SPECIFICATION OF THE MIDLINE OF THE UPPER BEAK OF THE CHICK EMBRYO

by

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A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree of

MASTER OF SCIENCE

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ABSTRACT

During embryonic development mesenchymal cells give rise to a number of different cell types including cartilage, muscle, and connective tissue. As these cell types differentiate, mechanisms must exist for establishing the spatial patterns of the forming tissues. In the chick embryo, the midline of the beak is defined by the differentiation of a rod of cartilage (the prenasal cartilage) in the centre of the frontonasal mass, and an egg tooth at its distal tip. The objective of this thesis is to explore the mechanisms involved in the specification of the midline of the chick embryo.

Initially, I investigated the chondrogenic potential of the chick frontonasal mass, from where most of the upper beak develops. At early stages of development, fragments of the primordium were able to give rise to midline structures.

Retinoic acid, a biologically active metabolite of retinol (vitamin A), causes severe defects in the upper beak when administered systemically to chick embryos. I analyzed the effects of retinoic acid on the face when released from a slow-release carrier placed in the nasal pit. The aim of this experiment was to augment the levels of endogenous retinoic acid and assess the changes in facial pattern. The increased concentration of retinoic acid in the nasal pit region, did not induce reduplication of midline structures because a mirror image of a suspected endogenous gradient was not created.

Tenascin, an extracellular matrix protein present in embryonic tissues, is associated with areas undergoing epithelial-mesenchymal interactions and chondrogenesis. Its distribution was mapped in the normal and abnormal embryonic face, during development. Tenascin distribution correlated with the establishment of the midline of the chick upper beak.

DEDICATION

To my Parents, *Jorge and Julieta*

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CHAPTER I

GENERAL INTRODUCTION

The purpose of this thesis is to study the mechanisms that lead to the specification of the mediolateral axis of the face of the chick embryo. I will explore this problem by investigating first how the frontonasal mass develops, and then examining the role of two molecules, retinoic acid and tenascin, in establishing the midline of the face. In this chapter I will review the development of the chick face, and also the role of retinoids and tenascin in embryological development.

I. Development of the embryonic chick

The stages of development of the embryo will be described using the classification of Hamburger and Hamilton (1951), which identifies and describes embryos on the basis of external characteristics. During development, different characteristics become prominent and these external features are particularly useful for assessing the stage of development.

In the next section the external features that are prominent from 2.5 days to 10 days of incubation (stage 15 to 36) are described. This time span encompasses all the stages of development used in the subsequent studies.

It takes approximate 2.5 days for the chick embryo to reach stage 15. By this stage the head is well developed but there is no sign of the future limb buds, and the embryo is very straight (Fig. I:1A).

One day later the embryo has reached stage 20 (3.5 days of incubation). The facial primordia are formed and the wing bud is easily distinguished as a discrete swelling on the flank (Fig. I:1B).

The developmental phase between 4 and 9 days of incubation is characterized by rapid changes in the wings, legs and visceral arches. The rapid progress in development of the limbs provides the most convenient diagnostic criteria from stage 15 to 32, (Fig. I:1C).

From the 8th to the 10 days (stages 33 to 36; Fig. I:1D), the number of scleral papillae in the eye, provide the most useful criteria for staging. The chick hatches after 20-21 days of incubation (stage 46).

II. Normal development of the chick face

The face of the chick is first recognized at stage 14 (50-53 hr incubation), with the development of the first branchial arch. This arch ultimately will form the entire lower jaw and may form part of the maxilla (Rowe *et al.*, 1992). At stage 15, the nasal placodes appear as paired thickenings of the cephalic ectoderm (Yee and Abbott,1978; Croucher and Tickle, 1990). By stage 18, the maxilla is a distinct prominence (Yee and Abbott, 1978). Between stages 18 and 28 the primitive oral cavity (stomodeum) is surrounded by the facial prominences in a square-shaped configuration (Fig. I:2A). The frontonasal mass is situated medial to the nasal pits and cranial to the stomodeum. Lateral to the nasal pits are the lateral nasal prominences. The maxillae lie caudal to the lateral nasal prominences

and flank the lateral edges of the stomodeum. The paired mandibular prominences that are fused together at the midline, are found caudal to the stomodeum.

A. Fusion of the facial prominences that contribute to the upper beak

The individual facial prominences of the upper beak become united into one structure between stage 26 and 30 (Yee and Abbott, 1978; Will and Meller, 1981; Tamarin *et al.*, 1984). At stage 26, the proximal part of the lateral nasal prominence is fused to the maxilla. Between stage 26 and 28 they "zip up" from deeper to more superficial aspects and ultimately are completely united. The palatal prominences have begun to form as medial enlargements of the maxillary prominences. The palatal processes are well developed, and they separate the nasal from the oral parts of the stomodeum laterally. These shelves fuse with the premaxilla at their distal end, and extend dorsally for a short distance as a united surface until interrupted by the choanal slit. This open palate, is the so called "schizognathic" condition characteristic of galliform birds (Bellairs and Jenkins, 1960; also see Fig. III:4:1A). At stage 30 the corners of the frontonasal mass (globular prominences) begin to fuse with the maxilla and lateral nasal processes and by stage 31 the oronasal groove has been obliterated. At stage 30, an ectodermal specialization called the egg tooth appears on the dorsal surface at the distal tip of the frontonasal mass (see Fig. III:3:3A,B). It is a transitory structure used by the chick on hatching to break the shell membrane; it is lost at hatching

or soon thereafter (Hamburger and Hamilton, 1951; Kingsbury *et al.*, 1953; Romanoff, 1960; Yasui and Hayashi, 1967).

The profile of the chick face remains flat until stage 31 and then the upper beak begins to protrude from the face surface while the lower beak is depressed into the stomodeal space. At stage 33, the entire bill extends well forward from the face. Figure I:2B, shows the essentially completed upper beak at stage 36.

B. Derivatives of the facial prominences

An exact fate map of all the regions of the facial primordia has not been constructed. Therefore this section contains hypothetical derivations for the primordia based on purely descriptive papers published many years ago. A critical experiment that has not yet been done is to label tissue in the primordia and follow its subsequent distribution in the fully developed beak.

The frontonasal mass gives rise to most of the upper beak including the prenasal cartilage, nasal septum and premaxilla (Tamarin *et al.*, 1984). The paired mandibular prominences form the entire lower beak. Therefore the prominences that make the biggest contribution to the bill are the frontonasal mass and the mandible. The lateral nasal prominences probably form the superior, middle and vestibular conchae (Romanoff, 1960) as well as tissue just dorsal and caudal to the external nares. The paired maxillae likely form the corners of the beak, including the lip ridge or tomium of the upper beak and tissue directly under the eye (Fig. I:2B). Inside the oral cavity, the maxillae form the palatal shelves.

III. Origin of facial tissues

The chick face develops from populations of cells derived from the neural crest with a small contribution from the paraxial mesoderm (review by Le Douarin 1982; Noden, 1988; and Hall, 1978). The neural crest cells are a population of ectodermal cells that originate from the dorsomedial aspect of the closing neural folds. Neural crest cells migrate into many regions of the embryo. Upon reaching their terminal destinations, members of this population undergo cytodifferentiation into a wide range of diverse cell types.

In order to study the fate of cranial neural crest cells, Johnston (1966) and Noden (1975), labelled avian crest cells with tritiated thymidine (^{3}H-TdR), and grafted the labelled tissue to a unlabelled host. These experiments were useful for mapping the distribution of ^{3}H-TdR labelled cells during migration and during their initial movements into the facial prominences. Neural crest cells in the head region begin to migrate away from the neural fold starting at 6 somites (stage 8.5) and first appear in the facial primordia at 22 somites (stage 14; Noden, 1975). However, progressive isotope dilution prevented a detailed analysis of the developing tissues. Therefore, in order to study the fate of neural crest cells in later beak development, a permanent nuclear marker was needed (Le Douarin, 1973). Quail nuclei can be distinguished from chick, and quail neural crest cells can migrate in a chick host and contribute to normal beak structures. These grafting experiments showed that in the face all cartilage, bone, tendon, dermal

component, pericytes and blood vessels walls, sensory and autonomic nerves, endothelium of the cornea, pigment cells and the investing fascia of muscles are of neural crest origin (Noden, 1978; Le Lièvre, 1978).

Non-neural crest derived structures in the head include: the craniofacial muscles, the cranial vault, the dorsal neurocranium, sclerotome of the cervical somites, the endothelial cells of blood vessels, and the motor nerves of the cranial ganglia (Noden, 1983a).

The myogenic cells of the facial muscles constitutes a separate cell lineage, originating from the paraxial mesoderm (Noden, 1983b).

While much work has been done to map the origin of facial tissues, there exists some ambiguity. This is because fine dissections can only resolve boundaries within the neural crest to a certain level. Also grafting procedures allow the possibility of introducing abnormalities by spatial and or temporal mismatch between graft and host. Wounding the neural epithelium and enforcing its repair may also disrupt the environment and migration of crest cells (Serbedzija *et al.*, 1991). Lumsden *et al.*, (1991), have further refined the chick neural crest fate map by labelling small regions of the premigratory crest *in situ*, using the fluorescent vital dye DiI. These experiments have confirmed the absence of migrating neural crest in the regions of rhombomeres 3 and 5. The significance of these crest-free regions is not known. In addition, the paths of migration of DI labelled cells were shown to be very similar to those reported by Johsnton (1966) and Noden (1975).

IV. Role of retinoids in embryological development

Vitamin A derivatives (collectively known as retinoids), are necessary for normal growth. Retinoids are not synthesized *de novo*, therefore they are obtained from the diet in the form of Vitamin A (retinol). Retinol is stored in the liver and distributed as needed to the cells via the blood. Retinol is enzymatically converted to retinoic acid via retinal according to the following pathway:

retinol ⇔ retinal → retinoic acid → polar metabolites (Thaller and Eichele, 1988). Retinoic acid cannot be converted back to retinal or retinol.

Both excess and deficiency of vitamin A can lead to defective development. Vitamin-A deficient quail embryos fail to develop a functional circulatory system, since the earliest defects were localized in the heart region (Heine *et al.*, 1985). In vitamin A deficient rats, it was found that proper differentiation of stem cells into mature epithelial cells failed to occur, and that abnormal cellular differentiation, characterized in particular by excessive accumulation of keratin, was a frequent event (Wolbach and Howe, 1925).

Excess vitamin A is embryolethal and teratogenic, in many species of animals including humans, and causes a high incidence of congenital malformations. In mouse embryos exogenous retinoic acid has teratological effects on limbs such as damaging cartilaginous elements (Sartre and Kochhar, 1989; Alles and Sulik, 1989). In chick embryos systemically administered retinoids lead to a bilateral cleft beak and limb truncations (Jelenik and Kistler, 1981). Retinoids

have also been observed to affect the development of the brain in amphibians (Durston *et al.*, 1989) and mammals (Morris and Steele, 1974). In pregnant women, 13-*cis*-retinoic acid (a treatment for acne) induced malformations involving craniofacial, cardiac, thymic and central nervous system structures (Lammer *et al.*, 1985). Most of the newborns born to mothers exposed to retinoic acid had craniofacial anomalies with the ear being severely malformed. Other abnormal features included a flat nasal bridge, mandibular hypoplasia, cleft palate and cranial defects.

Analysis of cleft lip and palate in chick embryos treated with retinoic acid can provide insights into the mechanisms of orofacial morphogenesis and how such abnormalities may arise in humans, since at early stages, the development of the face of chick and human embryos is similar. Application of all-*trans*-retinoic in a slow release form has striking effects on the pattern of connective tissue differentiation in the developing chick limb (reviewed by Tickle and Brickell, 1991), and chick face (reviewed by Wedden *et al.*, 1988). Local application of all-*trans*-retinoic acid to chick limb buds leads to mirror image duplication of digits (Tickle, *et al.*, 1982). Associated with the effect of retinoic acid in the treated limb, were truncations of the upper beak (Tamarin *et al.*, 1984 and Wedden and Tickle, 1986a). Local application of retinoic acid to the embryonic chick face has not previously been carried out, but there is the possibility that pattern changes as opposed to merely truncation of the upper beak will result from local release.

V. Tenascin: an extracellular matrix protein involved in embryonic development

The major groups of extracellular matrix molecules are proteoglycans, collagens and glycoproteins. Among the glycoproteins, fibronectin (Hynes and Yamada, 1982) and laminin (Timpl *et al.*, 1983) have been studied most extensively. Fibronectin is a major glycoprotein of mesenchymal tissues, whereas laminin is predominantly present in basement membranes. Both glycoproteins are important for cell attachment, cell migration, differentiation and morphogenesis (Hynes and Yamada, 1982; Timpl *et al.*, 1983).

Recently another extracellular matrix component has been implicated in the control of morphogenetic events taking place during embryonic development. Originally called myotendinous antigen (Chiquet and Fambrough, 1984a,b), this molecule was later renamed tenascin. The word tenascin is the combination of two latin verbs "tenere" (to hold) and "nasci" (to be born), which provided the roots for the English words "tendon" and "nascent", which describes its location and its developmental expression (Chiquet-Ehrismann *et al.*, 1986). Tenascin was shown to be very similar and possible identical to other molecules simultaneously discovered by other laboratories such as cytotactin (Grumet *et al.*, 1985), glioma mesenchymal extracellular matrix protein (Bourdon *et al.*, 1985) and hexabrachion (Erickson and Inglesias, 1984).

Tenascin is a disulphide-linked oligomer consisting of subunits of about 220, 200 and 190 kD (Chiquet and Fambrough, 1984b). Electron microscopic observation of tenascin and its analogs have shown that the molecule has a six-armed structure with a central core (Erickson and Inglesias, 1984; Vaughan *et al.*, 1987). Tenascin is synthesized by numerous cell types. For example tenascin is incorporated onto the cell surface of glial cells, and secreted into the basement membrane and extracellular spaces surrounding smooth muscle, lung and kidney cells (Grumet *et al.*, 1985). The structural complexity of this molecule and its reported capacity to differentially modulate adhesion of various cell types have led several authors (Grumet *et al.*, 1985; Mackie *et al.*, 1987; Lotz *et al.*, 1989) to suggest that tenascin modulates cell-substrate adhesion.

Tenascin protein is expressed in regionally specific domains in developing embryos (Crossin *et al.*, 1986; Riou *et al.*, 1988). The expression of tenascin in areas where morphogenetic rearrangements take place has suggested that this molecule is important for early embryonic development as well as for tissue differentiation.

VI. Specific aims

In the subsequent chapters I examined some mechanisms involved in the specification of the midline of the upper beak of the chick embryo.

In chapter II, I assessed the chondrogenic potential of fragments of the frontonasal mass at early stages in development, to determine whether cells can reestablish a single midline consisting of a prenasal cartilage and egg tooth.

In chapter III, the effects of locally released retinoic acid on the midline of the face were analyzed in detail in order to disturb endogenous retinoic acid gradients.

In chapter IV, the distribution of an extracellular matrix molecule involved in embryonic morphogenesis was mapped. I examined the correlation between expression of tenascin and the establishment of the midline of the upper beak.

Figure I:1

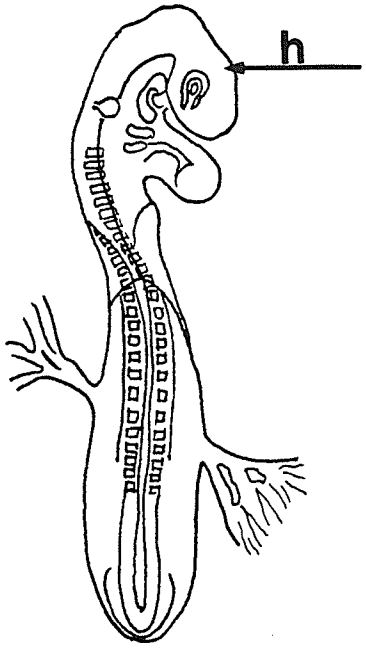
Diagrams of embryos at different stages of development.

(A) Chick embryo stage 15 (2.5 days of incubation). The embryo is very straight. The head is well developed, but there is no sign of future limb buds. h = head.

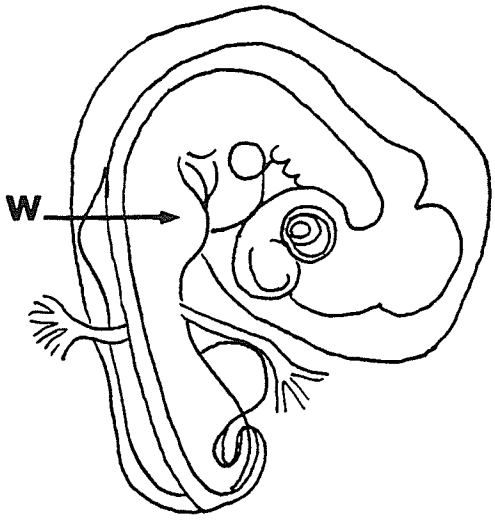
(B) Chick embryo stage 20 (3.5 days of incubation). The wing bud forms a discrete swelling on the flank. w = wing bud.

(C) Chick embryo stage 24 (4.5 days of incubation). The wing bud is longer than it is wide. w = wing bud.

(D) Chick embryo stage 36 (10 days of incubation). The pattern of cartilage elements has differentiated in the limb and beak.



A



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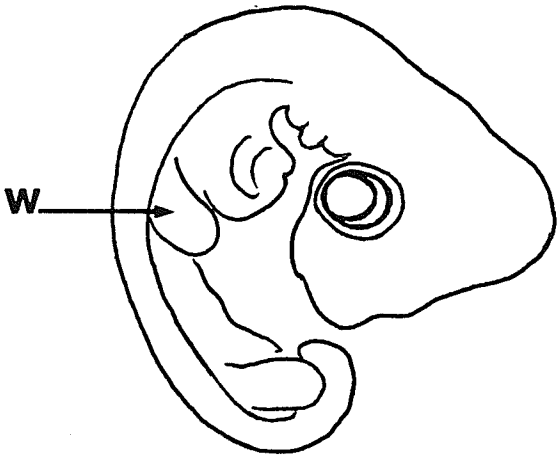

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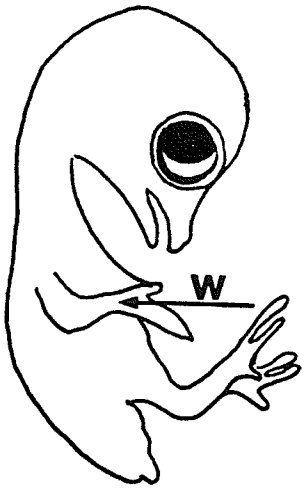
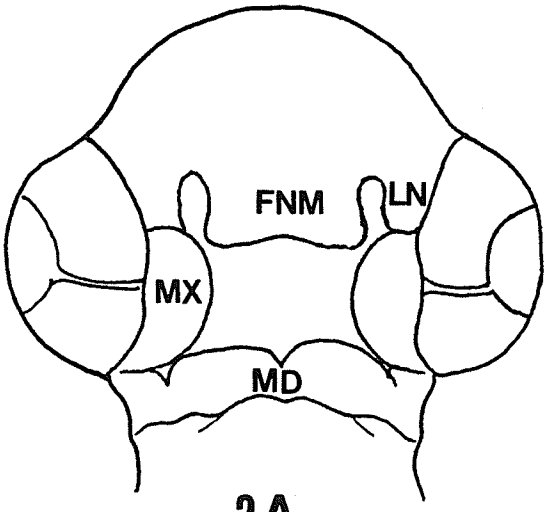


D

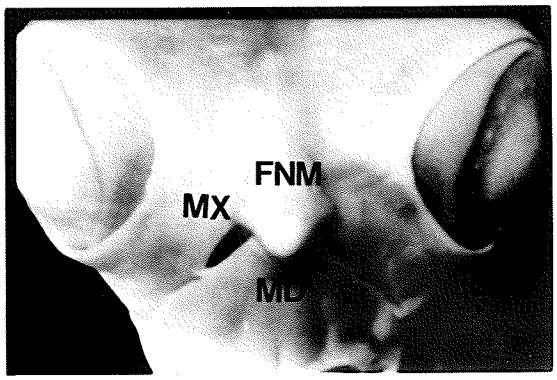
Figure I:2

(2A) Schematic diagram of the facial primordia of a stage 24 chick embryo (frontal view). The frontonasal mass (FNM) lies between the nasal pits, lateral to the nasal pits are the lateral nasal prominences (LN), the paired maxillae (Mx) are at each corner of the stomodeum, and the mandible (Md) is inferior to the stomodeum.

(2B) Photograph of a stage 36 embryo, showing the regions that the embryonic primordia hypothetically give rise to in the mature beak.



2A



2B

CHAPTER II

INTRODUCTION

The development of both upper and lower beaks involves considerable outgrowth, accompanied by the differentiation of cartilage and bone in spatially defined patterns. In the upper beak a central rod of cartilage, the prenasal cartilage develops. In the lower beak, paired rods of cartilage called Meckel's cartilage form. The midline of the upper beak is specified by the formation of this prenasal cartilage, and the midpoint is defined by a characteristic egg tooth at the distal tip (Kingsbury *et al.*, 1953; Yasui and Hayashi, 1967; Tamarin *et al.*, 1984). How this pattern of connective tissue is established is a major unanswered question. It is not known how cells are informed of their position with respect to this mediolateral axis.

There appear to be similarities between the development of the chick facial primordia and the limb. Although potentially chondrogenic cells are present throughout the limb bud (Ahrens *et al.*, 1977), and face (Wedden, *et al.*, 1986) cartilage normally forms only in the central core region. Therefore, in each case spatially organised patterns of cartilage are generated. While the mechanism involved in pattern formation in developing chick limb buds have been extensively studied the face has been less studied. There is evidence that the midline in the frontonasal mass is not established simultaneously with the initial development of the facial prominences (Wedden *et al.*, 1988). This evidence comes from studying

the development of fragments of the frontonasal mass of chick embryos isolated from influences of the surrounding facial primordia. These grafting experiments suggested that between stages 21 and 25 the ability to form midline structures gradually became restricted to more central regions of the frontonasal mass (Wedden and Tickle, 1986b). It was not until stage 29-30 that the ability to form the prenasal cartilage was completely restricted to the centre of the frontonasal mass. Lateral thirds of the frontonasal mass no longer gave rise to cartilage rods and egg teeth at this stage (Wedden and Tickle, 1986b).

In preliminary studies (Wedden, 1987), observations of double rods of cartilage were made in some of the younger frontonasal mass grafts. This chapter investigates in more detail the pattern of cartilage elements formed by discrete regions of the stage 20 frontonasal mass. The aim is to understand how fragments of the frontonasal mass reestablish a midline and to see whether multiple rods forming from 1 fragment are a rare or common event. The propensity to form double rods will tell us whether positional information is used to set up a new midline or whether the fragments have a memory of the original set of positional values in the intact primordium.

MATERIALS AND METHODS

I. Preparation of embryos

Fertile eggs from White Leghorn chickens were purchased from a breeder (Dept. of Poultry Science. U. of Manitoba). The day prior to the experiment, the embryos were staged in order to synchronize them. They were taken out from the incubator and each egg was turned slowly 180° to detach the embryo from the inner shell membrane. Then the egg was positioned horizontally on the egg holder. With a small pair of scissors a small hole was made into the air space at the one end of the egg. The embryo detaches from the shell membrane and sink. A piece of scotch tape was placed on the shell above the embryo, and with the scissors a small hole of ~10 mm in diameter was made on the shell.

Using the scheme of Hamburger and Hamilton (1951), the embryos were staged on the basis of external characteristics. If they were too advanced they were left at room temperature for several hours. After staging, the hole was covered with a piece of scotch tape, and the embryos were returned to the incubator.

II. Grafting of facial tissues

Normal stage-20 chick embryos (Hamburger and Hamilton, 1951) were used to provide the tissue for grafting. The frontonasal mass was dissected in culture medium (Minimum Essential Medium + 200 units/ml penicillin, 200 μg streptomycin and 0.05 μg fungizone (antibiotic-antimycotic Gibco). The frontonasal mass was divided into three fragments of approximately 0.59 mm (Fig.

II:1). Each of these tissue fragments was grafted into the dorsal surface of the wing bud of a host stage-22 chick embryo. The graft site was prepared by removing a square piece of tissue from the dorsal surface of the host right wing bud. Donor tissues were placed into the hole and the windowed eggs were resealed and reincubated at 38°C.

III. Examination of grafted tissues

The host embryos were examined the day after the operation and the presence or absence of the graft was recorded; then they were allowed to develop for a total of 6 days after grafting.

Right and left wings were excised from the embryo and fixed in 5% Trichloroacetic acid (TCA) or 10% buffered formalin overnight; rinsed with distilled water, stained in Alcian blue for 3-6 hr, differentiated in acid alcohol (1% HCl in 70% methanol); dehydrated in 100% methanol, and cleared in methyl salicylate.

Cleared specimens were examined under a dissecting microscope and photographed using FP4 35 mm film (Ilford) with substage illumination. The grafts were scored for the presence of typical midline structures such as prenasal cartilages and egg teeth or lateral structures such as nasal conchae. To quantify the amount of outgrowth, the cartilage rods were measured under a dissecting microscope using a calibrated eye-piece graticule. The wing bud was placed in a petri dish with methyl salicylate, and half of a coverslip was placed on top of the

specimen and gently pressed down, in order to flatten the wing and make it two dimensional. The extension of the rod was measured from the site most proximal to the humerus, to the most distal point (Fig. II:2C). In those cases where the rods presented a curvature, they were measured by segments following the shape, and subsequently added together to get the total length.

RESULTS

To investigate the ability of the frontonasal mass to form midline structures, the primordium was divided into 3 fragments (2 lateral and 1 middle fragment) and each piece grafted to a host limb bud. The results of these grafts are summarized in (Table II:1).

All fragments taken from the midline and lateral regions of the frontonasal mass of stage 20 chick embryos gave rise to a long central rods of cartilage, (the prenasal cartilage) and an egg tooth formed at the distal end (see quantitative data, Fig. II:4, and whole mounts Fig. II:2, lateral grafts not shown). Therefore from 1 frontonasal mass, 3 rods of cartilage and 3 egg teeth were obtained.

There was no significant difference in the outgrowth of fragments derived from either a lateral third or a middle third (3.75 mm $\pm$0.91 for middle thirds, compared to 4.36 mm $\pm$ 1.01 for lateral thirds, $p > 0.5$, students's t-test). Egg tooth formation occurred in grafts in which the length of the rod of cartilage was greater than 2 mm.

Two grafts gave rise to double rods of cartilage, one derived from a lateral fragment and one from a central fragment (not illustrated). Two other grafts, one central and one lateral fragment formed 3 rods of cartilage and 1 egg tooth (Fig. II:3). The total length of these 3 rods was equal to the average length of a single rod that normally developed from the middle third of the frontonasal mass.

In 5/15 grafts derived from lateral thirds of the frontonasal mass, lateral structures such as the scrolls of cartilage which normally develop around the rostral conchae, formed at the base of the cartilage rod (not shown).

DISCUSSION

I. Growth of facial primordia in isolation

The development of small fragments of facial primordia has been analyzed by grafting them to the dorsal surface of host chick wing buds. This grafting technique is relatively simple and has the advantage that the graft is rapidly vascularized and growth is not restricted. (Wedden, 1987; Richman and Tickle, 1989).

II. Specification of the mediolateral pattern in the frontonasal mass

The behaviour of grafts of the frontonasal mass was the same as described by Wedden (1987), and Richman and Tickle (1989). Grafts of fragments of the frontonasal mass developed into characteristic beak-like structures. This result suggests that fragments of the frontonasal mass, are able to regulate their development and that at stage 20, the ability to form characteristic midline structures is not an exclusive property of the tissue at the midline.

Another interesting feature of the regulatory behaviour of grafts of the frontonasal mass primordium was the development of duplicate prenasal cartilages and egg teeth from a single fragment. These double rods were similar to those reported by Wedden and Tickle (1986b), and Richman and Tickle (1989). The former investigators obtained double rods of cartilage in 4/4 experiments when the frontonasal mass of stage 20 embryos was also divided in 3 fragments, however the fragments were not identified as being central or lateral pieces. The

latter investigators, obtained double rods of cartilage in 3/5 experiments, when recombinations of the central third of the frontonasal mass mesenchyme and mandibular epithelium of stage 20 embryos were grafted. However, Richman and Tickle (1989) were more concerned in trying to fit the recombination into the graft site. Therefore some of the fragments probably involved more than the central third of the frontonasal mass (J. Richman, personal communication). The lower frequency of multiple rod formation in my experiments (4/29) indicates that duplication from a single fragment is a relatively rare event at stage 20.

The basis of the regulatory behaviour of lateral fragments of the frontonasal mass that result in the formation of midline structures is not clear. The new midline point could be formed by cells in lateral positions now taking on central characteristics following removal of the native midpoint. Therefore each fragment may behave as though it was an entire frontonasal mass with 1 midline and 2 lateral edges. This re-establishment of midline does not explain the formation of the double rods of cartilage occasionally observed. Therefore a second hypothesis can be formulated, which assumes that no regulation occurs but cells already have a fixed memory of where they were in the intact frontonasal mass. In this case the formation of the double rods maybe be related to the size of the fragments. For example if a lateral fragment is wider than usual, it will also involve part of the central third of the frontonasal mass. If a fixed, prepattern exists then a double rod of cartilage will form (i.e 2/3 of frontonasal mass should give rise to 2 rods of

cartilage). In order to determine if this is what is happening, the next crucial experiment is to graft halves of the frontonasal mass. If significant occurrences of multiple rods are observed, then we can say that regulation is not occurring, but rather that positional values are already fixed at stage 20.

Table II:1

Table 1, summarizes the development of grafts derived from either a middle or lateral fragment of the chick frontonasal mass (stage 20), fixed after 6 days of growth. FNM = frontonasal mass.

FNM fragment	# of exp.	grafts with rods > 1 mm	grafts with multiple rods	grafts with egg tooth
Midline	14	100 %	2	11 (85.7 %)
Lateral	15	100 %	2	12 (86.6 %)
TOTAL:	29			

Figure II:1

Diagram illustrating the grafting technique of facial tissues.

(A) Schematic frontal view of the "donor embryo" stage 20 (3.5 days of incubation). Dashed lines (---) represent lines of tissue dissection. FNM = frontonasal mass. MD = mandible.

(B) Frontonasal mass divided into thirds. Central and lateral fragments grouped in different dishes, containing culture medium.

(C) Schematic representation of "host embryo" stage 22 (4 days of incubation), showing the graft site (dashed lines ---) in the dorsal surface of the wing bud, with a fragment in place. g = graft.

(D) Embryos were allowed to continue developing for 6 more days, at which time (stage 36), wings are excised, fixed in 5% TCA, stained in Alcian blue, and cleared in methyl salycilate. g = graft.

(E) Schematic representation of a right wing bud which contains a graft. 2 3 4 = digits

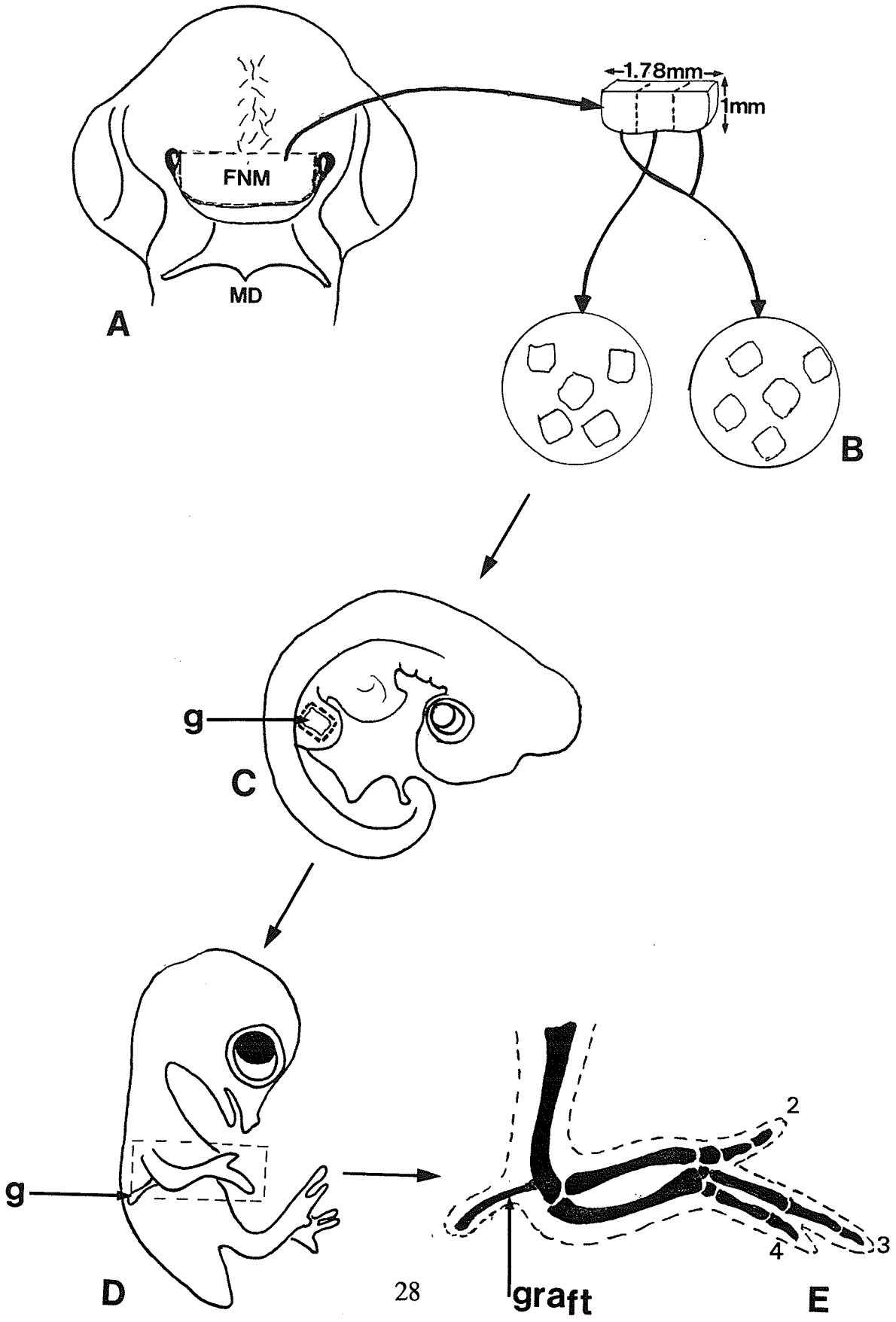

1.78mm
1mm
FNM
MD
A
B
g
C
g
D
graft
2
3
4
E

Figure II:2

Whole mounts of cleared grafts of stage 20 frontonasal mass have been excised from the host limb after 6 days of growth. Scale bars = 1 mm.

(A) Graft derived from a central fragment of the frontonasal mass. h = humerus, r = radius, u = ulna.

(B) Graft derived from a central fragment of the frontonasal mass. h = humerus, r = radius, u = ulna.

(C) Graft derived from a central fragment of the frontonasal mass. Dashed lines (---) illustrate how the graft was divided in fragments, in order to measure it. The total length of the graft was measured from the point of fusion to the humerus, or the point closer to this bone, to its distal tip.

(D) Graft derived from a central fragment of the frontonasal mass. Note the presence of an egg tooth (arrow), at the distal tip of the rod of cartilage.

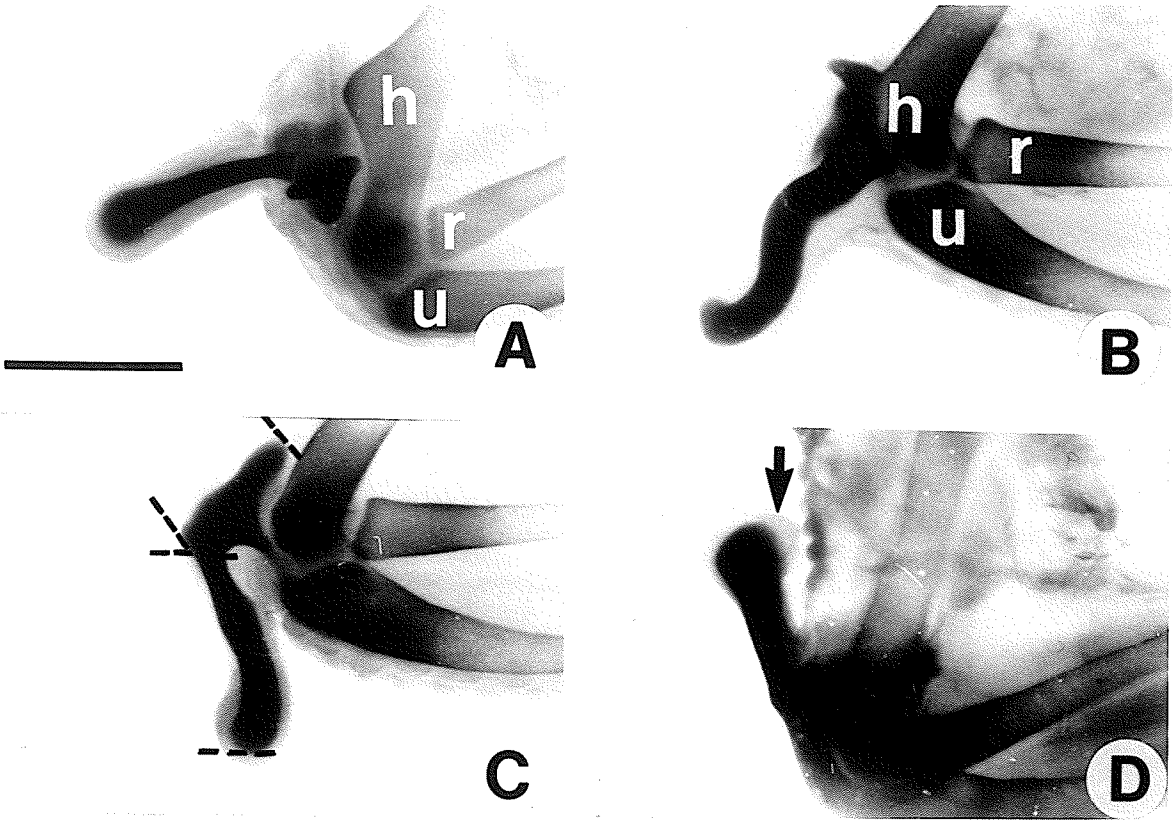

h
r
u
A
h
r
u
B
C
D

Figure II:3

Whole mounts of cleared grafts of stage 20 frontonasal mass from the host limb, after 6 days of growth.

1 A,B Whole mount of a graft derived from the middle of the frontonasal mass. Note the presence of 3 rods of cartilage (arrows). Scale bars = 1 mm.

2 A,B Whole mount of a graft derived from a lateral fragment of the frontonasal mass. Note the presence of 3 rods of cartilage (arrows). Scale bars = 1 mm.

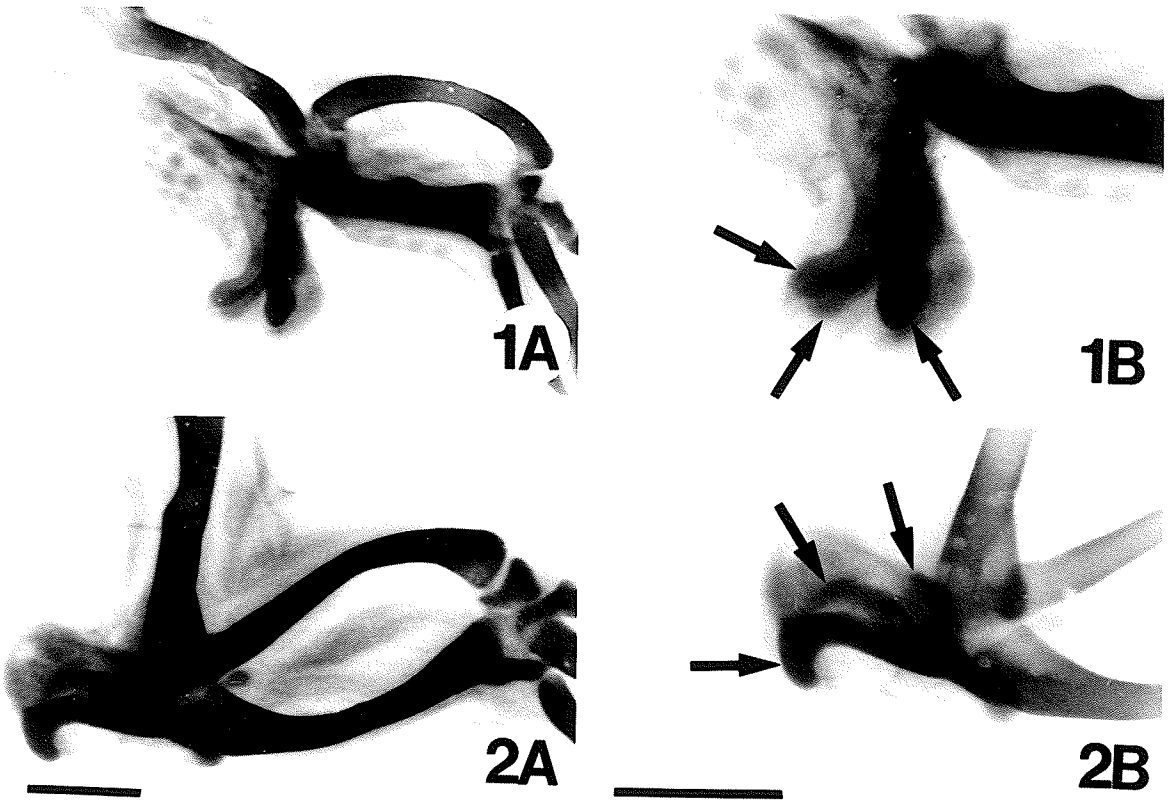
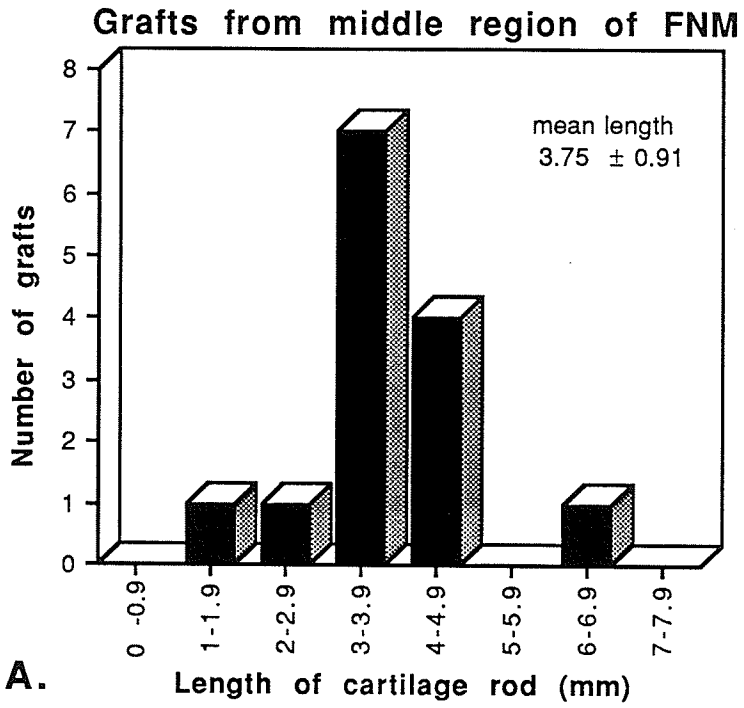

1A
1B
2A
2B

Figure II:4

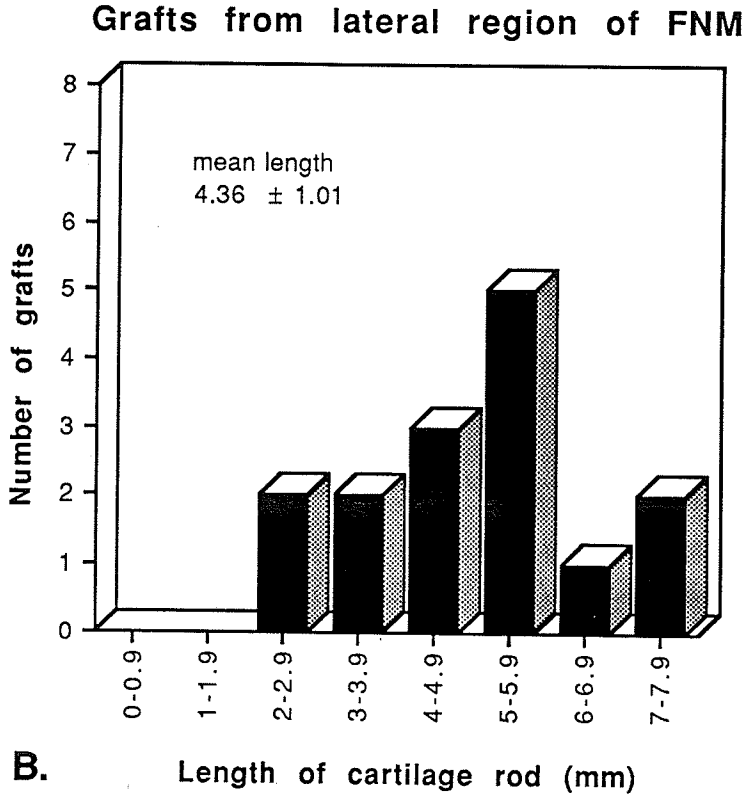
Histograms showing the absolute frequency of cartilage rod formation, against length (in mm), in cartilage containing grafts of stage 20 chick facial tissues fixed at 6 days. The mean lengths of the cartilage rods and the standard deviations are indicated in each histogram.

(A) Grafts from middle regions of the frontonasal mass. N = 14.

(B) Grafts from lateral regions of the frontonasal mass. N = 15


Grafts from middle region of FNM
mean length
3.75 ± 0.91
Number of grafts
0
1
2
3
4
5
6
7
8
0 -0.9
1-1.9
2-2.9
3-3.9
4-4.9
5-5.9
6-6.9
7-7.9
Length of cartilage rod (mm)

A.


Grafts from lateral region of FNM
mean length
4.36 ± 1.01
Number of grafts
0
1
2
3
4
5
6
7
8
0-0.9
1-1.9
2-2.9
3-3.9
4-4.9
5-5.9
6-6.9
7-7.9
Length of cartilage rod (mm)

B.

CHAPTER III

INTRODUCTION

The development of the chick limb has provided a powerful system to study pattern formation in vertebrates. Various experimental approaches have been used. The original approach, was to alter the spatial relationships under by transplanting tissue to different sites and observing the resultant disruption of the pattern. In the limb, this approach revealed organizing regions such as the zone of polarizing activity. This native signalling region was discovered by Saunders and Gasseling (1968), who transplanted a piece of posterior wing bud mesenchyme to an anterior position in a host wing bud. This manipulation resulted in a mirror-image, symmetrical duplication of the host wing pattern. Instead of the normal digit pattern 2 3 4 (Fig. II:1:E), digits 4 3 2 2 3 4 developed from the manipulated bud.

Saunders and Gasseling (1968), also found that when the leg polarizing region was grafted into a wing bud, duplicated wing structures would form. In other words, the polarizing activity of the leg graft induced the surrounding wing bud mesenchyme to form the extra digits rather than toes. These experiments lead to the hypothesis that the zone of polarizing activity releases a signalling molecule that diffuses into the limb bud and thereby forms a concentration gradient spanning the antero-posterior axis (Wolpert, 1969; Tickle *et al.*, 1975). Cells in different positions in the limb bud will be exposed to different concentrations of the

signalling molecule (morphogen), and will therefore assume different characters. This model has successfully predicted the results of many experiments. For example one would predict that as the anterior graft is moved closer to the polarizing region the gradient set up by the graft and the gradient set up by the natural signalling region would begin to overlap. There would be summation of the two gradients in the middle of the bud causing loss of middle structures. These result were obtained by Summerbell *et al.* (1973). Another prediction is that varying the source concentration of the signalling molecule, would result in specification of different digits. This experiment was carried out (Tickle *et al.*, 1980) and once again the results fit the prediction.

Other embryonic tissues such as ventral (but not dorsal) tail bud mesenchyme (Saunders and Gasseling, 1983) and Hensen's node (Hornbruch and Wolpert, 1986) induce duplications when grafted into the anterior portion of the chick wing or leg bud. Therefore it is possible that at the molecular and cellular level, the mechanisms of pattern formation in different parts of the embryo are related.

I. What is the nature of the morphogen with polarizing activity ?

In order to identify the substance made by the polarizing region, attempts were made to try to replace the polarizing region graft with various chemicals. These chemicals were placed directly opposite the natural signalling region and were locally released from small strips of paper. Finally, Tickle *et al.* (1982)

fortuitously discovered that all-*trans*-retinoic acid, a naturally occurring vitamin A derivative, could induce extra digits. It is possible to locally release retinoids from pieces of diethylaminoethyl (DEAE)-cellulose paper (Tickle *et al.*, 1982); newsprint (Summerbell, 1983) and from polystyrene beads (Eichele *et al.*, 1984). The result of local application of retinoic acid and grafts of the polarizing region tissue, showed striking similarities: 1) the effects on pattern are dose-dependent, 2) position-dependent, and 3) the length of exposure to produce pattern changes is the same (Reviewed by Smith *et al.*, 1989). Each digit of more posterior character (digit 4 being the most posterior) can be produced by increasing retinoic acid concentration or by increasing the length of exposure to it (Tickle *et al.*, 1985; Eichele *et al.*, 1985). An exposure of about 18 hr is sufficient to specify a complete set of additional digits with the appropriate dose of retinoic acid.

Subsequent work has demonstrated that retinoic acid is present in the developing limbs (Thaller and Eichele, 1987), and this has firmly established retinoic acid as a good candidate for an endogenous signalling substance. However, there is no direct evidence that cells read the local concentration of retinoic acid to find their position. Some preliminary data suggest that a local source of retinoic acid induces neighbouring tissue to become a polarizing region, which then determinates the antero-posterior axis by some mechanism not involving the graded activity of retinoic acid (Wanek *et al.*, 1991; Noji *et al.*,

1991). Thus retinoic acid may not be the final step in producing pattern changes but it is likely to be involved in the cascade of developmental events.

II. Pattern formation in the face

Little is known about the mechanisms involved in patterning of the face. It is possible that similar mechanisms to those used elsewhere in the embryo will also be used in the face. For example, retinoic acid may be an important signalling molecule in the face just as it is in the limb bud. Application of retinoic acid to the anterior margin of the limb bud also produces a specific facial defect in chick embryos. Patterning and morphogenesis of the frontonasal mass that gives rise to the upper beak are inhibited, while growth of the mandible is not affected (Tamarin *et al.*, 1984; Wedden and Tickle, 1986a). Thus the face and limb are both affected by retinoic acid.

Although retinoic acid has not yet been purified from facial tissues, recent investigations revealed that both retinoic acid receptors (Osumi-Yamashita *et al.*, 1990; Rowe *et al.*, 1991; Smith and Eichele, 1991) and cellular retinoic acid binding proteins (for example Vaessen *et al.*, 1990) are expressed in striking patterns in the embryonic face. The presence of these molecules implies that facial cells can detect and respond to retinoic acid. This evidence reinforces the idea that retinoic acid plays a role in the development of the face.

In order to determine if patterning of the frontonasal mass depends on cues provided by retinoic acid, experiments in this section will disturb endogenous

gradients of retinoic acid by locally releasing retinoic acid in the right nasal pit of the face of the chick embryo. It is possible that this local application of retinoic acid could cause reduplication of the frontonasal mass by disturbing endogenous gradients, as has been demonstrated in the limb (Tickle *et al.*, 1982). Also the effects produced by local application of retinoic acid will be analyzed and compared to those effects produced when the face receives a systemic dose from a retinoic acid-soaked bead implanted in the limb bud (Tamarin *et al.*, 1984; Wedden and Tickle, 1986a).

MATERIALS AND METHODS

I. Impregnation of beads with all-*trans*-retinoic acid

The method of retinoid application used, was developed by Eichele *et al.* (1984), and is the same described in other studies (Tamarin *et al.*, 1984; Tickle *et al.*, 1985; Wedden and Tickle, 1986a; Fig. III:1). AG1-X2 beads (BioRad, Formate form), of 100μm diameter (dry size) were selected under a dissecting microscope. Six beads were placed using forceps into a small petri dish containing 100 μl of all-*trans*-retinoic acid (Sigma, Lot # 40H0313 and 110H0234) dissolved in dimethylsulfoxide (DMSO) for 20 min at room temperature. Various concentrations of retinoic acid were used ranging from 0.005 mg/ml to 10 mg/ml RA. Then, each bead was rinsed in two 100 μl drops of tissue culture medium (Minimum essential medium with Hanks salts and L-glutamine (Gibco-Biocult), 10% fetal calf serum, antibiotic and glutamine) and, left to wash in 1ml of culture medium for 20 min at 38°C before they were implanted. The kinetics of retinoic acid uptake and release in the beads has been described previously (Eichele *et al.*, 1984). Retinoids are light sensitive compounds, therefore experiments were carried out with as little ambient light as possible.

Control embryos were treated with beads soaked in Dimethyl-sulphoxide (DMSO), following the above procedure.

II. Implantation of the bead in the right nasal pit of the embryo

An egg containing a embryo at the stage required (stages 15-24) was taken from the incubator, and the tape seal was removed. The egg was examined under the dissecting microscope, illuminated with a fibre optic light (Lumina-I). In the face region, the serosa and amnion membranes were torn away gently using forceps, to obtain direct access to the face. The right nasal pit was localized and with a fine sharpened, tungsten needle, a slit was cut in this area. Using forceps a bead was placed inside the egg, and pushed into position with the needle (Fig. III:2). A drawing of each embryo was made at this time, marking the position were the bead was positioned, and the time of implantation was recorded. The egg was sealed with tape, labelled with pencil on the shell and returned to the incubator, for a further 5-7 days. The embryos were checked the next day to verify the presence of the bead in the face, and reincubated immediately. All surgical manipulations were performed on a clean bench.

In a small group of embryos, beads soaked in 5 mg/ml retinoic acid were implanted in the middle of the frontonasal mass. Placement of the bead in this site was difficult, because the embryo develops in a lateral position, therefore there is not direct view of the front of the face. Eggs were sealed and reincubated for 6 more days.

III. Analysis of incidence and morphology of the facial defect

The incidence and morphology of the facial defect was examined in 10-day-old embryos (stage 36). The embryos were removed with forceps from the egg and sacrificed by decapitation. The heads were fixed overnight in 5% trichloroacetic acid (TCA) and stained for about 8 hr in Alcian Blue dye solution (1% of dye in 500 ml 70% ethanol containing 1% HCl). Excess dye was removed by washing the embryo for 6 hr in acid ethanol. Then the specimens were placed into absolute ethanol for examination under a dissecting microscope and photographed using FP4 35 mm film (Ilford). Some embryos were placed in methyl salicylate to examine cartilaginous structures.

In order to quantitate the severity of the upper beak defect, the scoring system termed "Severity of the beak defect Index" (Wedden, and Tickle, 1986a) was used as a reference. The original index was modified in order to register the presence or absence of egg tooth, as well as symmetry or asymmetry in the defect. Asymmetry was measured with an eye-piece graticule of a dissecting microscope, using the distal end of the lower beak as the reference point, and measuring from here the amount of deviation of the upper beak (Fig. III:3:3A,B), or middle of the frontonasal mass, when the beak was absent.

"Severity of Beak Defect Index"

Score	Morphology of the upper beak
0	Normal.
1a	Upper beak slightly short and symmetric both nostrils and egg tooth present.
1b	Upper beak slightly short and asymmetric both nostrils and egg tooth present.
2a	Upper beak slightly short; right nostril missing and egg tooth present.
2b	Upper beak drastically reduced or asymmetric; both nostrils and egg tooth present.
3	Upper beak drastically reduced or asymmetric; right nostril missing and egg tooth present.
4	Upper beak drastically reduced; no nostrils and egg tooth occasionally present.
5	Upper beak completely absent. Egg tooth absent.

In order to analyze dose-response relationships of retinoid treatment, beads soaked in a range of concentrations of all-*trans*-retinoic acid (10; 5; 1; 0.1; 0.5; 0.01 and 0.005 mg/ml RA) were implanted in the right nasal pit of embryos stage 20.

In order to analyze the length of exposure to produce facial defects, the beads were removed at various times (4 hr, 8 hr, 16 hr, 24 hr and 144 hr = 6

days), after implantation in the right nasal pit of chicken embryos at stage 20. The beads were removed by making a slit in the tissue that had healed over them with a tungsten needle and then removed with forceps. The eggs were reincubated and examined at stage 36 (10-day-old embryos).

In order to analyze the stage sensitivity to retinoid treatment, beads soaked in 5 mg/ml retinoic acid were implanted in the right nasal pit of embryos at different stages of development (stage 15, 20 and 24). Incidence and facial defects were examined in 10-day-old embryos (stage 36).

RESULTS

Facial defects were quantified by using a modified "Severity of beak defect Index" (Wedden and Tickle, 1986a). The face of each embryo was given a score. Figure III:3, illustrates the 5 major types of beak defects produced by retinoid treatment.

I. Dose-response relationships of retinoid treatment

The total number of embryos exhibiting a particular score in response to various concentrations of retinoic acid is graphically illustrated in figure III:6. All defects from the most severe to the most subtle are included in this figure.

A. Appearance of control embryos

All control embryos N = 16 were treated with DMSO soaked beads and all developed completely normal beaks (Fig. III:1A,B and III:6). The upper beak always overlapped the lower one. Complete right and left nostrils formed in all cases. An egg tooth on the dorsal surface at the distal end of the beak was always present. The eyelid formed a narrow ellipse surrounding the cornea. The intrastomodeal (intraoral) surface of the beak was composed of the fused primary and secondary palate rostrally and the narrow choanal slit between the palatal shelves dorsally (see chapter I).

B. Appearance of embryos treated with retinoic acid at various concentrations

Low concentrations of retinoic acid 0.005 mg/ml to 0.1 mg/ml produced mild upper beak defects (Fig. III:6). With the lowest concentration 1 out of 6 embryos had mild shortening of the upper beak. It is interesting that this very low concentration of retinoic acid did have some effect. Doses of 0.01 mg/ml to 0.05 mg/ml produced scores as high as 2b, but the majority of the embryos were unaffected (4/5 and 5/7 respectively). Exposure to a concentration of 0.1 mg/ml retinoic acid produced very few normal embryos (1/12). The defects produced by this dose were either scored as 1a or 1b.

Increased concentration of retinoic acid (1-10 mg/ml) lead to increased severity of defects in the upper beak. Treatment with 1 mg/ml lead to a wide range of responses in embryos, from scores as low as 1a-5 (Fig. III:6). Higher concentrations of 5 and 10 mg/ml produced severe defects (Fig. III:6), with the majority developing full defects (score 5); 17/20 and 29/32 respectively. In embryos with a full defect, the upper beak is completely missing and the front of the face is formed by a scalloped rim of tissue above the mouth (see Fig. III:4:2A,B). No nostrils were formed. In contrast, the lower beak appears to be essentially normal. The scalloped border of the mouth is derived from tissue masses that would normally fuse to form the upper beak (Fig. III:4:2A,B). Seven bulges can be distinguished at the upper margin of the mouth: these are a central

tubercle derived from the frontonasal mass, flanked laterally by tubercles in either side derived from the lateral nasal prominences, which in turn are flanked laterally by tubercles derived from the maxillary prominences. The minimum dose required to produce a full upper beak defect (score 5), was 5 mg/ml (Fig. III:7).

II. Length of exposure to retinoid required to produce beak defects

Controlled release beads soaked in 5 mg/ml retinoic acid were removed at various intervals after being implanted (4 hr, 8 hr, 16 hr, 24 hr and 144 hr = 6 days) in 73 stage 20 chicken embryos. In one embryo of the 4 hr, 8 hr and 16 hr groups, the bead was left in place for 6 days, in order to verify the reproducibility of the complete beak defect. All 3 controls had a completely absent upper beak.

Surprisingly, a 4 hr exposure to retinoic acid already produced a mild defect (score 1a) in 5/13 embryos (Fig. III:8). When beads were left 16 hr, 100% of embryos had a beak defects with scores ranging from 3 to 5 (Fig. III:8). A 24 hr exposure was necessary to give the full defect in 100% of the embryos (Fig. III:8).

III. Stage sensitivity to retinoid treatment

Beads soaked in 5 mg/ml retinoic acid were implanted in the right nasal pit of 41 chicken embryos stage 15, 20 or 24. The beads were left in place for 6 days. The data clearly illustrates the severity of the beak defect is stage-dependent (Fig. III:9). The most severe effects occurred with application at stage 20. Less severe defects occurred at earlier or later stages of development (15 and 24). When beads were implanted at stage 15, many of the embryos died (62.86%). The mortality

rate of embryos stage 20 and 24 was low (~10%). The higher mortality rate at stage 15 is most likely due to rupturing of important blood vessels during the implantation procedure, not due to the toxicity of retinoic acid.

IV. Unusual effects associated with local release of retinoids in the face

A. Retinoic acid induces midline deviations

In the retinoic acid treated embryos a difference in the effect on the right and left sides of the face was detected; the right side being more affected (Table III:1). With lower doses of retinoic acid (0.005 mg/ml and 0.1 mg/ml) few embryos had deviation of the upper beak to the right, and the degree of asymmetry was also low (0.3 mm -1.5 mm). With higher doses (1-10 mg/ml) the incidence and severity of the asymmetry increased (0.3 mm - 3.3 mm). Embryos with full defect did not have upper beaks but the frontonasal mass tubercle sloped toward the right side of the face (Fig. III:4:3B).

In order to verify that the asymmetry was not being produced as a result of the presence of the bead, a bead soaked in 5 mg/ml retinoic acid was implanted in 5 stage 20 embryos and removed 24 hr later. All 5 embryos developed the same degree of asymmetry as those in which the bead was left in place for 6 days.

After an 8 hr exposure to 5 mg/ml retinoic acid, 5 embryos out of 15 presented 0.3 mm to 2 mm of asymmetry, longer times of exposure produced asymmetry in all the embryos and the severity also increased (2.5 mm - 3.3mm).

All embryos treated at stage 15 and 24 also developed midline deviations, but the degree was always less that in the stage 20 embryos, compared to the younger and older stages in which the asymmetry ranged between 0.3 mm and 2.8 mm.

B. Retinoic acid-induced changes in the palate

Associated with the failure of formation of nostrils and upper beak in the severely affected embryos, the development of the secondary palate was also abnormal. The roof of the abnormal oral cavity had five tissue masses: a central bulge and two lateral bulges, flanked by the palatal shelves (Fig. III:4:2A,B). The central bulge of tissue was derived from the frontonasal mass. The two inner lateral bulges were derived from the primordia of the most rostral nasal conchae. The secondary palate, formed by the maxillary palatal shelves was separated by a wide space along the length of the shelves (Fig. III:4:2A,B). In addition the right maxillary tubercle was reduced in size (Fig. III:4:2A,B). A variation from the typical arrangement of tubercles was also observed in some specimens (Fig. III:4:2A,B). Apparently the maxilla and lateral nasal tubercles are missing on the right side in this less typical specimens.

C. Defects in the commissure of the eye

A right eye defect was noticed in those embryos that were treated with 1 mg/ml retinoic acid (Table III:1A). Embryos presented with slight discontinuities of the lower eye lid. With doses of 5 and 10 mg/ml retinoic acid, all the embryos

had defects, which involved the lower lid, plus the ventral and superior aspects of the nictitating membrane (Fig. III:3 :7A).

A 16 hr exposure to 5 mg/ml retinoic acid was enough to affect the right eye in 18 out of 20 embryos. With longer times of exposure all the embryos were affected, (Table III:1B). Stage 24 embryos treated with 5 mg/ml retinoic acid had a much lower incidence of right commissure defects (6/13) than stage 15 or 20 embryos (100 %); Table III:1C.

D. Egg tooth development correlated with severity of beak defect

Embryos with scores 0-3 always developed an egg tooth. Those embryos with a score 4, occasionally formed an egg tooth, while score 5 embryos never presented with an egg tooth (Table III:1). Thus less outgrowth was correlated with less frequent egg tooth formation.

V. <u>Beads implanted in the centre of the frontonasal mass give rise to more symmetrical defects</u>

The centre of the frontonasal mass was less accessible to the right nasal pit due to the fact that the embryo develops on its side, therefore only 5 embryos were treated in this way. Of the 5 embryos treated with beads in the centre of the frontonasal mass with 5 mg/ml retinoic acid, four developed full defects. In one case, the bead fell out of the face and therefore it was excluded from the sample. Two of the four embryos had asymmetrical facial defects including midline

deviations and incomplete right eye commissures (not illustrated). The other two embryos had both right and left eye defects and no midline deviations (Fig. III:5).

DISCUSSION

I. Differences in the response of facial tissues to systemic and local released retinoic acid

My experiments have shown that the face responds differently to locally released retinoic acid than it does to a systemic exposure; 1) with a systemic dose the full upper beak defect (score 5) was produced with a 200 μm bead, soaked in a solution of 10 mg/ml retinoic acid (Wedden and Tickle, 1986a), while the same defect was produced locally with a 100 μm bead soaked in 5 mg/ml retinoic acid; 2) the systemic effect of 10 mg/ml retinoic acid released from a bead implanted in the limb bud produced more symmetrically truncated upper beaks (Tamarin *et al.*, 1984), while in the present study local release of retinoic acid produced midline deviations towards the right and the medial corner of the right eye was affected; 3) systemic exposure to retinoic acid lead to an abnormal primary palate represented as individual tubercles (Tamarin *et al.*, 1984), while local treatment reduced the size and occasionally eliminated the maxillary tubercle. In addition the distance between the palatal shelves increased in locally treated specimens.

II. Defective midline formation

Retinoic acid released locally leads to aberrant midline formation. Deviation of the partially or completely truncated beak was always to the right side of the face. This effect is probably related to the increase exposure to the chemical in this area, due to the proximity to the implant site. The reduction in the size of the

maxillary prominence observed, may contribute to the asymmetry of the effect. It is known that the maxillary prominence gives rise to the corners of the beak including the lip ridge of the upper beak (Romanoff, 1960; Tamarin *et al.*, 1984). Therefore reduction in the size of the maxillary prominence could create tension in the base of the beak and skew the beak towards the right side of the face. The right eye defect observed, may also be related to this reduction in the size of the maxillary primordium, as it is suspected that the maxillary prominence gives rise to the tissue directly under the eye (C. Tickle, personal communication).

Wedden (1986) implanted control and retinoic acid soaked beads into the developing right eye between stages 15-24. This resulted in reduction of the size of the operated eye, and skewing of the upper beak towards the right side of the face. Even beads soaked in DMSO produced the same deviation. These experiments demonstrate the importance of the eye volume in facial symmetry. In contrast, a bead placed in the nasal pit has no direct effect on facial development. Moreover, asymmetry produced by retinoic acid does not require the bead to be present beyond 24 hours. Since fusion of facial primordia does not begin until stage 28, it is unlikely that the retinoic acid soaked bead is directly interfering with the fusion process.

It has been shown that retinoic acid receptors ß (RAR-ß), are expressed in the rostral (anterior) part of the maxillae in stage 20 embryos, and that retinoic acid treatment (beads soaked in 10 mg/ml placed in the limb bud) changed the

distribution of these receptors (Rowe *et al.*, 1991). Therefore abnormal growth in areas derived from the maxillary prominences may be mediated by the interaction of retinoic acid with this nuclear receptor.

III. Similarities between the effects of systemic and locally released retinoic acid

Similarities in length of exposure and stage sensitivity were found with both systemic (Wedden and Tickle, 1986a), and local retinoic acid treatment: 1) in both situations an exposure of at least 24 hr was required to produce the full defect. This time requirement may reflect the need for the retinoid to be present for a specific number of cell cycles as has been shown for the limb bud (Eichele *et al.*, 1985). The mean cell cycle time in the maxillary process of chick embryos is 12 hr (Minkoff, 1991); 2) embryos are most sensitive to retinoic acid between stages 20 and 21. Before and after these stages sensitivity drops sharply. It is possible that the increased sensitivity between days 3.5 and 4 is due to the development of retinoid-binding molecules in certain target cell populations. Thus far a correlation has not been made between initiation of expression of RARs or cellular retinoic acid binding proteins (CRABPs) and the onset of retinoid sensitivity (Rowe *et al.*, 1991; Maden *et al.*, 1991); 3) both local and systemic application of retinoic acid did not affect the development of the mandibular primordia, which give rise to the lower beak. Thus increased proximity of the bead and higher local concentration of retinoic acid in the mandibular primordia did not induce changes in outgrowth.

The reasons for the specificity of retinoic acid on the upper beak are unclear. Several possible mechanisms have been proposed and subsequently excluded.

Retinoids interfere with the migration of cranial neural crest cells into the facial primordia (Wiley *et al.*, 1983). This reduces the amount of facial mesenchyme and may cause clefting. However, it has been shown that the chick face is particularly sensitive to retinoic acid at stage 20, long after neural crest cells have reached the face (Johnston, 1966; Noden, 1975).

It is known that retinoic acid can prevent chondrocytes from differentiating *in vitro* (Wedden *et al.*, 1987; Langille *et al.*, 1989), but the *in vivo* inhibition must be working through a different mechanism, because both primordia, frontonasal mass and mandible contain large number of chondrocytes (Wedden *et al.*, 1986; Richman and Crosby, 1990), yet only the frontonasal mass chondrocytes are affected. Moreover, the period of greatest sensitivity to retinoic acid *in vivo* is at stage 20, and this is 48 hr before chondrocytes differentiate. My data show that the maxillae are also affected by retinoic acid although these primordia do not form cartilage (Wedden *et al.*, 1986; Richman and Crosby, 1990). Thus retinoic acid may also affect cells that belong to the osteogenic line. It is therefore unlikely that retinoic acid is directly affecting differentiation, but is probably preventing precursor cells from becoming committed to a differentiated phenotype.

Retinoic acid could be increasing the level of programmed cell death at the corners of the frontonasal mass. However, no increase in cell death was detected by Nile Blue sulfate staining in whole embryos or in sections in any part of the face (Wedden, 1991).

The frontonasal mass might take up more retinoic acid than other facial primordia. However, a radioactive analogue of retinoic acid (TTNPB) was taken up equally by the frontonasal mass and mandible from beads placed in the limb bud (Wedden *et al.*, 1987).

Finally retinoic acid treatment may affect the distribution of retinoic acid receptors which could lead to the facial defect. However, after retinoic acid treatment the distribution of RAR-ß is only affected in the maxillae, and not in the frontonasal mass where the major defect occurs (Rowe *et al.*, 1991). It is possible that other retinoic acid receptors such as RAR α, γ, or binding proteins are involved in generating the facial defect, but these have not be investigated.

IV. Evidence for a progress zone in the frontonasal mass

All doses of retinoic acid used in the present study caused some degree of truncation of the upper beak. Low concentrations of retinoid allowed the primary palate to fuse but prevented extensive outgrowth, an event that carries on for an additional 6 days after stages when retinoids were applied. Application of high concentrations of retinoid also inhibited the fusion of the primordia that form the primary palate, an event which does not occur for another 48 hr. One possible

explanation is that a high dose of retinoid significantly reduced the cells number leading to a reduction in growth of the primordia. A lower dose of retinoid may cause some initial reduction in cell number, but will permit cell numbers to be replenished and growth to occur.

This possible explanation for the inhibition of outgrowth of the upper beak in the face, can be compared to the "progress zone" of the limb bud (Summerbell *et al.,* 1973). The progress zone consist of a region of rapidly dividing mesenchymal cells at the tip of the limb bud. Cells continually spill out of the progress zone into the adjacent, proximal mesenchyme. The more time cells spend in the progress zone the more likely it is that they will contribute to the formation of distal structures (i.e digits). If a reduction of cells occurs in the progress zone at an early stage, proximal structures (i.e humerus, ulna, radius), will be reduced in size (Wolpert *et al.,* 1979). As the progress zone become repopulated with successive cell cycles, normal distal structures will be formed. Thus in the face low doses of retinoic acid stunted proximal growth of the beak, but allowed normal distal growth complete with egg tooth formation. Since we do not have good markers for proximal structures, it is difficult at present to verify the reduction of proximal cartilage.

V. Location of a signalling region in the face

In the present experiments, application of retinoic acid in the right nasal pit did not cause reduplication of the frontonasal mass cartilage, therefore, a mirror

image of a possible endogenous retinoic acid gradient was not created. The most likely explanation is that the bead was not placed opposite to a natural signalling region. It is possible that the signalling region in the frontonasal mass may be located in the midline, somewhere along the antero-posterior axis. In order to test this theory beads soaked in 5 mg/ml retinoic acid were placed in the midline. Although duplications did not occur, it is nonetheless possible that if lower doses are placed in the midline, duplication of cartilage structures may occur (also see chapter V, Figure V:1). Lower doses of around 0.1 mg/ml retinoic acid caused duplications in the limb (Tickle *et al.*, 1975).

In order to determine if a natural signalling region exists in the face, grafting of discrete pieces of frontonasal mass into the anterior margin of the limb bud can be performed. If polarizing activity is found in certain regions of the frontonasal mass this will support the hypothesis that the frontonasal mass and limb use similar patterning mechanisms. There is a recent study that has shown that signals passing between epithelium and mesenchyme are similar in the frontonasal mass and limb (Richman and Tickle, in press), therefore it would not be unexpected to find that other signalling processes are also shared between these two parts of the embryo.

Table III:1

Incidence of asymmetry, eye defects and egg teeth in embryos treated with retinoic acid

(A) Dose - Response

[] of RA (mg/ml)	# of embryos	Asymmetry	Eye defect	Egg tooth
0	16	0 (0%)	0 (0%)	16 (100%)
0.005	6	1 (16.7%)	0 (0%)	6 (100%)
0.01	5	1 (20%)	0 (0%)	5 (100%)
0.05	7	2 (28.6%)	0 (0%)	7 (100%)
0.1	12	5 (41.7%)	0 (0%)	12 (100%)
1	31	27 (87.1%)	14 (45.2%)	26 (83.8%)
5	20	20 (100%)	20 (100%)	0 (0%)
10	32	32 (100%)	32 (100%)	0 (0%)
TOTAL:	129			

(B) Length of exposure

Length of expo. hr	# of embryos	Asymmetry	Eye defect	Egg tooth
4	13	0 (0%)	0 (0%)	13 (100%)
8	15	5 (33.3%)	0 (0%)	15 (100%)
16	20	20 (100%)	18 (90%)	9 (45%)
24	5	5 (100%)	5 (100%)	0 (0%)
144=6 days	20	20 (100%)	15 (75%)	0 (0%)
TOTAL:	73			

(C) Stage sensitivity

Stage embryos	# of	Asymmetry	Eye defect	Egg tooth
15	8	8 (100%)	8 (100%)	8 (100%)
20	20	20 (100%)	20 (100%)	0 (0%)
24	13	10 (76.92%)	6 (46.1%)	13 (100%)
TOTAL:	41			

Figure III:1

The technique of local application of retinoic acid to chick embryos (stages 15-24).

(A) Schematic representation of a stage 20 chick embryo (3 1/2 days of incubation).

(B) AG1-X2 beads (Biorad, formate form, 100μm dry size), soaked for 20 min in different concentrations of all-trans-retinoic acid or DMSO (controls). Beads were of same variety as in Eichele *et al.*, 1984 and Tamarin *et al.*, 1984).

(C) Two quick rinses in droplets of tissue culture medium.

(D) Final wash in 1ml of tissue culture medium at 38°C (total time spent rinsing in steps C and D = 20 min).

(E) Slit made in the right nasal pit region with a tungsten sharp needle.

(F) Bead implanted in the right nasal pit.

(G) Embryos were allowed to continue developing for 5 to 7 days at which time (stage 36), the heads were removed, fixed in 5% TCA and stained in Alcian Blue.

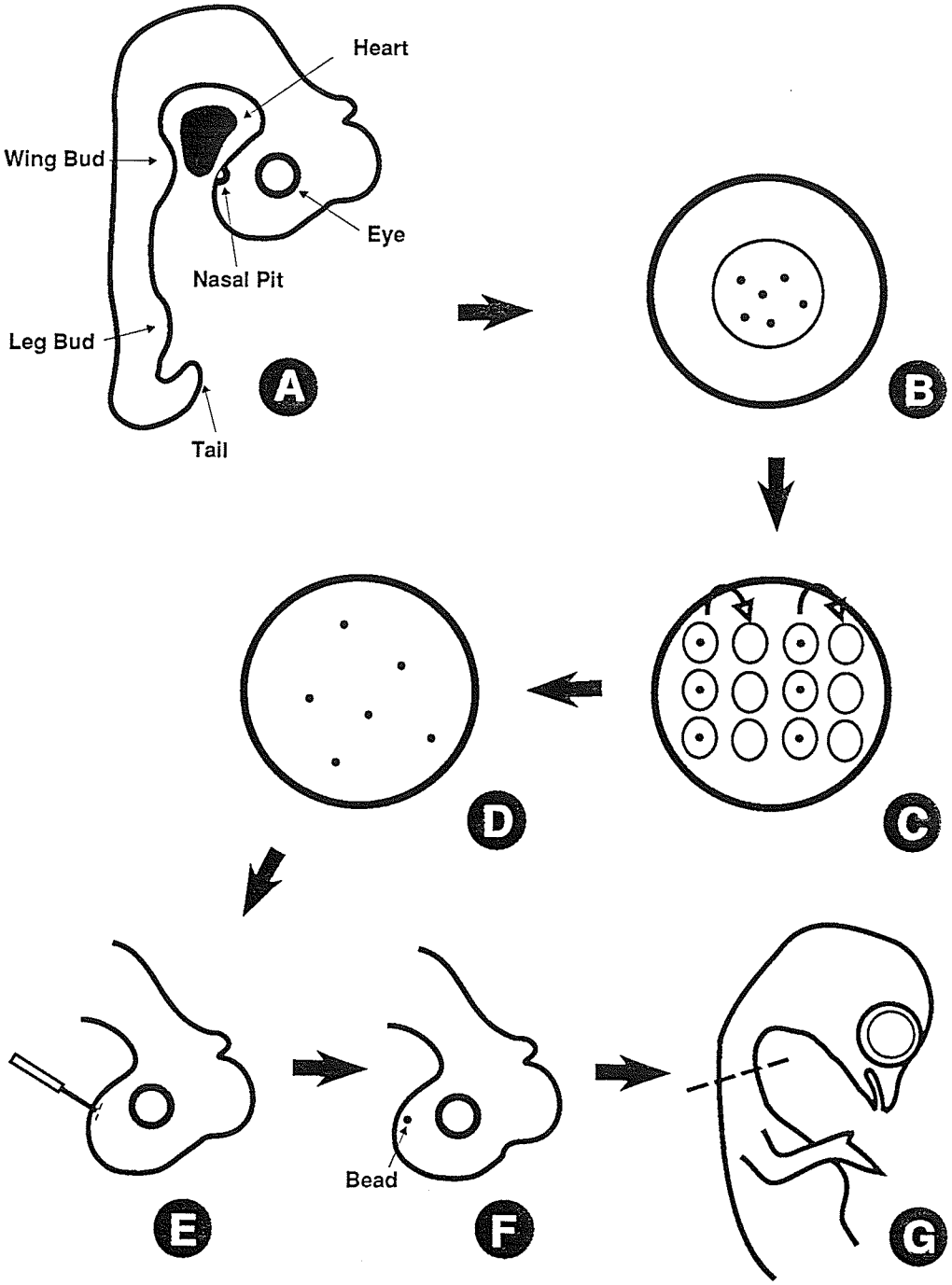

Heart
Wing Bud
Eye
Nasal Pit
Leg Bud
Tail
A
B
C
D
E
F
G
Bead

Figure III:2

Chick embryo stage 20 *in ovo*, photographed after the operation, showing the bead implanted in the right nasal pit. b = bead, e = eye, w = wing bud.

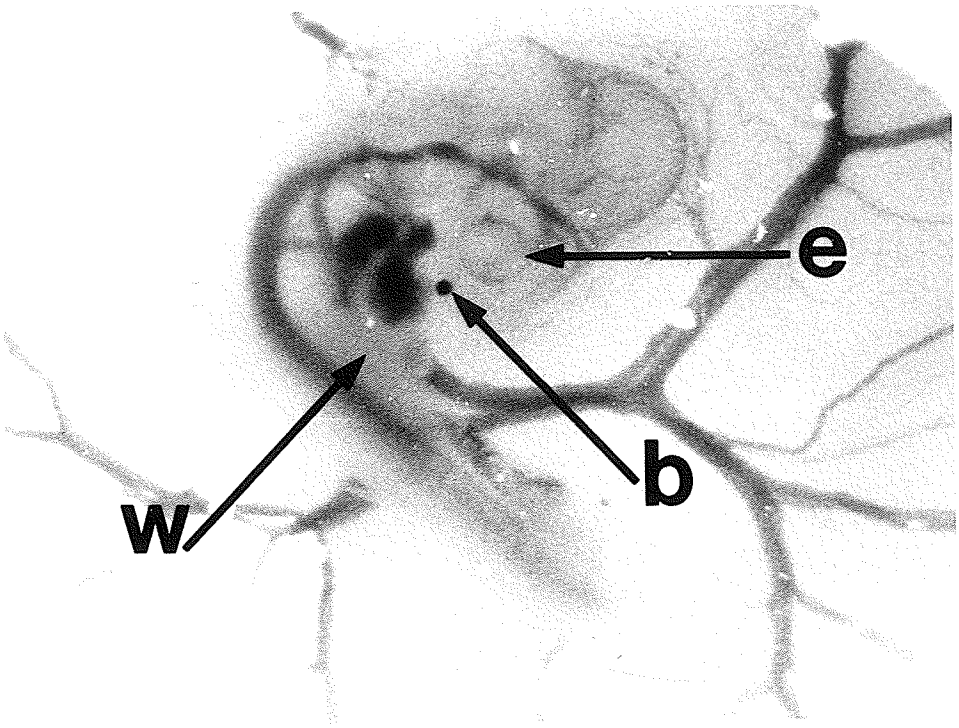

e
b
w

Figure III:3

Types of beak defects produced by retinoid treatment. All embryos have been fixed 6 days (stage 36) after retinoic acid-soaked beads were placed in the right nasal pit. Scale bars = 1 mm.

1A,B Score 0 = Normal. The upper beak overlaps the lower beak (dashed line).

2A,B Score 1a = Upper beak slightly short and symmetric (dashed line); both nostrils and egg tooth present.

3A,B Score 1b = Upper beak slightly short and asymmetric. Both nostrils and egg tooth present (arrows), n = nostril; e = egg tooth.

4A,B Score 2a = Upper beak slightly short, right nostril missing (arrow), and egg tooth present.

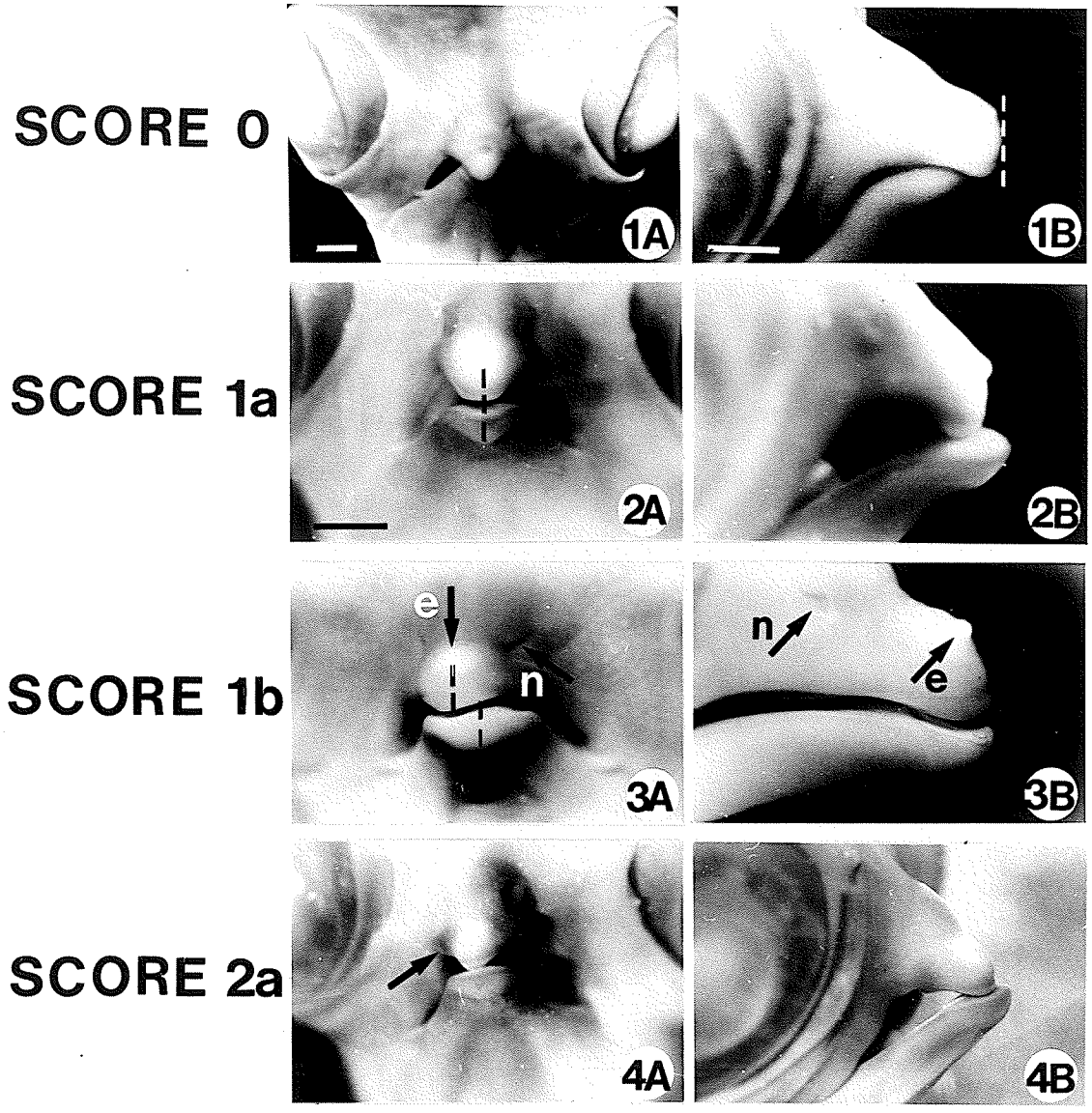

SCORE 0
1A
1B
SCORE 1a
2A
2B
SCORE 1b
e
n
3A
n
e
3B
SCORE 2a
4A
4B

Figure III:3 (continued)

Types of beak defects produced by retinoid treatment. Scale bars = 1 mm.

5A,B Score 2b = Upper beak drastically reduced or asymmetric. Both nostrils (arrows) and egg tooth present.

6A,B Score 3 = Upper beak drastically reduced and asymmetric. Right nostril missing (arrow); egg tooth present.

7A,B Score 4 = Upper beak drastically reduced. No nostrils and, egg tooth occasionally present. Right eye defect (arrow).

8A,B Score 5 = Upper beak completely absent; no egg tooth. Absence of the maxillary process in this specific specimen (arrow).

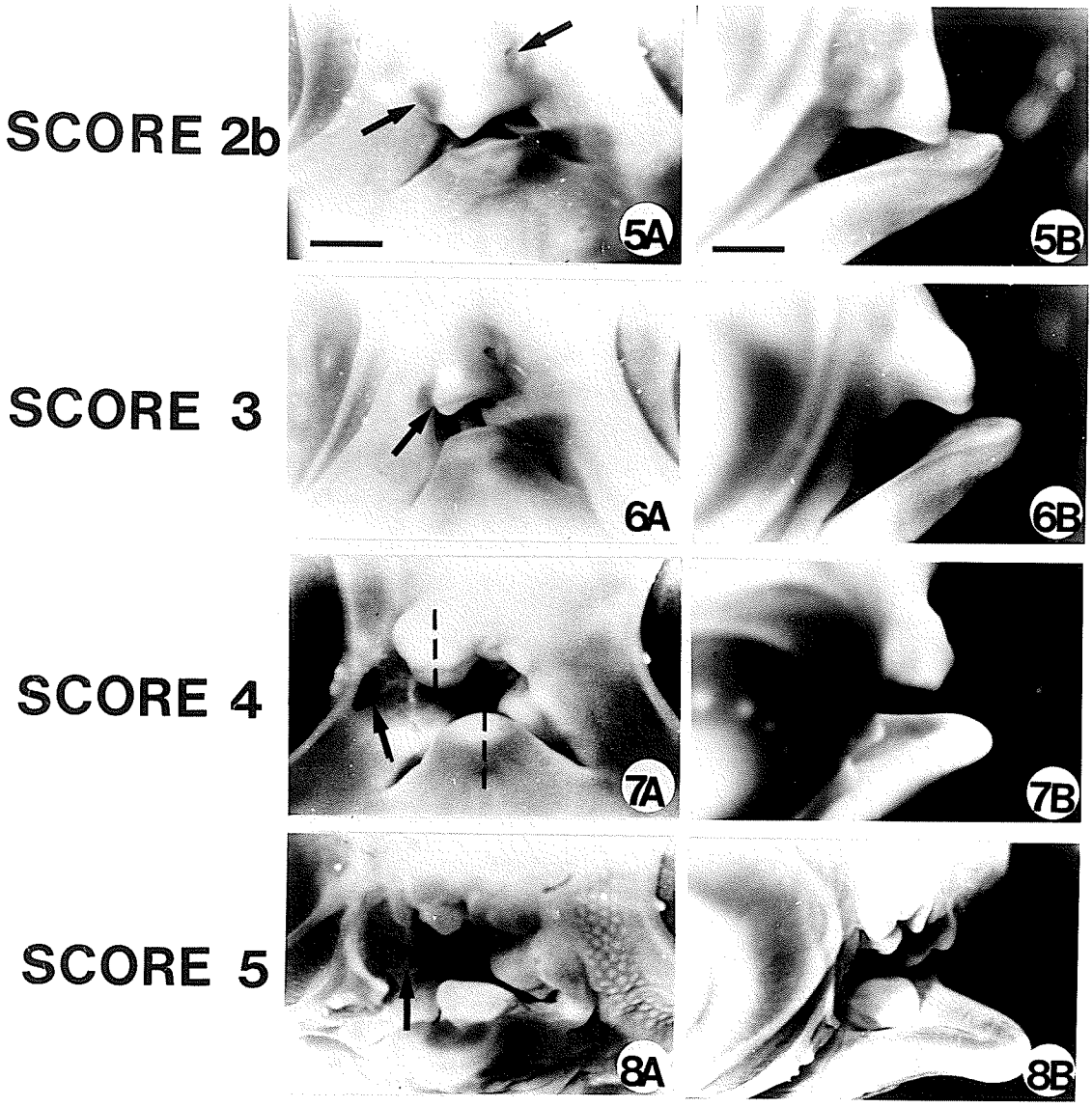

SCORE 2b
5A
5B
SCORE 3
6A
6B
SCORE 4
7A
7B
SCORE 5
8A
8B

Figure III:4

1A Normal palate of a control embryo (stage 36). The palatal shelves remain unjoined to form the choanal slit (arrow), which connects the oral and nasal chambers. This open palate is typical of galliform birds. Scale bar = 1 mm.

2A,B Palatal view of a stage 36 embryo treated with 5 mg/ml retinoic acid at stage 20. Both A and B are the same specimen. The palatal view shows one type of arrangement of the tubercles in the full defect (score 5).

The border of the mouth is formed by 7 tubercles:

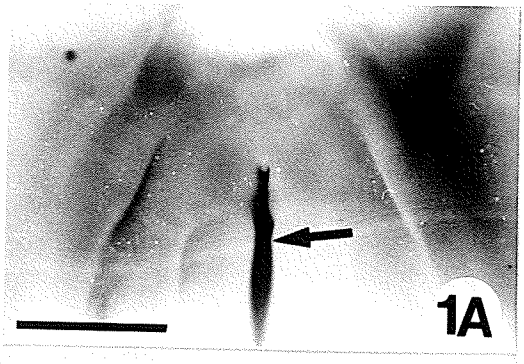
a) Frontonasal mass tubercle (unpaired).
b) Upper part of the lateral nasal tubercle (paired).
c) Lower part of the lateral nasal tubercle (paired).
f) Maxillary tubercle (paired).

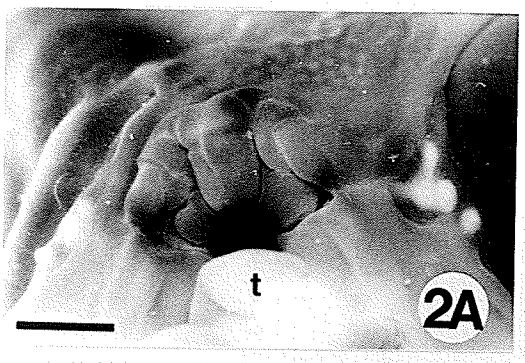
The roof of the abnormal palate has five tubercles:

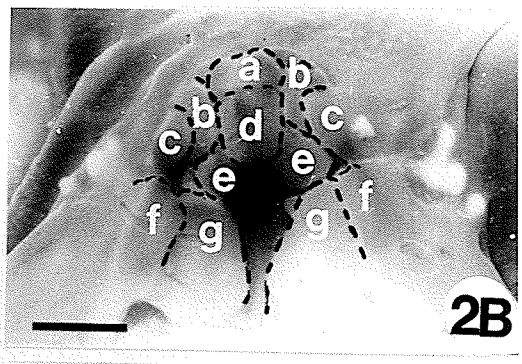
d) Palatal tubercle (unpaired).
e) Conchal tubercle (paired).
g) Palatal shelves (paired).

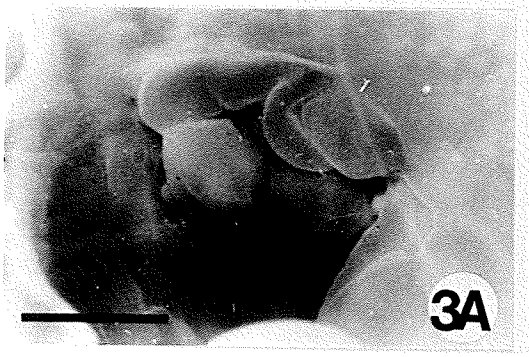
Note that the separation between the palatal shelves is wider than in the normal embryos.

3A,B Palatal view of a stage 36 embryo exposed to 5 mg/ml retinoic acid at stage 20. Variation from the typical arrangement of tubercles. Apparently, maxillary and lateral nasal tubercles are missing on the right side (see above for key to letters).


1A


t
2A


a
b
c
b
d
c
e
e
f
g
g
f
2B


3A

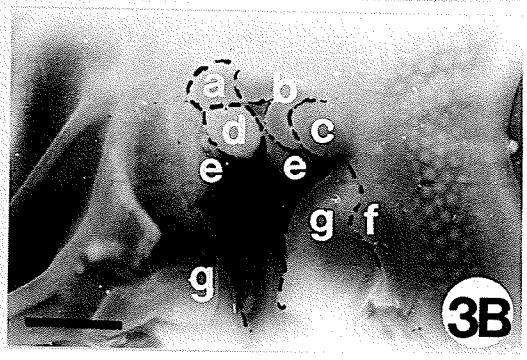

a
b
d
c
e
e
g
f
g
3B

Figure III:5

Chick embryo stage 36 (10-day old), in which the bead soaked in 5 mg/ml retinoic acid was implanted in the middle of the frontonasal mass instead of the right nasal pit. Scale bars = 1 mm.

(A) Frontal view. Medial corners of the right and left eyes are affected (arrows). The defect is symmetrical.

(B) High power view of A. A series of tubercles replaces the normal pointed upper beak.

(C) Lateral view. Upper beak completely missing. The lower beak developed normally.

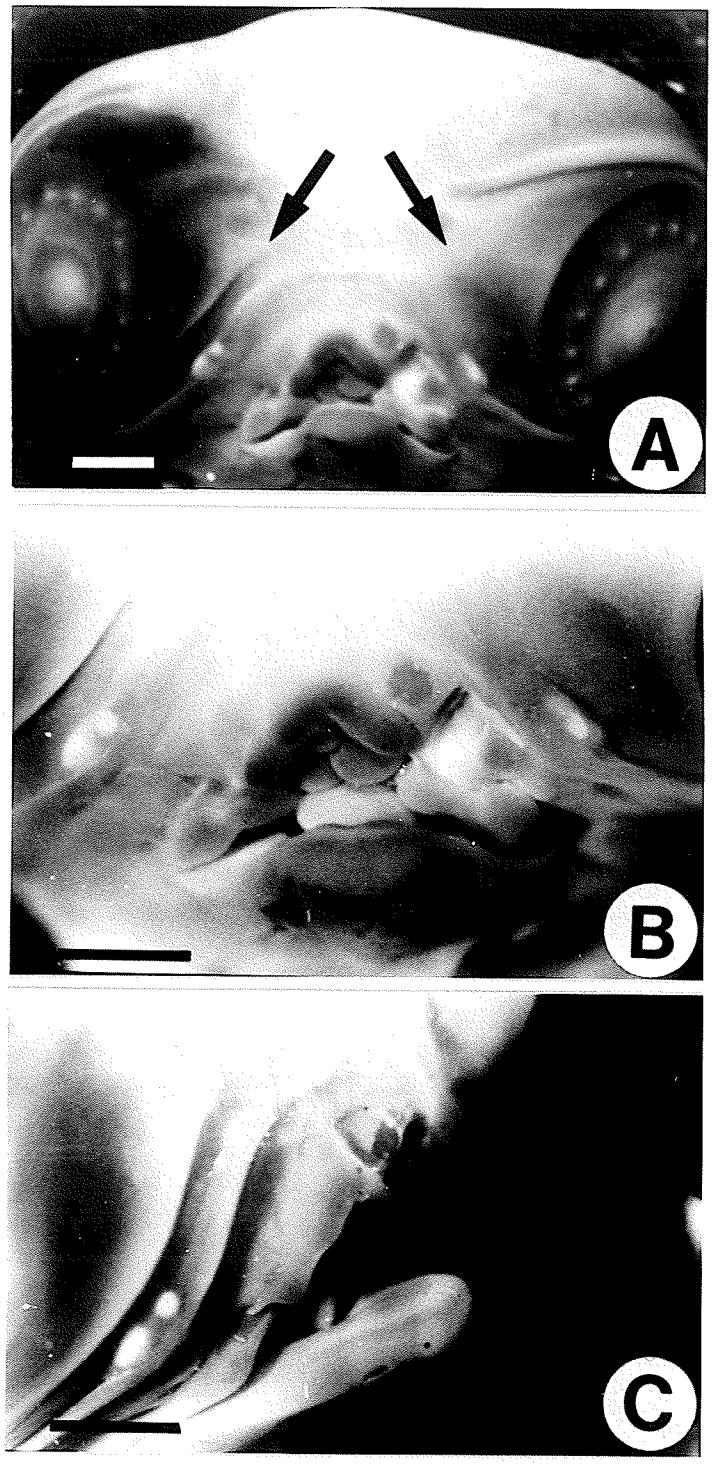

A
B
C

Figure III:6

Dose-Response relationships to retinoic acid treatment

Embryos were treated with 7 different doses of retinoic acid at stage 20 and examined 6 days later at stage 36 (N = 113). Control embryos were treated with the solvent for retinoic acid, dimethylsulfoxide (DMSO; N = 16). The morphology of each face was given a score according to criteria described in Figure III:3.

The severity of the beak defect increases with increasing doses of retinoic acid. It is interesting that even with extremely low doses (0.005 mg/ml RA) some abnormality in the face is observed. With 1 mg/ml of retinoic acid, embryos were found with a wide range of defects from the mildest to complete absence of the upper beak. Doses of 5 and 10 mg/ml gave rise to facial defects with scores of 4 or greater, therefore consistent severe facial defects occur with these higher doses of retinoic acid.

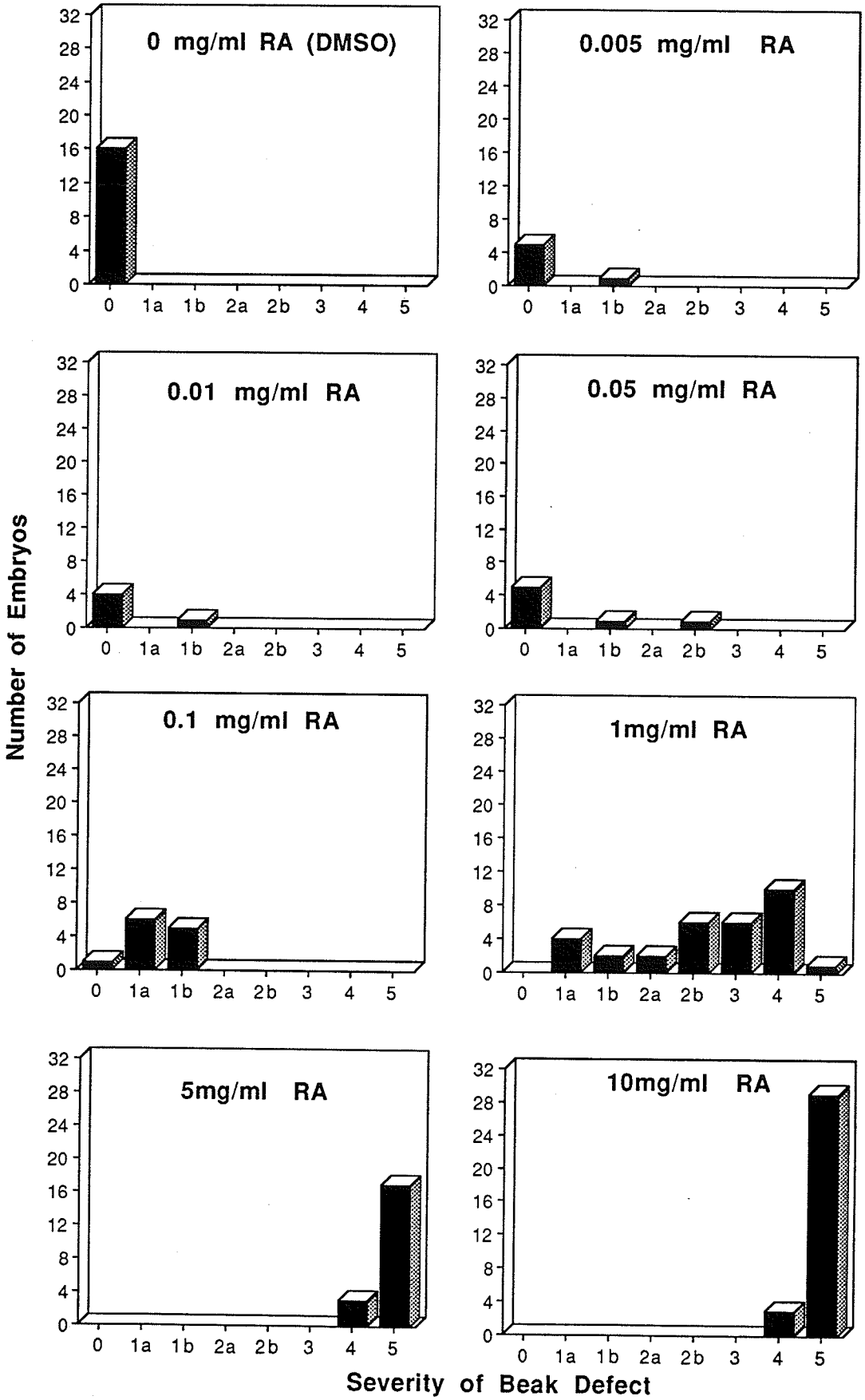

0 mg/ml RA (DMSO)
0.005 mg/ml RA
0.01 mg/ml RA
0.05 mg/ml RA
0.1 mg/ml RA
1mg/ml RA
5mg/ml RA
10mg/ml RA
Number of Embryos
Severity of Beak Defect
0 1a 1b 2a 2b 3 4 5
0 4 8 12 16 20 24 28 32

Figure III:7

Dose-Response relationships to retinoic acid treatment

Severity of beak defect index plotted as a function of the concentration of retinoid in which the beads were soaked. Each point represents the mean score of at least 5 embryos. Embryos with scores 1a and 1b were grouped as 1; and those with scores 2a and 2b were grouped as 2. Although the values on the y axis are not continuous scale, the data was illustrated in this way to show more clearly the minimum dose required to produce a full upper beak defect.

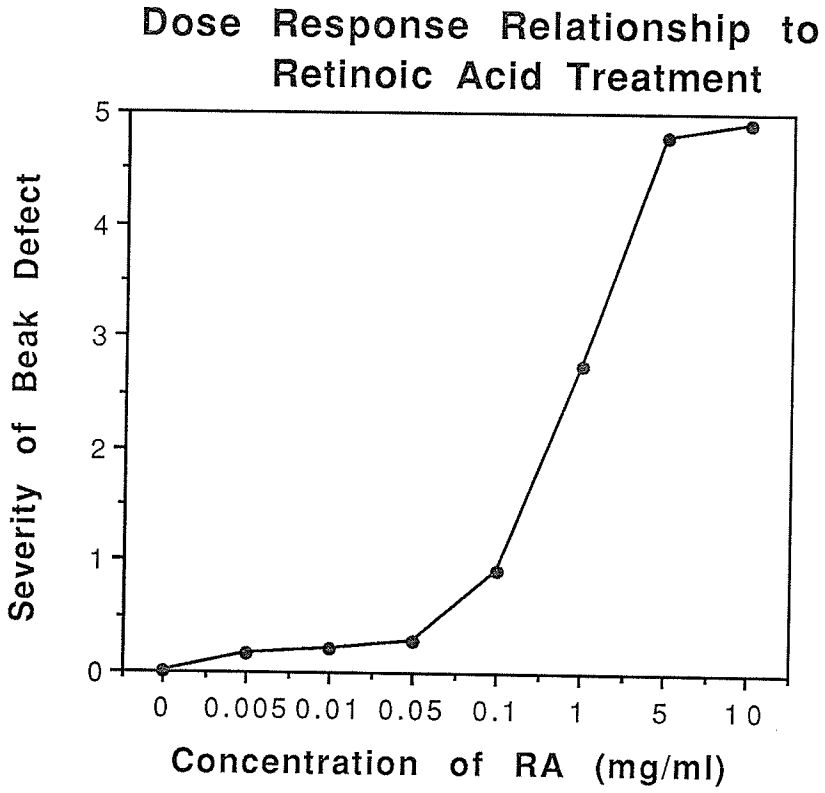

Dose Response Relationship to Retinoic Acid Treatment
Severity of Beak Defect
0
1
2
3
4
5
0
0.005
0.01
0.05
0.1
1
5
10
Concentration of RA (mg/ml)

Figure III:8

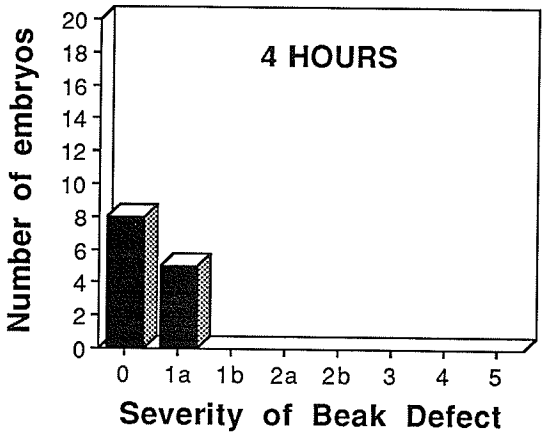
Length of exposure to retinoic acid treatment

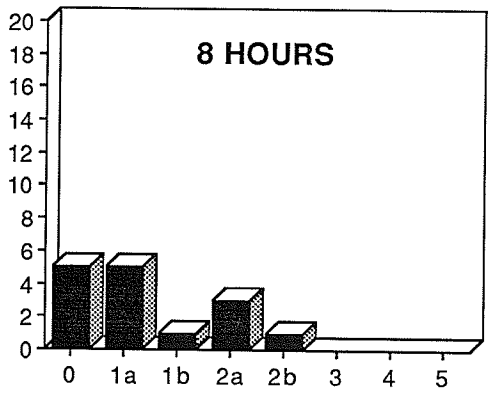
Controlled release beads soaked in 5 mg/ml of retinoic acid were removed at various times after implantation, in stage 20 chick embryos (N = 73).

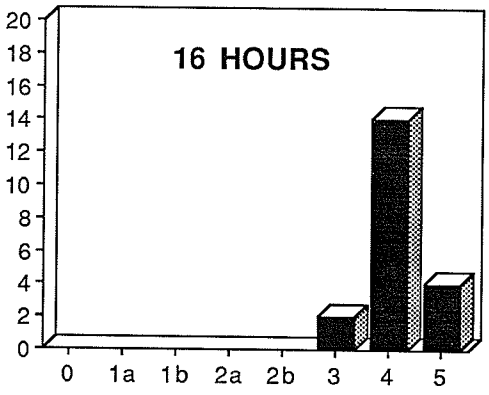
In one embryos of the 4 hr, 8 hr and 16 hr groups the bead was not removed at the specific time, instead it was left in place for a further 6 days, in order to verify the reproducibility of the effect. All 3 embryos had a absent upper beak (score 5).

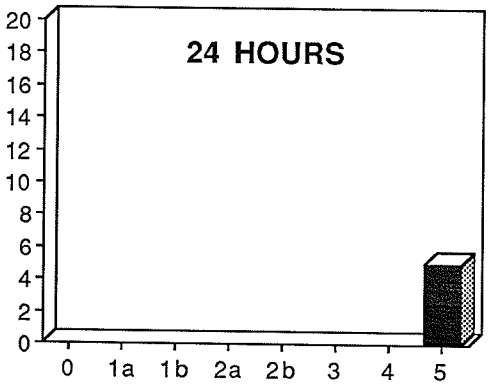
It is surprising, that 4 hr exposure to retinoic acid already produced a mild defect. With and exposure of 16 hr all embryos were affected (score 3-5); but a 24 hr exposure was necessary to give the full defect (score 5) in all the embryos. Wedden and Tickle (1986a), also required a 24 hr exposure to produce full defects.

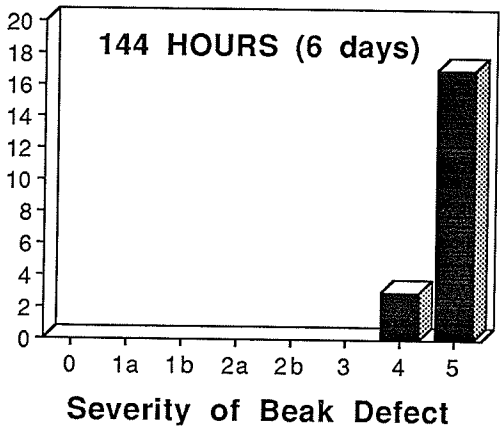
The graph in the right bottom corner illustrates the severity of beak defect index, plotted as a function of the length of time the beads presoaked in 5 mg/ml of retinoic acid were left in place. Each point represents the mean score of at least 5 embryos. (See Figure III:7 for explanation of Mean score).


4 HOURS
Number of embryos
20
18
16
14
12
10
8
6
4
2
0
0 1a 1b 2a 2b 3 4 5
Severity of Beak Defect


8 HOURS
20
18
16
14
12
10
8
6
4
2
0
0 1a 1b 2a 2b 3 4 5


16 HOURS
20
18
16
14
12
10
8
6
4
2
0
0 1a 1b 2a 2b 3 4 5


24 HOURS
20
18
16
14
12
10
8
6
4
2
0
0 1a 1b 2a 2b 3 4 5


144 HOURS (6 days)
20
18
16
14
12
10
8
6
4
2
0
0 1a 1b 2a 2b 3 4 5
Severity of Beak Defect

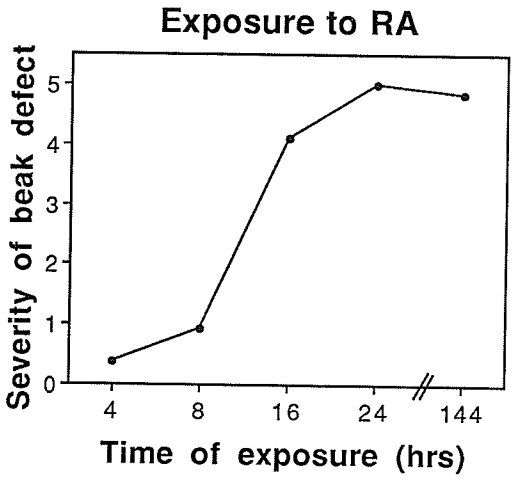
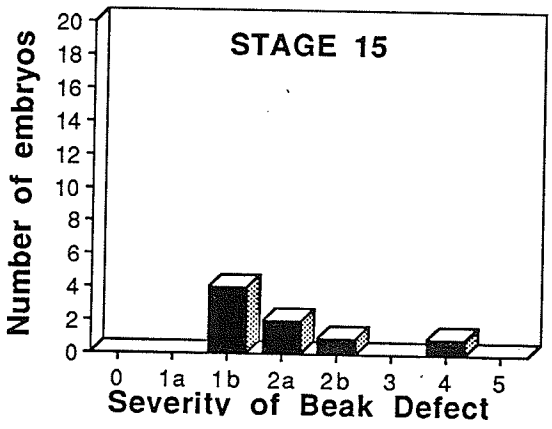

Exposure to RA
Severity of beak defect
5
4
3
2
1
0
4 8 16 24 144
Time of exposure (hrs)

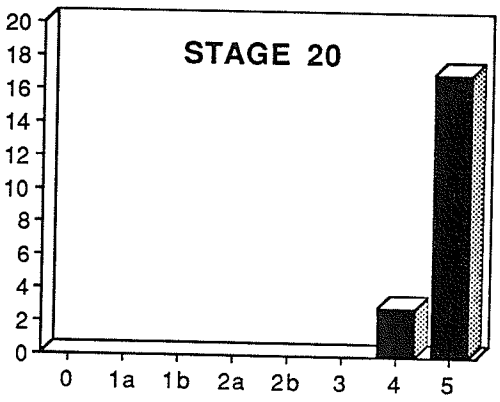
Figure III:9

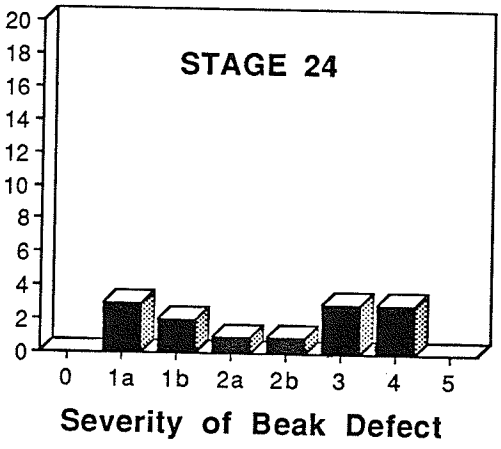
Stage sensitivity to retinoic acid treatment

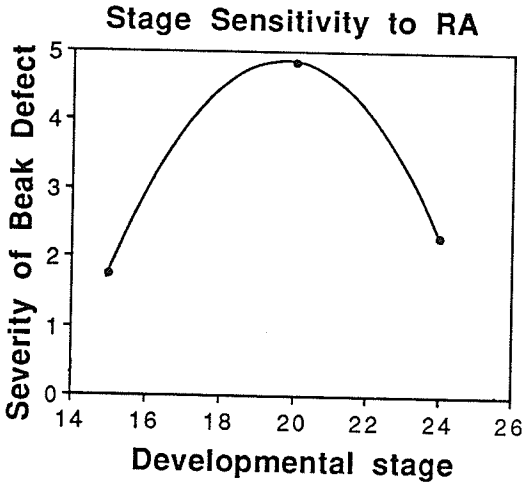
Beads soaked in 5 mg/ml retinoic acid were implanted in chick embryos at 3 developmental stages and examined 5-7 days later at stage 36 (N = 41). A full defect was obtained when retinoic acid is applied at stage 20; less severe defects occurred at earlier or later stages of development (stage 15 and 24). Wedden and Tickle (1986a), also demonstrated that stage 20 embryos are more sensitive, than younger or older stages when beads are implanted in the right limb bud.

The graph in the right bottom corner, illustrates the severity of beak defect index plotted as a function of the developmental stage at which the retinoid was applied. Each point represent the mean score of at least 8 embryos. (See Figure III:7 for explanation of Mean score).


STAGE 15
Number of embryos
20
18
16
14
12
10
8
6
4
2
0
0 1a 1b 2a 2b 3 4 5
Severity of Beak Defect


STAGE 20
20
18
16
14
12
10
8
6
4
2
0
0 1a 1b 2a 2b 3 4 5


STAGE 24
20
18
16
14
12
10
8
6
4
2
0
0 1a 1b 2a 2b 3 4 5
Severity of Beak Defect


Stage Sensitivity to RA
Severity of Beak Defect
5
4
3
2
1
0
14 16 18 20 22 24 26
Developmental stage

CHAPTER IV

INTRODUCTION

The extracellular matrix plays an important role in embryonic cell differentiation and morphogenesis. Interactions between many cells define matrix molecules have been associated with cell differentiation (Zannetti and Solursh, 1986). Such molecules include cell-cell adhesion molecules (Edelman, 1985), as well as extracellular matrix proteins (Timpl *et al.*, 1983; Yamada, 1983). Recently tenascin, another extracellular matrix glycoprotein has been implicated in the control of morphogenetic events that take place during embryonic development (Chiquet and Fambrough, 1984a,b).

In the embryonic chicken, tenascin first appears during gastrulation (Crossin *et al.*, 1986), and is present in neural crest pathways (Bronner-Fraser, 1988; Crossin *et al.*, 1986; Mackie *et al.*, 1988). Later in development it is present in the extracellular matrix of the central and peripheral nervous system (Chiquet, 1989). Tenascin is also found in non-neural tissues such as muscle and myotendinous structures (Chiquet and Fambrough, 1984a,b), cartilage (Vaughan *et al.*, 1987), eye (Bronner-Fraser, 1988; Kaplony *et al.*, 1989; Fyfe *et al.*, 1988), and in the lung and kidney (Crossin *et al.*, 1986). Tenascin has been found to be expressed in tissues undergoing epithelial-mesenchymal interactions such as

mammary glands, hair follicles (Chiquet *et al.*, 1986, Inaguma *et al.*, 1988) and teeth (Chiquet *et al.*, 1986; Thesleff *et al.*, 1987).

In adult life tenascin reappears in malignant tumours such as mammary adenocarcinoma in rats (Mackie *et al.*, 1987); during wound healing in the dermis and matrix underlying the migrating epithelium (Mackie *et al.*, 1988), and also during the process of repair that follows peripheral nerve injury (Daniloff *et al.*, 1989).

It has been shown that after the cartilage elements have differentiated, tenascin continues to be expressed in the perichondrium of vertebral and limb cartilage in chick fetuses (Chiquet and Fambrough, 1984a). Archer *et al.* (1983), have stressed the importance of the perichondrium for the morphogenesis of the long bones. Tenascin could be deposited together with type I collagen and fibronectin during the formation of a tough, cylindrical, perichondrial sheet. This might constrain the chondrogenic area and thereby allowing only lengthening of the long bones. Tenascin may similarly be necessary for extension of Meckel's cartilage and prenasal cartilage.

Retinoic acid locally released in the face leads to changes in the face shape (see chapter III). The face first begins to look abnormal 48 hr after retinoic acid treatment is initiated, therefore, there is a 24 hr period during which irreversible changes have taken place at the molecular level, but morphological changes have yet not occurred (Wedden and Tickle, 1986a). It is possible to use the retinoic acid

model system to study early changes in extracellular matrix molecules that lead to abnormal face shape.

Cartilage differentiation begins with the rounding and condensation of mesenchymal cells, followed by differentiation (Solursh *et al.*, 1982; Rooney *et al.*, 1984). In rodents it has been shown that tenascin is present in the early mesenchymal condensations of cartilage and bone, but not in the surrounding mesenchyme. Tenascin progressively disappeared with the accumulation of mature cartilage or bone matrix (Mackie *et al.*, 1987). A similar aggregation of cells occurs in chondrogenic regions of the frontonasal mass and mandible. Tenascin could therefore be involved in early cell-cell interactions that lead to cell aggregation *in vivo*.

The aim of experiments in this chapter is to describe the distribution of tenascin in the embryonic chick face of normal and abnormal embryos (retinoic acid treated), prior to and during cell differentiation. The goal is to determine if the distribution of tenascin protein correlates with the gradual establishment of the midline in the upper beak. For this purpose tenascin was localized by immunohistochemisty in the facial primordia of chick embryos.

MATERIALS AND METHODS

I. Preparation of embryos

The distribution of tenascin was determined in the facial primordia of 16 chick embryos stage 20, 24, 28, and 30. Facial defects were produced in 6 embryos by implanting beads soaked in 5 mg/ml retinoic acid following the same procedure described in chapter I. Beads were left for the entire duration of the experiment. Normal specimens did not receive any treatment (i.e no beads were implanted).

II. Preparation of tissues

Heads were dissected in ice cold phosphate buffered saline (PBS) on an ice cooled slab and fixed overnight in freshly made 4% paraformaldehyde in PBS at 4°C. Heads were rinsed in PBS at 4°C for 30 min, then dehydrated in a series of ethanols; 1 X 1:1 EtOH/PBS 30 min; 2 X 70% EtOH 15 min; 1 X 85% EtOH 15 min; 1 X 95% EtOH 15 min; 2 X 100% toluene, 30 min each, and 3 X in freshly melted and filtered paraffin (Histowax BaxterCanlab) at 60°C, for 20 min each. Blocks were stored at 4°C until required for sectioning.

III. Preparation of slides and sectioning of blocks

A. Slide cleaning and coating

Glass slides were cleaned by dipping in 10% HCL in 70% ethanol (EtOH) 10 sec; 95% EtOH 10 sec; and then dried in oven at 150°C 1 hr. Slides were dipped in a 2% solution of 3-Aminopropyltriethoxysilnane (TESPA, Sigma) in

acetone for 10 sec; 2 X 100% acetone, 10 sec each; 1 X in distilled water 10 sec, and baked dry at 42°C, 1 hr to 1 1/2 hr.

B. Sectioning

Five μm thick sections were made and sections from representative areas of the head were selected to be placed onto the slides. TESPA coated slides were placed on a hotplate (40°C) and a drop of distilled water was placed on top of each one of them. Sections were floated onto the water, and dried by leaving the slides overnight on the hotplate. Each slide contained serial sections from up to 4 different blocks. Sections were stored in slide boxes at 4°C until used. Slides were divided into 2 groups. Slide "a" was incubated with the experimental antibody and slide "b" with the control antibody. Thus it was possible to compare adjacent sections stained with the 2 antibodies.

IV. Immunohistochemical staining

Sections were dewaxed 2 times in xylene 2 min each, and hydrated in a series of alcohols, 2 X 100% EtOH 5 min each (a special pen DAKOPEN [Dakopatts, Dimension Labs] was used to circumscribe a line around each section which helped to limit the flow of the antibodies), 1 X 95% EtOH 2 min; 1 X 70% EtOH 2 min, 1 X 50% EtOH 2 min and 3 X PBS 2 min each.

To prevent masking by other matrix components sections were digested with hyaluronidase (2 mg/m)l solution in PBS containing protease inhibitors (10 μg/ml of Pepstatin A; 1 mM EDTA, 1 mM Iodoacetamide, 1 mM

Phenylmethylsulfonyl fluoride (PMSF), for 1 hr in humidified trays at room temperature, then rinsed 3 times in PBS 2 min each.

Sections were incubated for 30 min with PBS + 1% bovine serum albuminin (BSA) to block non-specific binding of antibodies, at room temperature. The excess was drained and primary antibodies applied to the sections.

Sections were incubated with the first antibody (Fig. IV: 1) that consisted of monoclonal antibodies to tenascin and or monoclonal antibodies to heparan sulfate proteoglycan (HSPG) obtained from the Developmental studies Hybridoma Bank). These antibodies have been used for immunocytochemistry by others in chick embryos (Chiquet and Fambrough, 1984a,b; Croucher and Tickle, 1989). Both antibodies were diluted 1:100 with PBS containing 1% Bovine serum albumin (BSA, Sigma) and 0.01% sodium azide, in humidified trays for 1 hr at 37°C. Slides were then washed 3 times in PBS for 2 min each.

In the present situation the use of pre-immune serum as the control would be contra indicated, because the process of generating monoclonal antibodies does not involve immunizing a mouse as does the fabrication of polyclonal antibodies. Anti - Heparan Sulfate Proteoglycan (HSPG) was used as the control, because this is a monoclonal antibody (product of a single immortalized B lymphocyte), that recognizes a different molecule than anti-tenascin and has a different distribution in the embryo. Biotinylated second antibody specific to mouse IgG (Amersham)

was placed on the sections at 1:20 dilution in PBS/ 1% BSA/ 0.01% sodium azide, for 1 hr at 37°C. Rinsed 3 times PBS 2 min each.

Antibody localization was visualized with the application of streptavidin - fluorescein isothiocyanate (Amersham) diluted 1:20 in PBS/ containing 1% BSA/ and 0.01% sodium azide, for 15 min at 37°C. Sections were given a final rinse 3 X PBS, 2 min each, and mounted under coverslips in glycerol/PBS (9:1 v/v), containing Paraphenoldiamine (DPDA, Sigma), to preserve the fluorescence during subsequent microscopy. Slides were stored at 4°C after staining.

Sections were examined with an Olympus microscope equipped with an epifluorescence attachment (BH2-RFCA) and 35 mm camera. Representative sections were selected, and in specific areas the amount of staining was recorded as strong, moderate, weak or absent. Sections were photographed with color slide film (Kodak Ektachrome 400).

RESULTS

The distribution of tenascin and Heparan Sulfate Proteoglycan (HSPG) was mapped in 10 normal and 6 abnormal (retinoic acid treated) embryonic faces at stages 20, 24, 28 and 30. The staining patterns produced by antibodies to tenascin and HSPG are summarized in (Table IV:1).

Tenascin was concentrated in a similar pattern in normal and retinoic acid treated embryos up to stages 28. At stage 30 a noticeable difference was observed between the normal and the treated embryos. In addition to areas previously expressing tenascin, stage 30 embryos also expressed tenascin in the notochord and vertebrae. In the retinoic acid-treated embryos only the mesenchyme medial to the eye labelled with tenascin antibodies.

The HSPG antibody in general colocalized with tenascin up to stage 28, in both normal and retinoic acid treated embryos. At stage 30 the distribution and intensity of HSPG was generally weaker than at younger stages, and HSPG staining was completely absent in sections of treated embryos.

I. Stage 20 embryos

A. Normal embryos

In sections incubated with tenascin antibodies there was weak staining in the following areas: primary stroma of the cornea of the eye, lateral mesenchyme of the frontonasal mass, lateral and central region of the second branchial arch where the hyoid cartilage is condensing (not illustrated).

In sections incubated with HSPG antibodies only the surface epithelium of the face and head exhibited slight staining, (Fig. IV:2:1D). The difference in distribution of HSPG antibodies compared to tenascin antibodies confirms the specificity of each staining pattern.

II. Stage 24 embryos

A. Normal embryos

The tissue distribution of tenascin seen at stage 20 was maintained through stage 24, but the intensity of the stain generally increased (Table IV:1A). The primary stroma of the cornea stained as a bright line (not shown). In addition, the mandibular primordia expressed tenascin in a inverted V distribution, the tip of the V pointing up towards the stomodeum (not shown); this shape corresponded to the condensing Meckel's cartilage.

In sections labelled with anti-HSPG the staining previously seen in the face epithelium disappeared, and now some stain was seen in the basement membrane surrounding the eye primordia (not shown).

B. Embryos treated with retinoic acid

The tissue distribution of tenascin in stage 24 abnormal embryos (Fig. IV:2 2B;3B;4B) was similar to that described in normal embryos. The only differences observed were the additional presence of tenascin in the basement membrane of the nasal pit and basement membrane of the notochord, as well as the mesenchyme

of the condensing vertebrae. These structures were not included in the sections of normal stage 24 embryos, therefore a comparison is not possible.

In sections incubated with anti-HSPG the only tissue that was positively labelled was the primary stroma of the eye (Fig. IV:2:2D).

III. Stage 28 embryos

A. Normal embryos

At stage 28 several additional areas expressed tenascin compared to earlier stages. A thick line of mesenchyme surrounding the eye expressed tenascin as well as a triangular shaped region in the centre of the frontonasal mass (not shown). The tip of the triangular domain of expression in the frontonasal mass points towards the stomodeum (Fig. IV:3A); the other 2 points of the triangle are medial to the nasal pits (Fig. IV:3A).

HSPG antibodies colocalized with tenascin at this stage including in the triangle-shaped chondrogenic region in the centre of the frontonasal mass (not shown).

B. Retinoic acid - treated embryos

Embryos treated with 5 mg/ml of retinoic acid and stained with tenascin were positive in similar areas to those of stage 28 normal embryos (Fig. IV:3B). The only difference noted was that the shape of the staining pattern in the frontonasal mass of the retinoic acid treated embryo was in the form of a band of fluorescence across the frontonasal mass, instead of a triangle, (Fig. IV:2:5B).

This difference in appearance was likely due to dimpling of the centre of the frontonasal mass which is the earliest morphologic change observed in the face following retinoic acid treatment (Rowe *et al.*, 1991).

The staining pattern in the frontonasal mass in sections incubated with HSPG antibodies (Fig. IV:2:5D) was similar that shown in sections incubated with tenascin (Fig. IV:2:5B).

IV. Stage 30 embryos

A. Normal embryos

The tissue distribution of tenascin at stage 30 (Fig. IV:2:6B; Fig. IV:3C) was similar that seen at stage 28. Both the basement membrane of the notochord and the perichondrium of the vertebral cartilage were labelled with anti-tenascin (not shown). The mandible was only present in one of the sections and curiously it did not express tenascin as expected. A possible explanation is that the section was very shallow and did not include the developing Meckel's cartilage.

Sections incubated in HSPG antibodies had weak staining around the eye and the centre of the frontonasal mass (Fig. IV:2:6D). The notochord and vertebrae areas were not stained with HSPG antibodies (not shown).

B. Retinoic acid - treated embryos

Surprisingly, the pattern of staining detected with tenascin antibodies changed significantly compared to the pattern observed in stage 28 retinoic acid-

treated embryos (Fig. IV:3B). Stage 30 treated embryos only presented with tenascin staining in the mesenchyme surrounding the eye (Fig. IV:3D).

HSPG antibody staining was also different compared to normal embryos of a similar stage. Retinoic acid treated-specimens did not exhibit staining with HSPG antibodies.

DISCUSSION

I. Tenascin involvement in establishment of prechondrogenic mesenchymal condensations

In the present experiments tenascin was found to be expressed in the frontonasal mass and mandible in areas where cartilage differentiates. Early in development (stage 20-24), tenascin was found to be distributed in the undifferentiated mesenchyme at the lateral edges of the frontonasal mass. This distribution in areas undergoing chondrogenesis correlates with the results obtained in chapter II, where it was shown that fragments taken from the lateral thirds of the frontonasal mass have the potential to form cartilage structures. Central thirds also can form cartilage, yet tenascin was not observed in this region. A possible explanation was that the sections were to deep in this area and did not include the tenascin rich region. Later in development (stages 28-30), tenascin was strongly expressed in the middle of the frontonasal mass; this corresponds to the time when the prenasal cartilage is specified in the centre of the frontonasal mass (Wedden and Tickle, 1986b). The maxillae was not included in many sections but data suggest that tenascin is not expressed in this primordium. Therefore tenascin expression seems to be restricted to parts of the face that develop cartilage.

Tenascin expression is not restricted to neural crest cell-derived cartilage. In addition other chondrogenic regions outside the head also expressed tenascin, such as the hyoid cartilage and the developing vertebrae. Tenascin expression in

these areas has also been shown by others (Chiquet and Fambrough 1984a; Crossin *et al.*, 1986; and Bronner-Fraser, 1988). This demonstrates that tenascin is a conserved protein involved in chondrogenesis from both neural crest and non-neural crest derived mesenchyme.

II. Tenascin is involved in epithelial-mesenchymal interactions

Patterning of the frontonasal mass is dependent on epithelial mesenchymal interactions (Wedden, 1987; Richman and Tickle, 1989). I did not observe tenascin at epithelial-mesenchymal interfaces in specific regions of the face. However, it is possible that sagittal sections will reveal a localized concentration of tenascin in the mesenchyme where the egg tooth will form. The formation of the egg tooth at the tip of the upper beak, requires an inductive signal from the underlying frontonasal mass mesenchyme (Tonegawa, 1973). Future experiments will be conducted to investigate this possibility.

III. Tenascin expression correlates with cell migration

I observed expression of tenascin in the developing cornea at all stages studied (20-30). This same result has been reported by Kaplony *et al.* (1991). These investigators found that tenascin expression (220 kD), correlated with corneal cell migration in chick embryos during development (stages 18-43). It has been suggested that the role of tenascin in cell migration is to "loosen the grip" of cells, which otherwise tightly adhere to substrata such as fibronectin (Chiquet-Ehrismann *et al.*, 1988).

IV. Tenascin is not directly responsible for retinoic acid induced changes in morphology

In the retinoic acid treated embryos, tenascin was expressed at stages 24-28 in the face, and then abruptly disappeared. This change in tenascin expression happened after morphologic changes have occurred (Tamarin *et al.*, 1984; Wedden and Tickle, 1986a; Rowe *et al.*, 1991). Thus, tenascin expression is secondarily affected by changes in morphogenesis induced by retinoic acid. A possible explanation for the disappearance of tenascin at stage 30, may be that retinoic acid leads to an inhibition of cartilage differentiation. Tenascin can be expressed in the undifferentiated aggregating mesenchyme, even though molecular changes have been produced in the cells by retinoic acid. However it is when cartilage differentiation does not occur, that tenascin is turned off. Therefore, retinoic acid may indirectly lead to cessation of tenascin expression in chondrogenic areas of the frontonasal mass. Other areas continued to express tenascin as usual, such as the notochord and vertebrae.

V. Tenascin colocalizes with other molecules expressed in the face *in vitro*

Not much is known about the molecular factors implicated in modulating the expression of tenascin. Transforming growth factor ß (TGFß), is known to be expressed in the face of mammalian embryos (Heine *et al.*, 1987). In addition TGFß can induce a four-fold increase in the synthesis of tenascin by chick embryo fibroblasts (Pearson *et al.*, 1988). Another growth factor, insulin like growth

factor-II (IGF-II) is expressed at high levels in the centre of the frontonasal mass at stage 28 (Ralphs *et al.*, 1990). Thus, TGF-ß and IGF-II could possibly interact with tenascin, to promote cell aggregation and proliferation of potential chondroblasts.

Finally, at least one other extracellular matrix molecule, HSPG is also expressed in regions undergoing chondrogenesis. Although these results are not central to the thesis, it is interesting that HSPG is expressed in many of the same regions as tenascin. HSPG is traditionally thought of as a basement membrane constituent (Rapraeger and Bernfield, 1985), however my results clearly show that HSPG must have other functions. An interaction between HSPG and tenascin is possible, and further cell culture experiments are necessary to confirm this.

Many other extracellular matrix molecules are probably expressed in a spatiotemporally restricted manner in the chick face as they are in the mouse (Richman and Diewert, 1987). Studying distribution of other molecules with *in situ* hybridization and immunohistochemistry will be necessary before we can propose a model of how facial pattern is set up.

Table IV:1

Expression patterns of tenascin and heparan sulfate proteoglycan (HSPG), in facial sections of normal and retinoic acid treated chick embryos.

A. Normal Embryos

		Tenascin								HSPG							
S	N	Ps	Me	Fe	Np	Fe	Md	Ha	N,V	Ps	Me	Fe	Np	Fn	Md	Ha	N,V
20	4	+	-	-	-	+	-	+	-	-	-	+	-	-	-	-	-
24	2	+ + +	+ +	-	*	+ +	+ +	+ +	*	+	-	-	*	-	-	-	*
28	2	-	+ + +	-	-	+ + +	*	*	*	-	+ + +	-	-	+ + +	*	*	*
30	2	-	+ + +	-	-	+ + +	-	-	+ + +	-	+	-	-	+	-	-	-

B. RA Treated Embryos

		Tenascin								HSPG							
S	N	Ps	Me	Fe	Np	Fe	Md	Ha	N,V	Ps	Me	Fe	Np	Fn	Md	Ha	N,V
24	2	+ + +	+ +	-	+ +	+ +	+ + +	+ +	+ + +	+ +	-	-	-	-	-	-	-
28	2	-	+ +	-	-	+ +	*	*	*	-	+ +	-	-	+ +	*	*	*
30	2	-	+ +	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Key: N = number of specimens, S = stage of development. PS = primary stroma of the cornea; Me = medial mesenchyme; Fe = face epithelium; Np = nasal pit; Fn = frontonasal mass; Md = mandible; Ha = hyoid arch; N,V = notochord and vertebrae. Extent of staining: strong (+ + +), moderate (+ +), weak (+), absent (-), and area not present in the section (*).

Figure IV:1

Diagram illustrating some of the steps involved in the immunohistochemical localization of protein molecules

(A) Incubation in primary antibody. Experimental sections were incubated with anti-tenascin monoclonal antibodies, and control sections were incubated with anti-HSPG monoclonal antibodies made from immortalized B lymphocytes.

Rinsed 3 times in phosphate buffered saline (PBS), 2 min each.

(B) Experimental and control sections were incubated in biotinylated second antibody, specific to mouse IgG.

Not shown is an additional step of applying streptavidin -fluorescein isothiocynate to detect the antibody location.

(C) Rinsed 3 time in (PBS), 2 min each.
Coverslip.

Immunolocalisation of Protein Molecules

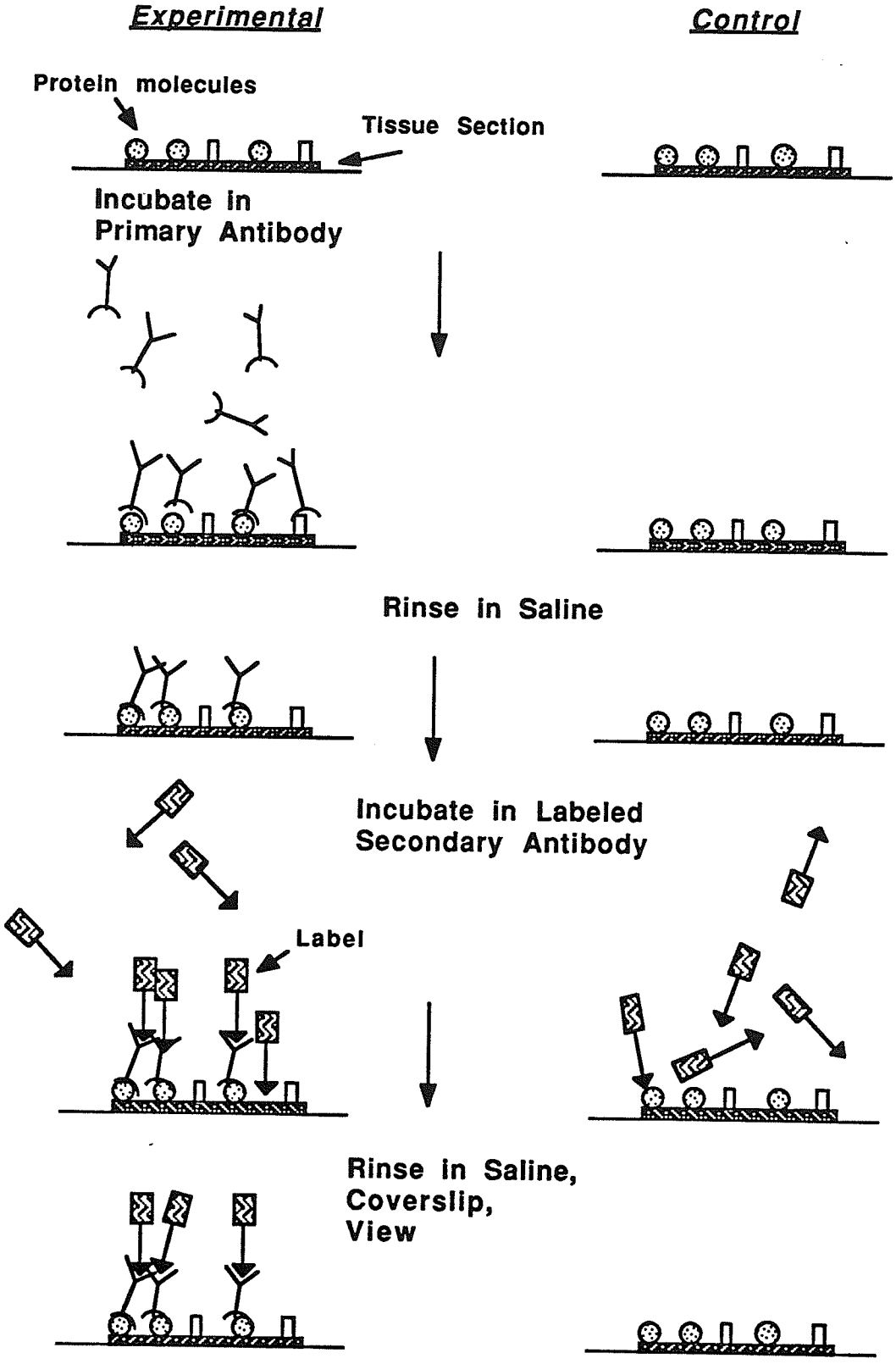


Figure IV:2

Tissue distribution of tenascin and heparan sulfate proteoglycan (HSPG), in serial sections of chick embryos at stages 20, 24, 28 and 30. Scale bars in photographs in column A = 1 mm. Scale bars for micrographs in B, C and D = 100 μm.

Column (A)- Low power of entire section. Column (B)- Tenascin antibody. Column (C)- phase photographs of exactly the same areas where antibody stain is present; all the phase photos show evenness of cell density in these areas, which confirms that the stain is not due to localized increase in cell density. Column (D)- HSPG antibody.

1 Normal embryo (non-treated), stage 20.
- (A) Dashed lines (---) show a lateral area of the head and face, where high power photos were taken. Scale bar = 1 mm.
- (B) Surface epithelium of head and face negative to tenascin. Scale bar = 100 μm.
- (D) Surface epithelium of head and face stain with antibody to HSPG. se = surface epithelium.

2 Stage 24 embryo treated with 5 mg/ml retinoic acid at stage 20.
- (A) Dashed lines indicating the disto-lateral area of the eye.
- (B) The primary stroma of the eye stained as a bright line with antibody to tenascin. ps = primary stroma, le = lens.
- (D) Same distribution of antibody to HSPG in the eye as seen with tenascin antibody.

3 Stage 24 embryo treated with 5 mg/ml retinoic acid at stage 20.
- (A) Dashed lines showing notochord and condensing vertebrae.
- (B) The basement membrane surrounding the notochord and the condensing vertebrae express high levels of tenascin. n= notochord, v= vertebrae mesenchyme.
- (D) Notochord and vertebrae are negative to HSPG antibody.

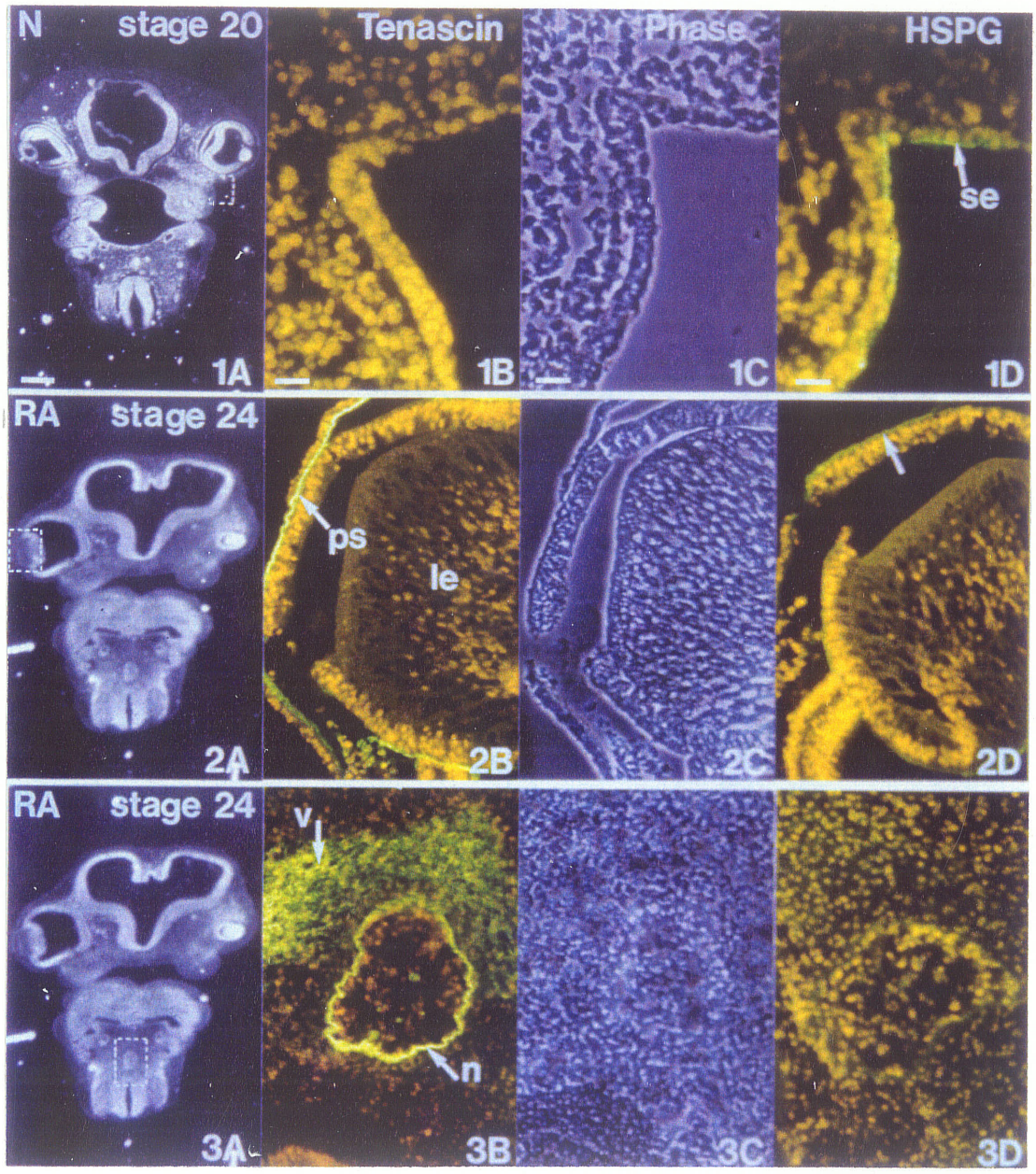

N
stage 20
Tenascin
Phase
HSPG
se
1A
1B
1C
1D
RA
stage 24
ps
le
2A
2B
2C
2D
RA
stage 24
v
n
3A
3B
3C
3D

Figure IV:2 (continued)

4 Stage 24 embryo treated with 5 mg/ml retinoic acid.

(A) Dashed lines indicating the central area from where high power views were taken.

(B) Staining pattern in a inverted v distribution; the tip of the v points upwards (arrow). md = mandible.

(D) Photo taken in a different orientation, but also showing the mandibular primordia which is negative to HSPG antibody.

5 Stage 28 embryo treated with 5 mg/ml retinoic acid.

(A) Dashed lines showing the left medio-lateral area of the frontonasal mass. No mandible is included in this section.

(B) The shape of staining pattern to tenascin antibody is in the form of a band of fluorescence across the frontonasal mass. fnm = frontonasal mass. oc = oral cavity.

(C) Same pattern of HSPG antibody expression as in B.

6 Normal embryo (non-treated), stage 30.

(A) Dashed lines showing the right medio-lateral area of the frontonasal mass.

(B) Strong stain with tenascin antibody in the frontonasal mass in a triangular shape configuration. The tip of the triangle points towards the oral cavity fnm = frontonasal mass.

(D) Very weak stain in the frontonasal mass with HSPG antibody.

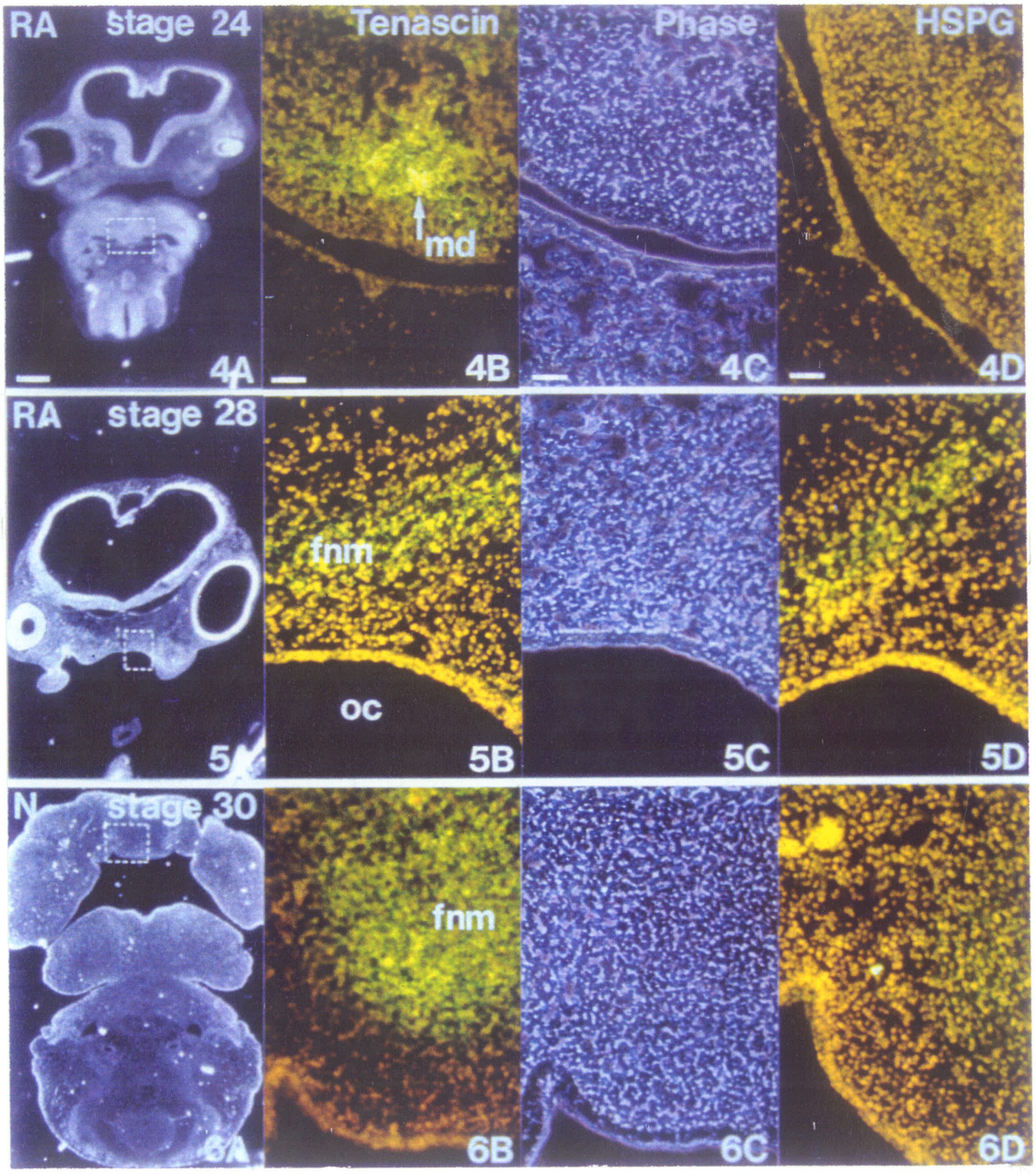

RA stage 24
Tenascin
Phase
HSPG
md
4A
4B
4C
4D
RA stage 28
fnm
oc
5
5B
5C
5D
N stage 30
fnm
6A
6B
6C
6D

Figure IV:3

Camera lucida drawings of tenascin expression in facial sections, of normal and retinoic acid treated embryos, stages 28 and 30.
The mandible is not included in any of the sections. Stippling indicates areas of tenascin expression. Key: e = eye, b = brain, ln = lateral nasal process, fnm = frontonasal mass, mx = maxilla.

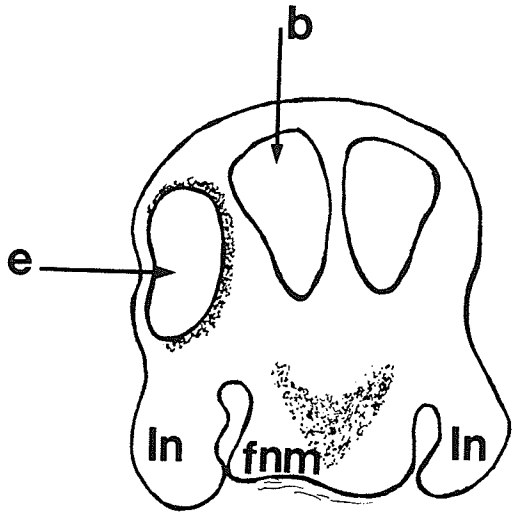
(A) Normal, stage 28 embryo. The mesenchyme around the eye is labelled, as well as the frontonasal mass, where the staining pattern was expressed in a triangular shape.

(B) Retinoic acid, stage 28 embryo. The expression of tenascin colocalized with that seen in 3A in the normal stage 28 embryo The shape of the staining pattern in the middle of the frontonasal mass, is in the form of a band across the primordium.

(C) Normal, stage 30 embryo. Tenascin is expressed in the same areas as at stage 28. The apparent decrease in the amount of staining in the middle of the frontonasal mass, is due to the shallowness of the section.

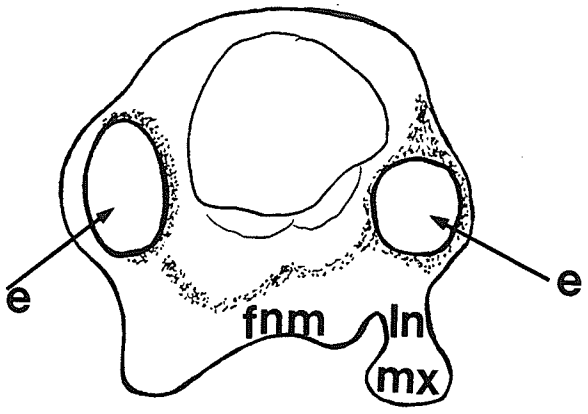
(D) Retinoic acid, treated stage 30 embryo. Tenascin was expressed only around the right eye. The frontonasal mass was negative, even though the plane of section was similar to that seen in 3C.

TENASCIN EXPRESSION



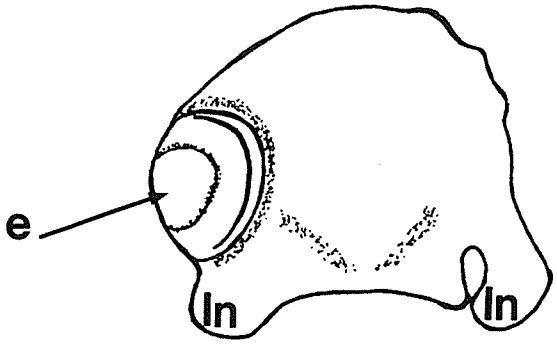
A

NORMAL STAGE 28



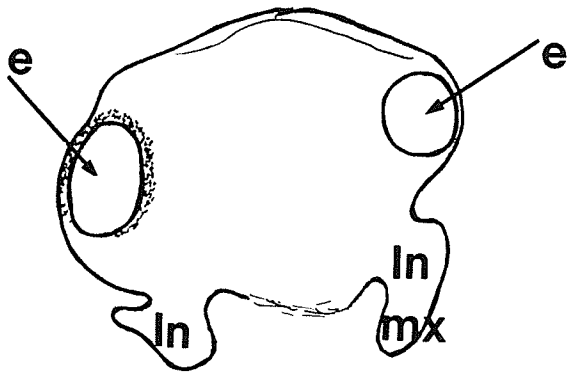
B

RA STAGE 28



C

NORMAL STAGE 30



D

RA STAGE 30

CHAPTER V

GENERAL DISCUSSION

In this chapter I will summarize some of the possible mechanisms involved in generation of the midline of the face, and link these mechanisms to clinical pathologies which include midline craniofacial defects.

I. Regulation of positional information

Positional information could help to generate the midline of the face. Wolpert (1969), hypothesized that mesenchymal cells are assigned positional values in a three dimensional co-ordinate system, and cells then interpret this positional information by differentiating into say, cartilage or connective tissue.

My results have shown that from one frontonasal mass it is possible to obtain at least 3 rods of cartilage (chapter II). This indicates that each fragment of the frontonasal mass can behave as though it were a complete frontonasal mass. Therefore positional values are still flexible at stage 20. The ability to regulate the positional values indirectly supports the premise that positional information operates in the frontonasal mass.

A concentration gradient is one way in which positional information conveys information to the cells. Each part of a structure will have a unique concentration of a signalling molecule (morphogen), which is distributed in a specific pattern.

The morphogen may be released by a small group of cells at one end of the structure (as for example the polarizing region of the limb bud), leading to the formation of a concentration gradient. Cells can interpret the morphogen as a signal to differentiate into for example, an anterior or posterior structure. The presence of concentration gradients within embryonic structures had been confirmed in hydra, drosophila and chick wing buds (reviewed by Wolpert, 1989).

I suspected that if the stage 20-24 frontonasal mass contains a gradient of a signalling molecule, it would be highest in the centre of the frontonasal mass at its caudal edge, and would gradually decrease towards the lateral edges of the primordium in a cranial direction (Fig. V:1). The concentration in the centre would be unique, and therefore cells in this region could use this information to form a single rod of cartilage in the midline. If endogenous retinoic acid is present in the face in the way just previously described, in order to get duplication of midline structures the soaked bead should be placed along the midline in a more cranial position (Fig. V:1); in this way the new gradient will be higher cranially and diffusing symmetrically in a caudal direction. A new mirror-image of the endogenous gradient is created and a second beak should form.

Another way to generate positional information in the face is by setting up a prepattern consisting of unequal distribution of molecules across the structure. This prepattern could be set up by two morphogens that diffuse at unequal rates

(reaction-diffusion model, Turing, 1952). A wave-like pattern which is set up by 2 morphogens could generate repeated structures. For example the digits in the hand could result from a wave-like distribution in a chemical morphogen with the peaks and troughs specifying the digits. A crucial feature is that all the peaks are the same. One could not alter one structure without affecting all, and this greatly limits the classes of patterns that can be generated. There is much less evidence for this model existing in the embryo than concentration gradients (Wolpert, 1989). The frontonasal mass may contain a wave-like prepattern with 3 peaks evenly spaced across the primordia. In chapter II, I discussed the ramifications of a fixed prepattern.

II. Epithelial - mesenchymal interactions

Epithelial-mesenchymal interactions are also involved in patterning of the frontonasal mass primordia and may be necessary for midline formation. Recombination experiments have shown that the facial epithelia contain a signal which encourages outgrowth and differentiation of the facial mesenchyme (Wedden *et al.*, 1987; Richman and Tickle, 1989; Hall, 1991), and the same signal is produced by the epithelium of each facial primordium (Richman and Tickle, 1989). Removal of facial ectoderm effectively truncates the development of fragments of the facial primordia. The effect is analogous to that obtained when

the apical ectodermal ridge (a pronounced thickening in the epithelium at the tip of the limb bud) is removed truncated limbs develop (Summerbell, 1974). The tip of the bud the limb has been called the progress zone (see chapter III). The progress zone model suggests that pattern along the proximo-distal axis of the limb may be specified by the length of time cells spend at the tip of the limb (Summerbell *et al.*, 1973). Such a progress zone mechanism may specify not only the pattern of cartilage differentiation but also that of membrane bone. If a progress zone is involved in upper beak development, then a prediction would be that the mitotic index of the mesenchyme at the distal tip of the frontonasal mass would be high. This experiment has not yet been done.

A second set of epithelial-mesenchymal interactions that may be involved in patterning during development of the facial primordia is suggested by experiments in culture. Epithelium from either mandibular or frontonasal primordia cam inhibit chondrogenesis when placed on top of facial mesenchyme cultures (Wedden *et al.*,1986). The zone of inhibition lies directly under the epithelium. The effect of epithelium on chondrogenesis could serve to confine cartilage differentiation to the core of the developing face. However, *in vivo* experiments in which limb buds have been permanently denuded of 70% of their dorsal epithelium by ultraviolet radiation, showed that this surprisingly has no

effect on the cartilage pattern that develops (Martin and Lewis, 1986). Thus the *in vitro* results in micromass culture may not be related directly to what is happening in the face.

III. Molecules that could be involved in patterning the midline

Over the last few years there has been a rapid progress in identifying molecules that are expressed in developing vertebrates and that could play an important role in patterning and morphogenesis. Molecules of potential interest include retinoic acid (discussed in chapter III). Growth factors can also function as signalling molecules. *In vitro* experiments have demonstrated that basic fibroblast growth factor (bFGF) and activin (a member of the TGFß family of growth factors) can induce mesoderm from *xenopous* animal caps (reviewed by Slack *et al.*, 1989). Animal caps will normally from epithelium in culture but in the presence of bFGF or activin particular types of mesoderm form. It is thought that bFGF and activin are responsible for very early embryonic decisions such as which end of the embryo will become the head. The face is also sensitive to bFGF. Addition of bFGF to cultures of facial mesenchyme has demonstrated that only frontonasal mass mesenchyme is stimulated to divide and differentiate in response to bFGF (Richman and Crosby, 1990). This specific stimulation of a subpopulation of potential chondrocytes in the frontonasal mass and not in other

facial primordia, could enhance cartilage formation in the centre of the face. The next step is to conduct *in vivo* experiments by locally releasing bFGF in the face.

Two other classes of molecules with potential roles in patterning are expressed in the face; nuclear receptors for retinoic acid (Rowe *et al.*, 1991) and homeobox genes. Homeobox genes regulate segment identity along the long axis of the embryo (Hunt and Krumlauf, 1991). So far the only homeobox containing genes specifically expressed at very high levels in facial mesenchyme are Hox 7 and 8 (Hiroaki *et al.*, 1991). However since these genes are not restricted to the midline they are unlikely to play a role in midline determination.

IV. Midline facial defects in humans

Some specific abnormalities in human facial form and jaw relationships can be traced to very early stages of development. Since most structures of the face are ultimately derived from migrating neural crest cells, it is not surprising that interferences with this migration produce facial deformities. Severe facial asymmetry in some people may be related to unequal amounts of neural crest migration on the two sides of the face, although it is difficult to be sure that this is the sole cause.

The most common congenital defect involving the face and jaws, is clefting of the lip and palate. Exactly where clefts appear is determine by the locations at

which fusion of the various processes failed to occur, and this is in turn influenced by the time in embryonic life when there was some interference with development. Retinoic acid induced facial clefting in the chick closely resembles a bilateral cleft lip in the human. Studying the chick embryo treated with retinoic acid may provide clues as to the molecular changes that lead to changes in lip morphology.

Other midline facial defects that occur sporadically include :1) holoprosencephalies, 2) frontonasal dysplasia and 3) frontofacionasal dysplasia. Holoprosencephaly is caused by two different kinds of midline deficiencies of the anterior neural plate. The result of these deficiencies are missing midline components in the brain (Sulik and Johnston, 1982) and face (Sulik and Johnston, 1983). The facial defects result from to the olfactory placodes being too close to the midline, thereby restricting the space for the medial nasal prominences (Sulik and Johnston, 1983). Median facial cleft syndrome, originally recognized by DeMyer (1967), and later renamed frontonasal "dysplasia" (Sedano *et al.*, 1970) has no known etiology. The main clinical manifestations are ocular hypertelorism, a broad nasal root, a bifid nasal tip, hypoplasia of the prolabium and premaxilla with medial cleft lip, and extension of the frontal hair line onto the forehead to for a pronounced widow's peak. Frontofacionasal "dysplasia", was originally described by Lyford and Roy (1974). In addition to midline facial clefts, eye

anomalies are present (palpebral fissures, ptosis, and iris colobomata). The facial defects present in the latter two syndromes involve the median nasal processes that contribute to the central part of the nose the philtrum of the lip; the lateral nasal process which form the outer parts of the nose and the maxillary process that gives rise to the upper jaw, bulk of the upper lip, cheeks and tissue below the eye. The involvement of these facial processes in humans corresponds to those areas affected by retinoic acid treatment in chick embryos. These similarity in the type of eye defect present in both frontofacionasal "dysplasia" and retinoic acid treated chick embryos is striking. In this thesis I have developed a model that closely resembles these two frontofacial "dysplasia" syndromes. Future investigations using locally released retinoic acid in the face may provide new insights into the etiology of these poorly understood human deformities.

Figure V:1

Hypothetical distribution of retinoic acid in the frontonasal mass

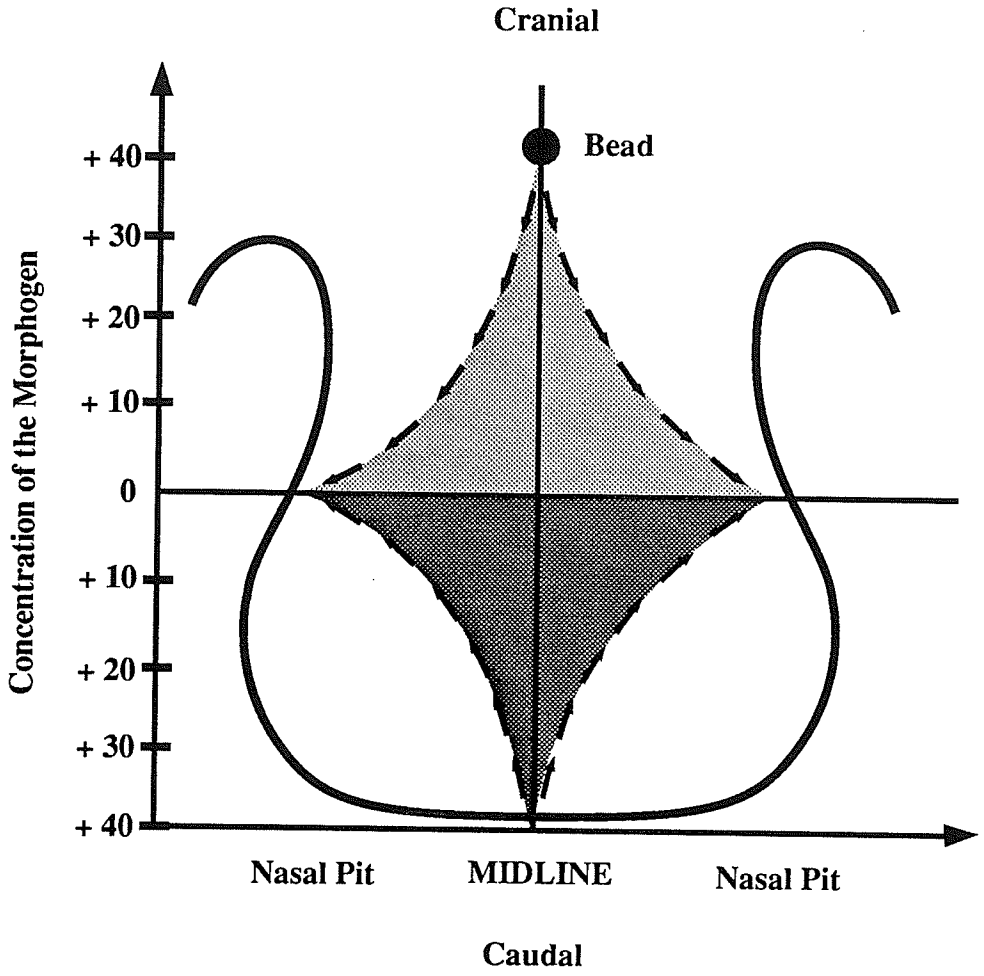
Schematic diagram of the frontal view of the frontonasal mass mesenchyme, showing the possible gradient distribution of a signalling molecule (morphogen).

(x) axis represents the medio-lateral axis of the primordium.
(y) axis corresponds to the concentration of the morphogen along the cranial-caudal axis. (Note that the values are arbitrary along this axis).

The concentration gradient of the morphogen would be highest in the centre of the frontonasal mass at its caudal edge, and gradually decreases in a cranial direction. The concentration in the centre would be unique, and cells could use this information to form a rod of cartilage in this region.


represents the possible endogenous gradient of retinoic acid in the face.


represents the distribution of exogenous retinoic acid in the face required to get a double beak. The bead soaked in retinoic acid would be placed in a more cranial position in the midline, in this way the concentration of retinoic acid will be higher there, and diffusing symmetrically in a caudal direction.



Legend: Mirror image gradient

Hypothetical natural gradient

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