

# Spatial and temporal expression pattern of retinoic acid receptor genes during mouse bone development

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Spatial and temporal expression pattern of retinoic acid receptor (RAR) genes was investigated in mouse finger bones during development by an *in situ* hybridization method with riboprobes synthesized from a human cDNA of the RAR- $\alpha$ . We found that the RAR genes are expressed intensively and specifically in calcifying fronts of the mouse finger bones, whereas the expression pattern is rather uniform in the limb buds and cartilage matrices of the embryonic fingers. Our findings are consistent with the fact that vitamin A is essential for normal mammalian bone development.

Retinoic acid receptor gene; Hybridization, *in situ*; Bone formation; (Mouse, Forelimb)

## 1. INTRODUCTION

Retinoic acid (RA) exhibits remarkable effects on cellular differentiation [1–3] and embryonic pattern formation, e.g. chick digits formation [4,5]. Recently, cDNAs of the retinoic acid receptor (RAR) genes were cloned by Petkovich et al. [6], Giguère et al. [7], de The et al. [8], Brand et al. [9], Benbrook et al. [10], and Zelent et al. [11]. Three subtypes of mouse RAR, designated RAR- $\alpha$ , - $\beta$  and - $\gamma$ , have been reported [11]. The RAR genes were found, by Northern blot analysis, to be expressed differentially in various tissues, e.g., brain, kidney, skin, liver, and testis [11–13]. Although it has been reported that RA plays important roles in bone formation, the presence of the RAR in the bone has not been reported yet.

In the present study, we performed *in situ* hybridization on sections of the developing mouse finger bones to detect mRNAs of the RARs. We demonstrated that the RAR genes are expressed in epithelia and mesenchymes of the limb buds and embryonic fingers, and most intensively in calcifying fronts of the finger bone (primary spongiosa) after birth.

## 2. EXPERIMENTAL

### 2.1. Tissue preparation

4-day-old male mice (C3H) were killed by cervical dislocation and perfused from the left ventricle with 0.07 M Sørensen's phosphate-

buffered saline containing 4% paraformaldehyde. Tissues cut into slices (2–3 mm thick) were post-fixed in the same fixative for 3 h at 4°C. For decalcification, the fixed tissues were incubated in 5% 2NaEDTA in 0.15 M NaCl at 4°C for one week, dehydrated in the usual manner, and embedded in paraffin (Paraplast).

### 2.2. *In situ* hybridization

Tissue sections of 5  $\mu$ m were collected on poly-L-lysine-coated slides. Section pretreatment and hybridization were performed according to Noji et al. [14]. Sections were deparaffinized in xylene, rehydrated, acetylated, and prehybridized in 2  $\times$  SSC, 50% formamide, and 10 mM dithiothreitol (DTT) at 50°C. 20  $\mu$ l of hybridization mixture (50% deionized formamide, 4 mg/ml BSA, 4 mg/ml tRNA, 10% dextran sulfate, 2  $\times$  SSC) was applied to each slide, and covered with a parafilm. Hybridization was carried out for 15–17 h in a humid chamber at 50°C with a probe concentration of 1  $\times$  10<sup>5</sup> dpm/ $\mu$ l. Slides were washed in 50% formamide, 2  $\times$  SSC, 10 mM DTT at 50°C for 80 min. The sections were then treated with 20  $\mu$ g/ml RNase A for 30 min at 37°C in NTE buffer (0.5 M NaCl, 10 mM Tris/HCl, pH 8, 1 mM EDTA) [15]. The slides were further washed with 0.1  $\times$  SSC at 50°C for 1 h, dehydrated and air-dried. For autoradiography, the slides were immersed in Kodak NTB2 emulsion (diluted 1:1 with water), air-dried and exposed for 14 days at 4°C in a dry, light-tight box. Slides were developed at 20°C in Kodak D19 developer for 3 min. Finally, slides were stained with hematoxylin and eosin for light microscopy.

### 2.3. Probe preparation

Templates for synthesis of riboprobes were constructed by subcloning the fragments of a RAR cDNA (a gift from Dr M. Petkovich and Professor P. Chambon) into pGEM vectors (Promega Co., WI, USA) (fig.1). A 341 bp fragment of mouse nerve growth factor (NGF) cDNA (from Dr Noboru Tomioka of the Mitsui Toatsu Chemicals, Inc.) and 500 bp fragment of elastin cDNA (from Dr Shingo Tajima of Keio University) were used as templates for control probes. The riboprobes (spec. act. approx. 5  $\times$  10<sup>8</sup> dpm/ $\mu$ g) was prepared from both strands with [ $\alpha$ -<sup>35</sup>S]UTP (400 Ci/mol, Amersham). After the removal of unincorporated labeled nucleotides, probes for the hybridization were subjected to limited alkaline hydrolysis [16] to shorten the transcripts to about 50–150 bp.

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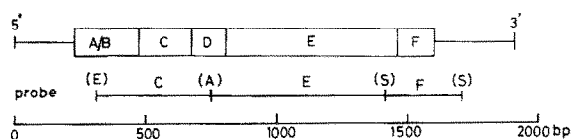


Fig.1. Structure of the RAR- $\alpha$  cDNA and templates used for synthesis of riboprobes. The RAR consists of five domains, A/B and C-F. C, DNA binding domain (zinc finger structure); E, ligand (RA) binding domain. Restriction enzymes, *Ava*I, *Eco*RI, and *Sma*I are designated by (A), (E), and (S), respectively.

### 3. RESULTS AND DISCUSSION

#### 3.1. Spatial expression pattern of the RAR genes in a finger bone

In order to analyze the expression pattern of the RAR genes during development of mouse forelimbs (11-day embryo to 1-week-old after birth), we conducted in situ hybridizations on thin (5  $\mu$ m thickness)

sections of the forelimb fingers. Since the nucleotide sequence of mouse RAR cDNA has a high homology with human RAR [11], we used a fragment of a human RAR cDNA (pTM61A), containing the DNA binding (C-domain), ligand binding (E) and C-terminal regions (F) (fig.1), as a template to synthesize both anti-sense and sense RNA probes. The most intensive accumulation of exposed silver grains was observed in endosteum of the finger bone of the 4-day-old mice, as shown in fig.2a,b. Although we used the sense probe as a negative control, a pattern of grain accumulation was observed to be indistinguishable from that obtained with the anti-sense probe. However, when we used two types of anti-sense riboprobes synthesized from heterologous DNAs of the NGF and elastin cDNAs, these control probes did not significantly hybridize with any of the regions where the hybridization signal was positive for the RAR mRNAs. Typical results with the riboprobe for the elastin mRNA are shown in figs 2c,d

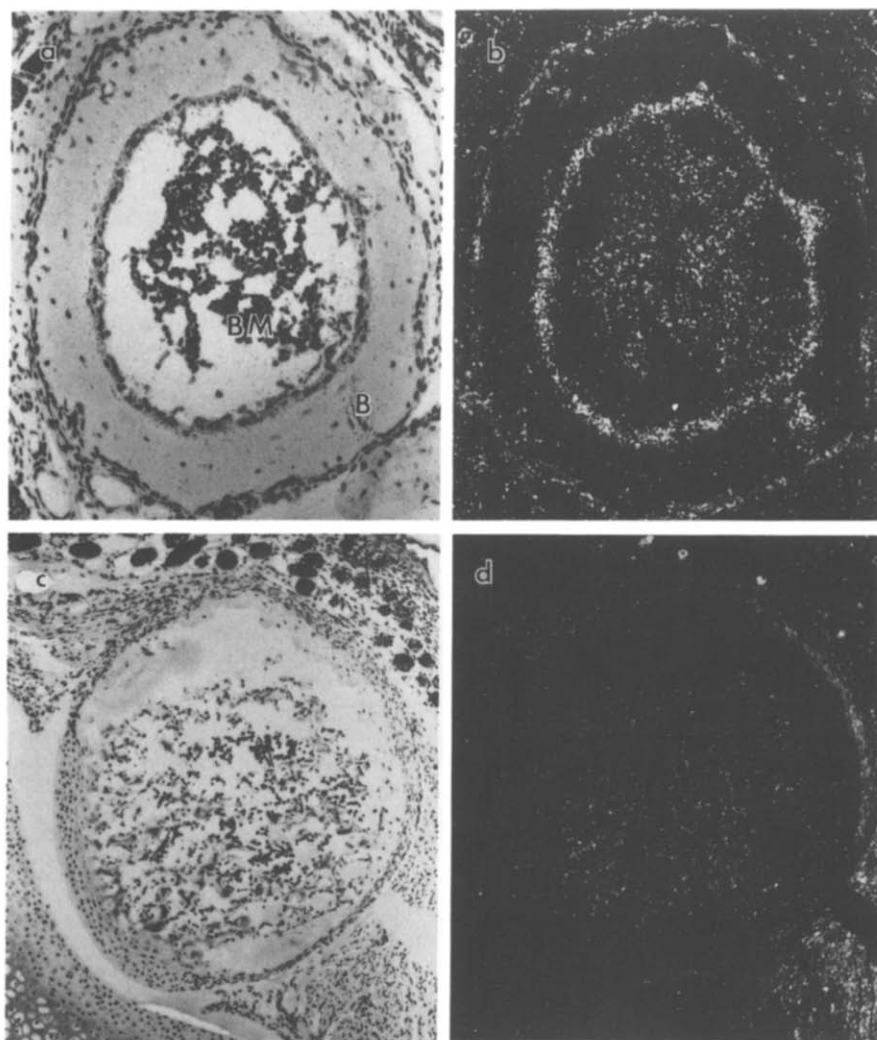


Fig.2. Expression of the retinoic acid receptor genes, as revealed by in situ hybridization on cross sections of the forelimb finger (4-day-old C3H mouse) with the anti-sense riboprobe from the RAR- $\alpha$  cDNA containing the C-F domains. Bright (a) and dark (b) field micrographs. As a negative control, the anti-sense riboprobe for the elastin mRNA was used. The results are shown as bright- (c) and dark- (d) field micrographs. B, bone; BM, bone marrow.

and 3c,f. With these probes, on the other hand, we detected the NGF mRNA in the adult mouse submandibular gland [14] and the elastin mRNA in the infant mouse aorta (data not shown). Furthermore, our Northern blot analyses with the riboprobe containing C–F domains indicated that the RAR- $\beta$  and/or - $\gamma$  genes were expressed with the concomitant expression of the RAR- $\alpha$  gene in the 4-day-old mouse skin and the osteoblastic clone MC3T3 cells (unpublished results), likewise as observed previously in adult rodents [10,11,13]. Judged from these results, we concluded that the anti-sense riboprobe hybridizes with RAR mRNAs. The presence of some transcripts hybridized with the sense probe may be due to some regulatory transcription of the RAR genes. Actually, the transcriptions of the opposite strand of the identical DNA fragment have been reported for various genes [17–21], though the function of complementary RNAs remains to be clarified.

### 3.2. Temporal change of the RAR-gene expression during bone development

In the forelimb buds (11-day-old embryo), the expos-

ed silver grains were rather uniformly distributed over the epithelia and mesenchymes (data not shown), indicating the presence of the RAR mRNA. This result supports the hypothesis that RA is an endogenous morphogen. In 15-day embryo fingers (fig.3a,b), cartilage tissues and perichondrium (fibroblasts) are developed. Although the accumulation of the grain indicates that the RAR genes are expressed over the tissues, no significant pattern of the RAR-gene expression was observed. In endochondral ossification of the finger bone, the cartilage tissues are progressively replaced by bones in the 4-day-old finger bones (figs 2a and 3d,e). The replacement in the course of development has been considered to depend at least on the activities of osteoblasts. In the newly formed layers of the bone, i.e., calcifying front (the primary spongiosa), we observed intense expression of the RAR genes, as shown in figs 1a,b and 3e. Thus, we concluded that osteoblasts of the calcifying fronts express the RAR genes, and thus possibly are target cells for RA.

Although the same expression patterns were throughout observed with both anti-sense and sense riboprobes synthesized from three different fragments

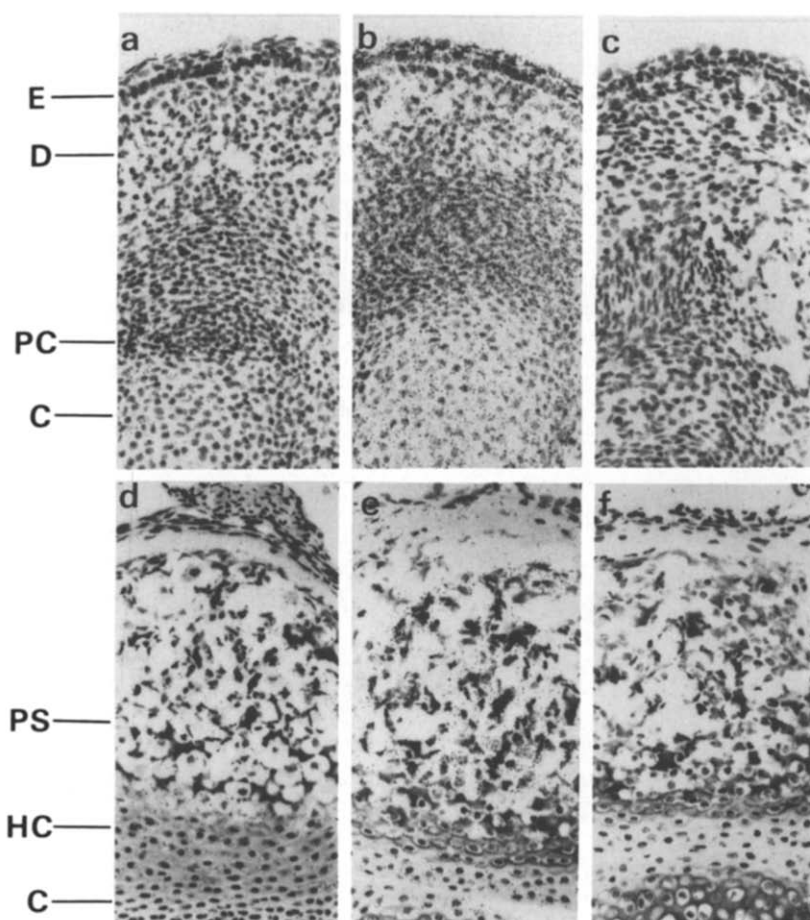


Fig.3. Expression of the RAR genes in the cross section of the finger of the 15-day embryo (a–c) and the 4-day-old (d–f) mouse. The grains are small black particles. (a,d) Probe for the E-domain; (b,e) for the C-domain; (c,f) for the elastin mRNA. E, epidermis; D, dermis; PC, perichondrium; C, cartilage cells (chondrocytes); HC, hypertrophic cartilage cells; PS, primary spongiosa.

of the RAR cDNA, i.e., C-, E-, and F-domain (fig.1), in the finger bones, the signal intensity depends on the used probes. With the probe synthesized from the E-domain, the signals were less intensive but significant in the primary spongiosa (fig.3a,d). On the other hand, with the riboprobes synthesized from the C- and F-domains (containing 100 bp 3'-noncoding region), the intensive accumulation was observed in the same regions (spongiosa: fig.3b,e for the C-domain). The same was observed in various mouse tissues expressing the RAR genes (data not shown). Since the nucleotide sequence homology of the C-domain among the cDNA sequences of the RAR subtypes is higher than that of the E-domain [11], it is likely that the probes for the C-domain should hybridize with the transcripts of the RAR gene family, while the probe from the E-domain of the RAR- $\alpha$  gene may hybridize predominantly with RAR- $\alpha$  mRNA under the highly stringent washing condition employed. Thus, it is probable that the RAR- $\beta$  and/or - $\gamma$  genes are expressed in addition to the RAR- $\alpha$  gene during bone formation.

The expression of the RAR genes during development of bone is quite consistent with the fact that deficiency of vitamin A causes defect of the bone formation [2,3]. Furthermore, in vitro, RA is known to control expression of the alkaline phosphatase genes in the osteoblasts [22], depending upon the differentiation stage. RA is also known to induce differentiation of various cells including keratinocytes [1-3]. These multiple functions of RA may be interpreted by taking the multiple expression of the RAR genes into account.

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