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DIFFERENTIAL EXPRESSION OF TWO MSH-RELATED HOMEOBOX GENES CHOX-7 AND CHOX-8 DURING CHICK LIMB DEVELOPMENT

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We have isolated two closely related cDNAs, Chox-7 and Chox-8, encoding homeodomain-containing proteins homologous to Drosophila msh. The Chox-7 and Chox-8 genes are chicken cognates of mouse Hox-7.1 and Hox-8.1, respectively. In situ hybridization using 3' regions of the cDNAs as probes revealed that the Chox-7 gene is highly expressed in the mesenchyme subjacent to the apical ectodermal ridge whereas Chox-8 expression is localized in the anterodistal mesenchymal region at early stages of limb formation, suggesting different roles during limb development. At later stages, both genes are expressed in the anterior and posterior mesenchymes and in the interdigital mesenchyme where programmed cell death occurs.

Homeobox-containing genes encode regulators of transcription which play key roles in pattern formation during vertebrate development (1-3). Expression of several homeobox genes was demonstrated in the limb bud with a specific spatiotemporal pattern. The homeobox genes at the 5' end of the Hox-4 (Chox-4 for the chick genes) complex are expressed in the developing limb of the mouse and chick embryos with an anteroposterior concentration gradient set up by a signal from the polarizing region (4-6). The members of the Chox-4 complex are ectopically induced in the anterior mesenchyme during digit duplication by grafting the polarizing region or a retinoic acid-containing bead (5,6). The homeobox genes at the 5' end of the Hox-1 (Chox-1 for the chick genes) complex are also expressed in the developing chick limb (7). The Chox-1 genes are implicated in the proximodistal pattern formation, since each gene is sequentially expressed with a proximodistal gradient in the limb bud.

A member of another class of homeobox genes, Hox-7.1, which encodes homeodomain homologous to the Drosophila msh, has been shown to be expressed in the distal mesenchyme of the mouse limb bud at early stages of development (8,9). The Hox-7.1 gene is suggested to play an important role in the

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proximodistal limb outgrowth resulting from short-range epithelial-mesenchymal interaction. In this study, we isolated two msh-related homeobox genes, Chox-7 and Chox-8, from the chicken embryo, and compared their expression patterns in the developing limb by in situ hybridization to differentiate their functions. We found that the expression pattern of the Chox-7 gene differs spatially and temporally from that of the Chox-8 gene, although the Chox-7 and Chox-8 genes encode similar homeodomain proteins.

Materials and Methods

Fertilized chick eggs (White Leghorn) were incubated at 38°C for 3 to 4 days, and poly(A) RNAs were extracted from either whole embryos or limb buds using Fast Track (Invitrogene). Double stranded cDNAs were synthesized according to the Gubler-Hoffman method (10) using mixture of random primer and oligo(dT), and ligated with EcoRI-XmnI adaptor (New England Biolabs). After kinasing ends, the cDNAs were cloned into lambda gt10.

Phage library DNA was prepared from liquid lysate, and used at first as a template for amplification of msh-related homeobox genes by polymerase chain reaction (PCR). The sequences of oligonucleotide primers were as follows: 5'-AACCGCAAGCCCAGGACGCCT-3' for upstream region, and 5'-CTTAGCGCGACGGTTCTGGAA-3' for downstream region. PCR was carried out at annealing temperature of 40°C for 35 cycles. The PCR products were cloned into pGEM7Zf(+) and sequenced by the dideoxy chain-termination method (11) using the Sequenase kit (USB).

Approximately 0.5 million recombinant phages were screened with the 165 nucleotides (nt) fragment obtained by PCR amplification under medium stringency conditions. Briefly, the filters were hybridized at 42°C in a solution containing 50% formamide, 5 X SSPE, 5 X Denhardt's solution, 0.1 mg ml^{-1} denatured salmon sperm DNA, 0.1% SDS and ^{32}P-labeled probe DNA for 16 h. The final washes of the filters were performed in 2 X SSPE and 0.1% SDS at 42°C.

The procedures for in situ hybridization were described previously (12).

Results and Discussion

Isolation and identification of Chox-7 and Chox-8 cDNAs

The PCR-amplified DNA, 165 nt, encodes msh-related homeodomain exactly identical to the mouse Hox-7.1. Using the DNA as a probe, we obtained total of six independent phage clones from the chick cDNA library, termed M1, M8, M9, M10, M11 and M13 (Fig. 1). The physical map (Fig. 1) and nucleotide sequence indicate that clones M8, M9 and M13 were derived from the same mRNA and the remainders were from another mRNA. We have designated the former as Chox-7 because of extensive similarity in the deduced amino acid sequence with the mouse Hox-7.1 sequence (8). The latter, designated here as Chox-8, was recently isolated from the chick library (13), and shown to be closely related to mouse Hox-8.1 and quail Quox-7 rather than mouse Hox-7.1 (Fig. 2). Thus, the DNA probe hybridized with two different cDNAs, because the homeobox sequence of Chox-7 is 85% homologous to that of Chox-8, especially 35 nt stretch of completely identical nucleotide sequence present in the 5'-half region of the homeobox (13).

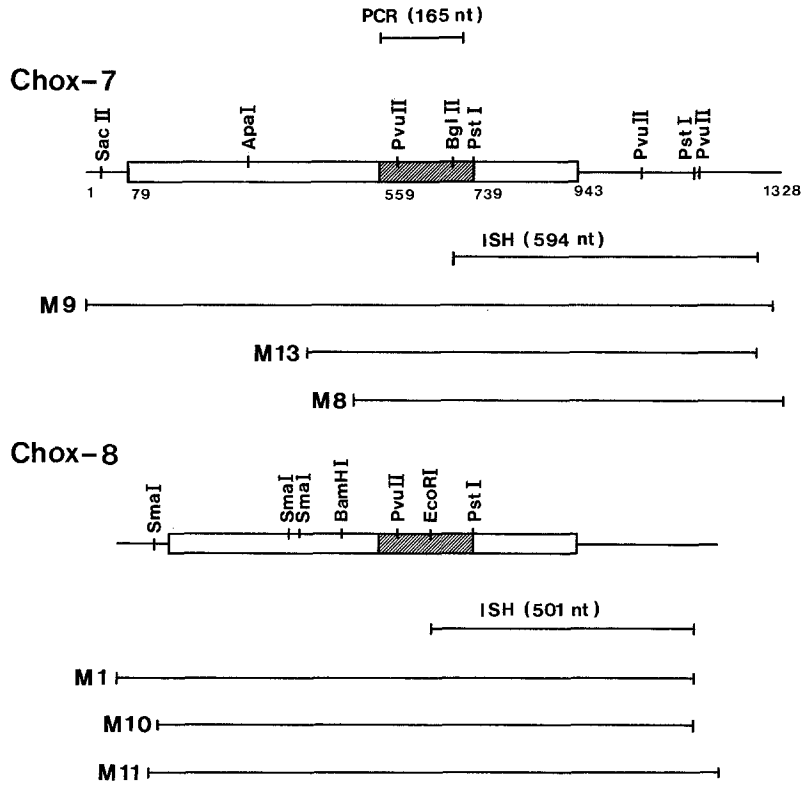

Fig. 1. Physical map of the Chox-7 and Chox-8 cDNAs. The coding region is boxed, and the homeobox is indicated by a hatched box. Clones M1 to M13 are aligned with respect to the PCR amplified probe shown on the top (PCR). These overlapping clones were used for sequencing, and differed only 5' and 3' ends in each subclass. Extents of probes used for in situ hybridization (ISH) are shown below the composite cDNA maps.

At amino acid level, the overall sequence of Chox-7 is only 55% homologous to Chox-8 (Fig. 2), whereas the Chox-7 sequence is 80% and 67% homologous to the Hox-7.1 and Xhox-7.1 sequences, respectively (8,14), and the Chox-8 sequence is 98%, 83% and 73% homologous to the Quox-7, Hox-8.1 and Xhox-7.1' sequences, respectively (13-16). Sequence conservation outside of the homeodomain suggest that the msh-related homeobox genes identified in the vertebrate can be subdivided into at least two subclasses, Hox-7 and Hox-8. Only two conservative substitutions are noted in the homeodomain sequence, but they do not contribute to the classification of Hox-7 and Hox-8 (Fig. 2).

In the mouse and zebrafish, three different homeobox genes of the msh class were identified by PCR amplification of genomic DNA (17). Our screening of the chick embryo cDNA library under low stringency condition, however, did not yield any additional clones other than Chox-7 and Chox-8. We do not know whether the third member of the msh class may not be expressed significantly during vertebrate development, and further elucidation is necessary.

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Chox-7      MAPAADMTTAPTGVRSDEPPASAFSKP.GGGL...PVAAA.....MGGEEESDKPKVSPSPLPFSVEALMADRRKPPGGR
Hox-7.1     -----A--SL-L--KV.-D..---A--A---VGQA-G---ATATA--TD--GA----PA-L----------H---GAKE
Xhox-7.1    ----LL-ASYQP--KIE-R-...LNRMQQT-V.............KPSLG-.-----P.GI----------.---GRD-
Chox-8                  MA-PSKAKEV-.SSDEE-PA.............A-A--HH-V---.-.---------S-.K---.KE
Quox-7                  MA-PSKAKEV-.SSDEE-PA.............A-A--HH-V---.-.---------S-.K---.KE
Hox-8.1                 MA-PTKGGDL-DSSDEE-PAVLAGPGPGPGGAE-SA--RR.V---.-.---------S-.K---.KE
Xhox-7.1'               MS-PRRIKEDL.SSDEE-Q.VHPTL.........SPS-DH-I-I-.-.----------.KRV-.KE

Chox-7      ...DGPEGSGPPLGSAR...ANLGALTT.EAPTSPLPLGGHFPSVGALGKLPEDALLKAESPEKPERSPWMQSP.RFSPP
Hox-7.1     SVLVAS--AQAAG--VQHLGTRP-S-GAPD--S--R---.--.---G-L-------V-------LD-T------.-----
Xhox-7.1    .DLSS-T--PLAGT-H...SPRV-SIAAG-T-N--IS--NRY-.---IMQ---ET---P----...--S-I---.S---S
Chox-8      ........LPLAAAGGNADG-TV-TS.RNLLLPGHGSRDA-S-P.---T-TFDT-SV-S-NS-..DGTS-I-EAG-Y---
Quox-7      ........LPLAAAGSSADG-TV-TS.RNMLLPGHGSRDA-S-P.---T-TFDT-SV-S-NS-..DGTS-I-EAG-Y---
Hox-8.1     ..........SPA.VPPDC-SA--VLRPLLLPGHGVRDA-S-..-P-V-PF-T-SV-S-NS-..DGA--I-E-G-Y---
Xhox-7.1'   ..........APP-RAVDSSAA.TS-PNRHL.HLGIRDSPS-.L-SQKG-KPRRSNRK.NS-..DGTS-SKDGGSY---

Chox-7      PPRRLSPPACTLRKHKT|NRKPRTPFTTAQLLALERKFRQKQYLSIAERAEFSSSLSLTETQVKIWFQNRRAKAKRLQ|E
Hox-7.1     -A---------------|-------------------------------------------------------------|-
Xhox-7.1    -T--M-----P------|----------S--------------------------N--------------------|-
Chox-8      -.-H---T---------|----------S--------------------------N--------------------|-
Quox-7      -.-H---T---------|----------S--------------------------N--------------------|-
Hox-8.1     -.-HM--TT--------|----------S--------------------------N--------------------|-
Xhox-7.1'   -.-H---SS--------|----------S--------------------------N--------------------|-

Chox-7      AELEKLKMAAKPMLPPAAFGISFPLGGPAVAGASLYGASSPFQRAGLPVAPVGLYTAHVGYSMYHLT*
Hox-7.1     -------------------L---------A------S--G-----A---------------------*
Xhox-7.1    -------------------.--------T-.-PT-----T-N----QA--MS-M------L-------S*
Chox-8      ---------------SG.-SLP--INS-.IQA-----T-Y--H-PV--IP-----ATP--------S*
Quox-7      ----------NA---SG.-SLP--INS-.IQA-----T-Y--H-PV--IP-----ATP--------S*
Hox-8.1     ---------------SG.-SLP--INS-.LQA--I----Y--H-PV--IP-----ATP---GI--VS*
Xhox-7.1'   --I---------I---G.-S-P--INS-.IQA-----S-YQ-H-PV--IP-M---ATP--------SEEGDMT*
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Fig. 2. Alignment of the amino acid sequence of Chox-7 and Chox-8 proteins with mouse, quail and Xenopus cognates. Sequences from the following sources are aligned by eyes with Chox-7: mouse Hox-7.1 and Hox-8.1 (8,16), chick Chox-8 (13), quail Quox-7 (15) and Xenopus Xhox-7.1 and Xhox-7.1' (14). Amino acid identities with Chox-7 are represented by dashes. Gaps are indicated by dots to improve the sequence alignment. Homeodomains are boxed.

Expression of the Chox-7 and Chox-8 genes during limb development

It is important to know the functional difference of the closely related Chox-7 and Chox-8 genes. We therefore examined whether the two genes are expressed differentially during development. From comparison of the entire nucleotide sequences, we used 3' regions of the Chox-7 and Chox-8 cDNAs, 594 nt and 501 nt long, respectively, as templates for synthesizing single-stranded RNA probes (Fig. 1). These probes do not cross-hybridize each other, since there is only a short stretch of similarity in the nucleotide sequence, which is not longer than 12 nt even in the 3' half of the homeobox owing to the differential usage of codons.

At stages 19 to 23 (18), differential expression of the Chox-7 and Chox-8 genes was most evident (Fig. 3). The Chox-7 transcripts were localized in the mesodermal region underneath the apical ectodermal ridge (AER), the progress zone, where mitogenic activity is known to be sustained by signaling from the

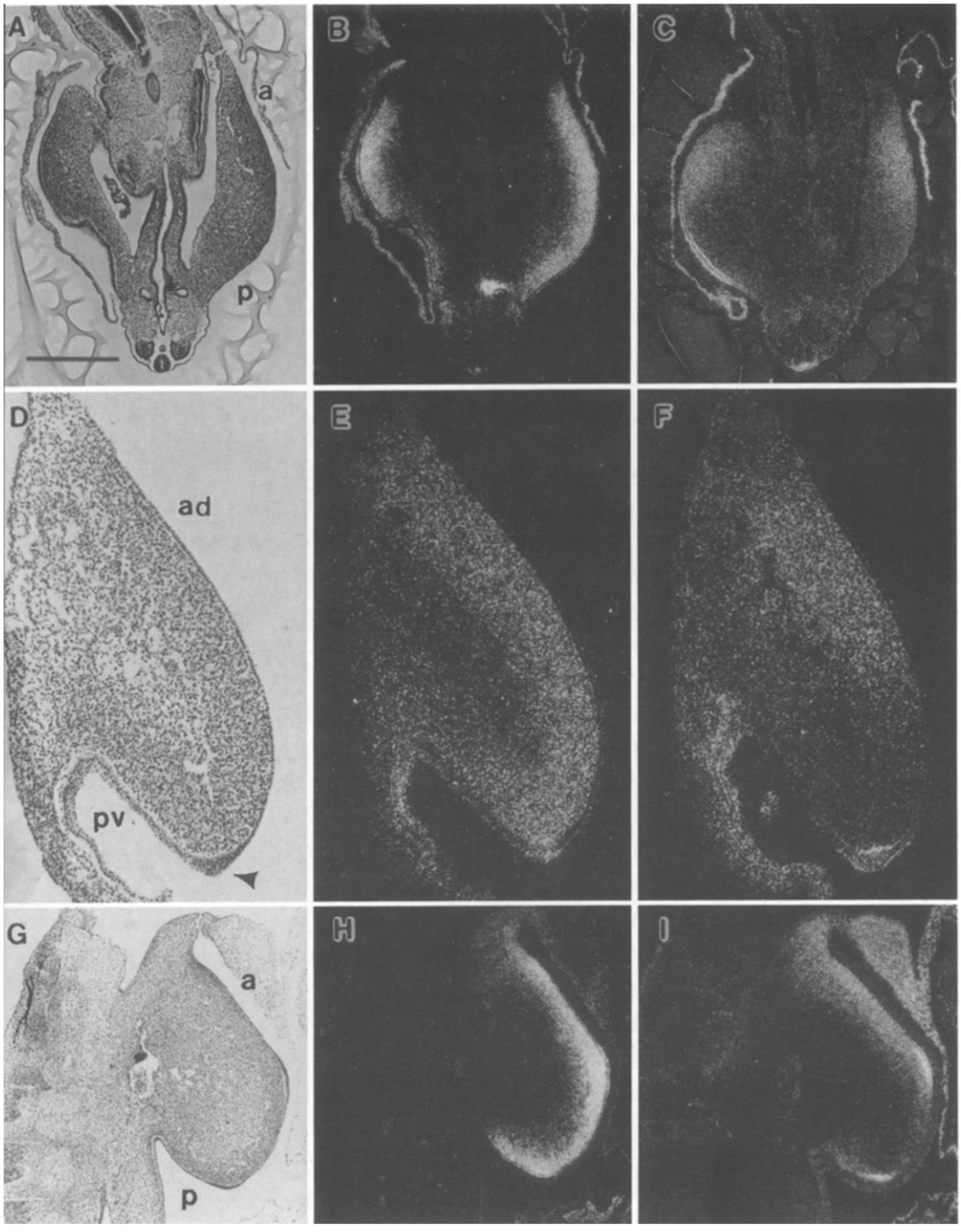

Fig. 3. Differential expression pattern of Chox-7 and Chox-8 at early stages of limb development. Left panels, bright-field views; middle panels, dark-field views for Chox-7; right panels, dark-field views for Chox-8. A-C, sagittal sections of the leg bud at stage 19; D-F, oblique-sagittal sections of the leg bud at stage 20; G-I, Sagittal sections of the wing bud at stage 23. Abbreviations: a, anterior; p, posterior; ad, anterodorsal; pv, posteroventral. Panels D-F, in which the AER is shown by an arrowhead, are magnified two fold as compared with the other panels. Scale bar = 0.5 mm.

AER. Slightly higher Chox-7 signals were observed in the dorsal mesoderm than in the ventral mesoderm, although the signals were much intense in the distal mesoderm subjacent to the apical ectodermal ridge. The Chox-7 expressing mesodermal cells are within the short distance from the AER, and gradient of the signal intensity is observed along proximodistal axis but not anteroposterior axis, as observed for the mouse limb bud (8,9). This indicates that the expression of the Chox-7 gene is correlated with patterning along proximodistal axis primarily under the control of the AER, and that signaling for the induction of Chox-7 expression is short range.

On the other hand, the Chox-8 expression domain in the early limb mesoderm is anteriorly localized within the Chox-7 expression domain (Fig. 3), implicating a different mechanism for regulating the Chox-8 expression. Similar but not identical results have been reported in the mouse limb bud (19). Following two mechanisms can be postulated to account for the localized expression of the Chox-8 expression. Firstly, cells containing a positive regulator for the Chox-8 gene are localized in the anterior region of the limb bud, independent of the AER signaling. Secondly, in addition to the AER factor which activates the Chox-8 expression as for the Chox-7 gene, another factor originating from the posterior region regulates negatively the Chox-8 transcription. Following lines of evidence suggest that the second mechanism is feasible. The anteroposterior limb axis is determined by signaling from the posterior mesoderm, the polarizing region, not from the anterior mesoderm (3). Retinoic acid treatment of the anterior margin cells conferring the posterior phenotype reduces the Chox-8 expression in the anterior mesoderm (13). Both Hox-7.1 and Hox-8.1 genes are activated by a signal from the AER (19). Reduced Chox-8 signals within the Chox-7 expression domain are correlated with the polarizing activity; differential expression of the Chox-7 and Chox-8 genes is most evident only when and where the polarizing activity is maximum (Fig. 3). It is likely, therefore, that the Chox-8 expression is under the positive control of the AER factor and the negative control of the polarizing factor.

After stage 26, the expression pattern of Chox-7 did not differ significantly from that of Chox-8, although the Chox-7 signals were distributed abundantly in the broader regions than the Chox-8 signals (Fig. 4). In addition to the mesenchymal region underneath the apical ectoderm, transcripts of both genes were observed in the anteroproximal and posterodistal regions and in the interdigital region, as observed in the other vertebrates (8,9,15). At stage 31 and later, the Chox-7 and Chox-8 expressions became progressively restricted to the mesenchyme surrounding cartilages and bones (Fig. 4). Since the expression domain overlaps the apoptotic zone (formerly called necrotic zone), both genes may be correlated with the programmed cell death. However, there is conflicting observation, as follows.

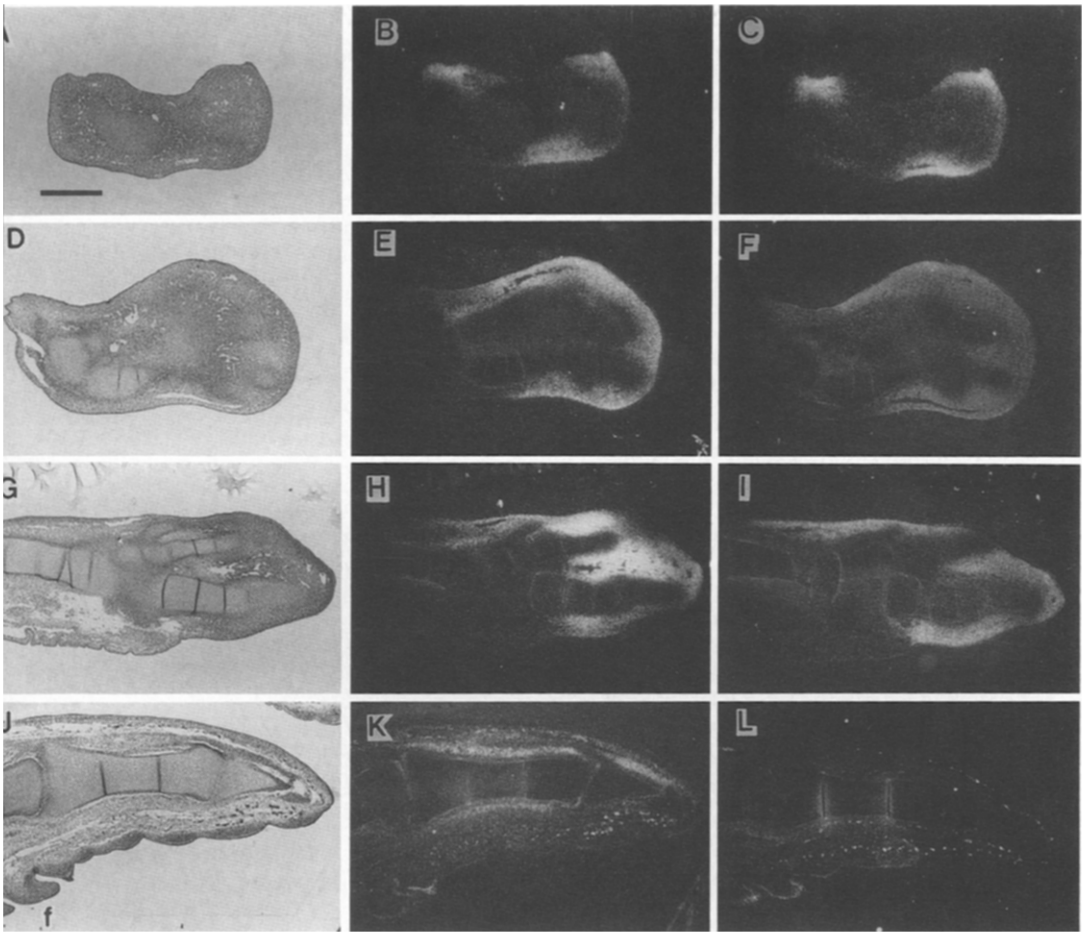

Fig. 4. Expression pattern of Chox-7 and Chox-8 at late stages of limb development. Left panels, bright-field views; middle panels, dark-field views for Chox-7; right panels, dark-field views for Chox-8. A-C, the wing bud at stage 27, D-F, the leg bud at stage 29; G-I, the wing bud at stage 31; J-L, the wing bud at stage 36. Abbreviation: f, feather bud. Scale bar = 0.5 mm.

The Chox-7 and Chox-8 transcripts are absent in the internal apoptotic zone between the presumptive radius and ulna possibly because of the absence of inductive signal from the AER. On the contrary, all the Chox-7 and Chox-8 expressing cells are not necessarily programmed to die, as evident in the early limb bud. An alternative explanation is that the Chox-7 and Chox-8 genes are responsible for preventing mesenchymal cells to enter cartilage-forming lineage. This can be effected by either maintaining undifferentiated state of mesenchymal cells or entering into cell death lineage.

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References

1. Kessel, M., and Gruss, P. (1990) Science 249, 374-379.
2. De Robertis, E. M., Morita, E. A., and Cho, K. W. Y. (1991) Development 112, 669-678.
3. Tabin, C. J. (1991) Cell 66, 199-217.
4. Dolle, P., Izpisua-Belmonte, J. C., Falkenstein, H., Renucci, A., and Duboule, D. (1989) Nature 342, 767-772.
5. Nohno, T., Noji, S., Koyama, E., Ohyama, K., Myokai, F., Kuroiwa, A., Saito, T., and Taniguchi, S. (1991) Cell 64, 1197-1205.
6. Izpisua-Belmonte, J. C., Tickle, C., Dolle, P., Wolpert, L., and Dudoule, D. (1991) Nature 350, 585-589.
7. Yokouchi, Y., Sasaki, H., and Kuroiwa, A. (1991) Nature 353, 443-445.
8. Hill, R. E., Jones, P. F., Rees, A. R., Sime, C. M., Justice, M. J., Copeland, N. G., Jenkins, N. A., Graham, E., and Davidson, D. R. (1989) Genes Dev. 3, 26-37.
9. Robert, B., Sassoon, D., Jacq, B., Gehring, W., and Buckingham, M. (1989) EMBO J. 8, 91-100.
10. Gubler, U., and Hoffman, B. J. (1983) Gene 25, 263-269.
11. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 5463-5467.
12. Noji, S., Takahashi, N., Nohno, T., Koyama, E., Yamaai, T., Muramatsu, M., and Taniguchi, S. (1990) Acta Histochem. Cytochem. 23, 353-366.
13. Yokouchi, Y., Ohsugi, K., Sasaki, H., and Kuroiwa, A. (1991) Development 113, in press.
14. Su, M. W., Suzuki, H. R., Solursh, M., and Ramirez, F. (1991) Development 111, 1179-1187.
15. Takahashi, Y., and Le Douarin, N. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 7482-7486.
16. Monaghan, A. P., Davidson., D. R., Sime, C., Graham, E., Baldock, R., Bhattacharya, S. S., and Hill, R. E. (1991) Development 112, 1053-1061.
17. Holland, P. W. H. (1991) Gene 98, 253-257.
18. Hamburger, V., and Hamilton, H. L. (1951) J. Morphol. 88, 49-92.
19. Davidson, D. R., Crawley, A., Hill, R. E., and Tickle, C. (1991). Nature 352, 429-431.