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Expression of retinoic acid receptor genes in neural crest-derived cells during mouse facial development

Noriko Osumi-Yamashita[1], Sumihare Noji[2], Tsutomu Nohno[3], Eiki Koyama[2], Hirofumi Doi[4], Kazuhiro Eto[1] and Shigehiko Taniguchi[2]

[1]*Faculty of Dentistry, Tokyo Medical and Dental University, 1-5-45, Yshima, Bunkyo-ku, Tokyo 113,* [2]*Okayama University Dental School, Okayama City 700,* [3]*Department of Pharmacology, Kawasaki Medical School, Kurashiki City 701-01* and [4]*IIAS-SIS, Fujitsu Ltd, 1-7-25, Shinkamata, Ohota-ku, Tokyo 144, Japan*

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Retinoic acid (RA) is known as a teratogen that induces abnormalities in facial structures which are made up mainly of neural crest-derived mesenchyme. We investigated expression patterns of RA receptor (RAR) genes (subtypes α, β, γ) during mouse facial development. The expression of the RARβ gene is specific for the mesenchyme around developing eyes and nose, whereas the RARγ gene is expressed in the mesenchyme differentiating to facial cartilages and bones. In contrast, the RARα gene is expressed weakly and uniformly over the facial region. These results suggest that crucial roles of endogenous RA in facial development depend on differential functions of the RAR subtypes.

Retinoic acid receptor gene; Expression pattern; In situ hybridization; Facial development; Neural crest cell; Embryo

1. INTRODUCTION

Retinoic acid (RA), a putative morphogen (see [1,2] for review), has long been known to be a teratogen in human and experimental animals [3–8]. In particular, RA induces abnormalities in large parts of facial structures including the facial skeleton, teeth, and middle and external ears [4,6]. It has been demonstrated that the facial structures are developed from the cranial neural crest cells which have migrated from the head neural folds [9] and that exogenous RA inhibits both migration of cranial neural crest cells from the cephalic neural tube and proliferation and differentiation of facial mesenchymal cells [5,7]. Thus, the developmental abnormality has been attributed to the perturbation, by exogenous RA, of normal differentiation of the facial neural crest cells or mesenchyme. It is, hence, expected that those neural crest cells and facial mesenchymal cells are targets for RA and both express RA receptor (RAR) genes during the period of the facial morphogenesis.

In the present study, we investigated the expression pattern of the three subtypes of the RAR gene (mouse RARα, β, and γ [10]) during mouse facial development by in situ hybridization to identify target cells for endogenous RA. Since the facial developmental process is very complicated, we summarize the process as follows: in 8–9-day-old embryos, the neural crest cells migrate from the neural folds to the presumptive facial region (reviewed in [9]). In 10–11-day-old embryos, the embryonic face is composed of a series of small prominences around the primitive mouth (Fig. 1). These facial primordia are made up mainly of mesenchyme originating from the cranial neural crest cells. At this stage, exogenous RA induces severe defects in the face [4]. After 12 days, the neural crest cells differentiate into the connective tissues (bone, cartilage, and muscle of the face), the peripheral nervous system and melanocytes.

Correspondence address: S. Taniguchi, Department of Biochemistry, Okayama University Dental School, 2-5-1 Shikata-cho, Okayama City 700, Japan

2. EXPERIMENTAL

2.1. *Preparation of mouse embryos*

Mouse embryos (C57BL/6) were fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) at 10–13 days post coitum (p.c.) (the day of vaginal plug = 0 day). The fixed embryos were dehydrated in ethanol series and embedded in paraffin.

2.2. *Probe preparation*

Templates for synthesis of riboprobes for RAR mRNA were constructed by subcloning a fragment (the ligand binding domain) of human RARα cDNA [11] (cross-hybridized with mouse RARα cDNA [11]) and whole cDNAs of mouse RARβ and γ [10] into pGEM7 vectors (Promega Co.). All cDNAs of RARs were kindly donated by Drs Pierre Chambon, Martin Petkovich, and Arthur Zelent (CNRS, Strasbourg, France). Both antisense and sense riboprobes were labeled with [α-^{35}S]UTP (Amersham Co.). The probes were subjected to limited alkaline hydrolysis to shorten them to 50–150 bases for in situ hybridization.

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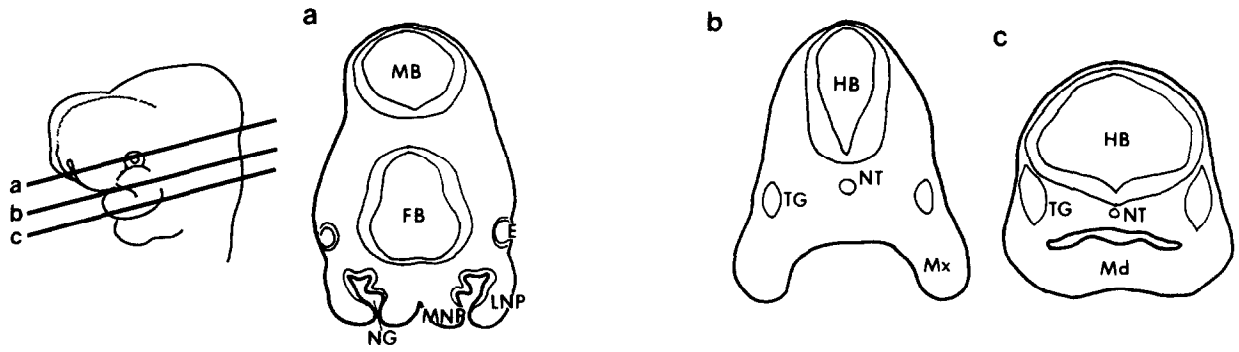

Fig. 1. Lateral features of the 11.5-day-old mouse embryo and transverse sections at nasal (a), maxillar (b) and mandibular (c) levels. LNP, lateral nasal prominence; MNP, medial nasal prominence; NG, nasal groove; MB, midbrain; FB, forebrain; HB, hindbrain; E, eye; Mx, maxillar prominence; Md, mandibular prominence; NT, notocord; TG, trigeminal ganglion.

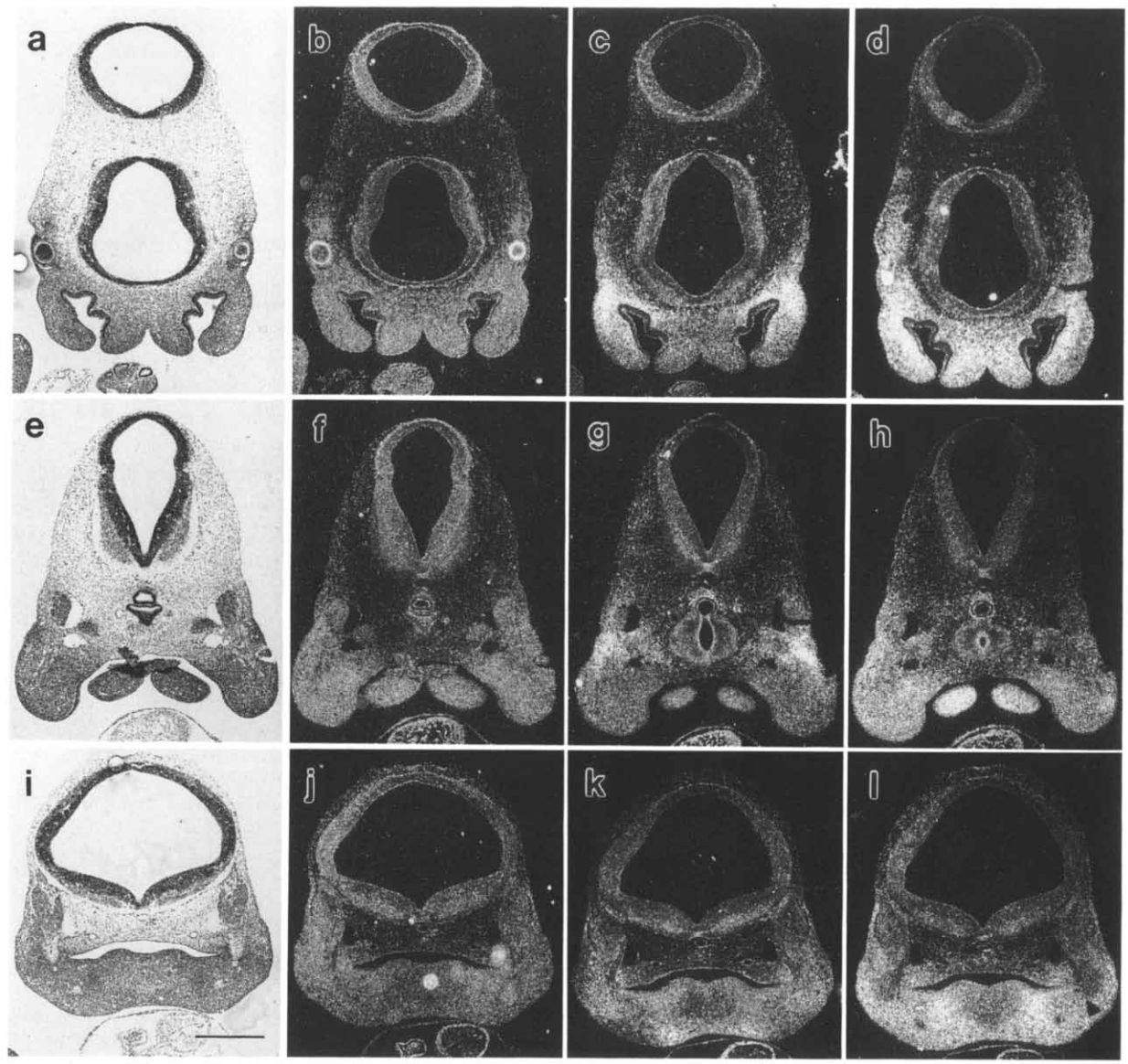

Fig. 2. Expression patterns of the RARα, β, and γ genes in the craniofacial region of the 11.5-day-old embryo in transverse sections at nasal (top), maxillar (middle), and mandibular (bottom) levels (see Fig. 1). Bright-field micrographs (left) and corresponding dark-field images (right). In situ hybridization was performed with the riboprobes for the RARα (b, f, j), β (c, g, k), and γ (d, h, l) transcripts. Bar = 500 μm.

2.3. *In situ hybridization*

In situ hybridization methods were described previously [12,13]. Briefly, 5-μm serial sections were taken on slides coated with poly-L-lysine. The slides were hybridized with either antisense or sense riboprobe at 50°C for 16 h, washed with 2 × SSC containing 50% formamide at 50°C, treated with 20 μg/ml RNase A, and further washed with 0.1 × SSC at 50°C. The slides were dipped in photographic emulsion (Kodak NTB2) and exposed for 7–10 days before developing in Kodak D19 developer. After staining the slides with hematoxylin and eosin, the slides were analyzed on an Olympus microscope using bright- and dark-field optics.

3. RESULTS

To examine the expression pattern of RAR genes in mouse facial development, we performed in situ hybridization on serial sections of the developing faces. At 10–11 days p.c., although the transcripts of the RARα were distributed uniformly in the craniofacial region (Fig. 2b, f, j). The RARβ transcripts were observed specifically around developing eyes and trigeminal ganglia and also in lateral nasal prominences, but not in maxilla or mandible (Fig. 2c, g, k). In contrast, RARγ mRNA was detected in maxilla (Fig. 2h), mandible (Fig. 2l), and both lateral and medial nasal prominences (Fig. 2d). The RARβ transcripts decreased in the anterior region of the lateral nasal prominences at 11 days p.c. At 12–13 days p.c., the RARβ transcripts were confined to the mesenchyme related to the retina, optic nerve and olfactory epithelium (Fig. 3b,e). The expression of the RARγ gene was found in the chondrogenic mesenchyme condensing to differentiate into the nasal and mandibular bones (Fig. 3c,f). In this stage, the transcripts of the RARα gene were still uniformly distributed in the face region (data not shown).

4. DISCUSSION

Expression patterns of the RAR genes have been investigated in mouse bone [13], skin [14], and developing limbs [15] by an in situ hybridization method. Although the expression of the RAR genes was observed in any stage of the developing limb, their expression patterns changed drastically with the differentiation stage [13–15]. Furthermore, the expression patterns of

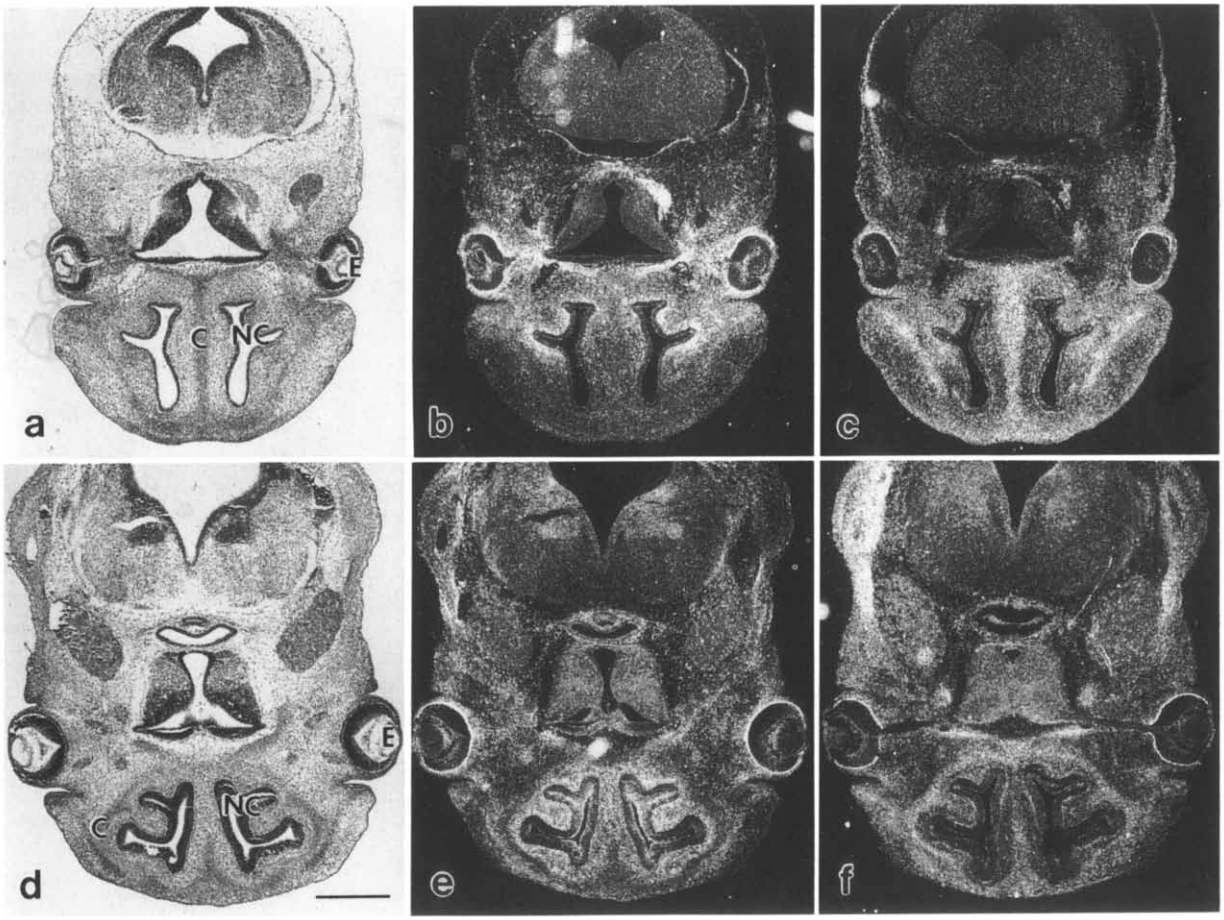

Fig. 3. Complementary expression patterns of the RARβ (b, d, e) and γ (a, c, f) genes in the craniofacial region of the 12.5- (top) and 13.5- (bottom) day-old embryos. Bright-field micrographs (left) and corresponding dark-field images (right). E, eye; NC, nasal cavity; C, chondrogenic mesenchyme. Bar = 500 μm.

the RAR subtype genes differ from each other [15]. Reconciliation of the published data with our results substantiates general rules for the RAR gene expression pattern: (i) the RARα gene is expressed rather weakly and uniformly in various tissues; (ii) the RARβ gene is expressed in cells committing programmed cell death [15] or cells developing into parts of sensory organs; (iii) the RARγ gene is expressed in differentiating skin, cartilage and bone.

It is intriguing to note that the expression patterns of the RARβ and γ genes at 10–11 days p.c. resemble the migration patterns of the neural crest cells from developing midbrain and anterior hindbrain, respectively. Morriss-Kay and Tan [16,17] recently investigated the pathway of the cranial neural crest cells by microinjection of labeled cells into rat embryos in vitro and found that (i) the neural crest cells settling to form maxillary and mandibular prominences migrate from the region of anterior hindbrain, (ii) the cells around eyes and trigeminal ganglia migrate from the midbrain region, and (iii) the cells migrate both from the midbrain and the anterior hindbrain in the facial primordia. In the morphogenetic stage (10–11-day-old embryo), we observed the overlapped region where both RARβ and γ transcripts are present (Fig. 2), although the mesenchymal cells in this area could not be distinguished morphologically from those in other facial regions. However, in the following differentiation stage (12–13-day-old embryo), the cells expressing either the RARβ or γ gene were sorted out from each other (Fig. 3), i.e. the region where either the RARβ or γ gene was expressed was distinctly specific for the various tissues. Since the neural crest cells migrating from midbrain and anterior hindbrain are settled together in such facial regions as the lateral nasal prominences during the morphogenetic stage [16,17], and since they migrate probably further to the terminal area specific for the differentiation fate, the change in the expression pattern of the RAR genes can be explained rather simply when we assume that the fates of the neural crest cells, including expression of the RAR genes, are specified before the cells start migrating. If the position of the cells determined the RAR gene expression, we cannot explain with a simple assumption, the occurrence of the overlapped expression of both RARβ and γ genes. Hence, we propose a working hypothesis that the differential expression of the RAR subtype genes correlates to the origin of the neural crest cells, i.e., the mesenchymal cells derived from the midbrain and anterior hindbrain express the RARβ and γ genes, respectively.

The role of RA during face development is still uncertain, although RA appears to be a putative morphogen working a positional cue in limb buds [1,2]. However, it is quite clear that the RA level in the developing face is an important factor for morphogenesis, because exogenous RA induces severe facial defects [4]. In any case, the endogenous level of RA and the distribution of the RARs are concluded to play crucial key roles during facial morphogenesis.

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