

## Retinoic acid treatment alters the distribution of retinoic acid receptor- $\beta$ transcripts in the embryonic chick face

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### Summary

Retinoic acid is a metabolite of vitamin A that can act as a signalling molecule in a number of developmental systems. Retinoic acid is also known to be teratogenic in mammals, causing a range of defects including abnormalities in craniofacial development. Exposure of the developing chick face to retinoic acid released slowly from a bead implanted in the wing bud results in a specific facial defect, in which outgrowth of the frontonasal mass is inhibited. This results in clefting of the primary palate and absence of the upper beak. To investigate the role of nuclear retinoic acid receptors in normal and abnormal chick face morphogenesis, we isolated chick retinoic acid receptor- $\beta$  (RAR- $\beta$ ) cDNA clones and probed northern blots of RNA isolated from chick embryos at stages 22, 24 and 25 and from adults. RAR- $\beta$  transcripts of 2.8 and 3.5 kb were present in several regions of the embryo, including the facial primordia, and were also present at much lower levels in adult tissues. *In situ* hybridisation showed that RAR- $\beta$  transcripts were present in all of the facial primordia at embryonic stages 20, 24 and 28, but that their distribution was not uniform. Transcripts were abun-

dant in the lateral nasal processes, at the edges and corners of the frontonasal mass and in the anterior part of the maxillary primordia. Lower levels were present elsewhere. Treatment of stage 20 embryos with retinoic acid altered the distribution of RAR- $\beta$  transcripts in the maxillary primordia, such that high levels of transcripts were present throughout, rather than being confined to the anterior part. This change was detectable at stage 24, before any alterations in the morphology of the facial primordia were apparent. By stage 28, when the morphology of the facial primordia was clearly abnormal, there were more widespread changes in the distribution of RAR- $\beta$  transcripts. These results show that RAR- $\beta$  transcripts are particularly concentrated in regions of the primordia that give rise to the upper beak, the development of which is specifically affected by retinoic acid. In addition, they demonstrate that retinoic acid can induce changes in the pattern of expression of RAR- $\beta$  transcripts *in vivo*.

Key words: retinoic acid receptor, RAR- $\beta$ , chick embryo, facial primordia, gene expression.

### Introduction

Retinoic acid is a metabolite of vitamin A that affects differentiation and patterning in a number of developmental systems. For example, application of retinoic acid to the anterior margin of the chick limb bud results in duplication of the digits, and there is evidence that retinoic acid may be a natural signalling substance in the limb bud (Tickle *et al.* 1982; Thaller and Eichele, 1987; Brickell and Tickle, 1989). Retinoids are potent teratogens in mammals, causing a range of malformations including abnormalities in limb and craniofacial development (Satre and Kochhar, 1989; Morriss and Thorogood, 1978; Lammer *et al.* 1985). These teratogenic effects may be related to the proposed normal activity of retinoic acid as a signalling molecule in embryonic development.

Local application of an appropriate dose of retinoic acid to the chick wing bud between embryonic stages 18 and 21 results in an absence of the upper beak in 100% of treated embryos (Tamarin *et al.* 1984; Wedden and Tickle, 1986; Wedden *et al.* 1988). The chick face develops from a series of primordia populated by mesenchymal cells originating in the cranial neural crest. The frontonasal mass, the lateral nasal processes and the maxillary primordia give rise to the upper beak, whilst the mandibular primordia give rise to the lower beak. The basis of the retinoic-acid-induced defect is a failure of outgrowth of the frontonasal mass, which then fails to make contact with the lateral nasal processes and the maxillary primordia, resulting in severe bilateral clefting of the primary palate. Experiments in which the epithelium and mesenchyme from normal and retinoic-acid-treated embryos were recom-

bined have shown that retinoic acid acts on the mesenchymal cells of the frontonasal mass, rather than on the epithelium (Wedden, 1987). The development of the mandibular primordia, which form the lower beak, is unaffected.

It has become clear that cells interpret retinoic acid signals by means of nuclear retinoic acid receptors, which are members of the steroid/thyroid hormone superfamily of nuclear receptors (Green and Chambon, 1988). Three closely related retinoic acid receptors, encoded by different genes, have been identified in humans and mice. These are RAR- $\alpha$  (Petkovich *et al.* 1987; Giguère *et al.* 1987), RAR- $\beta$  (Brand *et al.* 1988; Benbrook *et al.* 1988) and RAR- $\gamma$  (Zelent *et al.* 1989; Krust *et al.* 1989). Studies in mice have shown that each of these receptors has a distinct pattern of expression (Dollé *et al.* 1989; Ruberte *et al.* 1990; Zelent *et al.* 1989; Osumi-Yamashita *et al.* 1990). Murine RAR- $\alpha$  and RAR- $\gamma$  transcripts were found to be uniformly distributed in the early facial primordia, with RAR- $\gamma$  transcripts becoming progressively restricted to regions of chondrogenesis (Ruberte *et al.* 1990; Osumi-Yamashita *et al.* 1990). In contrast, RAR- $\beta$  transcripts were found to be regionally localised (Osumi-Yamashita *et al.* 1990).

The RAR proteins each comprise six domains, designated A to F. Binding of retinoic acid to the E domain converts the receptor into an active transcription factor which can then regulate the expression of target genes containing a suitable response element (de Thé *et al.* 1989, 1990). The receptor binds to DNA via its C domain. The amino acid sequences of RAR- $\alpha$ , - $\beta$  and - $\gamma$  are very similar in the B, C, D and E domains, but are divergent in the A and F domains. A fourth nuclear receptor, hRXR- $\alpha$ , which also has the capacity to regulate gene expression in response to retinoic acid, shows amino-acid sequence similarity only to the C domain of RAR- $\alpha$ , - $\beta$  and - $\gamma$ , and thus represents a distinct class of nuclear receptor (Mangelsdorf *et al.* 1990).

A knowledge of the pattern of retinoic acid receptor gene expression in chick facial primordia could assist our understanding of the mechanism by which locally applied retinoic acid causes upper beak defects, and could indicate the role played by retinoic acid in normal face development. We therefore isolated cDNA clones encoding chicken RAR- $\beta$  and studied the distribution of RAR- $\beta$  transcripts in normal chick facial primordia, using *in situ* hybridisation. Since retinoic acid is able to induce transcriptional activation of the human RAR- $\beta$  gene *in vitro*, we also examined retinoic-acid-treated facial primordia for changes in the distribution of RAR- $\beta$  transcripts.

## Materials and methods

### Isolation of cDNA clones

Approximately  $2 \times 10^5$  recombinant bacteriophage from a 10 day (stage 36) chick embryo cDNA library constructed in  $\lambda$ gt11 (Clontech) were screened with a 380 bp *KpnI*-*PstI* fragment of the human RAR- $\alpha$  cDNA clone p63 (Petkovich *et*

*al.* 1987), which had been labelled with [ $\alpha$ - $^{32}$ P]dCTP (New England Nuclear) by random priming (Feinberg and Vogelstein, 1984). This probe contained sequences encoding the A, B and most of the C (DNA-binding) domain of human RAR- $\alpha$ . Plaque lifts on to Hybond-N (Amersham International) were performed according to the manufacturer's instructions. Hybridisation to duplicate filters was performed for 16 h at 65°C in a solution containing 6 $\times$ SSC, 1% (w/v) SDS, 5 $\times$ Denhardt's solution, 0.1 mg ml $^{-1}$  yeast total RNA and 10 $^6$  cts min $^{-1}$  ml $^{-1}$  of radiolabelled probe. The most stringent post-hybridisation wash was for 30 min at 55°C in 1 $\times$ SSC, 0.1% (w/v) SDS. The filters were autoradiographed and positive clones  $\lambda$ RAR1 and  $\lambda$ RJO5 were purified by rescreening under the same conditions. The *EcoRI* inserts of these clones were subcloned into the plasmid vector pBluescript SK $^+$  (Stratagene) for subsequent analysis, yielding plasmids pRAR1 and pRJO5, respectively.

### Nucleotide sequencing

Dideoxy sequencing was performed using Sequenase (US Biochemicals) according to the manufacturer's instructions, either from double-stranded plasmid DNA or following transfer of selected fragments into M13, mp18 or mp19. Oligonucleotide primers were synthesised in our laboratory. Nucleotide sequences were analysed using the Microgenie programme (Beckman).

### Chick embryos and retinoic acid treatment

Fertilised chicken eggs were obtained from Poyndon Farm, Waltham Cross, Herts, and were incubated at 38 $\pm$ 1°C. The embryos were staged according to Hamburger and Hamilton (1951), dissected into fresh PBS and processed for RNA isolation or *in situ* hybridisation as described below. For retinoic acid treatment, a bead soaked in 10 mg ml $^{-1}$  all-*trans* retinoic acid (Sigma) was implanted beneath the ectoderm at the anterior margin of the right-hand wing bud of a stage 20 embryo, as described previously (Wedden and Tickle, 1986; Eichele *et al.* 1984). This dose of retinoic acid produces facial defects in 100% of treated embryos (Tamarin *et al.* 1984; Wedden and Tickle, 1986). Heads were dissected 24 h (stage 24) or 48 h (stage 28) after treatment and were processed for RNA isolation or *in situ* hybridisation as described below.

### RNA isolation and northern blotting

Total RNA was isolated from snap-frozen tissue by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987) and was then fractionated on a 1% agarose MOPS-formaldehyde gel (10  $\mu$ g per track) and blotted on to Genescreen Plus membrane (Dupont). Sample quality and quantity were checked by ethidium bromide staining of non-denaturing agarose gels and by hybridisation of filters with probes for type I collagen transcripts, as described previously (Devlin *et al.* 1988). Filters were hybridised with a  $^{32}$ P-labelled RNA probe synthesised with T7 RNA polymerase (BCL), as previously described (Devlin *et al.* 1988), using pRAR1 DNA as a template after linearisation with *Bam*HI. This probe contained sequences complementary to those encoding part of the A domain, the B,C and D domains, and part of the E domain (Fig. 1a,b, nucleotides 1-937). Hybridisation was performed for 16 h at 65°C in a solution containing 60% (w/v) formamide, 6 $\times$ SSC, 20 mM sodium phosphate, 1% (w/v) SDS, 5 $\times$ Denhardt's solution, 7% (w/v) dextran sulphate, 0.1 mg ml $^{-1}$  denatured sonicated herring testes DNA, 0.1 mg ml $^{-1}$  yeast total RNA, 0.01 mg ml $^{-1}$  poly(A) and 10 $^6$  cts min $^{-1}$  ml $^{-1}$  of radiolabelled probe. The most stringent post-hybridisation wash was for 30 min at 80°C in 0.1 $\times$ SSC, 0.1% (w/v) SDS. Transcript sizes

were estimated by reference to the 18S and 28S ribosomal RNAs in total RNA samples run in adjacent gel tracks and stained with ethidium bromide, and by reference to size standards (actin mRNA and type I ( $\alpha$ -2) collagen mRNA), following reprobing of blots.

#### In situ hybridisation

Normal and retinoic-acid-treated chick embryo heads were fixed and embedded in wax as described by Davidson *et al.* (1988). 7  $\mu$ m sections were then cut, collected on slides coated with 3-aminopropyltriethoxysilane (TESPA, Sigma) and baked for 6–16 h at 60°C. *In situ* hybridisation was performed essentially as described by Hogan *et al.* (1986), using a <sup>35</sup>S-labelled antisense RNA probe synthesised from the same template as for the northern hybridisation probe. Hybridisation was performed at 55°C. As a negative control, adjacent sections were hybridised with a <sup>35</sup>S-labelled sense-strand RNA probe. After post-hybridisation washes and treatment with 40  $\mu$ g ml<sup>-1</sup> RNAase A (Wilkinson *et al.* 1987), the slides were autoradiographed (Hogan *et al.* 1986) and stained with 0.05% (w/v) malachite green.

## Results

#### Isolation of chicken RAR- $\beta$ cDNA clones

Low-stringency screening of the 10 day chick embryo cDNA library with the human RAR- $\alpha$  cDNA probe led to the isolation of two clones ( $\lambda$ RAR1 and  $\lambda$ RJO5), which had identical nucleotide sequences in the region of overlap (Fig. 1a). The composite nucleotide sequence and the predicted amino acid sequence of the protein it would encode are shown in Fig. 1b. Comparison with human and murine RAR- $\alpha$ , - $\beta$  and - $\gamma$  sequences showed by far the strongest homology with RAR- $\beta$  (Table 1). This homology was maintained in the A and F domains, which are highly diverged in the RAR- $\alpha$ , - $\beta$  and - $\gamma$  molecules of human and mouse, indicating that  $\lambda$ RAR1 and  $\lambda$ RJO5 are chicken RAR- $\beta$  cDNA clones.

#### Expression of RAR- $\beta$ transcripts in embryonic and adult chicken tissues

A subclone of the  $\lambda$ RAR1 insert was used as a template

for synthesis of a radiolabelled RNA probe for chicken RAR- $\beta$  transcripts (Fig. 1b, nucleotides 1–937). In northern blotting experiments, this probe hybridised to two transcripts, of 3.5 and 2.8 kb, which were abundant in limb buds, facial primordia and trunks dissected from chick embryos at stages 22 to 25 (Fig. 2, tracks a–k). An oligonucleotide probe complementary to sequences encoding part of the RAR- $\beta$  A domain (Fig. 1b, nucleotides 1–37) also hybridised to these two transcripts (data not shown). Since the A domains of chicken RAR- $\alpha$ , - $\beta$  and - $\gamma$  are likely to be highly diverged, like those of human and murine RAR- $\alpha$ , - $\beta$  and - $\gamma$ , this indicated that both transcripts derived from the chicken RAR- $\beta$  gene, rather than representing cross-hybridisation to transcripts of related RAR genes. The relative abundance of the two chicken RAR- $\beta$  transcripts varied between developmental stages and between tissues, although the 3.5 kb transcript was generally the more abundant of the two (Fig. 2). Both transcripts were present in adult tissues, including liver and lung (Fig. 2, tracks l and m), but at much lower levels than in the embryo.

#### Distribution of RAR- $\beta$ transcripts in normal chick embryo facial primordia

RAR- $\beta$  transcripts were expressed in stage 24 frontonasal mass and mandibular primordia (Fig. 2, tracks b and c). Retinoic acid causes upper beak defects when applied to the wing bud between stages 18 and 21, with retinoic-acid-induced morphological changes becoming visible by stage 28 (Wedden and Tickle, 1986). We examined the distribution of RAR- $\beta$  transcripts in the facial primordia during this period of development by performing *in situ* hybridisation to sections of normal chick embryo faces at stages 20 (Fig. 3), 24 (Fig. 4) and 28 (Fig. 5), using the same probe as in the northern blotting experiments. In all cases, adjacent sections were incubated with a negative control sense strand RNA probe, and no specific hybridisation was detectable (as in Fig. 3F). In parasagittal sections of stage 20 and stage 24 heads, facial primordia expressed RAR- $\beta$

**Table 1.** Identity of chick RAR cDNA clones

(a) Comparison (% homology) of predicted amino acid sequence derived from  $\lambda$ RAR1 and  $\lambda$ RJO5 with those of human and murine RAR- $\alpha$ , RAR- $\beta$  and RAR- $\gamma$ .

	A	B	C	D	E	F
human RAR- $\alpha$	n/s	75%	97%	67%	89%	28%
mouse RAR- $\alpha$	n/s	75%	97%	65%	89%	n/s
human RAR- $\beta$	90%	96%	98%	89%	97%	86%
mouse RAR- $\beta$	79%	96%	98%	91%	99%	78%
human RAR- $\gamma$	n/s	89%	95%	59%	90%	n/s
mouse RAR- $\gamma$	n/s	89%	95%	59%	90%	n/s

(b) Comparison (% homology) of nucleotide sequence derived from  $\lambda$ RAR1 and  $\lambda$ RJO5 with those of human and murine RAR- $\alpha$ , RAR- $\beta$  and RAR- $\gamma$  mRNAs.

	A	B	C	D	E	F
human RAR- $\alpha$	n/s	74%	81%	72%	75%	n/s
human RAR- $\beta$	89%	85%	88%	86%	87%	84%
human RAR- $\gamma$	n/s	74%	78%	69%	76%	n/s

Human and murine sequence data were taken from Petkovich *et al.* (1987), Brand *et al.* (1988), Krust *et al.* (1989) and Zelent *et al.* (1989). A to F denote the different domains of proteins in the steroid/thyroid hormone superfamily of nuclear receptors. Since the chicken sequence lacks some 5' coding sequence, the A domain compared here is incomplete (see Fig. 1). n/s: not significant.

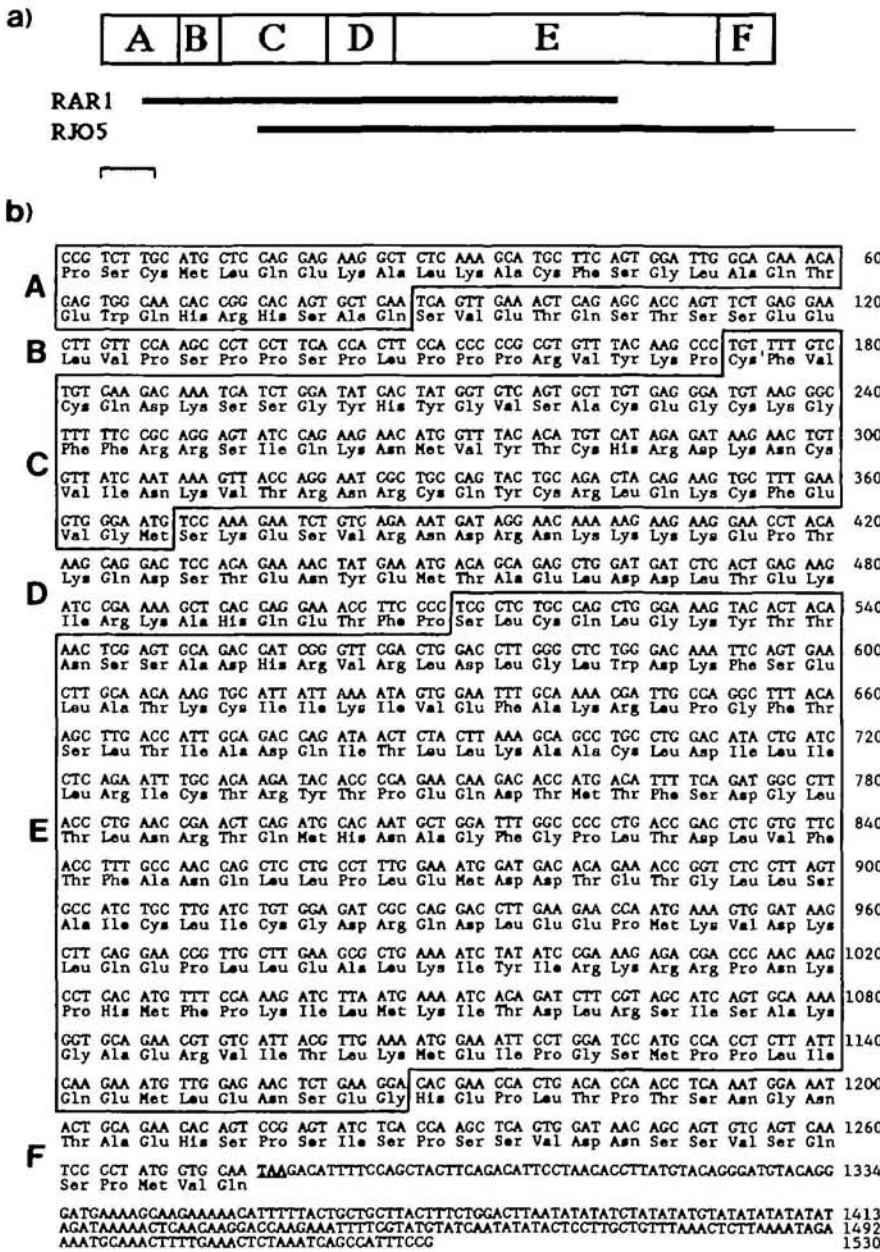


Fig. 1. (a) Putative structure of chicken RAR- $\beta$  mRNA, showing regions encoding domains A-F of human and murine RAR- $\beta$ . C, DNA-binding domain; E, retinoic-acid-binding domain. Coding sequences are represented by thick lines and 3' untranslated region by a thin line. Scale bar: 100 nucleotides. (b) Nucleotide sequence and predicted amino acid sequence derived from clones  $\lambda$ RAR1 and  $\lambda$ RJ05. Domains A-F are indicated. Comparison with human and murine RAR- $\beta$  indicates that  $\lambda$ RAR1 lacks sequence encoding 23 amino-terminal amino acids, including the initiation codon.

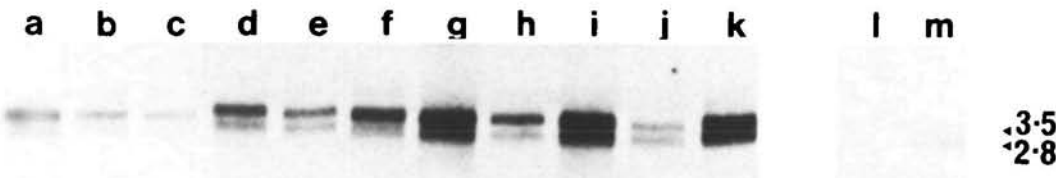
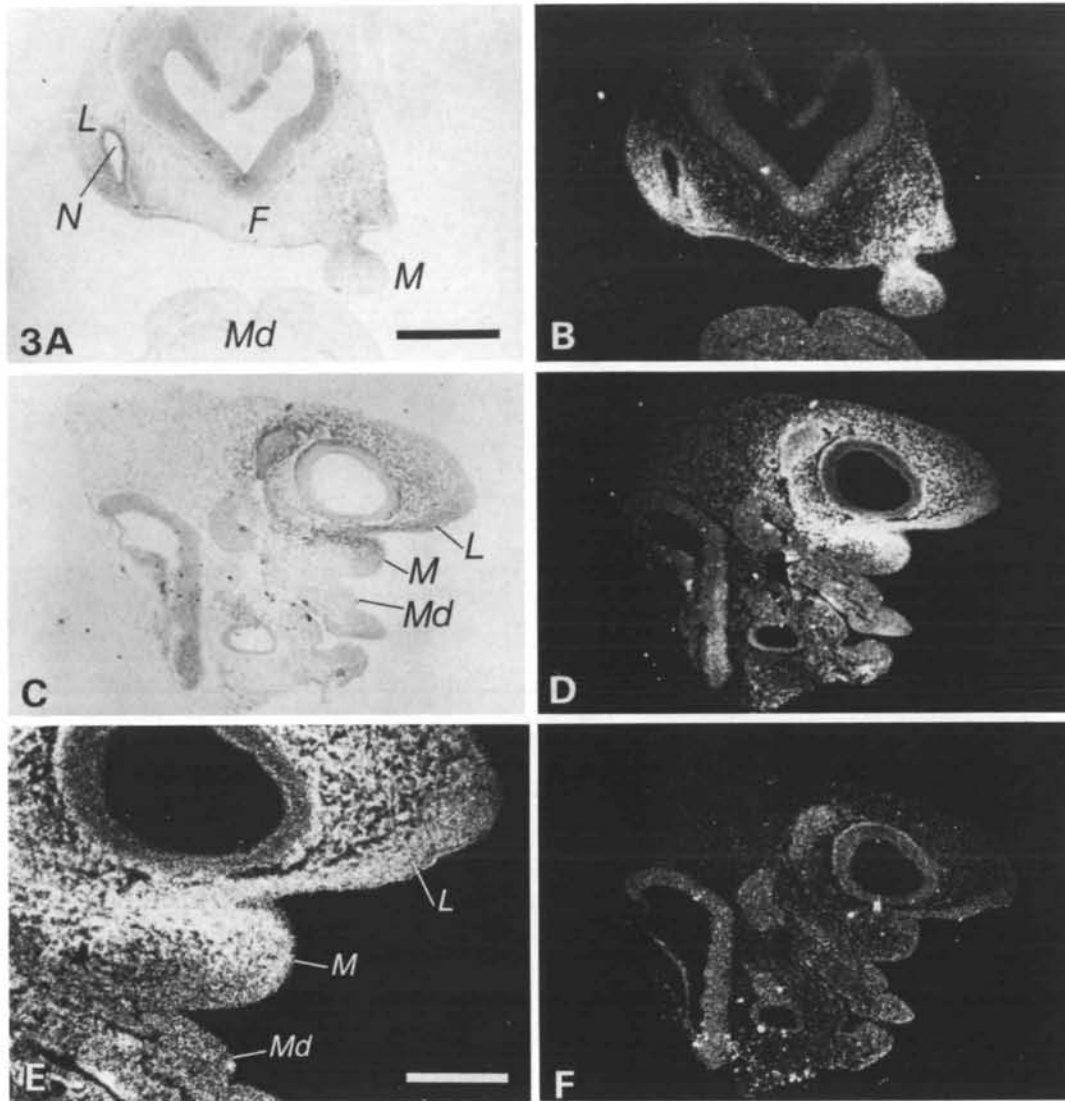


Fig. 2. Northern blotting analysis of RAR- $\beta$  transcript expression in chick tissues. Tracks contain 10  $\mu$ g of total RNA isolated from: a, whole 10 day embryo; b, stage 24 frontonasal mass; c, stage 24 mandibular primordia; d, stage 25 head; e, stage 22 head; f, stage 24 leg bud; g, stage 22 leg bud; h, stage 24 wing bud; i, stage 22 wing bud; j, stage 24 trunk; k, stage 22 trunk; l, adult liver; m, adult lung. Tracks l and m were exposed for 5 times longer (two weeks) than tracks a-k.

transcripts at higher levels than more dorsal head tissue (Fig. 3C,D; Fig. 4I,J). However, the distribution of RAR- $\beta$  transcripts in the facial primordia was not uniform at stages 20, 24 or 28 (Fig. 3A-E; Fig. 4A,B,E,F; Fig. 5A,B).

At all three stages, there was strong hybridisation to the anterior part of the maxillary primordia, with much weaker hybridisation to the posterior part. This was striking in both frontal (Fig. 3A,B; Fig. 4A,B and Fig. 5A,B) and parasagittal (Fig. 3C-E; Fig. 4E,F)



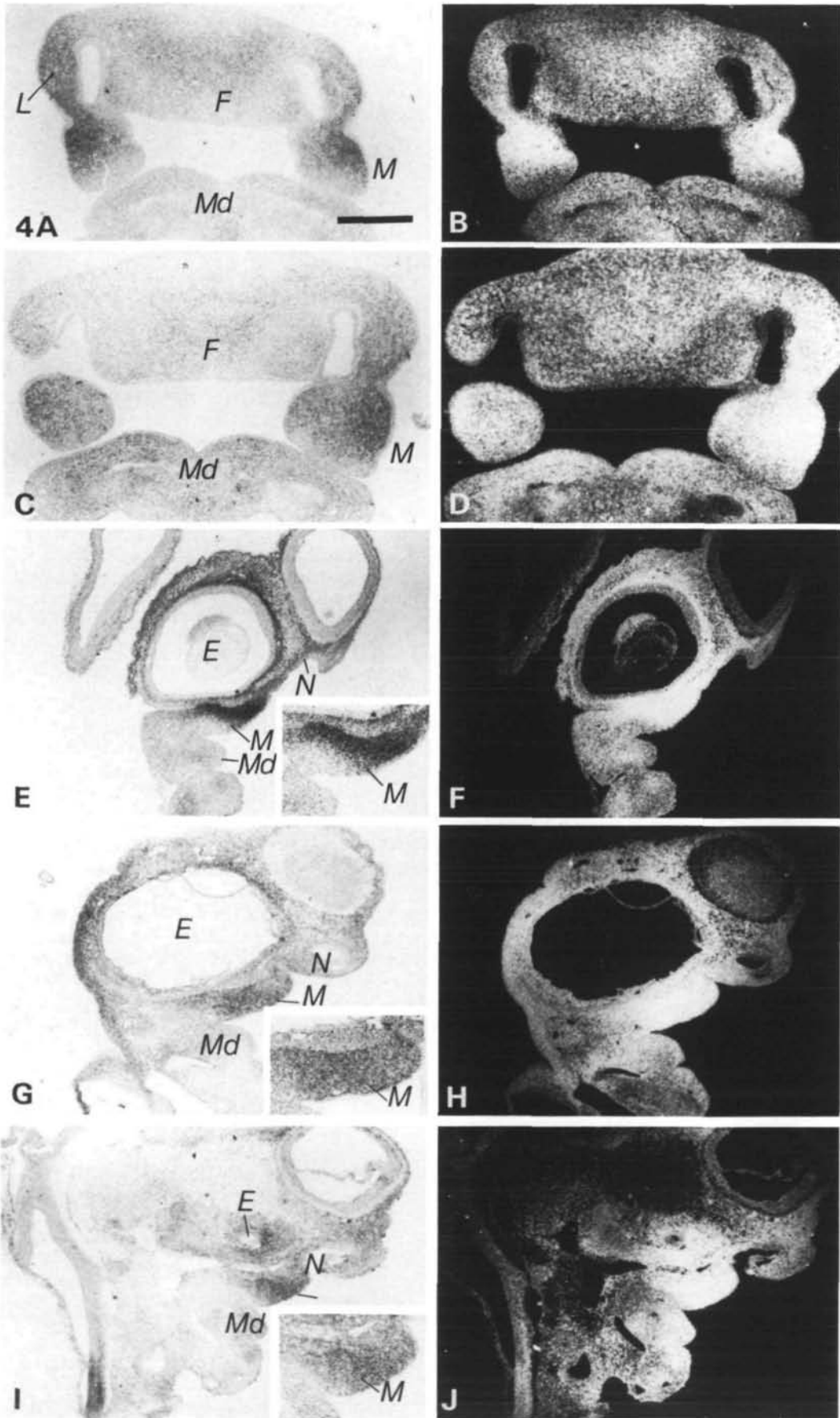
**Fig. 3.** Distribution of RAR- $\beta$  transcripts in normal stage 20 embryonic chick face. Autoradiographic signal is visible as black grains under bright-field illumination (A,C) and as white grains under dark-field illumination (B,D,E,F). Sections A–E were hybridised with the probe for RAR- $\beta$  transcripts. (A,B) Frontal section of stage 20 embryo face. The maxillary primordium is present only on the right-hand side, and the nasal slit only on the left-hand side, due to the plane of section. (C,D) Parasagittal section of stage 20 embryo head, including the lateral nasal process and the maxillary and mandibular primordia. (E) High-power view of part of section D, showing the boundary between the strongly hybridising upper region and the weakly hybridising lower region of the maxillary primordium. (F) Section adjacent to section D, hybridised with the negative control sense strand RNA probe. Six embryos were examined. Key: E, eye; F, frontonasal mass; L, lateral nasal process; M, maxillary primordium; Md, mandibular primordium; N, nasal slit. Scale bar for A–D and F: 500  $\mu\text{m}$ . Scale bar for E: 200  $\mu\text{m}$ .

sections. The boundary between regions expressing high and low levels of RAR- $\beta$  transcripts was canted in both planes examined. There was also strong hybridisation at all three stages to the lateral nasal processes and to the lateral edges and outermost corners of the frontonasal mass (Fig. 3A–E; Fig. 4A,B,E,F; Fig. 5A,B). In addition, at stages 24 and 28 there was a broad V-shaped area of strong hybridisation across the upper aspect of the nasal slits and extending into the central third of the frontonasal mass (Fig. 4A,B; Fig. 5A,B). In contrast to these clear regional differ-

ences in the upper beak primordia, RAR- $\beta$  transcripts were relatively evenly distributed in the mandibular primordia at all three stages. In all primordia, at all stages, RAR- $\beta$  transcripts were expressed in mesenchyme, rather than in epithelia.

#### *Distribution of RAR- $\beta$ transcripts in retinoic-acid-treated chick embryo facial primordia*

Implantation of a retinoic-acid-soaked bead to the anterior margin of a stage 20 wing bud consistently results in irreversible changes in facial development.



**Fig. 4.** Distribution of RAR- $\beta$  transcripts in normal and retinoic-acid-treated stage 24 embryonic chick face. Sections were hybridised with the probe for RAR- $\beta$  transcripts and viewed under light-field (left column) and dark-field (right column) illumination. (A,B) Frontal section of normal stage 24 embryo face. (C,D) Frontal section of retinoic-acid-treated stage 24 embryo face. There are no visible changes in morphology. (E,F) Parasagittal section of normal stage 24 embryo head. The plane of section includes the nasal slit and the maxillary and mandibular primordia. The inset shows the maxillary primordium at higher magnification, with strong hybridisation to the upper part only. (G,H) Parasagittal section of retinoic-acid-treated stage 24 embryo head (right-hand side). The inset shows the maxillary primordium at higher magnification, with strong hybridisation over the whole primordium. (I,J) Parasagittal section of retinoic-acid-treated stage 24 embryo head (left-hand side). The plane of section is deeper than in section G,H. The inset shows the maxillary primordium at higher magnification, with strong hybridisation over the whole primordium. Six normal embryos and five retinoic-acid-treated embryos were examined. Sections E–J were hybridised on the same slide. Key: E, eye; F, frontonasal mass; L, lateral nasal process; M, maxillary primordium; Md, mandibular primordium; N, nasal slit. Scale bars: 500  $\mu$ m.

Morphological changes are not visible until 48 h after retinoic acid application (stage 28), but clear changes in the distribution of RAR- $\beta$  transcripts were detectable as early as 24 h after retinoic acid treatment (stage 24). In retinoic-acid-treated embryos at stage 24, transcripts were evenly spread throughout the maxillary primordia (Fig. 4C,D,G–J), whereas in normal stage 24 embryos they were confined to the anterior part of the maxillary primordia (Fig. 4A,B,E,F). Administration of retinoic acid to the right wing bud induced similar changes in the distribution of RAR- $\beta$  transcripts in both right and left maxillary primordia (Fig. 4G–J). The distribution of RAR- $\beta$  transcripts in the frontonasal mass, lateral nasal processes and mandibular primordia was unaffected by retinoic acid treatment.

By stage 28, there were obvious changes in the facial morphology of retinoic-acid-treated embryos. The frontonasal mass was constricted and its corners were rounded. The lateral nasal processes were bilobed and the maxillary primordia were rotated. There were also striking changes in the distribution of RAR- $\beta$  transcripts at this stage (Fig. 5C,D). As at stage 24, RAR- $\beta$  transcripts were evenly distributed across the maxillary primordia. In the frontonasal mass, the hybridisation signal was more evenly spread than in normal embryos, but there remained a region of slightly stronger hybridisation in the centre of the primordium. Retinoic acid treatment had no obvious effect on the distribution of RAR- $\beta$  transcripts in the mandibular primordia. In order to determine whether there were any changes in the overall levels of RAR- $\beta$  transcripts at this stage, we analysed northern blots of RNA isolated from the pooled upper beak primordia and the mandibular primordia of normal and retinoic-acid-treated stage 28

embryos. The normal primordia contained the 3.5 kb transcript and relatively low levels of the 2.8 kb transcript (Fig. 5E). Retinoic acid treatment induced a slight elevation in levels of both the 3.5 kb and the 2.8 kb RAR- $\beta$  transcripts in the upper beak primordia, and a slight increase in levels of the 2.8 kb transcript in the mandibular primordia (Fig. 5E,F). However, neither of these changes was dramatic.

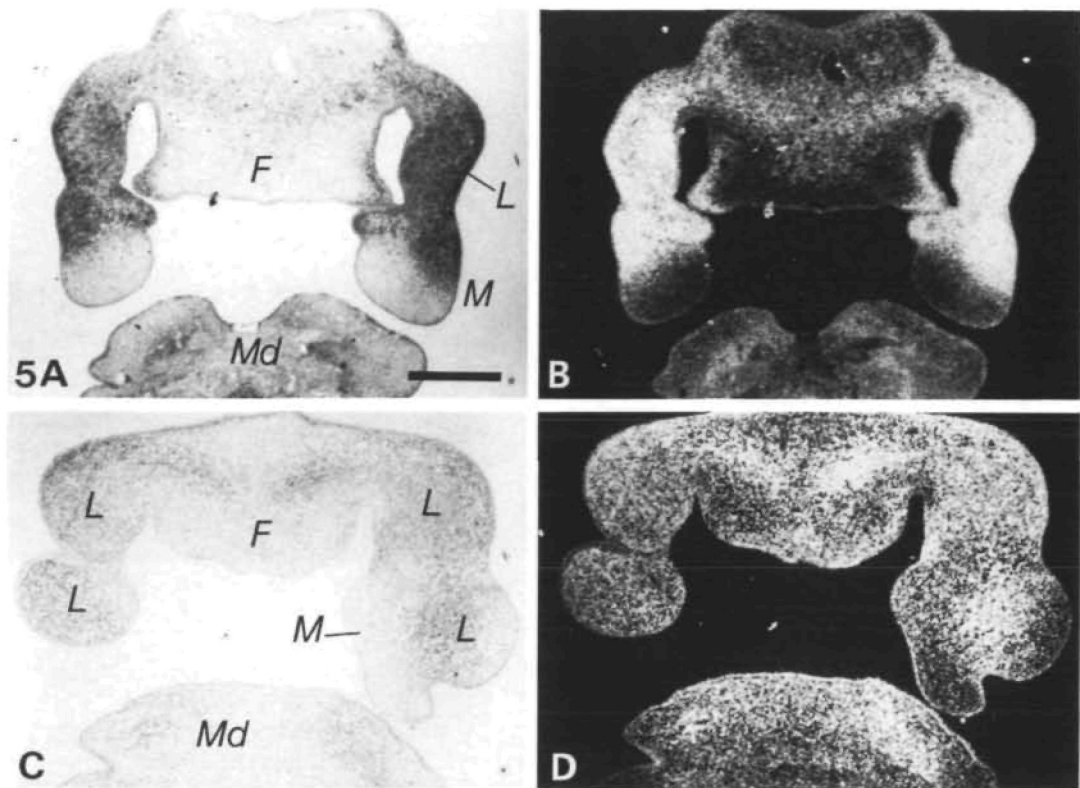
## Discussion

In this report, we have described the isolation and characterisation of chicken RAR- $\beta$  cDNA clones. The predicted amino acid sequence of chicken RAR- $\beta$  exhibited a high level of similarity with those of human and murine RAR- $\beta$ .

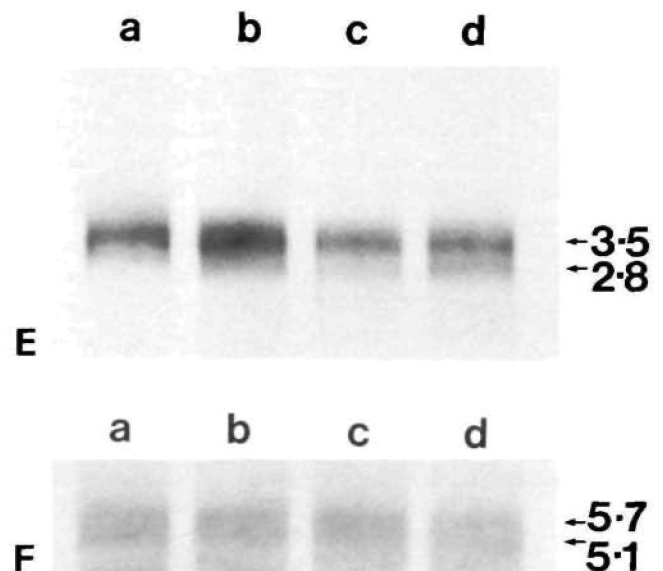
Studies of the distribution of RAR- $\alpha$ , - $\beta$  and - $\gamma$  transcripts in the embryonic mouse face have been described recently. RAR- $\alpha$  and - $\gamma$  transcripts were found to be uniformly distributed, with RAR- $\gamma$  transcripts becoming progressively restricted to chondrogenic regions (Osumi-Yamashita *et al.* 1990; Ruberte *et al.* 1990). RAR- $\beta$  transcripts were found to be restricted spatially, being expressed at higher levels in the lateral nasal processes than in the other primordia (Osumi-Yamashita *et al.* 1990). We have shown here that RAR- $\beta$  transcripts are distributed in a regionally specific pattern in the developing chick face. The distribution of RAR- $\beta$  transcripts in the facial primordia of normal chick embryos was intriguing in two respects.

First, there were boundaries between regions of high and low expression within single primordia. This was particularly striking in the frontonasal mass and the maxillary primordia. For example, RAR- $\beta$  transcripts were abundant in the anterior part of the maxillary primordia, but were undetectable in the posterior part. A similar distribution of RAR- $\beta$  transcripts in the chick maxillary primordia has recently been described by Smith and Eichele (1991). It is possible that these two regions of the maxillary primordia are populated by cells originating from different levels of the neural crest, which differentially express RAR- $\beta$  transcripts. The developmental fates of the cells in these two regions of the maxillary primordia have not been mapped.

Second, RAR- $\beta$  transcripts were highly concentrated in specific regions of the primordia which contribute to the upper beak, the development of which is affected by retinoic acid treatment. In contrast, low levels of RAR- $\beta$  transcripts were evenly distributed in the primordia that form the lower beak, the development of which is unaffected by retinoic acid treatment. Morphological studies of retinoic-acid-treated embryos led to the conclusion that the frontonasal mass was the primary target for retinoic acid and that the subsequent failure of expansion of the maxillary primordia was a secondary effect resulting from the lack of fusion with the frontonasal mass (Wedden, 1987). If the presence of high levels of RAR- $\beta$  transcripts identifies regions that



**Fig. 5.** Distribution of RAR- $\beta$  transcripts in normal and retinoic acid-treated stage 28 embryonic chick face. All sections were hybridised with the probe for RAR- $\beta$  transcripts and viewed under light-field (A,C) and dark-field (B,D) illumination. (A,B) Frontal section of normal stage 28 embryo face. (C,D) Frontal section of retinoic acid-treated stage 28 embryo face. Morphological changes are clearly visible (see text). Due to the plane of section, the maxillary primordium is present on the right-hand side only. Five normal and three retinoic acid-treated embryos were examined. Key: F, frontonasal mass; L, lateral nasal process; M, maxillary primordium; Md, mandibular primordium. Scale bars: 500  $\mu$ m. (E) Northern blot of total RNA (10  $\mu$ g per track) isolated from the pooled upper beak primordia (frontonasal mass, lateral nasal processes, maxillary primordia) or the mandibular primordia of normal or retinoic acid-treated stage 28 embryos. a, normal upper beak primordia; b, retinoic acid-treated upper beak primordia; c, normal mandibular primordia; d, retinoic acid-treated mandibular primordia. The blot was probed for RAR- $\beta$  transcripts, the sizes of which are shown in kilobases. (F) Filter E was stripped of probe and rehybridised with a probe for type I collagen transcripts, whose abundance does not change following retinoic acid treatment. Sizes of transcripts are shown in kilobases.



are sensitive to retinoic acid, this interpretation may be incorrect, since high levels of RAR- $\beta$  transcripts were present not only in regions of the frontonasal mass but also in the lateral nasal processes and the anterior part of the maxillary primordia. The localisation of RAR- $\beta$  transcripts to the mesenchyme rather than to the epithelium is consistent with the results of recombination experiments, which show that retinoic acid acts on the mesenchyme of facial primordia rather than on the epithelium (Wedden, 1987). These results therefore leave open the possibility that RAR- $\beta$  is involved in

mediating the effects of applied retinoic acid upon facial development.

Retinoic acid treatment of stage 20 chick embryos resulted in a dramatic increase in levels of RAR- $\beta$  transcripts in the posterior part of the maxillary primordia. This was detectable 24 h after retinoic acid treatment, before any retinoic acid-induced morphological changes were visible, and is the earliest biochemical change to have been detected in the facial primordia following retinoic acid treatment. Retinoic acid applied to the right-hand wing bud affected both



the right- and left-hand maxillary primordia, whilst cells in the frontonasal mass and mandibular primordia were unaffected. Thus, cells in the mesenchyme of the facial primordia differ not only in the levels of RAR- $\beta$  transcripts which they contain, but also in their response to retinoic acid treatment.

Retinoic acid treatment also resulted in a slight increase in the levels of RAR- $\beta$  transcripts in the upper and lower beak primordia after 48 h. However, this small effect was difficult to interpret in view of the widespread morphological changes induced in retinoic-acid-treated embryos by this time.

The increase in levels of RAR- $\beta$  transcripts in the posterior part of the maxillary primordia at stage 24 is the first demonstration that retinoic acid can induce a change in the distribution of retinoic acid receptor transcripts *in vivo*. This change cannot be due to an effect of retinoic acid on the migration of neural crest cells into the maxillary primordia, since this is completed by stage 20, when the retinoic acid was applied. It is also unlikely that retinoic acid induces differences in the proliferation rate of cells in the anterior and posterior parts of the maxillary primordia, since this would be expected to alter the morphology of the primordia, and no such alteration is seen 24 h after treatment. It therefore seems likely that retinoic acid induces changes in RAR- $\beta$  gene expression in the cells in the posterior part of the maxillary primordia. Experiments on cell lines have shown that retinoic acid can directly induce transcription of the human RAR- $\beta$  gene as a result of the presence of a specific retinoic acid response element in its upstream region (de Thé *et al.* 1989, 1990). Whilst our data do not exclude other regulatory mechanisms, it is possible that the changes in RAR- $\beta$  transcript expression that we have detected *in vivo* are also a result of transcriptional regulation by retinoic acid. It remains to be determined why this response is spatially restricted.

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