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cDNA cloning of a quail homeobox gene and its expression in neural crest-derived mesenchyme and lateral plate mesoderm

(quail embryo/developmental gene/*in situ* hybridization)

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ABSTRACT **We report here the cloning of a quail cDNA related to the *Drosophila* gene *msh* and to the mouse genes *Hox-7* and *Hox-7.1*. For this reason we called this cDNA Quox-7. The amino acid homology of Quox-7 cDNA with the above mentioned genes is high (83%) for the homeobox and its 5′- and 3′-flanking sequences, and the homology is medium (43%) for another stretch of amino acids upstream of the homeobox; elsewhere the sequences of quail and mouse cDNAs have diverged significantly. In quail embryos of day 2–5, Quox-7 transcripts were found essentially in the ventral mesenchyme (neural crest-derived mesectoderm of the face and hypobranchial structures, somatopleure, and limbs) and also in a narrow dorsomedial band of cells of the superficial ectoderm and neural tube. This pattern is fundamentally similar to that reported for *Hox-7/7.1*, suggesting that the products of these genes play a similar role in the development of the different classes of vertebrates.**

Certain genes involved in homeotic mutations in *Drosophila* have been found to share a highly conserved 180-base-pair (bp) stretch of DNA, the homeobox (1, 2). Subsequently, the homeobox was also found in other developmental genes, such as maternal genes and those controlling segmentation of the fly (3–5). All these genes encode nuclear proteins, and the peptide domain corresponding to the homeobox has structural similarity with the helix-turn-helix DNA-binding region of some regulatory proteins in yeast and prokaryotes (3, 4). The finding that homeobox-containing genes also exist in vertebrates has aroused considerable interest, based on the assumption that such genes might play critical roles in the control of developmental processes throughout the animal kingdom.

Homeobox genes have been identified in amphibians (6), human (7), and mouse, in which a number of them are under intensive research; they have been grouped into several so-called *Hox* gene families according to their chromosomal localization and are thought to be transcription factors (8–10). Investigations on mouse homeobox genes have so far essentially dealt with description of their spatial and temporal expression, which in many cases is extremely suggestive of an involvement in patterning—i.e., in encoding positional values in vertebrate development. This is particularly striking as far as development of the nervous system (8, 9, 11) and limb (12–16) is concerned.

However, to dissect the role of homeobox genes in construction of the body plan during development, embryonic manipulations are required. As many examples have shown, the avian embryo is particularly suitable to study this type of problem in higher vertebrates. Moreover, systematic comparisons have shown that the regions of the embryo in which certain homeobox genes are expressed seem to be similar in different vertebrates (17–19). This is why it seemed worthwhile to search for homeobox genes in avian species.

Our long-standing interest in the neural crest and its role in the genesis of facial structures (20) prompted us to focus on the equivalent of a homeobox-containing gene related to the *Drosophila msh* (muscle segment homeobox) gene (12, 13). Part of this gene has been isolated from the mouse by Robert *et al.* (12) and called *Hox-7*. A cDNA (Hox-7.1) was obtained from the same species by Hill *et al.* (13). We will refer to these mouse DNAs as Hox-7/7.1 because of their similarity. Expression of Hox-7/7.1 in the mouse embryo has been reported to be strong in the cephalic neural crest and its mesectodermal derivatives in the branchial arch region. The limb bud is also the site of Hox 7/7.1 expression in both its mesenchymal and ectodermal components.

We report here the sequence of Quox-7 cDNA, which we have cloned and found to be the quail equivalent of mouse Hox-7/7.1.* We also describe its expression, as analyzed by *in situ* hybridization, in quail embryos from the neurula stage up to embryonic day 5 (E5).

MATERIALS AND METHODS

Isolation of cDNA Clones. An oligo(dT)-primed cDNA library was prepared in λgt10 from poly(A)$^+$ RNA isolated from limb buds of E5 quail embryos, as described (21). Approximately 10^6 phage recombinants were screened with a 150-bp fragment of Hox-7 (12), which included 95 bp in the 5′ region of the homeobox and 55 bp upstream from the homeobox. Mild-stringency hybridization was performed at 42°C in buffer containing 45% formamide, followed by washing in 0.2× SSC (1× SSC is 0.15 M sodium chloride/0.015 M sodium citrate, pH 7)/0.1% SDS at 55°C. One positive clone was obtained and used for rescreening the same library to obtain a longer cDNA fragment. Nucleic acid sequences were determined on both strands by the dideoxynucleotide chain-termination method with 7-deazaguanine instead of guanine.

Northern (RNA) Blot Analysis. Total cellular RNA was isolated by the lithium chloride/urea method (22), followed by purification of poly(A)$^+$ RNA. RNA was submitted to electrophoresis on 1% agarose/formaldehyde gel, transferred to GeneScreen nylon membrane (NEN), and hybridized with [^{32}P]dCTP-labeled DNA probe at 42°C in buffer containing 50% formamide followed by washing in 0.2× SSC/0.1% SDS at 55°C.

***In Situ* Hybridization.** Paraffin sections (5 μm) of quail embryos, fixed in 4% paraformaldehyde/phosphate-buffered saline, were prepared. The procedures applied for section treatment, hybridization, and washing were those reported by Wakamatsu and Kondoh (23). The 320-bp fragment (probe C, Fig. 1*A*) was subcloned into pGEM-4z and pGEM-3z

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Abbreviation: E directly followed by number, embryonic day.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M37164).

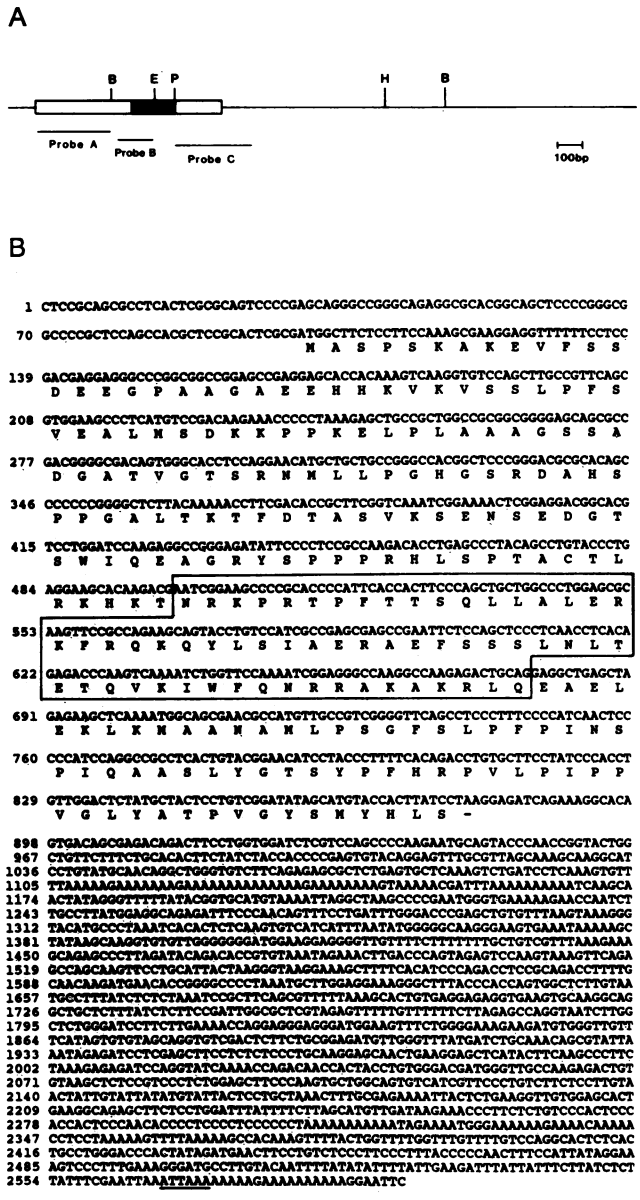

FIG. 1. (*A*) Restriction map of quail Quox-7 cDNA. The open box and solid box indicate the open reading frame and the homeobox, respectively. B, *Bam*HI; E, *Eco*RI; P, *Pst* I; H, *Hin*dIII. (*B*) Nucleotide and deduced amino acid sequences (in one-letter code) of Quox-7. The homeodomain is boxed. A potential polyadenylylation site is underlined.

vectors (Promega). Antisense and sense RNA probes were prepared by *in vitro* transcription using SP6 RNA polymerase with uridine [^{35}S]thiotriphosphate (800 Ci/mmol; 1 Ci = 37 GBq; Amersham). Probes were partially hydrolyzed to give an average length of 150 nucleotides and used at a concentration of 10^4 cpm/μl in hybridization buffer. After hybridization, the washed sections were autoradiographed with NTB-2 emulsion (Kodak) and exposed for 8–10 days. The sections were then counterstained with cresyl violet.

RESULTS

Isolation and Characterization of Quox-7 cDNA. An E5 quail limb bud cDNA library was screened with a fragment of the Hox-7 probe (12) from which 80 bp had been eliminated from the 3′ end of the homeobox region because of its very high similarity to Antennapedia-type homeoboxes (see *Materials and Methods*). Of several overlapping clones, the longest insert (2.6 kilobases) was selected for further analysis. The simplified restriction map of Quox-7 cDNA is shown in Fig. 1*A*. The size of the cDNA corresponds to that of mRNA detected by Northern blot analysis (see below).

The entire nucleotide sequence of the Quox-7 cDNA clone and the deduced amino acid sequence are shown in Fig. 1*B*. The sequence of 2595 nucleotides (nt) contains an open reading frame of 777 nt that begins with an ATG codon that meets the requirement for the initiation codon as defined by Kozak (24) and encodes 259 amino acids. Unlike the other homeobox genes, Quox-7 cDNA has a long 3′-untranslated sequence of 1710 bp. The homeobox sequence, 180 bp long, is found between positions 499 and 678.

Compared with Hox-7.1 (13), the amino acid sequence of Quox-7 contains two highly conserved regions encoded by nt 166–359 and nt 411–747, displaying 43% and 83% identity, respectively. The region that exhibits the greatest conservation, including the homeobox and its N-terminal and C-terminal flanking regions of 29 amino acids and 23 amino acids, respectively, is shown in Fig. 2. This similarity allows us to conclude that Quox-7 and Hox-7/7.1 represent related genes. Interestingly, this region also shows a remarkable homology to the *msh* gene of *Drosophila* (12, 13). The consensus sequence for the putative helix-turn-helix structure is well conserved (3). The hexapeptide sequence, which is found upstream from the homeobox in many other genes (25), was not seen in Quox-7 nor is it present in the Hox-7.1 cDNA (13). However, in the stretch of protein to the C terminus of the Quox-7 homeobox, no cysteine residues were found, whereas seven such residues appear in the corresponding region of Hox 7.1. Except for the two conserved regions shown above, no sequence was found to exhibit significant homology to Quox-7 cDNA. In Northern blot analysis (Fig. 3), Quox-7 cDNA probe A (Fig. 1*A*), which stretches 400 bp downstream from the ATG codon and lacks the homeobox region, hybridized with a single mRNA species that migrated at a position corresponding to 2.8-kb size. When the 200-bp fragment (probe B, Fig. 1*A*), containing the 5′ half of the homeobox sequence—i.e., the region divergent from the Antennapedia sequence, was used as probe, a faint band of 1.8 kb of mRNA was detected in addition to the major 2.8-kb signal. Probing with another fragment located upstream from the polyadenylylation site resulted again in a single band of 2.8 kb (data not shown). Therefore, it is unlikely that the mRNAs of 2.8 kb and 1.8 kb are derived from alternative splicing of a common precursor RNA.

Expression of Quox-7 in Quail Embryo. To study the expression pattern of Quox-7 transcripts during quail embryogenesis, we prepared paraffin sections of embryos and

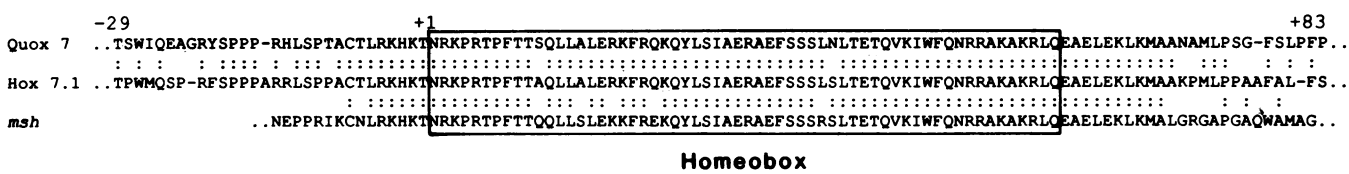

FIG. 2. Comparison of the homeodomain and its flanking sequence between Quox-7 (quail), Hox-7.1 (mouse) (13) and *msh*-encoded (*Drosophila*) (13) proteins. The first amino acid (in one-letter code) in the homeodomain is numbered as +1.

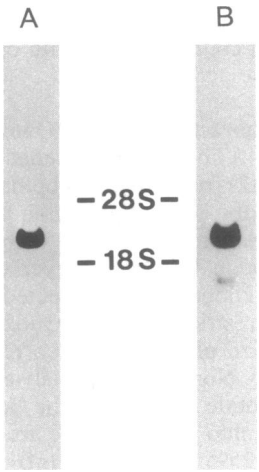

FIG. 3. Northern blot analysis. Ten micrograms of poly(A)$^+$ RNA was resolved in agarose/formaldehyde gel followed by hybridization with probe A (*A*) and with probe B (*B*) (Fig. 1*A*). Positions of 28S and 18S ribosomal RNAs are shown.

performed *in situ* hybridization with an antisense probe of Quox-7 (probe C, Fig. 1*A*). Alternate sections were hybridized with the sense probe and did not show any labeling signal over the background level (data not shown). The embryos were examined from 30 hr (eight somites) to E5.

In the trunk region of the eight-somite embryo, Quox-7 was expressed in the neural folds before and after closure of the neural tube (Fig. 4 *a* and *b*). At the level of the mesencephalic vesicles, the neural tube is closed, and the neural crest cells have already started migrating (Fig. 4*c*). The dorsal part of the neural tube and overlying ectoderm also produced a hybridization signal (Fig. 4*d*). The label was also detected in the population of migrating neural crest cells proximal to the neural tube, whereas crest cells more distant from the neuraxis were negative (Fig. 4 *c* and *d*). Quox-7 expression in the dorsal part of the rhombencephalon extended laterally in the neuroepithelium (data not shown). Intense signals were seen in the somatopleural mesoderm and in the overlying ectoderm. In contrast, the somites, the intermediate cell mass, and splanchnopleure were negative (Fig. 4 *a* and *b*) and remained so throughout development except for a small amount of dorsal superficial mesoderm at E3.5–4.5, as indicated below. Quox-7 transcripts were detected in the somatopleure and its derivatives over the whole length of the anteroposterior axis at all stages concerned (see below). Extraembryonic membranes of somatopleural origin (particularly the amnios) were also labeled (Fig. 4*f*).

In the 25-somite embryo, strong labeling was observed in the mesenchymal cells in the branchial arches, which are mostly derived from cephalic neural crest cells (Fig. 4 *g* and *h*) (20). It is noteworthy that at later stages no neuronal tissue of neural crest origin gave any signal. In the trunk region, Quox-7 was expressed strongly in the somatopleure that forms the body wall and in the extraembryonic membranes (Fig. 4 *e* and *f*). There was a clear-cut boundary of expression between the somatopleure and the developing mesonephros. It should be noted that the ectoderm that overlies the somatopleure expressing the gene remained labeled at least until E5 (Fig. 4 *e* and *f*). In the dorsal part of the body, no somite-derived mesenchymal tissue and no neural crest derivatives were labeled. In contrast, the dorsal part of the neural tube retained its expression even after all the neural crest cells had left (Fig. 4 *f* and *h*).

At E3, the branchial arches are well developed and showed a strong positive signal. When the first branchial arches were on the point of fusing, the label was particularly intense on their medioventral margins (Fig. 5 *a* and *b*), whereas in their proximal portions the mesectodermal cells did not exhibit Quox-7 mRNA. The mandible at E4.5, which is derived from the first branchial arch, also showed strong signal (Fig. 5 *c* and *d*). At this stage, the maxillary processes, which are

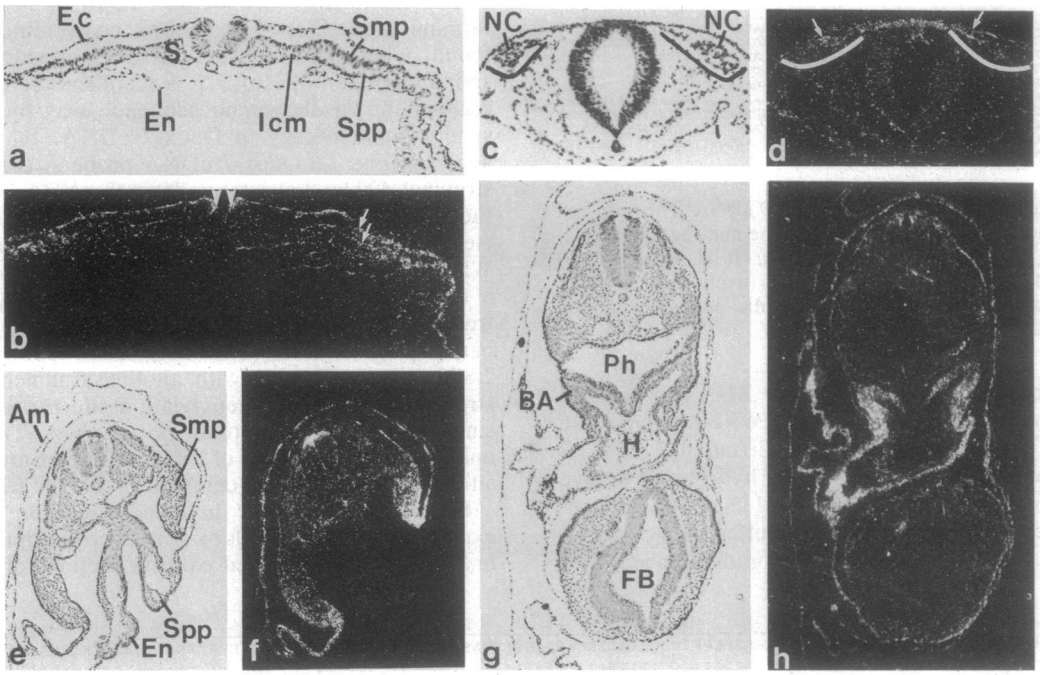

FIG. 4. *In situ* hybridization of Quox-7 transcripts on transverse sections of 8-somite and 25-somite embryos. (*a*, *c*, *e*, and *g*) Bright-field photographs. (*b*, *d*, *f*, and *h*) Dark-field photographs. (*a* and *b*) Trunk level of an 8-somite embryo. Labeling in the neural fold is indicated by arrowheads and in the ectoderm and somatopleure by arrows. (*c* and *d*) Cephalic level of an 8-somite embryo. Lines show the border between migrating neural crest cells and the mesoderm. Arrows indicate Quox-7 expression in the neural crest. (*e–h*) Sections through the trunk (*e* and *f*) and heart (*g* and *h*) of a 25-somite embryo. Am, amnios; BA, branchial arch; Ec, ectoderm; En, endoderm; FB, forebrain; H, heart; Icm, intermediate cell mass; NC, neural crest; Ph, pharynx; S, somite; Smp, somatopleure; Spp, splanchnopleure.

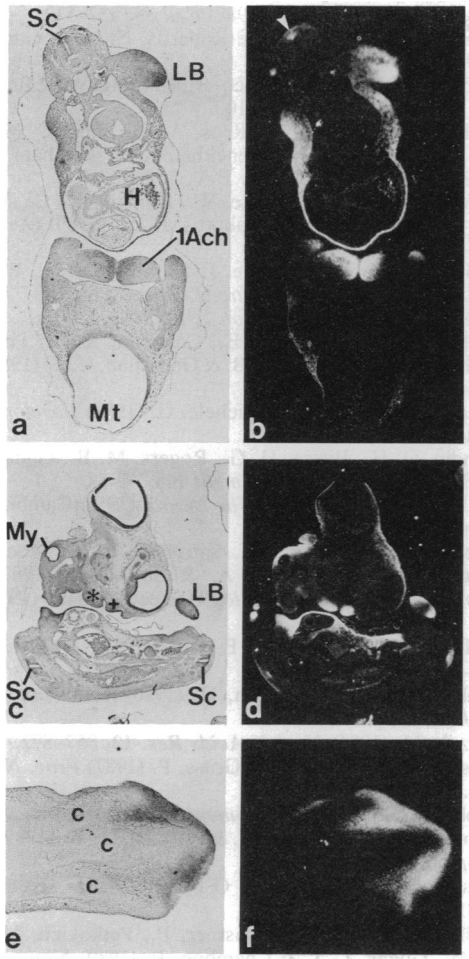

FIG. 5. *In situ* hybridization of Quox-7 on sections from E3 to E5 embryos. (*a*, *c*, and *e*) Bright-field photograph. (*b*, *d*, and *f*) Dark-field photograph. (*a* and *b*) Transverse sections of an E3 embryo. Label in the dorsal mesenchyme of the neural tube is indicated by an arrowhead. (*c* and *d*) Parasagittal sections of an E4.5 embryo. (*e* and *f*) Horizontal sections of the limb bud of an E5 embryo. 1Ach, first branchial arch; C, cartilage; H, heart; LB, limb bud; Mt, metencephalon; My, myelencephalon; Sc, spinal cord; +, maxillary process; *, mandible.

derived from the cephalic neural crest, were clearly positive for Quox-7 expression. Fig. 5*a–d* show that in the trunk region at E3 and E4.5, the somatopleure retained its intensive expression while the splanchnopleure, which had by then completely surrounded the gut endoderm, remained negative. At E3–E5, two other regions were strongly labeled. One was the distal part of the developing limb buds, including the apical ectodermal ridge (Fig. 5 *a* and *b*). Expression in the limb bud was later progressively restricted to the interdigital region (Fig. 5 *e* and *f*), while the cartilage primordia were always negative. The second region was the mesenchyme surrounding the dorsolateral part of the brain (Fig. 5*a–d*). Along the dorsal aspect of the spinal cord, a narrow band of mesenchyme also expressed the Quox-7 mRNA, and a signal was still observed at E5 (Fig. 5*a–d*). The strong expression observed at the eight-somite stage, at the level of the dorsal rhombencephalic neuroepithelium, was maintained throughout development in the metencephalon and the myelencephalon (Fig. 5 *b* and *d*). In the mesencephalon and the prosencephalon (including the optic vesicles) no signal was detected.

DISCUSSION

We have isolated a cDNA from quail using mouse Hox-7 probe (12), and have named it Quox-7 (*Qu*ail homeob*ox*). In our view, the cloning of developmental genes in avian species that are already known in mammals and *Drosophila* presents at least two interesting aspects. The first consists in comparing the genes and determining the degree of conservation of certain protein structures throughout evolution. The second is to compare patterns of expression of related developmental genes in amniotes. Because mechanisms of development are extremely similar in higher vertebrates, resemblances are likely to occur if these genes are, indeed, important in development.

Embryonic manipulations, carried out essentially in the avian embryo, have shown the paramount importance of positional information in patterning vertebrate development. This has been particularly well analyzed for limb bud development in the chicken. As a result of signals operating within the developing limb bud, cells acquire positional values that are then interpreted so that they develop according to their position (26). Similar observations have been made for cells originating from the neural crest (20). Because homeobox-containing genes are involved in patterning in *Drosophila*, their homologues are good candidates for similar roles in vertebrates.

The conservation of the nucleotide sequence between Hox-7/7.1 (12, 13) and Quox-7 cDNA is particularly high for the homeobox and for its flanking regions. It has been proposed that the DNA-binding specificity of the homeoproteins is defined by the helix-turn-helix domain encoded by the homeobox and by the neighboring amino acid sequences (3, 10, 27, 28). This, therefore, suggests that Hox-7/7.1 and Quox-7 may play similar roles in mammals and birds. Such an assumption is further supported by the fact that they are expressed at virtually identical sites during embryogenesis (12, 13). Thus, Quox-7 is expressed first in the neural folds and the neural crest and subsequently in the branchial arches, as well as in limb bud, body wall, and head mesenchyme, according to a spatiotemporal pattern analogous to that of Hox-7/7.1 in the mouse embryo (12, 13).

At E3.5, branchial arch mesenchyme appears homogeneous by morphological criteria. However, Quox-7 is expressed only in its midventral part, indicating that at the level of gene expression, heterogeneity is already established in the mesenchyme, although the rudiments of cartilage appear only later in development.

We found that Quox-7 is already detectable in the somatopleure at the neurula stage (E2) and persists up to at least E5, although it was not mentioned that Hox-7/7.1 was expressed in somatopleure (12, 13). The absence of signal in the somitic and splanchnopleural mesenchyme shows that the expression of Quox-7 is not a common feature of all mesenchymal cells. The overlying developing epidermis is also labeled, both in the presumptive body wall and in the limb bud, showing that ectodermal structures also transcribe this gene, a fact confirmed by the finding that Quox-7 is expressed in certain regions of the neuroepithelium (i.e., as a narrow band in the dorsal spinal cord and the rhombencephalon).

The dorsal head mesenchyme at the forebrain level, which expresses Quox-7 and Hox-7/7.1 (12, 13) in quail and mouse embryo respectively, is derived from the neural crest (20), and its fate is to provide meninges as well as membrane bones. Dorsal head mesenchyme at the mid-hind-brain level, which also exhibits Quox-7, is derived from the mesodermal germ layer and also differentiates into membrane bones. Therefore, this shows that certain mesenchymal structures express Quox-7 and Hox-7/7.1 (12, 13), irrespective of their germ layer of origin.

In the limb bud, Quox-7 expression first concerns the distal mesenchymo–ectodermal region and thereafter becomes restricted to the mesenchyme located in the interdigital areas. Neither Hox-7/7.1 (12, 13) nor Quox-7 mRNA could be

detected within the precartilaginous and cartilage rudiments. Interestingly, there is a considerable overlap between the patterns of expression of Quox-7 and those of retinoic acid receptor β gene (29), the cellular retinoic acid-binding protein (CRAB) gene (29, 30), and N-*myc* gene (31). It has to be underlined that the pattern of Quox-7 expression has a spatial distribution alternating with that of retinoic acid receptor γ gene in the developing limb bud (29). Given the established role of retinoic acid in the patterning of limb bud and facial development (32–34), our observations suggest that there may be important interactions between the Quox-7- or Hox-7/7.1-encoding genes and those controlling the distribution and the morphogenetic effects of retinoic acid during development of both limb buds and facial structures. It is interesting to notice in this respect that a positive statistical correlation has been found in humans between congenital malformations of the limb and those of facial structures (35), indicating that development of these structures is controlled, at least to a certain extent, by common mechanisms.

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