Title
Effect of exogenous retinoic acid on syndecan expression along the anterior-posterior axis of the mouse embryo

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EFFECT OF EXOGENOUS RETINOIC ACID ON SYNDECAN EXPRESSION ALONG THE ANTERIOR-POSTERIOR AXIS OF THE MOUSE EMBRYO

by

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THESIS

Submitted in partial satisfaction of the requirements for the degree of

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in

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in the

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of the

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DEDICATION

This Thesis is dedicated to my family whose guidance, love, and support have made this all possible

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I would like to especially thank Dr. Caroline Damsky for her guidance, support, and unending assistance throughout not only the entire research project but from my entrance into post-Doctoral studies. Her never ending patience, enthusiasm for teaching, and truly insightful direction continually stimulated my interest in research. With her help I was able to navigate the many issues and frustrations which arose from the Articulated graduate program in orthodontics and oral biology.

I would also like to thank Dr. Ann Sutherland for her never ending help, guidance and tolerance.

Lastly, I would like to thank Dr. Robert Boyd for his always very positive support and encouragement.

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INTRODUCTION & BACKGROUND

In most vertebrate embryos the specification of the body axes and of different tissue types depends on a series of inductive interactions (Slack 1983). Work in experimental embryology has resulted in a fairly detailed picture of the processes of regional specification. Indeed, these inductive interactions have been well documented by transplantation experiments. Understanding the molecular mechanisms underlying regional specification and axis determination requires the identification of proposed inducing factors, as well as the responses of component cells to these inducing factors.

Recently, interest in mesoderm induction has been stimulated by the discovery that homologues of several growth factors have the capacity to induce mesodermal structures (Slack 1987). Heparin binding factors such as transforming growth factor type-β (TGF-β), basic fibroblast growth factor (bFGF), embryonal carcinoma derived growth factor (ECDGF), and acidic fibroblast growth factor (aFGF), have been shown to be active as mesoderm inducing factors (MIF's) (Slack, 1989).

FGF is capable of inducing muscle, kidney, mesenchyme, and mesothelium but not notochord, the most dorsal mesodermal tissue. This finding has led to the suggestion that the inductive signal for dorsal mesodermal structures is TGF-β -like and that the inductive signal for ventral mesodermal structures is FGF-like (Dale & Slack 1987).

Genetically vertebrates possess four clusters of homeobox containing genes known as Hox genes. These genes are related to genes of the Drosophila

antennapedia and bithorax complexes (Akam 1987). Vertebrate Hox genes show general spatial restrictions in expression along the anterior-posterior axis in the central nervous system and somitic mesoderm (Duboule & Dolle, 1989). Analysis of the expression of these genes has demonstrated a potential role for them in the axial patterning of a number of embryonic systems (Wilkinson et al., 1989). Furthermore Hox-2 genes are thought to have a role in neural crest development (Hunt et al., 1991), thereby influencing the ultimate development of other embryonic tissues. The proposed role of Hox genes in craniofacial development suggests that they are involved in two interlinked processes that produce the structures of the branchial region (Hunt et al 1991). The first is the specification of the rhombomeres and the second is the formation of the branchial arches themselves. Hypothetically a developmental process results in the establishment of differential Hox gene expression in the neural plate when there are no other intrinsic differences in the existing cell populations. The particular combinations of Hox gene expression that exist in the different regions of the neural plate initiate a chain of events that give rise to the region specific patterns of other gene activities that eventually result in regional tissue identity. The specific signals involved in regulating Hox gene expression have yet to be well characterized, although there is evidence that MIF's can influence Hox gene expression in the frog (Melton et al., 1989)

Additionally, retinoic acid (RA) has been shown to play a role in regulating anterior-posterior patterning. A retinoic acid based signaling system which specifies position along the anterior-posterior axis of the developing embryo has been suggested (Conlon & Rossant 1992). Vertebrate embryos including xenopus, chick, and, mouse all contain endogenous RA (Thaller & Eichele1987). Vertebrate embryos have also been shown to contain retinoic acid and retinoid X receptors (Smith & Eichele1991) as well as retinoic acid and

retinoid binding proteins (Maden et al 1991) all of which could cooperate to transduce an RA signal.

An RA responsive transgene (RAREhsplacZ) designed to reveal RA mediated transcriptional activity at the early neural plate stage of mouse development generates a domain of β-galactosidase staining that is limited to the posterior half of the embryo, and has a sharp anterior boundary across all germ layers (Rossant et al., 1991). It has been suggested that members of the Hox-C gene clusters might normally be spatially regulated by endogenous RA (Simeone et al., 1991). Certain Hox-2 gene expression boundaries resultant to the addition of exogenous RA manifest rapid anterior displacement whereas others examined were relatively insensitive to RA treatment (Conlon and Rossant, 1991). The expression of the RAREhsplacZ transgene presumably represents an integrated response to an RA signal thereby supporting a role for RA in patterning along the A-P axis in embryonic development.

Retinoids have long been recognized as teratogens for vertebrate embryos with effects on a wide variety of structures (Hale, 1933). Shenenfelt (1972) demonstrated that the responding organ or structure typically has a period of sensitivity to exogenous retinoids that corresponds approximately to the time of that organs establishment. The morphology of retinoid induced abnormalities has been interpreted as a transformation of anterior cell fates to more posterior cell fates. One example is the morphologic transformation of the otic vesicle as a result of exogenous retinoids. It has been demonstrated in the mouse that the otic vesicle is positioned more anteriorly in embryos treated with retinoic acid (RA) than in untreated normal controls (Sulik et al., 1988). More recently application of exogenous RA to mouse embryos causes somites to form at levels more anterior than normal as well as causing axial skeletal alterations suggestive of anterior to posterior transformations of cell fates (Kessel & Gruss., 1991). The

transformation of cell fates implies that developmental patterning particularly along the anterior-posterior (AP) axis can be disrupted by exogenous retinoids. It is further possible that exogenous RA causes anterior to posterior transformations by disrupting an endogenous RA based signaling system which specifies the identity of various cell types along the A-P axis of the embryo.

Syndecan-1 is an integral membrane proteoglycan isolated from mouse mammary epithelial cells containing both heparin sulfate and chondroitin sulfate glycosaminoglycans. Syndecan is expressed differentially along the A-P axis in the 7.5 day mouse embryo. As the embryo develops syndecan-1 is expressed at the basolateral surface of ectodermal cells and their derivatives, endoderm, and the undifferentiated mesodermal cells emerging from the primitive streak (Sutherland et al., 1991). Syndecan-1 is then lost from the mesenchyme in an asymmetric pattern; it continues to be expressed strongly on posterior and lateral mesoderm cells but becomes barely detectable on anterior mesoderm cells. In older embryos it is not expressed by mesenchyme anterior to the otic sulcus (Sutherland et al., 1991).

Recent studies have shown that syndecan may also participate in cellular interactions with basic fibroblastic growth factor (bFGF). Syndecan binds to bFGF in-vitro (Bernfield and Saunderson, 1990), and has been shown to be homologous to a low-affinity FGF receptor identified by ligand-affinity cloning (Keifer et al. 1990). The binding of FGF to cell surface heparin sulfate proteoglycans is required for its biological activity. Treating cells with heparitinase to remove cell surface heparin sulfate glycosaminoglycans (GAG's), or with chlorate, to inhibit their sulfation, prevents binding of bFGF to a high affinity receptor (Rapraeger et al., 1985). The role of heparin sulfate proteoglycans was found to be more than stabilization of bFGF, and may involve changing the conformation of FGF in a way that allows it to interact with high-

affinity receptors (Yayon et al., 1991). These results indicate that the ability of cells to respond to FGF can be regulated by changes in the expression of cell surface proteoglycans (Klagsburn, 1990). Additionally on the syndecan gene a promoter region showing homology to an antennapedia-like homeobox gene has been identified. Theoretically syndecan could bind Hox type transcription factors therby mediating A-P positional information.

In summary, tissue patterning in vertebrate organisms results via mesoderm-inducing factors such as FGF. FGF is capable of inducing muscle, mesenchyme, and mesothelium and has therefore been suggested as being a ventral mesodermal inductive signal. Recent studies have shown that syndecan participates in cellular interactions with bFGF and that in fact the binding of FGF to cell surface heparin sulfate proteoglycans is critical to its biological activity. Analysis of Hox gene expression demonstrates a role for them in axial patterning. A Retinoic acid based signaling system has been suggested. In evaluating syndecan's expression in the 7.5 day mouse embryo it is expressed differentially along an A-P pattern. Syndecan is expressed strongly on posterior and lateral mesoderm cells but becomes barely detectable on anterior mesoderm cells a distribution reminiscent of FGF expression. There are three components then proposed to be part of an anterior-posterior patterning system. Mesoderm inducing factors (MIF's), retinoic acid, and Hox genes. Retinoic acid has been proposed as an endogenous A-P signaling system. It further has been shown to influence Hox gene expression anteriorposterly. Syndecan-1 may be a downstream Hox gene product target and may also be related to FGF a member of the MIF component of this system. We therefore hypothesize that syndecan-1 may be regulated by an endogenous RA based signaling system . This is the question we have addressed in the below described work.

HYPOTHESIS

The hypothesis being tested is that the asymmetric anterior-posterior distribution of syndecan in the mouse embryo can be altered by the addition of exogenous retinoic acid. This hypothesis was tested by treating the mouse embryo with exogenous retinoic acid at day 7.0 of development, and then evaluating the expression and distribution of syndecan at subsequent stages in the postimplantation embryo.

If the hypothesis is correct, it would suggest that syndecan expression is regulated by transcription factors (possibly of the Hox type) that are responsive to retinoic acid. If not, it would suggest that syndecan is not responsive to a retinoic acid based signaling system.

EXPERIMENTAL DESIGN & MATERIALS AND METHODS

Materials

Tween 20 was from Sigma Chemicals St. Louis, MO. Diethylene glycol distearate and conical micromolds were from Polysciences, Warrington PA. Normal donkey serum, biotinylated secondary antibodies to rabbit IgG, and streptavidin-Texas Red were from Amersham, Arlington Height IL. Biotinylated secondary antibodies to rat and to rabbit IgG and horseradish peroxidase detection kit were obtained from Vector laboratories, Burlingame CA. Retinoic acid was obtained from Sigma. TB syringes were from Becton Dickinson and Co. Rutherford, New Jersey, and polypropylene catheters were from Sherwood Medical, St. Louis, MO.

METHODS

Animal colony: Female ICR mice (12-w-old, Harlin) were superovulated by injection with 5 IU of pregnant mare's serum gonadotropin (Teikoku Hormone Mfg. Co., Japan) followed after 48 hours by an injection of 2.5 IU of human chorionic gonadotropin (hCG, Sigma Chemical Co., St Louis, MO) and were then caged with ICR males. Females were checked the next morning for an insemination plug indicating that copulation had occurred, then removed and housed separately. Pregnant females were treated in both the experimental group and the control group on day 7, hour 6 with retinoic acid in vegetable oil or vegetable oil alone by gavage.

Dissection and fixation of embryos: The treated and control mice were sacrificed at day 8.5 of pregnancy and implantation sites were dissected out of the uterus. The embryos within each implantation site were removed and fixed without further dissection in Carnoy's fixative (6V ethanol: 3V chloroform: 1V glacial acetic acid), or Bouin's fixative (picric acid, formalin, acetic acid) for 30-45 minutes at room temperature.

Embedding of embryos: All steps were done at 4° C. First the embryos were dehydrated through an ethanol series, and then transferred into 100% butanol. (50%, 70%, 80%, 95%, 100% EtOH; EtOH: butanol (2:1), EtOH: butanol (1:2) and finally into 100% butanol overnight). The dehydrated embryos were then transferred into diethylene glycol distearate (DGD), and embedded. The DGD was melted at 65° C, dimethylsulfoxide (DMSO) was added to 0.5% (v/v), and the mixture was filtered through Whatman #1 paper in a 65° C oven (overnight). The filtered DGD was then kept in the oven, ready for use.

The embryos were then transferred through a gradient to 100% DGD. The embryos were first placed into butanol: DGD (2:1), then butanol: DGD (1:2), and lastly into 100% DGD. All steps were done in the 65° C. oven. Embryos were appropriately oriented (using a dissecting microscope) in the melted DGD, and then the blocks were allowed to cool and harden for 24 hours.

Immunohistochemistry on sectioned embryos: DGD embedded embryos were sectioned on glass knives fitted with water troughs. Sections were cut at 1 μm thickness and then transferred with an eyebrow hair from the water trough to a drop of water placed on a slide previously coated with 0.1 % poly-L-lysine. Slides were then placed on a warm plate (35°-37° C.) allowing the water to evaporate. As this happened the sections would flatten and melt onto the

surface of the slide adhering them for rehydration and subsequent staining. The sections were then rehydrated (100% butanol overnight, butanol: EtOH (2:1), butanol: EtOH (1:2), 100%, 80%, 70%, EtOH, and then PBS. All steps were done at room temperature). Following rehydration, non-specific binding sites within the section were then blocked using PBS containing Ca and Mg, 3% BSA, 2.5 % normal goat serum (NGS) and 0.5 % Tween 20, for 1 hour at 37° C.

The rat monoclonal antibody (mAb) to syndecan, 281-2, was used a purified rabbit IgG. Control antibodies included crude serum preparations of normal goat serum

The primary antibody was diluted in 3 % BSA in PBS. The incubation period was for one hour at room temperature, or overnight at 4° C. in a humidified chamber. The sections were then rinsed with stirring for 30 min. at room temperature in 3% BSA in PBS, 2.5% (NGS), 0.5 % Tween-20 in a slide dish. The secondary antibody was also diluted in 3 % BSA in PBS, 2.5% (NGS), 0.5 % Tween-20. Incubation was for 30 min. at room temperature or overnight at 4° C. in a humidified chamber. The secondary antibody was again rinsed as described above. Finally, the sections were incubated in streptavidin reagent diluted in 3 % BSA in PBS, 2.5% (NGS), 0.5 % Tween-20. Incubation was for 15 min. at room temperature in a humidified chamber and the rinse again was as described above.

The slides were then mounted in medium containing p-phenylenediamine to prevent quenching of the fluorescent signal. The edges of the coverslip were immediately coated with fingernail polish to prevent drying of the mounting medium and oxidation of the quencher. The mounted slides were then stored in the dark at 4° C.

Protocol summary: Pregnant female mice were treated with retinoic acid on day 7.5 of gestation. At 8.5 days the implantation sites were dissected from the uterus. The embryos within were removed and fixed without further dissection. All embryos were fixed in either Carnoy's or Bouin's fixative, dehydrated and embedded in diethylene glycol distearate and then sectioned at 1um thickness and mounted on slides coated with poly-L-lysine. The sections were then rehydrated, processed and examined for syndecan expression by immunofluorescence. The results were photographed using Kodak Technical Pan film with a blue filter to enhance the image.

Syndecan expression staining data was compared between Sagittal and para-sagittal sections of treated and control 8.5 day embryos. Some embryos in both groups were allowed to develop further in order to observe the more gross morphological changes associated with exogenous RA treatment. Changes in the embryo overall and the effects on the craniofacial region specifically were thus evaluated.

Immunohistochemistry on whole mouse embryos: Embryos were dissected at day 10.5 of pregnancy as described above from the uterus and fixed in methanol: DMSO (4:1) overnight at 4° C. Fixed embryos were transferred into methanol: DMSO: 30% H2O2 (4:1:1) for 4-5 hours at room temperature to bleach the embryos and block endogenous peroxidases. Embryos were then rehydrated through a methanol gradient to PBS, (80%, 50%, 15%, methanol to PBS, for 30 minutes each), followed by two 1 hour baths in 2% instant skim milk, 0.5% triton X-100 in PBS (PBSMT) at room temperature.

Whole embryos were then incubated overnight in PBSMT containing a 1:200 dilution of a polyclonal antibody against Hox 2.1a. The total volume used was 800ul. The embryos were incubated upright with nutation overnight at 4° C.

Embryos were then washed in PBSMT twice at 4° C and 3 times at room temperature for 1 hour each time.

The embryos were again incubated overnight at 4° C with the secondary antibody (HRP coupled donkey anti-rabbit IgG [Jackson Immunoresearch]) diluted 1:200 in PBSMT with a total volume being 800ul. Embryos were again washed in PBSMT twice at 4° C and 3 times at room temperature for 1 hour each time. A final 20 minute wash in PBT at room temperature was done prior to HRP-coupled secondary antibody detection.

Embryos were incubated in a solution containing .5mg/ml diaminobenzidine (DAB) in PBS containing 0.2% BSA, and 0.5% triton X-100 (PBT) at room temperature for a minimum of 30 minutes, and usually for 40-60 minutes. The stain color was enhanced in some cases by adding 1% cobalt chloride, and 1% nickel ammonium to a concentration of 2.5% and 2% of the of the total volume respectively. Specimens were photographed using a Minolta X-370N camera mounted on a dissecting microscope and Kodak TMAX 100 black and white film. Throughout the entire protocol embryos were gently rocked to improve penetration of the tissue.

Lastly, after examination, the embryos were dehydrated through a methanol series: (30%, 50%, 80%, 100% methanol) for 30 minutes each and stored at -20 ° C.

RESULTS

We wished to examine the effects of exogenous retinoic acid (RA) on the pattern of syndecan expression along the anterior-posterior axis of the postimplantation embryo. The goal of this study was to test the hypothesis that the anterior boundary of syndecan-1 expression in the mesoderm observed at the late gastrula and early headfold stage in the mouse embryo (7.5-9.0 days) is influenced by exogenous retinoic acid. Retinoic acid in vegetable oil (treated group), or vegetable oil alone (control group), was administered via gavage to pregnant mice on embryonic day 7.5 of gestation. Control and RA treated mothers were then sacrificed at day 8.5 and embryos were analyzed for syndecan expression. Some treated and control embryos were allowed to develop further (9.5-11.0 days) and were examined morphologically and by immunostaining in order to determine the extent of exogenous RA effect on embryonic development.

A retinoic acid based morphogenetic signaling system is hypothesized to exist along the anterior-posterior, (AP) axis in the developing mouse embryo (Conlon and Rossant 1992). The distributions of retinoic acid receptors and binding proteins during embryogenesis are complex and dynamic, and the distribution of retinoic acid itself is unknown within the developing organism. However, the domains of RA action almost certainly depend not just on RA distribution, but on the integration of receptor, and binding protein distribution, and possibly interaction with other transcription factors (Husmann et al., 1991). Destruction of this RA signaling system by the addition of exogenous RA is

known to have a posteriorizing effect on embryonic development (Morriss-Kay et al., 1991). That is, the location of a structure characteristic to a particular position on the A-P axis (e.g. the otic vesicle) is displaced anteriorly resulting from the dysmorphic development or lack of development of structures even more anterior. Furthermore, the anterior boundary of expression of many DNA binding (homeodomain containing) transcription factors (HOX genes) is extended in the presence of exogenous RA (Conlon and Rossant, 1991) suggesting that RA may regulate the expression of these HOX genes. HOX gene products in turn affect the expression of other genes which ultimately form the structural features of the embryo (Simeone et al., 1991). Some of these downstream HOX gene product targets should also have a patterned expression along the A-P axis. Previous data have shown that syndecan-1, a low affinity receptor for FGF's has an asymmetric patterned expression in the mesoderm (Sutherland et al., 1991). Also evidenced is a regulatory region within the syndecan gene showing homology to an antennapedia-like homeobox containing promoter region. Both these findings are suggestive of A-P patterning functions. If our hypothesis is correct, the anterior boundary of syndecan-1 expression in the mesoderm should extend more anteriorly in RA treated embryos with respect to anatomical or biochemical markers that are not affected by the addition of exogenous retinoic acid, or alternatively, extend anteriorly in concert with features or markers that are anteriorly displaced by the addition of exogenous RA treatment. If no such displacement is observed, it would suggest that syndecan-1 expression is regulated by Hox gene products not affected by RA, or by a different class of regulatory genes.

Our data indicate that no difference was detected in the expression pattern of syndecan-1 examined by monoclonal antibody (281-2) immunostaining in RA treated or control embryos treated on embryonic day 7.5 and sacrificed at 8.5

days. To appreciate and better understand the structures and orientation of the 8.5 day mouse embryo viewed in sagittal section, a schematic representation is presented (figure 1.). Syndecan staining of the embryonic ectoderm and definitive endoderm was uniform along the entire A-P axis in both treated (Fig. 2 A,B,C,) and control (Fig. 3 A,B,C,) postimplantation (8.5 day) embryos. This was not unexpected, as previous results on untreated 8.5 day embryos showed a uniform expression of syndecan-1 on embryonic ectoderm and endoderm (Sutherland et al., 1991). In the ectodermal layer of treated as well as control embryos syndecan-1 was found to be expressed on basolateral cell surfaces. No regional differences were detected in the staining pattern of syndecan within the ectoderm along the anterior-posterior axis (Treated fig. 2 B, C; Control Fig. 3 B, C,). Also, within the definitive embryonic endoderm in both treated (Fig. 2 B, C,) and control (Fig. 3 B, C,) embryos syndecan-1 was distributed on the basal and lateral cell surfaces again with no regional differences in its expression noted along the embryonic anterior-posterior axis.

Syndecan staining in the mesodermal layer, however, displays marked asymmetry along the anterior-posterior axis. Posterior and lateral mesoderm stained more intensely for syndecan than did anterior mesoderm. This finding is consistent in both the RA treated and control embryos. More specifically, syndecan staining in the primitive streak and the distal and the lateral mesoderm of both the 8.5 day RA treated (Fig. 4 A), and control embryos, (Fig. 4 B) is intense. In the presomitic mesoderm and in the somites of treated (Fig. 5 A) as well as control embryos (Fig. 5 B), syndecan staining is also intense. In contrast, syndecan staining is barely detectable within the anterior mesoderm of the head fold which underlies the anterior neural ectoderm in both RA treated (Fig. 6 A, B, C) and control embryos (Fig. 6i A, B, C). In both the treated and control groups, this change in syndecan expression occurs rather abruptly within the mesoderm

at the level of the otic flexure. Just distal to this landmark there is a low level of syndecan staining detectable but anterior to this landmark, syndecan staining is not detected (Fig. 6 & 6i A, B, C).

A similar pattern of expression was detected in all embryos treated with RA at day 7.5 and examined at 8.5 days. These finding were consistently observed in three separate experiments each of which included embryos from at least two pregnant animals. A minimum of two embryos per experiment were sectioned and stained. Likewise for the control results described above, three separate experiments (minimum two pregnant animals per experiment) with at least two embryos per experiment were sectioned and stained. The findings were consistent among all experiments and among all embryos within each experiment.

To confirm that retinoic acid treatment truly affected these embryos in the experimental group, some embryos from both the treated and control groups were allowed to develop further to day 9.5-11.0 of gestation to assess the frequency of morphologic developmental anomalies characteristic of exogenous RA treatment. To appreciate the morphology and structures of interest in the whole mouse embryo, Figure 7 is a schematic representation of a 9.5 day normal mouse embryo in sagittal view and Figure 8 is a schematic representation of a 9.5 day normal mouse embryo viewed dorsally. One hundred and forty one embryos were dissected from twelve RA treated mothers and seventy one embryos were dissected from six control mothers. In the experimental group, of these 141 embryos 112 (79.4%) displayed severe gross morphological alterations associated with the addition of exogenous retinoic acid. In the control group, of the seventy one embryos, only three embryos (4 .2%) appeared somewhat abnormal.

In the experimental group a cluster of morphological defects is detectable at day 9.5-11.0 resulting from a teratogenic dose of retinoic acid administered at the headfold stage (day 7.5). An anterior displacement of the otic vesicle and anterior somites is observed relative to the mandibular and hyoid branchial arches. Telencephalic and mesencephalic development of the brain further appear grossly aberrant. A loss of rhombencephalic segmentation is also noted. These defects are clearly seen in (Fig. 9 A, treated; B, control). Also seen very strikingly, is an incomplete or lack of closure of the anterior neuropore in treated embryos further resulting in altered head morphology (Fig. 10 A, treated; B, control).

Experimentally, exogenous retinoic acid induces anterior cells to adopt more posterior fates (Figs. 9 & 10 A treated: B control). The above described defects correlate with this direction of morphological transformations due to RA treatment. Twenty nine (20.6%) of the 141 treated embryos however did not show these gross morphological aberrations. The head morphology in these embryos appeared normal with a closed anterior neuropore, and appropriate brain segmentation. The otic vesicle and anterior somites generally appeared to be positioned appropriately on the A-P axis (Fig. 11 A: treated, B: control).

This is not to say that these embryos were not affected by the RA treatment, however. They may be only moderately affected, with changes detectable by biochemical means, but not by gross morphology. Therefore, to further evaluate the penetrance of RA in this treated sample of embryos the expression pattern of Hox-2.1a was evaluated.

Previous studies have characterized the expression pattern of Hox-2.1a in the early mouse embryo (Wall et al., 1992). It is first detectable at approximately the 7-9 somite stage corresponding to day $\approx$ 8.0 of development. The anterior limit of Hox 2.1a staining in the somites was determined to be the posterior half

of somite 5 with expression in all cells of more caudal somites. In contrast, at this stage expression in the neural tube extends more anteriorly and can be detected at the level of the first few somites. By day 9.0 (≈ 12 somites) and onward, however, the anterior border of expression in the neural tube and in the somites is coincident and located at the posterior half of the fifth somite. Thus, neural tube and somitic staining are in register at this stage (Wall et al., 1992). Also at 9.5 days the lung bud, gut, and anterior proximal region of the forelimb buds stain positively for Hox-2.1a (Wall, et al 1992).

In 9.5 day control embryos examined by whole mount Immunohistochemistry, a similar pattern of Hox-2.1a expression to that described by Wall (1992) was observed (Fig. 12 A & B). The anterior limit of staining detected in both somites and neural tube was at the posterior half of the fifth somite. Furthermore, the staining within the neural tube and the somites appeared to be in register having a coincident anterior limit. Additionally, the lung bud, gut, and anterior proximal region of the forelimb buds stained positively for Hox-2.1a.

In the 9.5 day RA treated embryos however, the anterior limit of Hox-2.1a staining appears to have been advanced anteriorly. The intense staining in the neural tube has advanced to nearly the level of the otic vesicle. The anterior limit of Hox 2.1a staining in the somitic and lateral plate mesoderm having been advanced also is not as clearly defined in embryos with severe as well as moderate morphological abnormalities (Fig. 13 A & B). The anterior shift of Hox2.1a staining within the neural tube is clearly evidenced when comparing the treated embryo to the unaffected embryo (Fig. 14 A: treated, B: control,). Hox 2.1a staining appears to have shifted anteriorly to within the first few somites as seen in oblique views of two embryos (Fig. 15 A: severe effect, B: moderate effect). Interestingly, the neural tube and somite staining are no longer in

register in RA treated embryos; the staining within the neural tube extended more anteriorly than the stain within somites. This is in direct contrast to control embryos where Hox2.1a staining was evidenced to be in register (Fig. 16 A: treated, B: control). This change in Hox-2.1a expression pattern indicates that Hox 2.1a is responsive to exogenous RA and further, its altered expression is seen to be consistent with the known effect of exogenous retinoic acid in posteriorizing anterior cell fates.

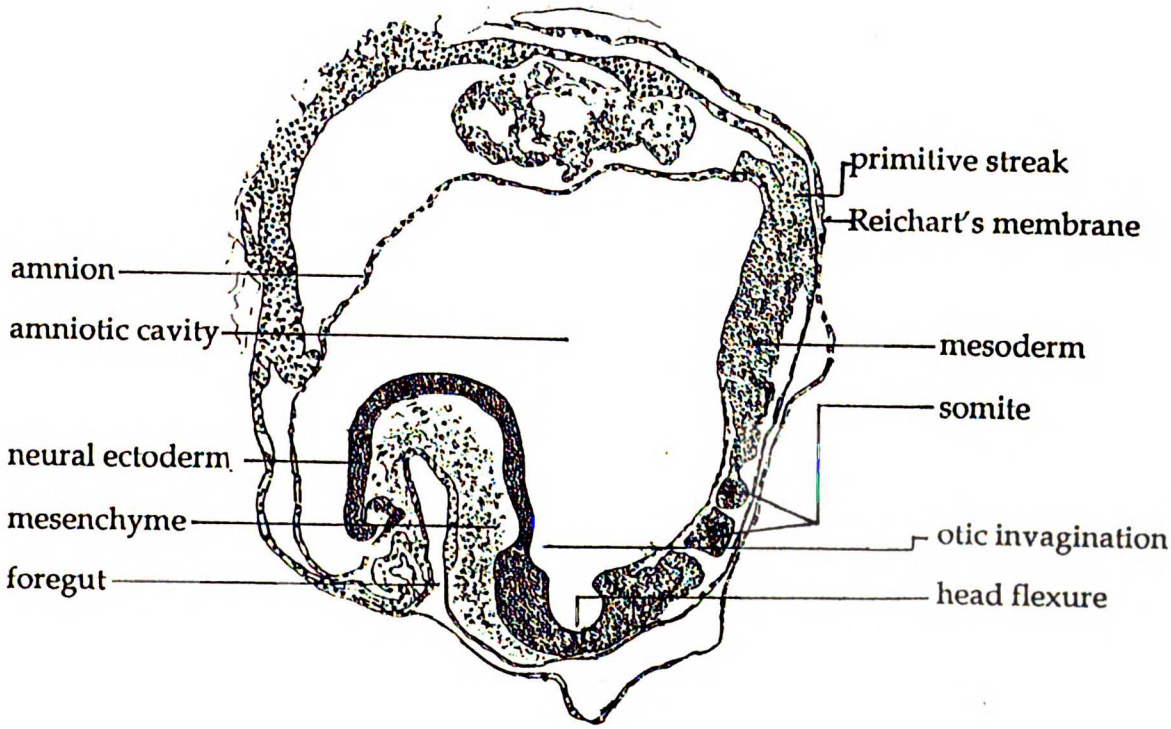
Some RA treated 9.5 day embryos that did not display gross morphological changes may yet have been affected by retinoic acid as evidenced by the altered staining pattern of Hox-2.1a (Fig. 17 A sagittal & B dorsal). The anterior limit of Hox-2.1a expression within the neural tube was shifted anteriorly more so than the somitic and lateral plate mesoderm, whose boundary became somewhat ill defined. Seven of nine embryos RA treated yet morphologically unaltered embryos showed this Hox 2.1a anterior shift. Biochemically these embryos appear to have been affected by the RA treatment yet morphologically they appear unaltered. Two embryos examined from the treated group did not manifest an anterior shift in Hox 2.1a expression within the neural tube or the somites, and may therefore have been truly unaffected by the RA treatment. These two embryos were the only ones of all embryos examined from the treated group not to show any effects of RA treatment. Otherwise, the responses to RA treatment seen morphologically and by Hox 2.1a staining were observed in all embryos examined.

Of particular importance for our study was this observation that more than three fourths of the RA treated embryos that appeared normal morphologically (20% of the treated embryos) showed altered Hox 2.1a staining. Both the anterior shift in the extent of staining as well as the lack of register in the anterior boundary of Hox 2.1a in neural tube and somite staining were observed.

Thus, if these embryos are included as part of the affected group, the penetrance of RA treatment in our experiments becomes 92-97%. This increases significantly the likelihood that the relatively small number of embryos that were sectioned and stained for syndecan-1 at 8.5 days were in fact affected by the RA treatment. This strengthens our conclusion that the spatial pattern of syndecan-1 expression was not affected by retinoic acid.

Figure. 1. Schematic representation of 8.5 day mouse embryo viewed in sagittal section

Figure. 1



8.5 day mouse embryo sagittal section

Figure. 2. Darkfield overview of parasagittal section of a DGD-embedded, 8.5 day retinoic acid treated embryo. (A) Lower magnification view showing the otic flexure (of), amnion (am), and primitive streak (ps). The boxes marked b and c indicate the regions shown in higher magnification showing monoclonal antibody staining for syndecan in the panels B and C, respectively (B) Sagittal view of the posterior region showing monoclonal antibody staining for syndecan-1 in the presomitic mesoderm (mes), the somites (s), the ectoderm (ec), and the endoderm (en). (C) Sagittal section of the head region showing staining for syndecan in the neural ectoderm (ec), but only faint staining in the underlying mesoderm (mes).

Figure. 2

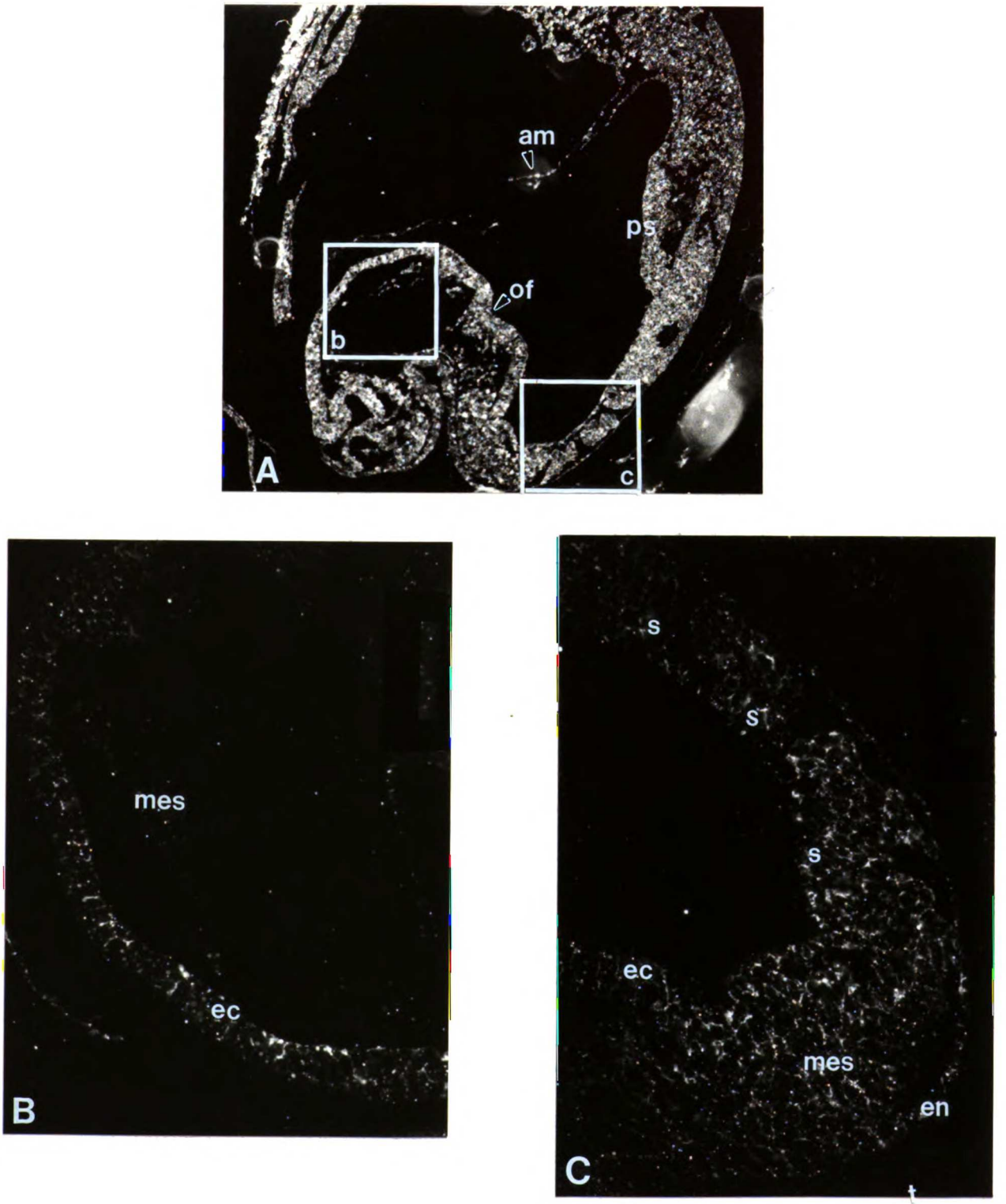


Figure. 3. (A) Darkfield overview of a parasagittal section of a DGD-embedded 8.5 day control embryo. (A) Lower magnification view showing the otic flexure (of), amnion (am), and primitive streak (ps). The boxes marked b and c indicate the regions shown in higher magnification showing monoclonal antibody staining for syndecan in the panels B and C, respectively. (B) Sagittal view of the posterior region of a DGD-embedded 8.5 day control embryo showing monoclonal antibody staining for syndecan in the presomitic mesoderm (mes), the somites (s), the ectoderm (ec), and the endoderm (en). (C) Head region showing staining for syndecan in the neural ectoderm (ec), but only faint staining in the underlying mesoderm (mes).

Figure. 3

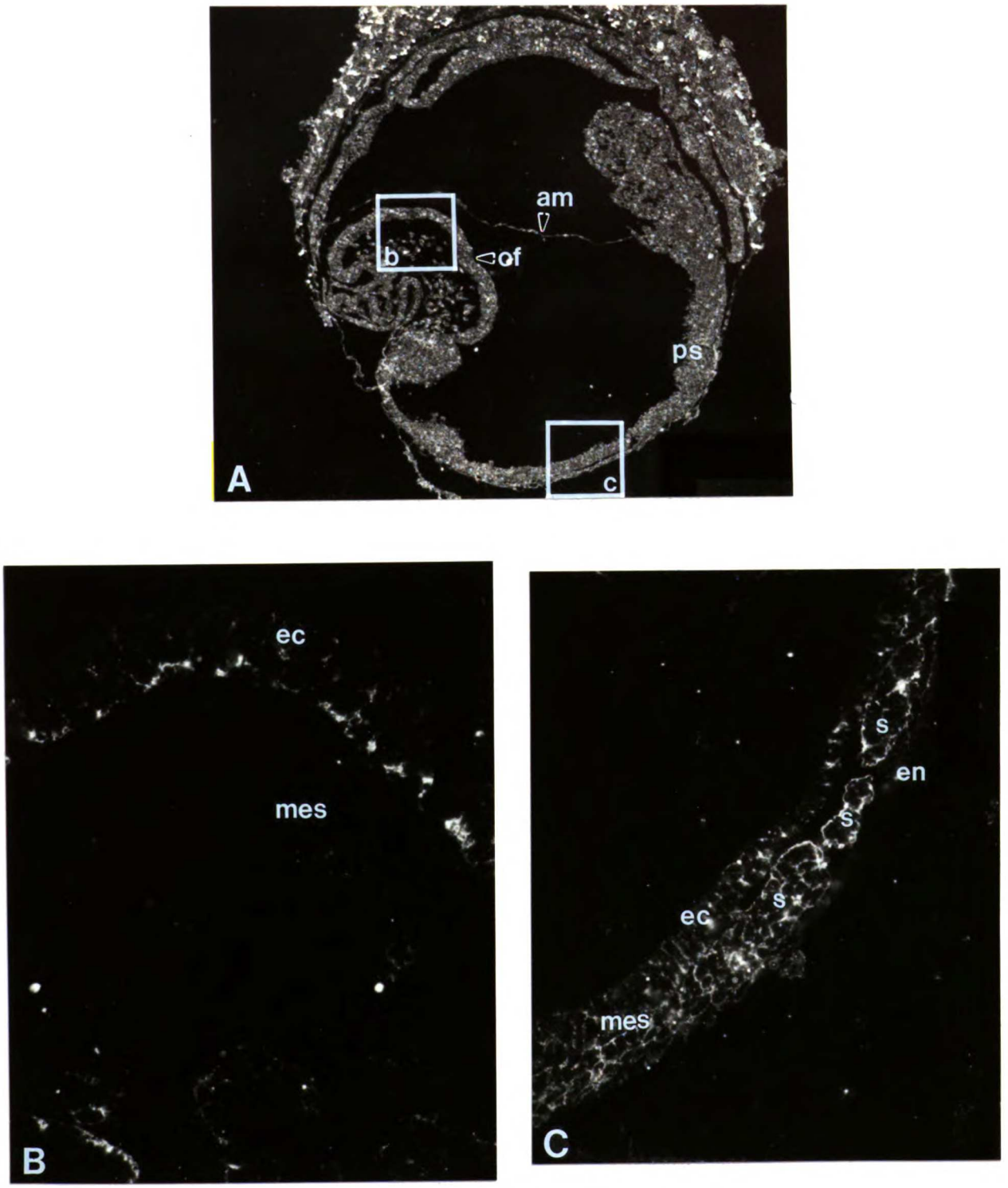


Figure. 4. (A) Sagittal section of RA treated embryo posterior region at high magnification showing monoclonal antibody staining for syndecan in the distal and lateral mesoderm (mes). (B) Sagittal section of the posterior region of a control embryo at high magnification showing monoclonal antibody staining for syndecan in the distal and lateral mesoderm (mes).

Figure. 4

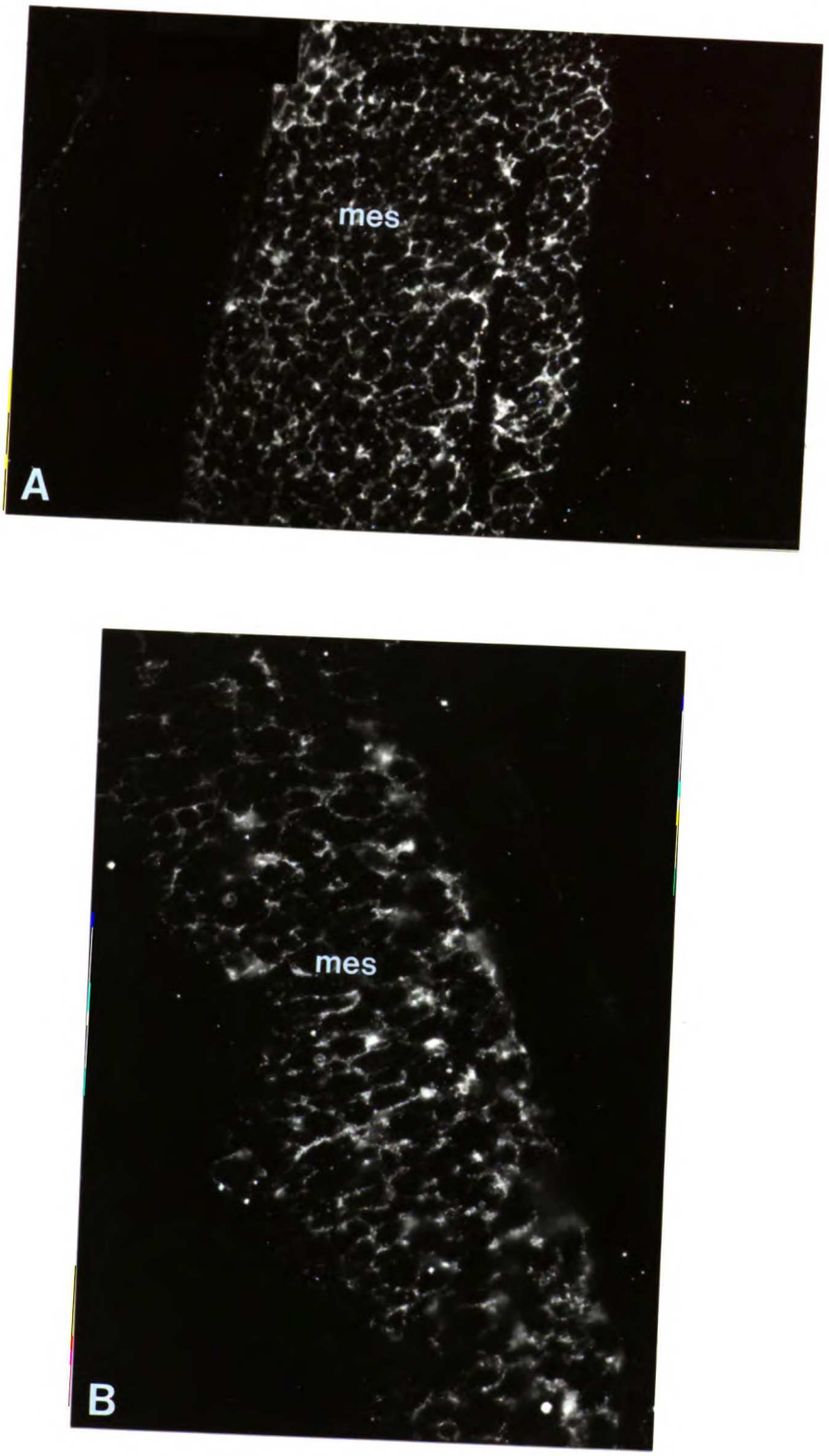
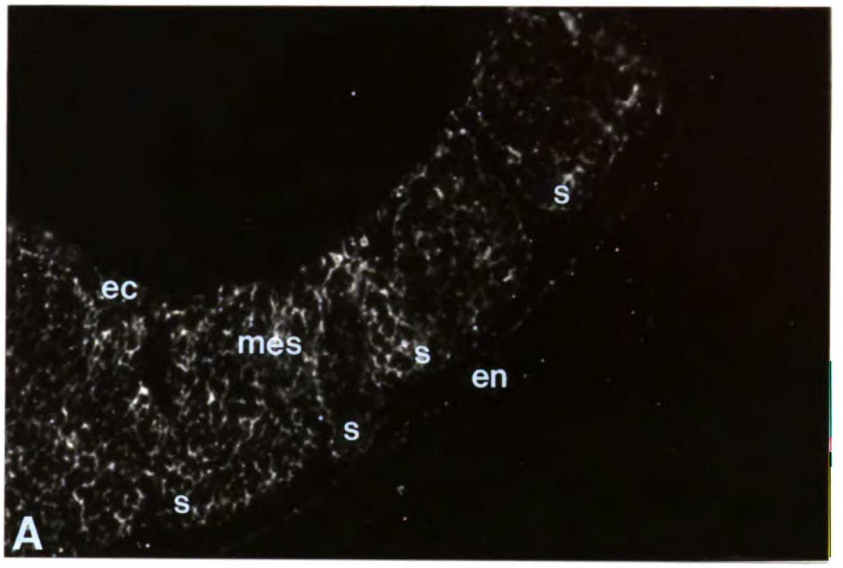


Figure. 5. (A) Sagittal section at high magnification of the somitic region of a RA treated embryo showing monoclonal antibody staining for syndecan in the presomitic mesoderm (mes), the somites (s), the ectoderm (ec), and the endoderm (en). (B) Sagittal section of the somitic region of a control embryo at high magnification showing monoclonal antibody staining for syndecan in the presomitic mesoderm (mes), the somites(s), the ectoderm (ec), and the endoderm (en).

Figure. 5



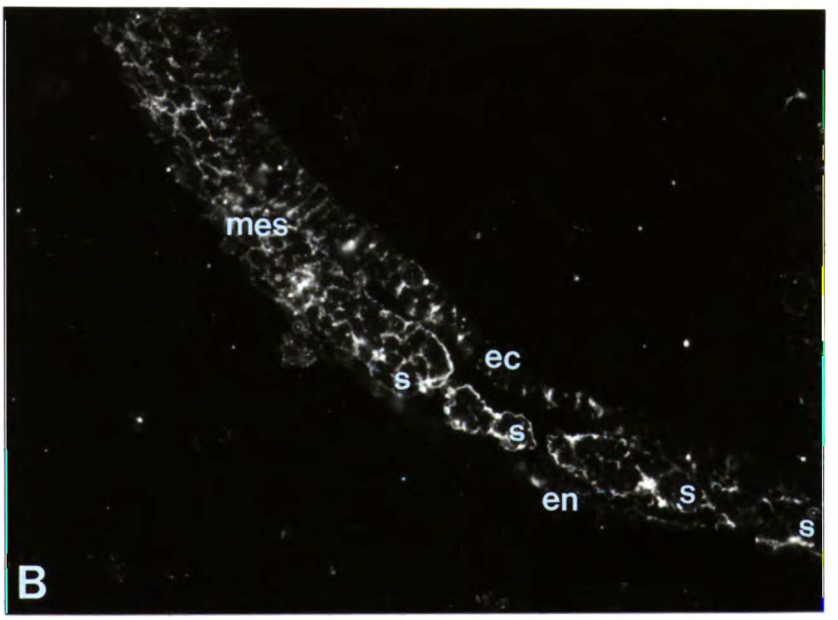


Figure. 6. (A) and (B) Sagittal section of the headfold region of RA treated embryos at moderate magnification showing monoclonal antibody staining for syndecan in the neural ectoderm (ec) on the basal and lateral surfaces and the anterior limit boundary of syndecan staining in the mesoderm (mes), fading distal to the otic flexure (small arrow head). and barely detectable staining anterior to the otic flexure as seen in high magnification view in panel (C).

Figure. 6

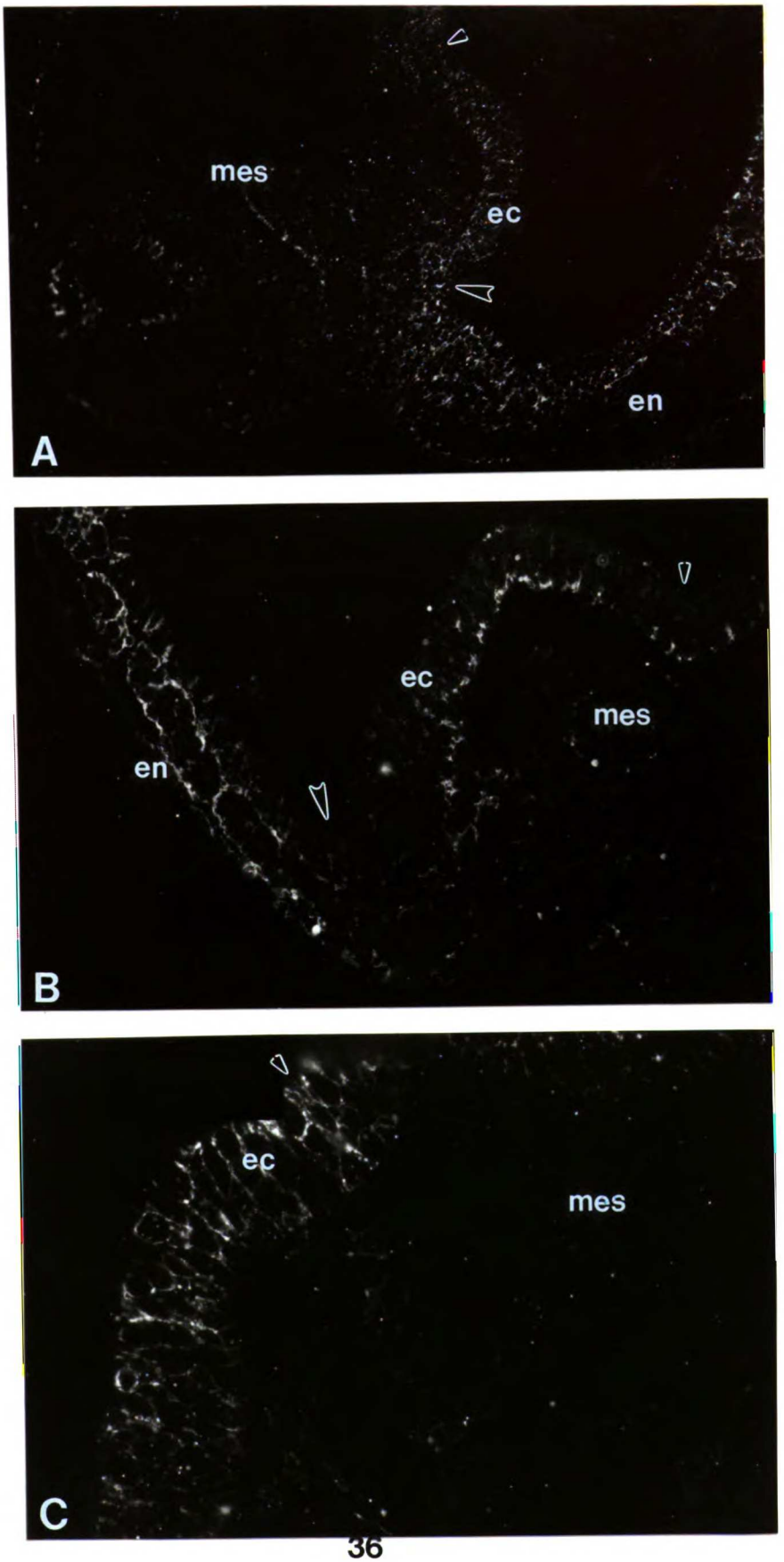


Figure. 6i. (A) and (B) Sagittal section of the headfold region of a control embryos at moderate magnification showing monoclonal antibody staining for syndecan in the neural ectoderm (ec) on the basal and lateral surfaces and the anterior limit boundary of syndecan staining in the mesoderm (mes), fading distal to the otic flexure (small arrow head) and barely detectable staining anterior to the otic flexure as seen in high magnification view in panel (C).

Figure. 6 i

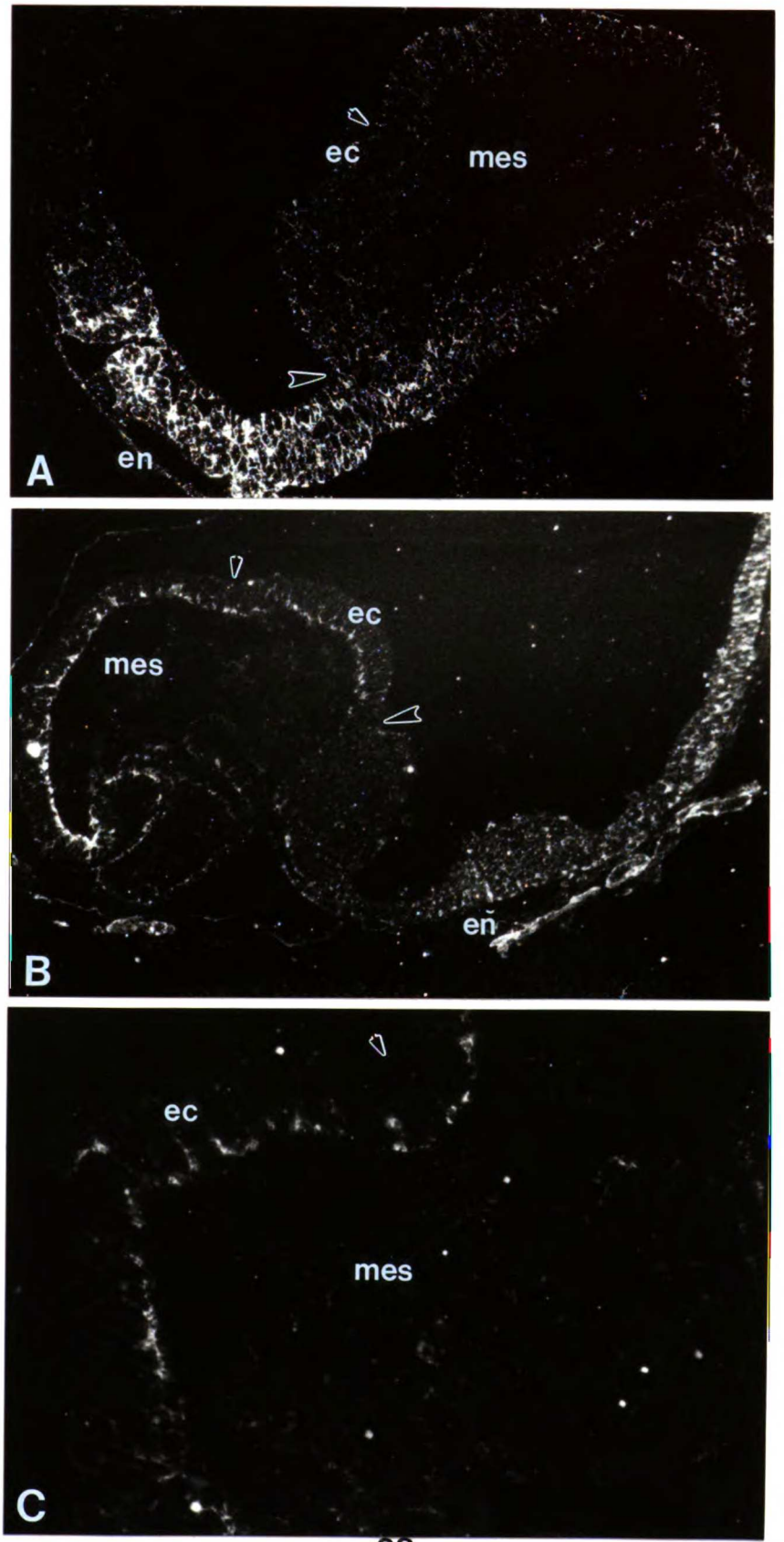
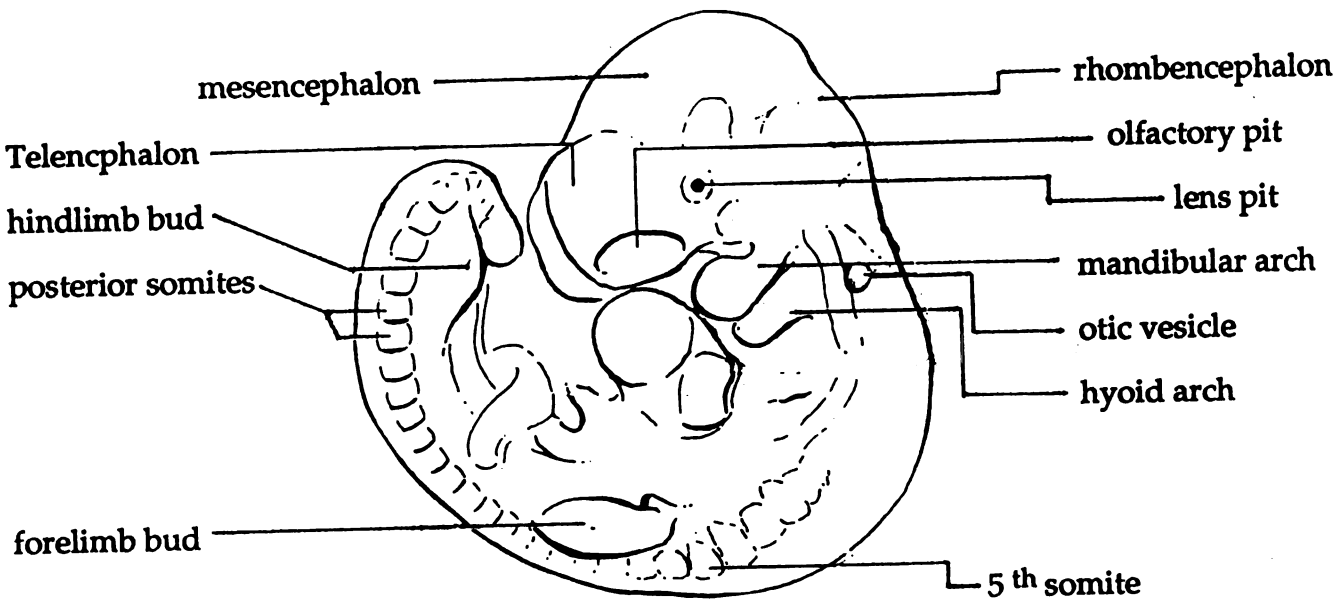


Figure. 7. Schematic representation showing Sagittal view of a whole 9.5 day mouse embryo.

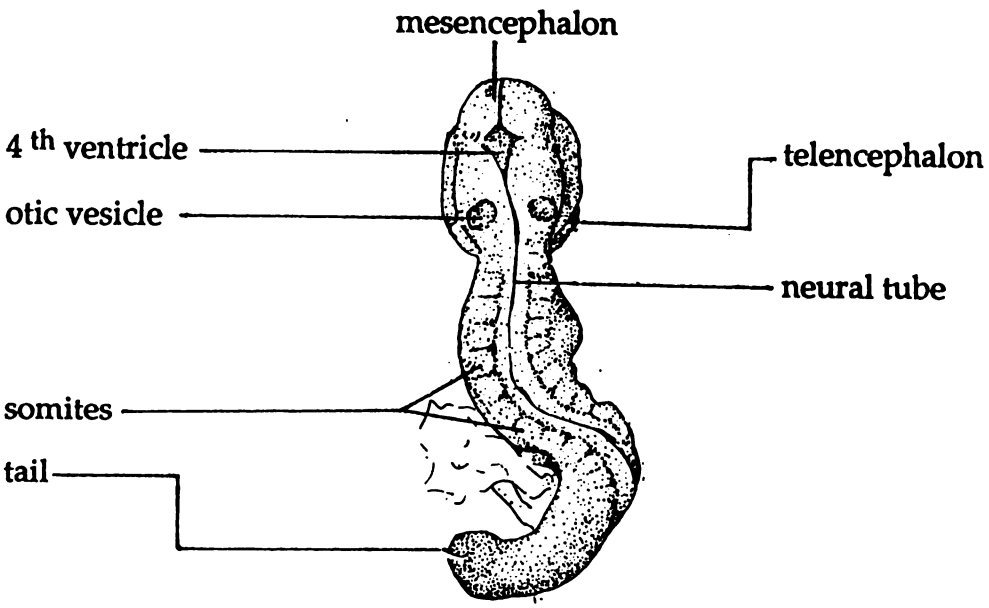
Figure. 7



9.5 day mouse embryo whole mount segittal view

Figure. 8. Schematic representation showing dorsal view of 9.5 day mouse embryo.

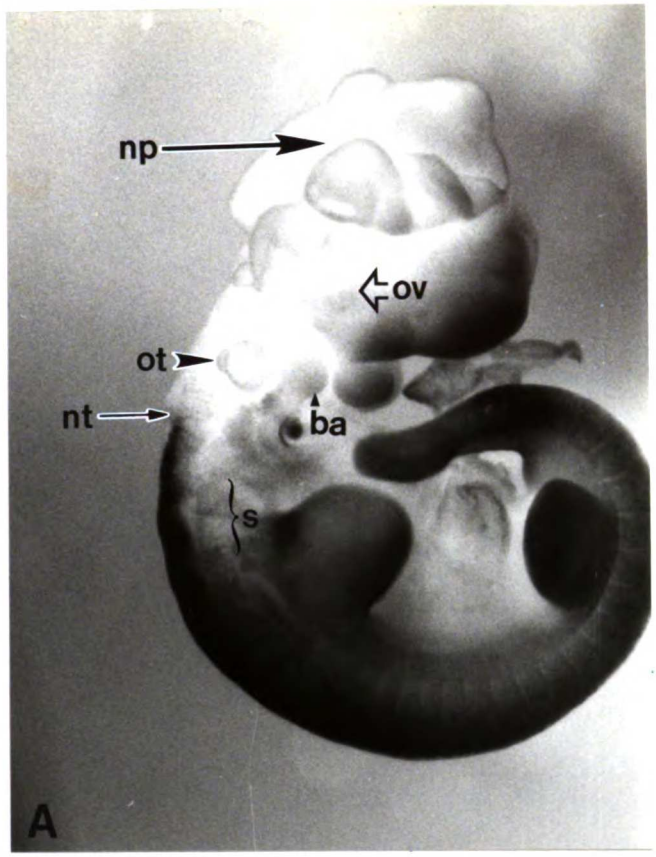
Figure. 8



9.5 day mouse embryo whole mount dorsal view

Figure. 9. (A) Sagittal view of a whole RA treated embryo immunostained with α Hox 2.1a showing the anterior limit of expression in the neural tube (nt), and the somites (s). Also demonstrated is the anterior displacement of the optic vesicle (ov), the 2nd branchial arch (ba), and the otic vesicle (ot). Note also the lack of brain development and the open anterior neuropore (np). Panel (B) a normal control embryo stained with α Hox 2.1a showing the anterior limit of expression in the neural tube (nt), and the somites (s). Further seen is the normal position and development of the optic vesicle (ov), the 2nd branchial arch (ba), and the otic vesicle (ot). Lastly, note the closed anterior neuropore (np).

Figure. 9



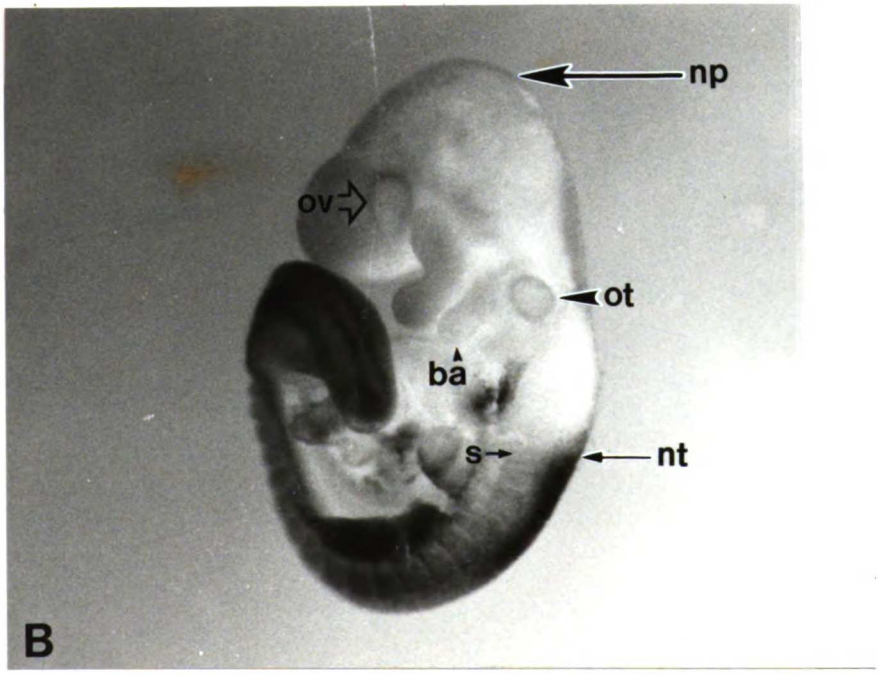
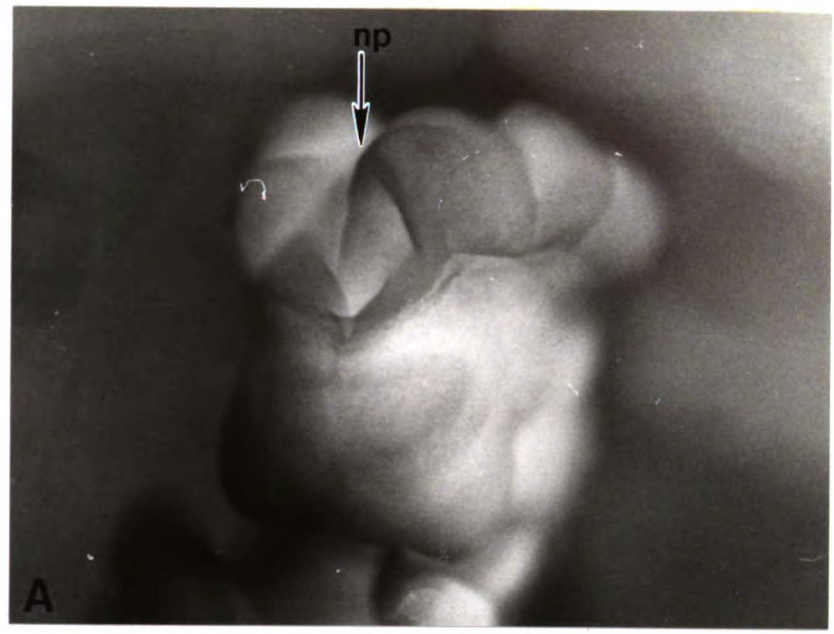


Figure. 10. (A) Frontal view of whole experimental embryo showing open neuropore (np) and lack of anterior brain formation. (B) Frontal view of control embryo showing normal development and morphology of craniofacial structures.

Figure. 10



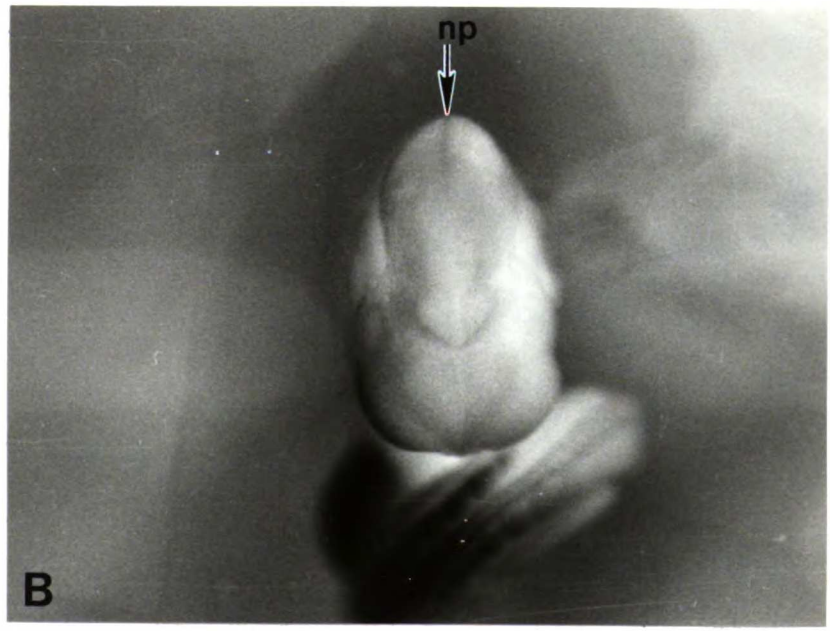


Figure. 11. (A) sagittal view of whole 9.5 day experimental embryo showing closed neuropore (np) and normal brain formation but evidencing an unregistered anterior shift in Hox 2.1 staining within the neural tube (nt) and the somites (s). (B) Sagittal view of control embryo showing normal development and morphology of craniofacial structures and unaltered registered anterior boundary of Hox 2.1 staining within the neural tube (nt) and the somites (s).

Figure. 11

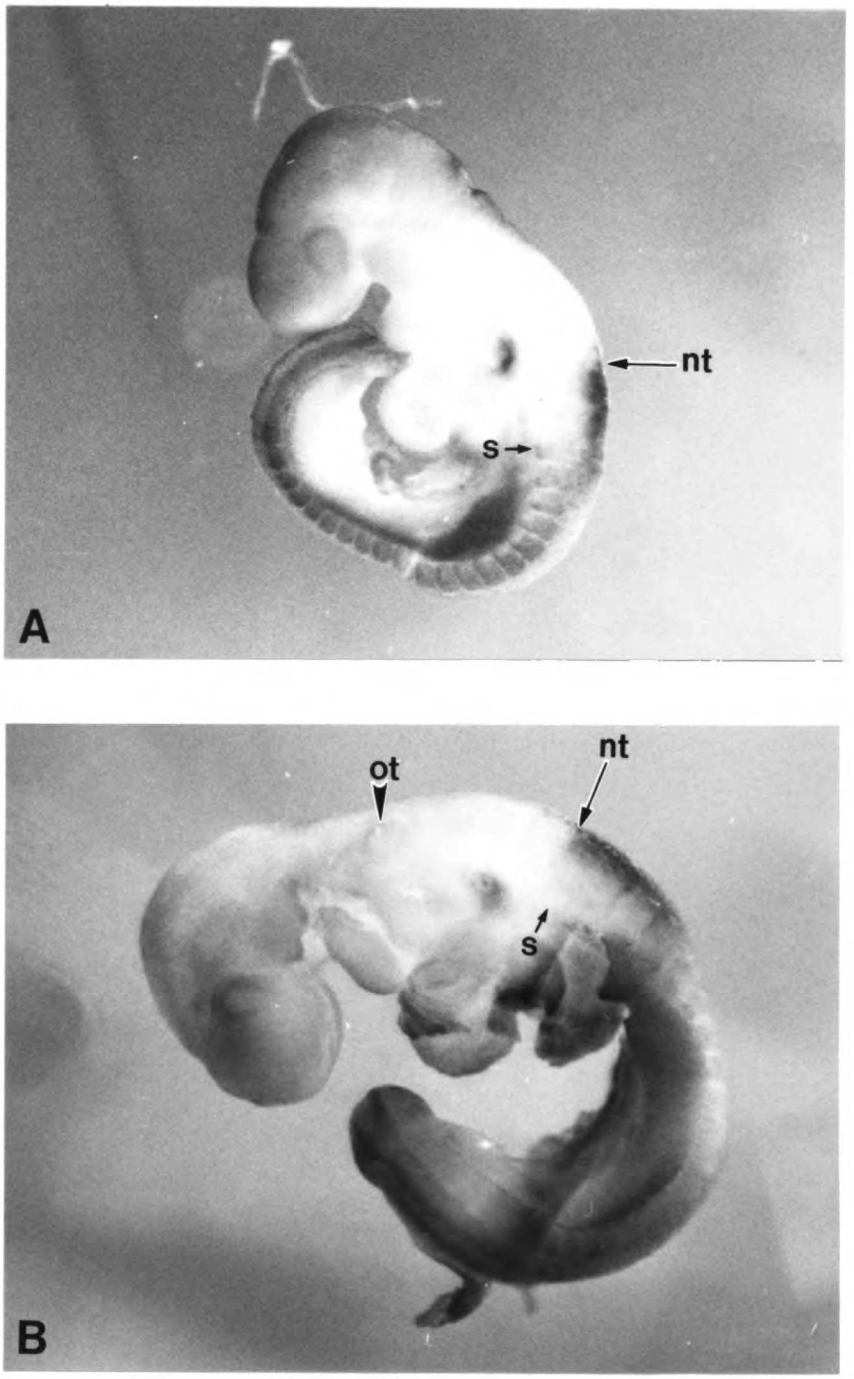
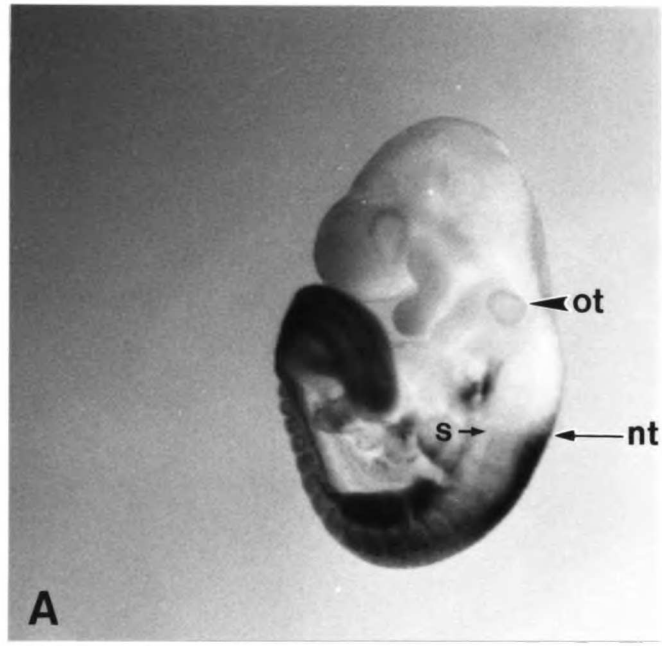


Figure. 12. (A & B) Sagittal and dorsal view of whole 9.5 day control embryo immunostained with α Hox 2.1 showing the coincident anterior limit boundary of expression in the neural tube (nt), and somites (s). Also demonstrated is the placement of the otic vesicle (ot) relative to the branchial arches (ba) and the normal brain development.

Figure. 12



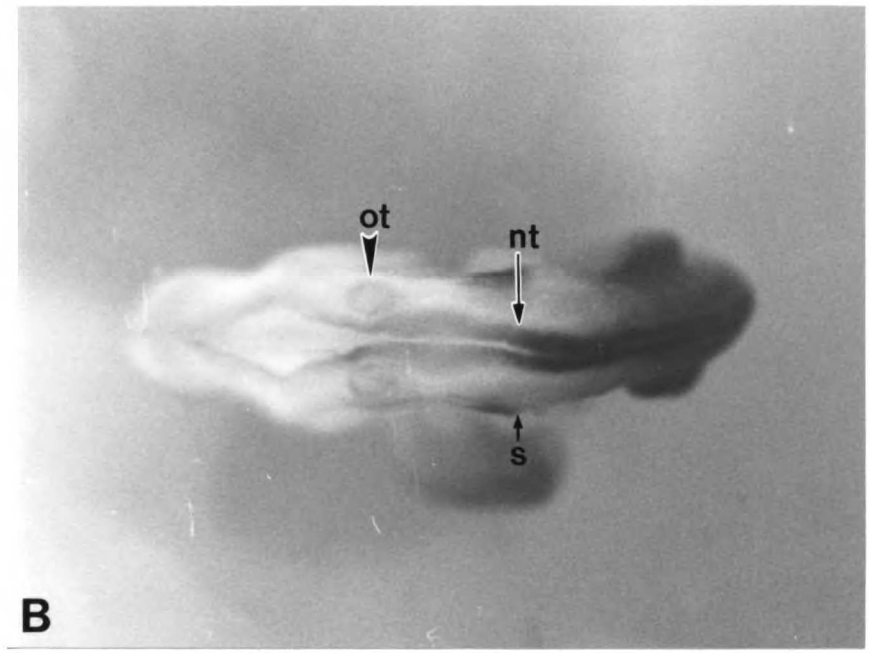


Figure. 13. (A & B) Dorsal oblique views of two RA treated 9.5 day embryos stained with α Hox 2.1 demonstrating the arteriorly advanced and unregistered shift of Hox 2.1 expression within the neural tube (nt) and somites (s). The anterior boundary of Hox 2.1 stain within the somites is less well defined in RA treated embryos. Also seen is the open anterior neuropore (np) and the anteriorly displaced otic vesicle (ov).

Figure. 13

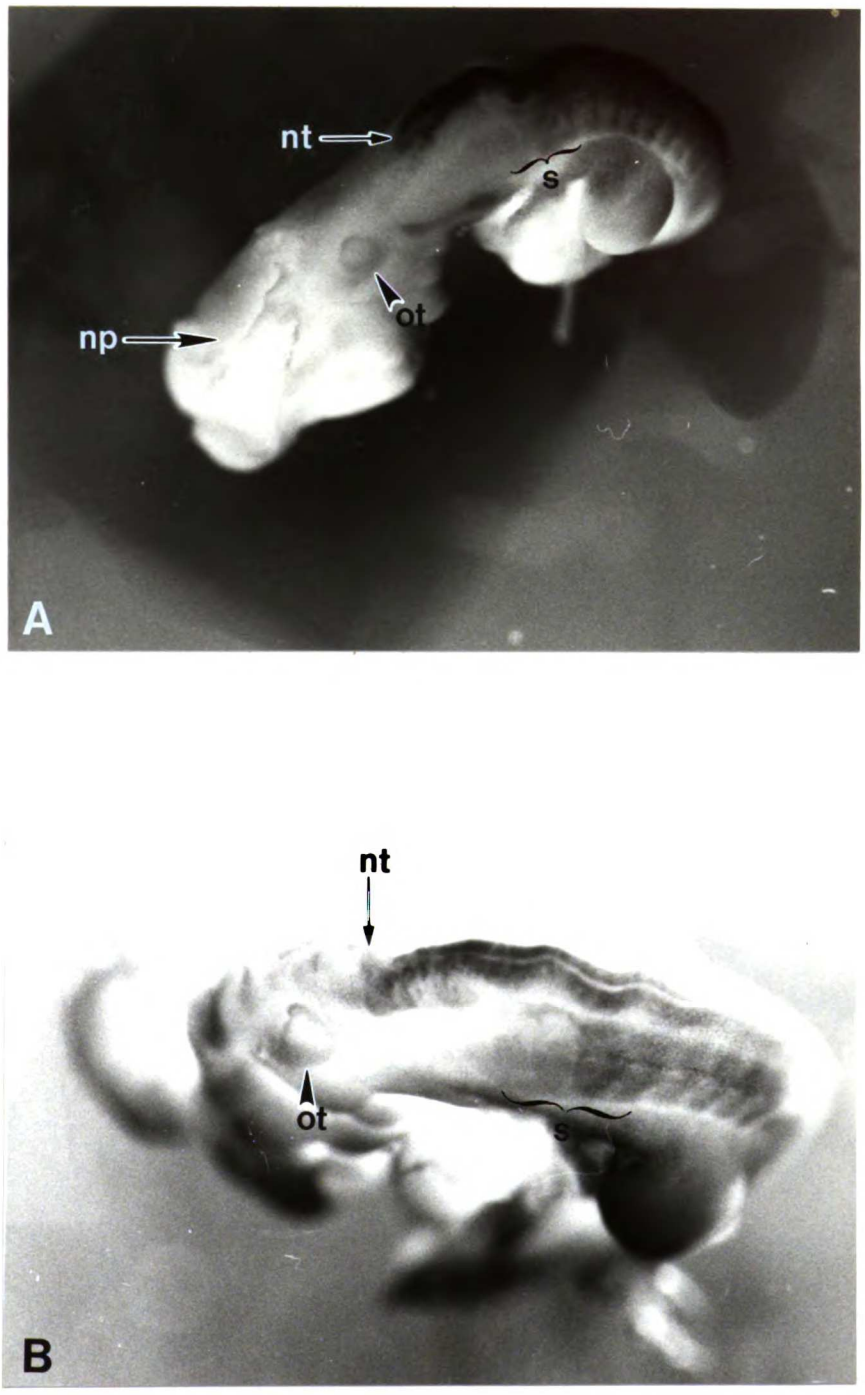


Figure. 14. (A) RA treated 9.5 day embryo dorsal view showing altered α Hox 2.1 expression in the neural tube. Note the proximity of the anterior limit boundary of Hox 2.1 staining to the otic vesicle (ot). (B) Control 9.5 day embryo dorsal view showing normal α Hox 2.1 expression in the neural tube.

Figure. 14

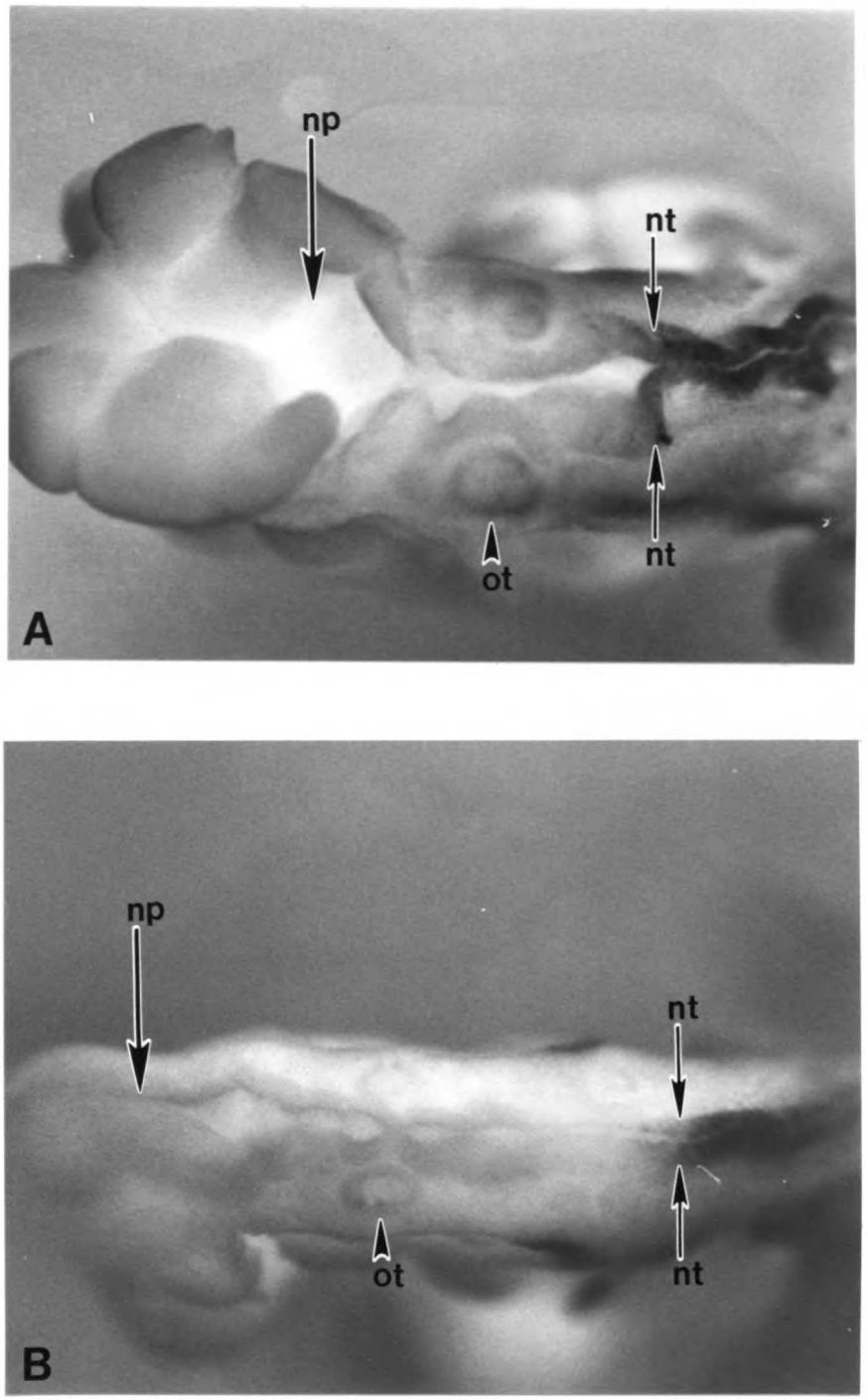


Figure. 15. (A & B) Dorsal oblique views of two RA treated 9.5 day embryos showing unregistered anterior shift in Hox 2.1 stain within the neural tube (nt) and less clearly defined anterior limit of Hox 2.1 staining within the somites (s). Note the proximity of the anterior boarder of staining within the neural tube and somites to the otic vesicle (ov).

Figure. 15

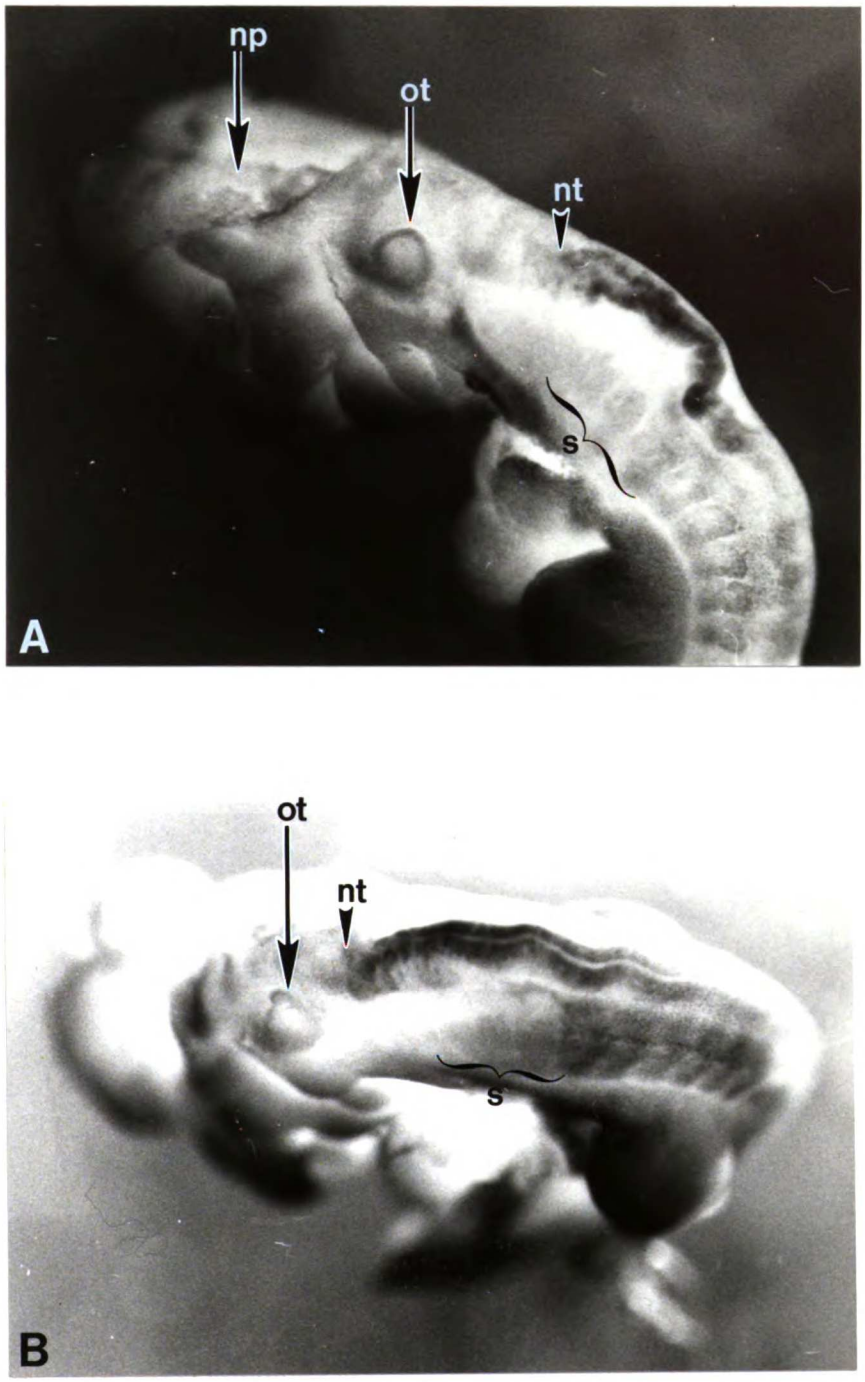
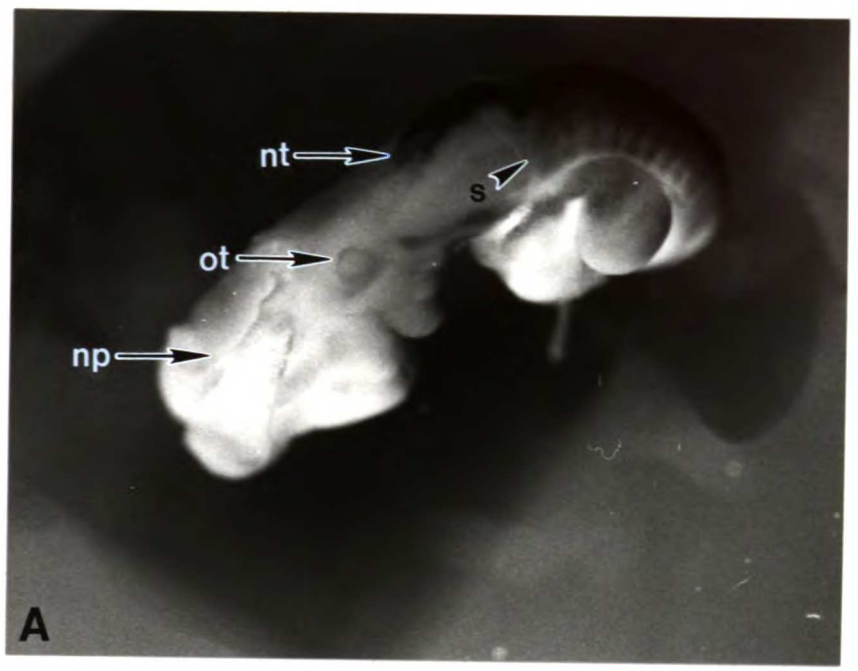


Figure. 16. (A) Dorsal oblique view of RA treated 9.5 day embryo demonstrating unregistered anterior shift in Hox 2.1 stain within the neural tube (nt) and the somites (s). (B) Sagittal view of control embryo demonstrating normal registered anterior limit of Hox 2.1 stain within the neural tube (nt)and somites (s).

Figure. 16



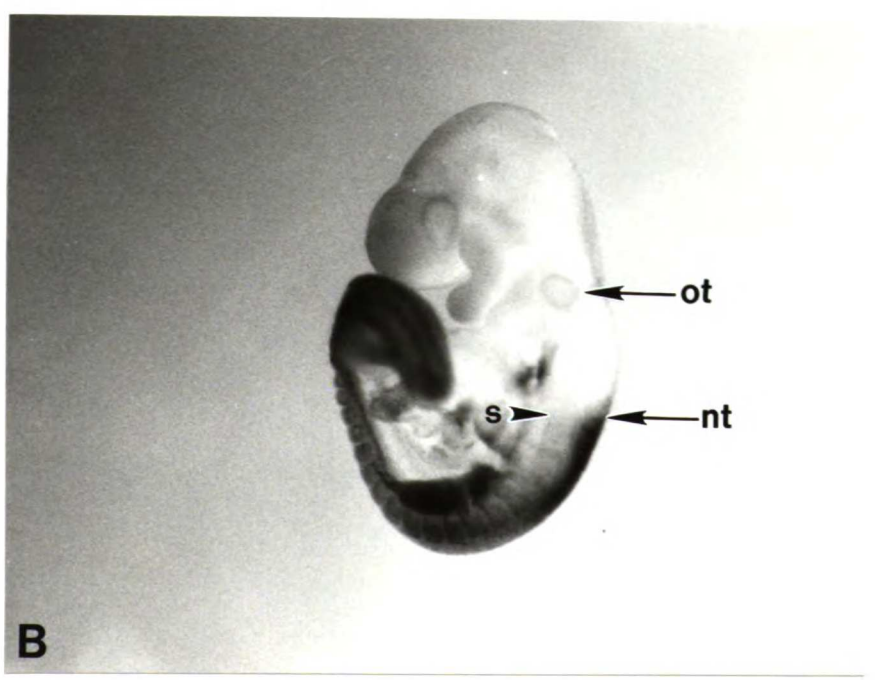
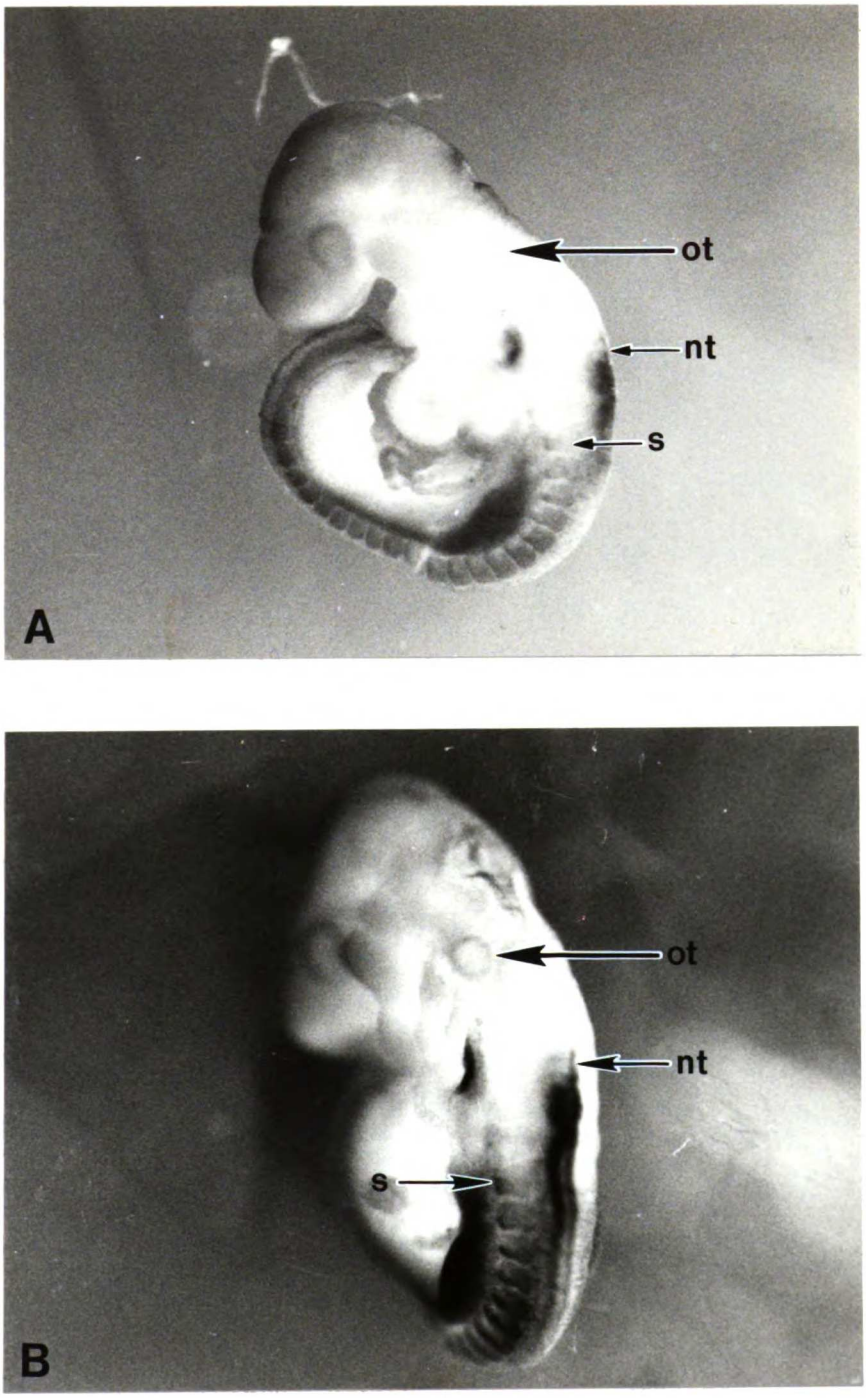


Figure. 17. (A) Sagittal view of RA treated 9.5 day embryo showing normal morphologic development but altered Hox 2.1 expression. Note the unregistered anterior shift of Hox 2.1 staining within the neural tube (nt), and the less clearly defined anterior boundary of Hox 2.1 staining within the somites (s). (B) Dorsal view of same treated embryo showing normal morphologic development but altered Hox 2.1 expression.

Figure. 17



DISCUSSION

Hypothesis and goal of this study

The hypothesis tested in these experiments was that the anterior-posterior distribution of syndecan may be related to anterior-posterior patterning homeobox gene expression, and may thus be altered by the addition of exogenous retinoic acid. This hypothesis is supported by previous data showing that syndecan-1, has a patterned expression along the A-P axis in the mesoderm. as might be expected of a downstream Hox gene product target, as well as by evidence that a regulatory region in the syndecan promoter shows homology to the consensus sequence for binding antennapedia homeobox protein. If the hypothesis is correct, treating embryos with exogenous RA should cause the anterior boundary of syndecan to move anteriorly with respect to anatomical or biochemical markers that are not affected by the addition of exogenous retinoic acid, or alternatively, move anteriorly in concert with markers that are anteriorly displaced by the addition of exogenous RA. Our findings demonstrate that there is no change in the A-P asymmetry of syndecan expression in RA treated embryos as compared to untreated control embryos examined similarly.

Syndecan

Syndecans are developmentally regulated transmembrane proteoglycans with marked heterogeneity of the heparin sulfate chains on the cell surface and a

highly conserved polypeptide in the cytoplasm (Rapraeger et al., 1986). The core protein also shows extensive divergence in the extracellular domain (Rapraeger et al., 1985). This difference in domains is similar to that seen in signal transducing receptors, which bear conserved enzymatically active domains in the cytoplasm but have less well conserved ligand binding domains extracellularly (Doolittle 1991).

The glycosaminoglycan (GAG) chains on syndecan are critical to its function . The heparin sulfate chains allow a multitude of various molecules to adhere to the cell surface while the chondroitin sulfate may modify the protein binding characteristics of syndecan. In addition to serving as a receptor to multiple molecules, syndecan may also serve as a co-receptor for FGF by acting in concert with the high affinity receptors to form a functional receptor complex which binds the ligand and mediates its action. Assuming that syndecan's structural variability modifies its binding characteristics this versatility in the co-receptor model would yield a combinatorial system allowing a wide variety of specific interactions. Such interactions are likely to be important to the process of development. developmental interactions.

Asymmetric distribution of syndecan in both experimental and control groups.

Previous data have shown that syndecan expression changes upon mesenchymal differentiation. For example, the loss of syndecan expression in association with mesenchymal differentiation has been seen in the developing tooth and limb (Solursh, 1990). The loss of syndecan appears indeed to be the initial change in the differentiation of central core mesenchymal cells of the limb into myoblasts and chondroblasts (Solursh, 1990). From these observations, the spatio-temporal pattern of syndecan expression might be expected to follow morphogenetic rather than histologic boundaries. Immunohistochemical

staining for syndecan observed in the mesoderm of gastrulating control and experimental embryos alike was strong on the more posterior and lateral structures (primitive streak, somites) and weak on anterior structures (anterior head mesoderm). Syndecan's anterior limit boundary of expression was no different in either the experimental or the control groups, and appeared to coincide with the otic invagination. This asymmetric pattern of expression in both experimental and control embryos still suggests a role for syndecan in both mesodermal differentiation and anterior-posterior patterning.

Retinoic acid morphogenetic signaling system

A retinoic acid based morphogenetic signaling system is hypothesized to exist along the anterior-posterior (AP) axis in the developing embryo (Conlon and Rossant, 1990). Destruction of this retinoic acid system by the addition of exogenous RA is known to have a posteriorizing effect on embryonic development (Sulik, 1988). Also, the anterior boundary of expression of many, but not all, homeodomain containing transcription factors (HOX genes) has been observed to be extended anteriorly in the presence of exogenous RA, suggesting that retinoic acid might regulate the expression of these HOX genes (Simeone, 1991). HOX gene products in turn affect the expression of other genes which ultimately form the structural features of the embryo. The distribution of syndecan in the 8.5 day embryo and the presence of an antennapedia consensus binding sequence in it's promoter suggest that syndecan may be a downstream target of Hox genes. The results of this work demonstrate that syndecan-1 expression is not likely to be regulated by those Hox genes that are affected by retinoic acid (Conlon and Rossant, 1991).

Exogenous RA effects on morphology and Hox 2.1 expression

Administration of a teratogenic dose of RA to early gastrula mouse embryos resulted in a syndrome of defects in the craniofacial region as has been previously demonstrated (Cook, 1991). Most strikingly, an open anterior neuropore was seen in severely affected embryos, along with a reduction in the size, or even complete absence, of the forebrain and midbrain and loss of hindbrain segmentation. A resultant anterior displacement of the otic vesicle and anterior somites was noted. Our data show that RA treatment at this stage results in the anterior displacement of the boundary of Hox 2.1 expression in both the neural tube and anterior somites. The induced changes in Hox 2.1 expression were evident even in embryos that lack gross morphological defects; retinoic acid treated embryos that appeared morphologically normal upon examination, displayed an anterior boundary shift in Hox 2.1 expression.. This suggests that these changes in gene expression are a primary response to retinoic acid rather than sequelae of the disruption of normal development. The altered expression of Hox 2.1 may further be partly, but not necessarily, responsible for the noted induced morphological changes, in that the anterior displacement of the ectopic expression of Hox 2.1 staining correlates with the direction of morphological transformations observed.

Conclusions

It appears from the evidence presented that although the asymmetric pattern of syndecan expression suggests a role for syndecan in anterior-posterior patterning, that this expression pattern is not solely regulated by an endogenous retinoic acid gradient. It remains to be elucidated what factors control syndecan's expression pattern within the mesoderm along the anterior-posterior axis. Likewise although the Hox 2.1 expression pattern was altered by

exogenous RA it remains to be demonstrated conclusively that the altered expression pattern of Hox 2.1 and likely other Hox genes induced by RA are directly responsible for the craniofacial aberrations noted. The correlation between the ectopic expression of Hox 2.1 staining and the observed morphological effects resulting from RA treatment do support some sort of connection. Ideally, Hox gene knockout experiments would provide more definitive evidence supporting this connection.

Methodology

In evaluating the methods employed in gathering our data several issues stand out. First, a technical issue of ensuring that the animals harvested from the colony and impregnated were truly pregnant. On average only half of those animals sacrificed were pregnant in both the experimental as well as the control groups, for reasons that are not understood Both super-ovulated and natural matings were done, with similar results. In addition, in those animals that were pregnant the observed stage of embryonic development was quite variable between embryos and embryos were often as much as 15-20 hours premature. This complicated the gathering of appropriate stage embryos for examination.

Second, obtaining true sagittal and parasagittal sections of embryos for immunostaining was difficult, as great skill was required in orienting and then sectioning the embedded embryos. Also contributing to this issue is a developmental process that occurs between day 8.0 and 9.0 of development known as turning. This is a process by which the embryo rotates 180° around its A-P axis. Embryos that were embedded and sectioned with less than ideal orientation, or while in the process of rotation could not provide useful information.

Third, establishing a positive control ensuring that treated embryos were truly affected by the addition of exogenous retinoic acid was somewhat difficult in that no gross morphologic changes are evident when syndecan expression was evaluated (8.5 day). Having a second biochemical marker which was affected by the addition of exogenous RA identifiable within the same section or adjacent sections would have been ideal. Unfortunately, the Hox gene for which we were able to obtain antibodies were not expressed at 8.5 days. Therefore, some treated and control embryos were allowed to develop further in order to observe the type of gross morphological alterations induced, and their frequency in the litters. At this later time, we were able to stain with an antibody against Hox 2.1a (a marker that is affected by RA). These later stage embryos enabled us to quantify the penetrance, within our sample, of the retinoic acid treatment. The penetrance based upon gross morphological inspection appeared to be approximately 80% . By biochemical analysis of Hox 2.1a staining, 96% of the embryos treated showed signs of RA treatment effect. Thus, the retinoic acid treatment was quite effective.

It would have been ideal to directly compare syndecan and Hox 2.1 expression at both the 8.5d. and 9.5-10.0d. periods. As indicated above, Hox 2.1 expression was not detected at day 8.5. Future studies could compare expression of syndecan and other Hox genes by in situ hybridization at this stage. At 9.5d., Hox 2.1 was detected by whole mount Immunohistochemistry. Syndecan was also localized in whole mount embryos. However, staining of ectoderm and endoderm was uniform and intense, making it difficult to determine staining pattern in the mesoderm. In the future, one could embed, section, and stain for syndecan whole mount embryos previously stained for Hox 2.1 expression. The stability of Hox 2.1 whole mount staining during the embedding and sectioning process, however, is not known. These approaches would ensure a direct

positive control for RA effects on syndecan expression and possibly provide further evidence concerning the regulation of syndecan expression by retinoic acid.

BIBLIOGRAPHY

Akam, M. 1987. The molecular basis for metameric pattern in drosophila embryo. Development 10, 1-22.

Akam ,M. 1989. Hox and HOM. Homologous gene clusters in insects and vertebrates. Cell 5, 347049.

Conlon, A. and Rossant, J. 1992. Exogenous retinoic acid rapidly induces anterior ectopic expression of murine Hox-2 genes in vivo. Dev. 116, 357-368.

Dale, L. and Slack, J. 1987. Fate map for the 32 cell stage of Xenopus laevis. Development. 99, 527-51.

IBID 1987. Regional specification within the mesoderm of early embryos of Xenopus laevis. Development. 100, 279-95.

Dale, L. Smith, J. and Slack, J. 1985. Mesoderm induction in Xenopus laevis: a quantitative study using cell lineage label and tissue-specific antibodies. J. Embryol. exp. Morph. 89, 389-312.

Duboule, D. Dolle, P. 1989. The structural and functional organization of the murine Hox gene family resembles that of Drosophila homeotic genes. EMBO J. 8, 1497-505.

Hunt, P. et al., 1991. The branchial Hox code and its implications for gene regulation, patterning of the nervous system and head evolution. Development. 113, 63-67.

Hunt, P. et al., 1991. Patterning of the vertebrate head: Murine Hox 2 genes mark distinct subpopulations of premigratory and migrating neural crest. Development 11, 43-51.

Hussmann, M. et al., 1991. Antagonism between retinoic acid receptors. Mol. Cell. Biol. 11, 4097-4103.

Maden, M. et al., 1992. Retinoic acid binding protein, rhombomeres and the neural crest. Development. 111, 35-44

McGinnis, W. and Krumlauf, R. 1992. Homeobox genes and axial patterning. Cell 68, 283-382.

Melton, D. A. et al., 1989. Growth factors in early embryogenesis. Ann. Rev. Cell Biol. 5, 93-118.

Morriss-Kay, G. M. et al., 1991. Effects of retinoic acid excess on expression of Hox-2.9 and Krox-20 and on morphological segmentation in the hindbrain of mouse embryos. EMBO J. 10, 2985-2995.

Nakamura, O. and Matsuzawa, T. 1967. Differentiation capacity of the marginal zone in the morula and blastula of Triturus pyrrhogasrter. Embryologia 9, 223-237.

Nieuwkoop, P. 1969. The formation of mesoderm in the Urodelean amphibians. I. Induction by the endoderm. Wilhelm Roux' Arch. EntwMech. Org 162, 341-373.

Ogi, K. 1969. Regulative capacity in the early amphibian embryo. Res. Bull. Dept. Gen. Ed. Nagoya Univ. Biol. 33, 239-47.

Rapraeger, A. et al., 1986. The cell surface proteoglycan from mouse mammary epithelial cells bears chondroitin sulfate and heparan sulfate glycosaminoglycans. J. Biol. Chem. 260, 11046-11052.

Rossant, J. et al., 1991. Expression of a retinoic acid response element-hsplacZ transgene defines specific domains of transcriptional activity during mouse embryogenesis. Genes Dev. 5, 1333-41.

Conlon, R. and Rossant, J. 1992. Exogenous retinoic acid rapidly induces anterior ectopic expression of murine Hox-2 genes in vivo. Dev. 116, 357-368.

Slack,J. et al 1987. Mesoderm induction in early Xenopus embryos by heparin-binding growth factors. Nature, Lond. 326, 197-200.

Slack, J. and Isaacs, H. 1989. Presence of basic fibroblast growth factor in early Xenopus embryo. Dev. 105, 147-53.

Slack, J. Isaacs, H. and Darlington, B. 1988. Inductive effects of fibroblast growth factor and lithium ion on Xenopus blastula ectoderm. Dev. 103, 581-90.

Smith, J. 1987. A mesoderm inducing factor is produced by a Xenopus cell line. Dev. 99, 3-14.

Smith, J. and Slack, J. 1983. Dorsalization and neural induction: properties of the organizer in Xenopus laevis J. Embryol> exp. Morph. 78, 299-317.

Smith, S. and Eichele, G. 1991. Temporal and regional differences in the expression pattern of distinct retinoic acid receptor-β transcripts in the chick embryo. Dev. 111, 245-52.

Smith, J.1989. Mesoderm induction and mesoderm inducing factors in early amphibian development. Development 105, 665-677.

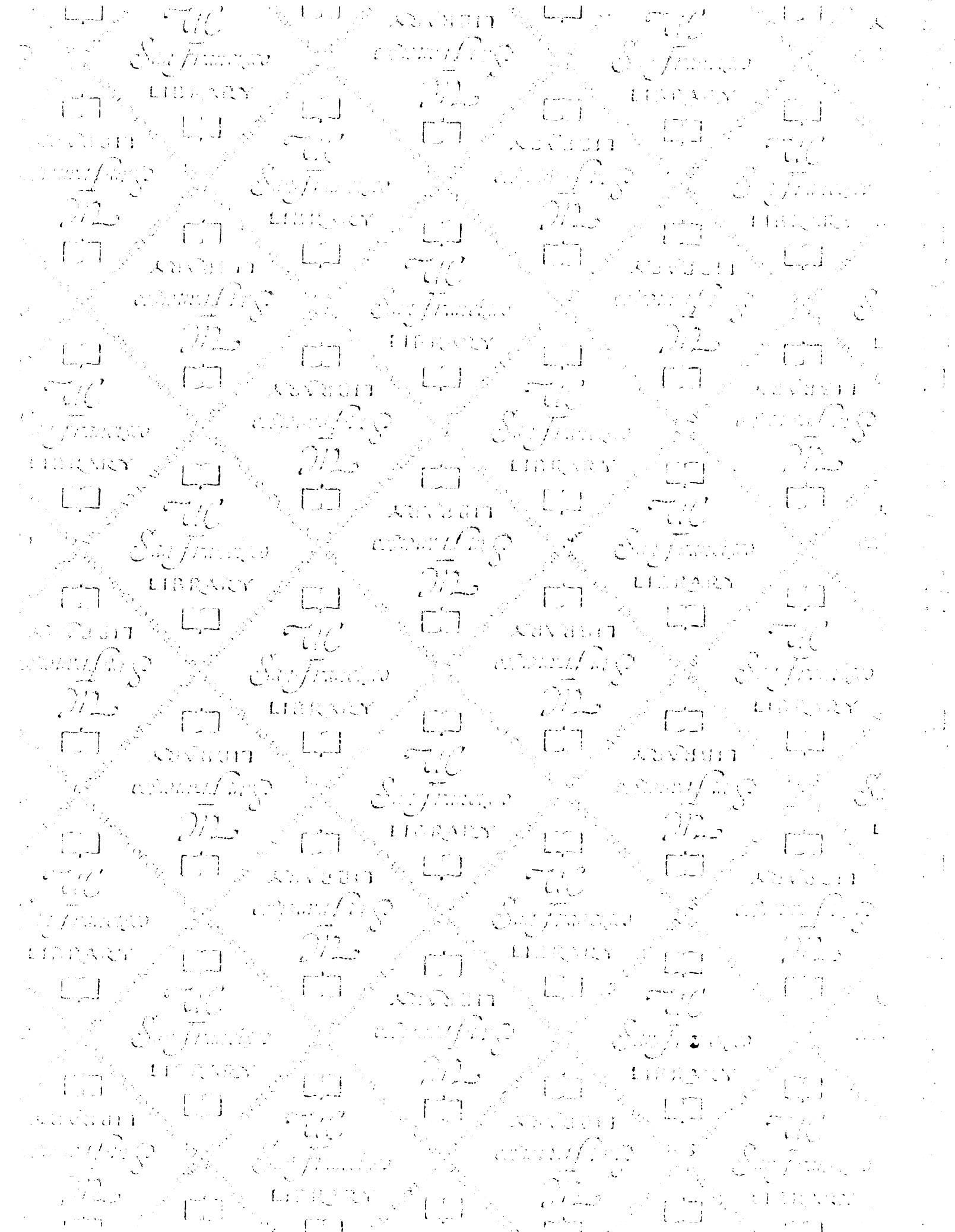
Stuart, J. et al 1991. A deficiency of the homeotic complex of the beetle Tribolium. Nature 350, 72-74.

Sutherland, A. E., et al., 1991. Expression of syndecan, a putative low affinity fibroblast growth factor receptor, in the early mouse embryo. Development. 113, 339-351.

Thaller, C. and Eichele, G. 1987. Identification and spatial distribution of retinoids in the developing chick limb bud. Nature 343, 177-80.

Wall, N. et al 1991. Expression and modification of Hox 2.1 protein in mouse embryos. Development 37, 111-120.

Wilkinson, D. et al 1989. Segmental expression of Hox 2 homeobox containing genes in the mouse hindbrain. Nature 34, 405-9.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