integrity of the embryos.

One of the problems encountered early on was that several *in situ* hybridizations resulted in only the control (treated with sense probe) embryos having dark (background) staining. The rest of the embryos, which were treated with the antisense probe, had little or no staining. One possibility was that the probes had been reversed, but after very careful plasmid mapping and probe making, the problem persisted. Finally, it was realized that when the antibody was mixed with the antibody solution in the vial containing the control embryo, and then distributed evenly to all of the tubes, most of the antibody was sticking to the control embryo. Once this practice was stopped, and the antibody was mixed with antibody solution in a separate vial, the control embryos had much lower background staining levels, and the embryos treated with antisense again showed positive staining.

The Digoxygenin (DIG) staining system worked well, and had several advantages. It is much easier to use, and there is much less worry when handling DIG than when handling radioactivity. There are also ways of determining the staining intensity with the DIG system, as can be done with radioactivity. The one disadvantage is that the DIG system is less sensitive than radioactivity. For faint messages this can be a problem, as it was with the Northerns. I would recommend that if anyone were to continue with this work, they use radioactively labeled probe.

Initially, one litter was hybridized at a time, so that the staining among littermates could be compared. Since litters rarely contained a *splotch* homozygote and a wildtype embryo of the same somite stage (Sp/Sp embryos tend to be several somites stages behind their +/+ littermates), this did not allow for good comparisons to be made. It was concluded that since our *splotch* stock had been kept for over 20 generations by brother-sister mating, there was enough similarity between embryos from different litters for good comparison. The protocol was changed to collecting many litters at once and grouping the embryos by somite stage. The genotype of all the embryos in the group was determined by PCR analysis, and the *in situ* hybridization was run on a group of wildtype and *splotch* embryos of the same somite stage. After switching from staining an entire litter all at once to staining a group of +/+ and *Sp/Sp* embryos of the same somite stage, much better comparisons were possible, even when one or two embryos disintegrated or were mangled during the *in situ* procedure.

Over twenty embryos of each genotype (+/+ and *Sp/Sp*) between the ninth and twenty-sixth somite stages were successfully stained, and in all cases the staining in the *splotch* embryo was darker than in its wildtype counterpart. Neural crest cells which express CRABP (Maden *et al.*, 1989a, 1991, 1992; Denker et al., 1990; Momoi *et al.*, 1990a,b; Ruberte *et al.*, 1991, 1993; Lyn and Giguère, 1994) are delayed in their migration out of the neural tube in the *splotch* embryos (Moase and Trasler, 1990), and their continued presence in the neural tube could account for the increase in the staining intensity in the

hindbrain regions of the *splotch* embryos compared to their wildtype littermates. However, this cannot account for the increased staining documented in the frontonasal mass, branchial arches, posterior neuropore or limbbuds.

The difference in staining suggests that there is an overexpression of CRABP mRNA in the *splotch* embryos during development. It was not possible to determine the earliest stages for which the difference in staining was apparent, as there were difficulties in staining embryos smaller than 8-9 somites. Lyn and Giguère (1994) were able to detect CRABP staining in wildtype embryos of 4-6 somites using *in situ* staining, and so studies could continue to determine if there is a difference in CRABP levels between *splotch* and wildtype embryos below the 8-9 somite stage. Staining between *splotch* and wildtype embryos larger than 26 somites was also not compared, so it was not determined when, or if, the overexpression of CRABP ceases. It would be interesting to continue this study on embryos smaller than 9 somites, and larger than 26 somites, and adult tissues as well, to determine the exact window of time during which CRABP is overexpressed. The increased CRABP expression in the younger (9-14 somites) *splotch* embryos may be implicated in the development of exencephaly and the delayed neural crest cell migration, and the persistence of the CRABP overexpression in older *splotch* embryos may be involved the elevated incidence of spina bifida (posterior neuropore closes at 33-35 somites; Morriss-Kay et al., 1994).

The simplest explanation for the CRABP overexpression in the *splotch* homozygotes is: CRABP acts to transport RA to the nucleus and the CRABP gene is downstream from *Pax-3*. Overexpression of CRABP mRNA in the *splotch* embryos most likely means they have an excess of CRABP protein as well. (Maden (1992) examined the localization of both CRABP mRNA and protein and found they had identical patterns of distribution, although this should be ascertained for the *splotch* embryos using *in situ* antibody staining.) Excess CRABP protein would mean higher levels of the RA-CRABP complex in the cytoplasm. In this scenario, the RA-CRABP complex is responsible for

transporting RA to the nucleus, and excess CRABP-RA leads to excess retinoic acid being transported to the nucleus. An exaggerated level of RA in the nucleus could hamper RA signals required for the normal developmental processes of that cell, and may induce other inappropriate RA-dependent developmental changes. In order to determine whether excess RA is reaching *splotch* nuclei, mRNA levels of RA-responsive genes such as retinoic acid receptor ß (RARß; de Thé *et al.*, 1990; Hoffmann *et al.*, 1990; Sucov *et al.*, 1990) or laminin B1 (De Luca, 1991) could be compared between *splotch* homozygotes and their wildtype littermates. This could be done using either *in situ* or Northern hybridization.

A group of experiments were done in which RA was administered at day 9 p.c. to *Sp/+* females who had been mated with *Sp/+* males, resulting in a decreased incidence of neural tube defects in offspring (Kapron-Bras and Trasler, 1985; Moase and Trasler, 1987). Moase's study included genetic markers, so she was able to attribute the decreased neural tube defects to an RA induced selective mortality of the *Sp/Sp* embryos. The mechanism of the RA induced selective mortality is not known. If we continue under the above hypothesis that CRABP transports excess RA to the nucleus, then the Trasler experiments can be explained very simply. The excess RA coupled with the overexpression of CRABP yielded a lethal dose of RA for the *Sp/Sp* embryos.

Most literature supports the opposing theory, that CRABP acts to sequester RA, thus protecting the nuclei of RA sensitive tissues from excess (Boylan and Gudas, 1991; Fiorella and Napoli, 1991; Bass, 1993). If this is the case, the delayed development of untreated *splotch* embryos can be easily explained. The increased levels of CRABP result in a decreased amount of RA reaching the nucleus. With lower nuclear RA levels, there is a weakened RA signal. This weakened RA signal may result in a delay or reduction in RA-induced differentiation of cells, causing delayed or aberrant development of *splotch* embryos. The experiment by Kapron-Bras (1988a) supports this theory. She measured necrosis in the posterior neural tube by counting pyknotic nuclei

in that region. She found programmed cell death to be highest in RA treated embryos, next highest in wildtype embryos, and lowest in *Sp/Sp* embryos. Since programmed cell death is one of the developmental processes which is RA induced (Alles and Sulik, 1989), the Kapron-Bras findings suggest that the RA pathway is inhibited in *splotch* embryos, possibly by excess CRABP sequestering and catalyzing the metabolism of the RA required for proper development.

This role for CRABP, however, does not easily explain the experiment by Moase and Trasler (1987). If CRABP is sequestering and catalyzing the metabolism of the RA required for proper development, an exogenous dose should rescue the *splotch* embryos. It does not: a dose of RA selectively kills those embryos which should have the most protection from the teratogen. It is possible to reconcile CRABP as a sequesterer of RA and the Moase and Trasler experiment if we assume that CRABP is not the rate limiting step in the metabolism of RA. In this case, the overexpression of CRABP would not be correlated with an increase in the rate of RA metabolism. If the protein which is the rate limiting step is not upregulated in conjunction with the upregulation of CRABP, then despite their excess CRABP, *splotch* embryos receive the same dose of RA as their heterozygote and wildtype littermates, and yet are still more sensitive to the exogenous RA. The implication of this possibility is that there are other RA responsive genes which are misregulated by aberrant *Pax-3* expression.

It is also a possibility that the cellular mechanisms which control CRABP protein levels are aberrantly regulated by the lack of Pax-3 protein in the *splotch* embryos. If the homozygotes are unable to translate CRABP mRNA into protein, they would have increased RA levels relative to their +/+ littermates. Studies have shown that over long periods of time (greater than 24 hours) an increase in RA levels can increase the concentration of CRABP (Harnish *et al.*, 1992; Adamson *et al.*, 1993; Leonard *et al.*, 1995). The *splotch* homozygotes would respond to the increased free cytosolic RA with the overexpression of

CRABP mRNA that I have documented. This possibility corresponds well with the Moase and Trasler experiment (1987) as little or no CRABP protein in would result in unprotected nuclei, and any exogenous RA would be a lethal dose for the *splotch* embryos. To test this theory, CRABP protein levels should be compared between wildtype and *splotch* embryos using either Western blot or *in situ* immunohistochemical analysis. If *splotch* embryos are deficient in CRABP protein, then the proteins involved in CRABP mRNA transcription are downstream from *Pax-3*. This would be very difficult to establish, as translation involves many proteins, not all of which are known. This possibility is unlikely, since this machinery is crucial to cellular function, and would probably not be regulated by a gene like *Pax-3* which has such a limited spatial and temporal distribution.

Finally, it is also possible that CRABP overexpression is a by-product of excess endogenous RA. An over expression of CRBP or some other protein involved in the conversion of retinoids into retinoic acid would lead to increased cellular levels of RA. In this scenario, the *splotch* homozygotes respond to the overload of endogenous RA with a delay in development, spina bifida with or without exencephaly, and finally death at or around day 14 of gestation. The selective mortality observed in the Moase and Trasler (1987) experiment can be explained by the exogenous dose of RA hastening the retinoic acid-induced death of the *splotch* embryos. To test this hypothesis, endogenous levels of RA, and the levels of the proteins involved in the production of RA can be compared between *splotch* embryos and their wildtype counterparts. This would be troublesome because exact RA levels are difficult to ascertain, and not all of the proteins involved in RA synthesis are known.

CRABP is expressed in the neural crest cells and tips of the neural folds (Denker *et al.*, 1990). As can be seen in my figures 1 through 4, the staining intensity in wildtype neural folds decreases as development progresses. In homozygotes, the decrease is less obvious. This implies that normally CRABP expression is reduced as the neural folds fuse, and that somehow the reduction

is hampered in the *splotch* embryos. This extra CRABP is associated with RA teratogenesis-like malformations in untreated *splotch* embryos, and selective mortality in RA treated embryos. There are many possibilities for how this overexpression of CRABP comes about, how it can account for the defects seen in the homozygotes, and how it can account for their RA-induced selective mortality. Although the function of CRABP is still ambiguous, it is certain that it plays a significant role in the RA pathway. It is associated with RA sensitive tissues, and its concentration can affect RA's ability to mitigate differentiation. The data collected in this study does not provide conclusive evidence for CRABP either as a sequesterer or a transporter of RA. The results obtained raise more questions than they answer. The original question of this study was "Is RA-induced selective mortality of *splotch* embryos associated with an aberrant expression of CRABP?". The expected answer was "Yes, reduced levels of CRABP in *splotch* embryos can explain the RA hypersensitivity of *splotch* embryos". Unfortunately, the answer is not so simple. Rather than having the expected reduction in CRABP mRNA levels, *splotch* embryos have excess CRABP mRNA. In trying to understand the etiology and implications of the CRABP overexpression, we are faced with several questions. Is CRABP downstream from *Pax-3*, and if so, what are the intervening genes? Is the CRABP-RA complex transferred to the nucleus as suggested by Takase (1986)? Are other genes in the RA pathway downstream from *Pax-3*? There exists much evidence to support both theories: that CRABP is involved in the transport of RA to the nucleus, and in its metabolism. In all likelihood, the function of CRABP is complex, and is involved in both of these processes.

Retinoic acid is very important in development. It plays a role in the development of the many systems, including the nervous system and limb buds. When present in too high or too low of concentrations, RA can cause embryonic malformations and in some instances, death. It is clear that retinoic acid binds nuclear receptor proteins, and effects cellular changes by altering the mRNA levels of many genes. Although many proteins involved in this pathway have

been identified, there is still much more we do not understand about how RA induces cell specific differentiation. It is unknown how RA gets from the cytoplasm into the nucleus. One suggestion is that there are receptors on the nuclear membrane which transfer RA from CRABP to the nuclear receptors. Once inside the nucleus, RA can upregulate, downregulate, turn on or turn off a wide range of genes. How the RA-RAR and RA-RXR DNA binding complexes have such a wide range of signals is still unknown.

Through mutations and aberrant expression patterns, we begin to understand the role each individual protein plays in the formation of such complicated structures as the brain and spinal cord. Pax-3 and CRABP are two of the many proteins involved in the development of the nervous system, and understanding these and other individual proteins and genes and their role in development helps in understanding more complicated processes like neural tube formation. As these processes become understood, we gain a better understanding of the development of the entire nervous system, which will hopefully lead to an understanding of the brain and spinal cord, and possibly ourselves.

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IMAGE EVALUATION
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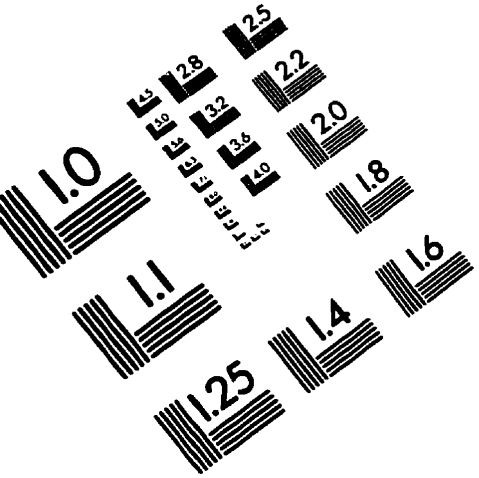

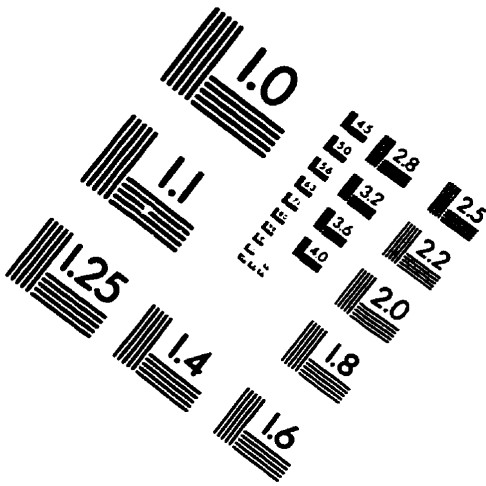

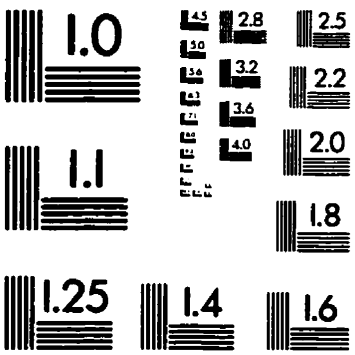

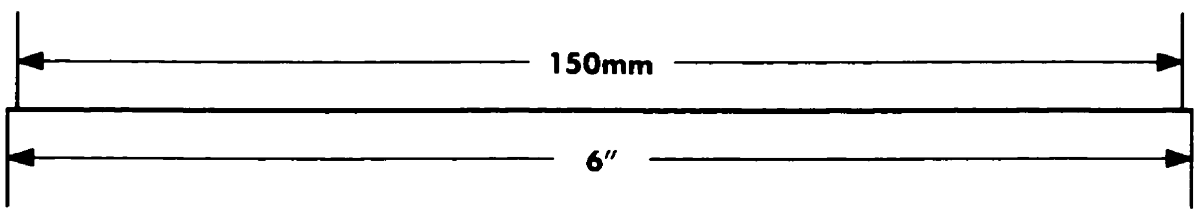

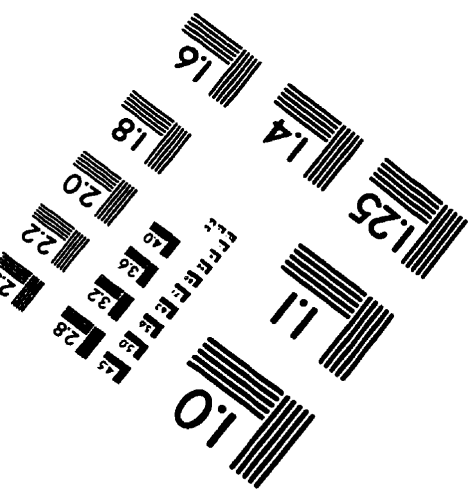

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