Mox-1 and Mox-2 define a novel homeobox gene subfamily and are differentially expressed during early mesodermal patterning in mouse embryos

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Summary

We have isolated two mouse genes, Mox-1 and Mox-2 that, by sequence, genomic structure and expression pattern, define a novel homeobox gene family probably involved in mesodermal regionalization and somitic differentiation. Mox-1 is genetically linked to the keratin and Hox-2 genes of chromosome 11, while Mox-2 maps to chromosome 12.

At primitive streak stages (approximately 7.0 days post coitum), Mox-1 is expressed in mesoderm lying posterior of the future primordial head and heart. It is not expressed in neural tissue, ectoderm, or endoderm. Mox-1 expression may therefore define an extensive ‘posterior’ domain of embryonic mesoderm before, or at the earliest stages of, patterning of the mesoderm and neuroectoderm by the Hox cluster genes.

Between 7.5 and 9.5 days post coitum, Mox-1 is expressed in presomitic mesoderm, epithelial and differentiating somites (dermatome, myotome and sclerotome) and in lateral plate mesoderm. In the body of midgestation embryos, Mox-1 signal is restricted to loose undifferentiated mesenchyme. Mox-1 signal is also prominent over the mesenchyme of the heart cushions and truncus arteriosus, which arises from epithelial-mesenchymal transformation and over a limited number of craniofacial foci of neural crest-derived mesenchyme that are associated with muscle attachment sites.

The expression profile of Mox-2 is similar to, but different from, that of Mox-1. For example, Mox-2 is apparently not expressed before somites form, is then expressed over the entire epithelial somite, but during somitic differentiation, Mox-2 signal rapidly becomes restricted to sclerotomal derivatives.

The expression patterns of these genes suggest regulatory roles for Mox-1 and Mox-2 in the initial anterior-posterior regionalization of vertebrate embryonic mesoderm and, in addition, in somite specification and differentiation.

Key words: mesoderm, mesenchyme, regionalization, homeobox, somitogenesis, sclerotome, embryogenesis.

Introduction

The precise construction of the mesodermal germ layer is essential for proper embryonic development. Its derivatives contribute to most of the major organ systems and it induces neural tissue from ectoderm. Although receiving intense study, the mechanism whereby mesoderm is induced has not been clearly determined. However, several genes have been implicated. Mutations in the murine Brachyury gene result in defects in the axial mesoderm and notochord (Wilkinson et al., 1990; Rashbass et al., 1991; Herrmann et al., 1990; Herrmann, 1991). Experiments with amphibian embryos suggest that signalling molecules of the TGF-β (activin and bone morphogenetic proteins), wnt and bFGF families represent components of the cascade leading to commitment to particular mesodermal fates (see Green and Smith, 1991; Sokol et al., 1991; Smith and Harland, 1991; Christian et al., 1992; Kimelman and Mass, 1992; Jones et al., 1992; Dale et al., 1992).

Convincing experimental evidence has been gathered supporting the idea that homeobox genes represent key genetic elements of the blueprint for the vertebrate embryo
Regulating cells at different axial positions with region-specific developmental programs. Other homeobox genes residing outside these four Hox clusters are very likely involved in patterning many other parts of the vertebrate body and head, including the forebrain and midbrain (Duboule, 1992; Holland, 1992; Wright, 1991).

Here we report the isolation of two closely related murine homeobox genes, Mox-1 and Mox-2 (for Mesoderm/Mesenchyme homeobox gene). Based on their highly divergent homeodomain sequences and expression patterns, Mox-1 and Mox-2 define a new homeobox gene subfamily. We report the deduced amino acid sequence of Mox-1 and the partial sequence of Mox-2. We also present the spatiotemporal expression patterns of Mox-1 and Mox-2 as determined by in situ hybridization, their genomic structures and chromosomal localizations. The expression patterns reported here suggest that, in coordination with genes like Brachyury, activin and vnt-related genes, Mox-1 and Mox-2 are additional elements of the network responsible for mesoderm induction and its earliest regional specification, and that they may have important additional roles in somitogenesis, and in myogenic and sclerotomal differentiation.

Materials and methods

Isolation of Mox-1 cDNA and gene

An amplified 8.5 days post-coitum (d.p.c.; noon on the day of vaginal plug being defined as 0.5 d.p.c.) C57BL/6J mouse 2gt10 cDNA library (kindly provided by Dr B. Hogan, Vanderbilt University Medical School) was hybridized with a short region of the XHbox8 gene, comprising the 3' end of the homeobox and most of the downstream C-terminal coding region (the region used as probe for the RNAase protection assays reported in Wright et al., 1988), at 30°C in low-stringency hybridization buffer (30% formamide, 0.02% sodium pyrophosphate, 5x SSC, 5x Denhardt's, 200 µg/ml Torula RNA, 0.1% SDS, 8% dextran sulfate, 125 µ/ml heparin, 0.03 M sodium phosphate, pH 6.5). Following hybridization, filters were washed twice with 2x SSC, 0.1% SDS at 30°C. From 1.5×10⁶ plaques, 104 positives were obtained, of which 94 were purified, their inserts subcloned into Bluescript KSII+ and sequenced using Pharmacia or USB T7 sequencing kits. cDNAs were identified by priming sequencing reactions with a degenerate oligonucleotide designed against the most conserved third helix region of the homeobox (sequence provided by T. R. Burglin and B. Blumberg). Three novel groups of cDNAs were initially isolated: five Mox-1 cDNAs, one Mox-2 cDNA and six cDNAs corresponding to a new caudal-related gene, cdx-3 (L. Gamer and C. V. E. W., unpublished data). Based on northern data, we concluded that all our initial Mox-1 and Mox-2 cDNAs were deficient at the 5' and/or 3' end(s). To obtain full-length Mox-1 and Mox-2 cDNAs, the same 8.5 d.p.c. cDNA library was rescreened at high stringency using 5' end fragments of the longest original cDNAs. Twelve overlapping Mox-1 cDNAs were isolated allowing the construction of a composite 2.2 kb cDNA sequence (see Fig. 1). In some Mox-1 cDNAs, the poly(A) signal is followed by the sequence TCAACAAAAATGC and then a poly(A) tail - probably representing an artefact during cDNA priming. Three overlapping Mox-2 cDNAs were isolated. The entire Mox-2 sequence has not been finalized, primarily because of G-C richness and compressions. Thus, we present only the Mox-2 homeodomain sequence here. Although the library was amplified, no duplicated clones were isolated.

A C3H/RI genomic library in λ2001 (kindly provided by Dr G. Rinchik, Oak Ridge National Laboratory) was screened using Mox-1 and Mox-2 cDNAs at high stringency. Several overlapping genomic clones of Mox-1 and Mox-2 were obtained. cDNAs and appropriate regions of genomic clones were sequenced on both strands.

Chromosomal localization of Mox-1 and Mox-2

Mox-1

Somatic cell hybrids. Production and characterization of the somatic cell hybrid panel has been described (Minna et al., 1975; Popp et al., 1981). The progenitors were a CHO cell line E36 and a BALB/c cell line. This panel has been used previously in conjunction with interspecific crosses to map several genes and loci in the mouse (Nadeau et al., 1989, 1990; T. Koizumi, P. Lalley and J. Nadeau, unpublished data).

Interspecific mapping panel - Mox-1. An interspecific cross between (C57BL/6J × M. spreptus) F1 hybrid females and C57BL/6J males was used. This panel has been used previously to map loci on several mouse chromosomes (Nadeau et al., 1989, 1990, 1991; Koizumi et al., 1991).

Mox-2

Recombinant inbred strains. Genomic DNAs from inbred and recombinant inbred strains were obtained from the Jackson Laboratory DNA Resource. Methods described by Silver and Bucker (1986) were used for calculating recombination frequencies and confidence limits for loci typed in recombinant inbred strains.

Restriction fragment variants, probe labeling, Southern blotting and hybridization conditions. Genomic DNA (4 µg) was digested with restriction endonucleases for 3 hours under conditions recommended by the enzyme suppliers. Digested DNA was electrophoresed in horizontal 1% agarose gels at 46 volts for 14 hours in E buffer (0.04 M Tris-HCl pH 7.2, 0.02 M sodium acetate, 0.001 M EDTA). Gels were prepared for transfer by soaking in denaturing buffer (0.02 M NaOH, 0.6 M NaCl, 0.015% thymol blue) for 1 hour with gentle shaking. DNA was then transferred to Zetabind nylon membranes overnight. Membranes were rinsed in 2× SSC buffer, blotted dry for 5 minutes at room temperature and DNA was UV-crosslinked to the filter for 30 seconds using a 254-nm ultraviolet transilluminator.

Fractions (50 ng) from Mox-1 and Mox-2 cDNAs were labelled using the random hexamer method (Feinberg and Vogelstein, 1983). The 550 bp Mox-1 fragment corresponded to the sequence between the PstI site, centered at nucleotide 1640 and the 3' end of the cDNA. The approx. 420 bp fragment from the Mox-2 cDNA contained the first two-thirds of the homeobox and 300 bp upstream.

Membranes were prehybridized in 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA, 7% SDS, 10 µg/ml poly(A), 10 µg/ml poly(C) at 65°C for 4 hours or overnight. Radiolabelled probes were hybridized overnight at 65°C in prehybridization buffer. Mem-
branes were blotted dry and exposed to Kodak XAR5 film with Dupont LightningPlus intensifying screens at −70°C for 48 hours.

**Northern blot analysis of Mox-1**

Litters of C57BL or ICR mouse embryos were dissected out of the deciduum at various stages in Dulbecco's Modified Eagles Medium and RNA extracted from them using a lithium chloride/urea method (Auffray and Rougeon, 1980). For each stage of development, eight embryo-equivalents of total RNA was electrophoresed through a 1.2% agarose/2.2 M formaldehyde gel and transferred to nitrocellulose (Sleckieher and Schuell). The filter was baked in vacuo for 90 minutes followed by hybridization (50% formamide, 50°C; all other components as in low-stringency hybridization buffer above) with the same 3′ probe used for the chromosome mapping experiments. The filter was washed in increasing stringencies, with a final wash at 65°C in 0.1× SSC, 0.1% SDS. Perhaps because of the lower level of *Mox*-2 transcripts (see below and Results), northern blot data for *Mox*-2 have not been obtained.

**In situ hybridization**

In situ hybridizations were carried out according to Pelton et al. (1989), with minor modifications. Briefly, paraffin-embedded mouse embryos were sectioned (7 μm), dried onto microscope slides coated with poly-L-lysine (Sigma), dewaxed, treated with proteinase K (Sigma; 20 μg/ml, 7.5 minutes) and acetylated. High-stringency hybridization, washings, RNAase A treatment and further manipulations are described in Pelton et al. (1989). We used T7 or T3 RNA polymerase to produce 35S-labelled in vitro transcribed RNA probes corresponding to the sense or anti-sense orientation, respectively, of a Bluescript KSII+ subclone of the 3′ *Mox*-1 fragment described above. Several different probes were used for the *Mox*-2 in situ hybridizations. Short probes from various regions of the cDNA, including or omitting homeobox sequences, resulted in low signal to background ratios. The in situ results for *Mox*-2 presented here were obtained with the longest (2.2 kb) *Mox*-2 cDNA, hydrolyzed after labelling to an average length of 300 nucleotides. After washing, exposure to emulsion for *Mox*-1 was for 4-7 days (7.0-9.0 d.p.c. embryos), or 10-14 days (9.5-16.5 d.p.c. embryos). The exposure times for *Mox*-2 were substantially longer, averaging 20-28 days. Sense strand contamination of this methionine as the initiating codon is suppressed by recently generated *Mox*-1 antibodies (data not shown).

We also isolated three cDNAs for a related gene, *Mox*-2, although the full-length sequence has not been finalized (see Materials and methods). The *Mox*-2 homeodomain is 95% identical to that of *Mox*-1 (100% similar allowing for conservative substitutions).

**Results**

**Isolation of Mox-1 and Mox-2**

In attempting to isolate the murine homolog of *XlHbox 8*, an endoderm-specific frog homeobox gene, a fragment of *XlHbox 8* DNA (see Materials and methods) was used to screen an amplified 8.5 d.p.c. murine cDNA library under low-stringency conditions. Positives were subcloned and sequenced (see Materials and methods). Many cDNAs corresponding to previously described *Hox* genes expressed at 8.5 d.p.c. were isolated, as well as three new types of sequence: *Mox*-1 and *Mox*-2 (and *cdx-3*, L. Gamer and C. V. E. W., unpublished data). Five overlapping cDNAs encoded different regions of *Mox*-1, the longest of which was 1.3 kb. Because northern analysis detected a 2.3 kb transcript (see below), the library was rescreened with a 5′ terminal fragment of the longest cDNA to obtain longer *Mox*-1 cDNAs. There is no evidence for alternative splicing in any of these cDNAs. The longest cDNA was 2.2 kb, with the sequence presented in Fig. 1A. An open reading frame in phase with the homeodomain predicts a 254 amino acid protein with a calculated relative molecular mass of 28×10^3 (Fig. 1A). The initiator methionine codon is not in ideal context (Kozak, 1991), as previously observed in other homeobox mRNAs (e.g. Wright et al., 1987). The designation of this methionine as the initiating codon is supported by fusion protein studies, in vitro translation of various synthetic *Mox*-1 mRNAs and detection of the endogenous protein by recently generated Mox-1 antibodies (data not shown).

**Genomic structures**

Single loci for *Mox*-1 and *Mox*-2 were identified on high-stringency Southern hybridizations of total genomic mouse DNA (e.g. Fig. 2A for *Mox*-1). Using cDNA probes, overlapping lambda clones spanning the *Mox*-1 and *Mox*-2 loci were isolated from a C3Hf/RI genomic library. Both genes comprise three exons (Fig. 1A,C) and have two identically located introns. One intron lies just upstream of the homeobox, while the other, located after nucleotide 132 of the homeodomain, interrupts the homeodomain between amino acids 44 and 45 (ERQ/VKVFQNR). Other homeobox genes in a wide variety of higher eukaryotes have an intron at this latter position. These are: *Hlx* and *NK-1* from mouse, *labial*, *proboscipedia*, *H2.0*, *s59*, *Distal-less* and *Abdominal-B* from *Drosophila*, and *cel-1*, *2*, *7*, *9* and *12* from *Caenorhabditis elegans* (summarized by Allen et al., 1991). It is premature to speculate about ancestral connections between *Mox*-1/*Mox*-2 and any or all of these genes.

**Chromosomal localization of Mox-1 and Mox-2**

We set out to determine the linkage of *Mox*-1 and *Mox*-2 to other homeobox loci and, if they map near known developmental mutations, in particular those showing phenotypes that might be consistent with the expression patterns of the two genes.

**Mox-1**

The chromosomal location of *Mox*-1 was initially determined in a hamster/mouse hybrid panel. A single variant fragment was detected in Southern hybridizations of
Fig. 1. Primary nucleotide sequence, deduced amino acid sequence and genomic structure of Mox-1. (A) The sequence of the longest Mox-1 cDNA is presented here. Stop codons in the same phase as the homeodomain are indicated by asterisks and arrowheads show intron positions. The homeodomain sequence, and a poly(A) addition signal sequence near the 3′ terminus, are underlined. Comparison with northern blots (Fig. 2B) suggests that a maximum of 50-100 nucleotides are missing from the 5′ terminus. (B) The prototypical homeodomain sequence of the Drosophila Antennapedia protein lies at the top of the figure. Asterisks indicate amino acid residues that are most conserved in alignments of all known homeodomain protein sequences (Scott et al., 1989). Mox-1 and Mox-2 homeodomain sequences are then compared with homeodomains from genes whose expression is either mesoderm-specific or predominantly in mesoderm, or else which bear sequence similarity to Drosophila mesoderm-specific homeodomain proteins. Sequence sources are: S8 (mouse - Opstelten et al., 1991), H2.0 (Drosophila - Barad et al., 1988), S59 (Drosophila - Dohmann et al., 1990), msh-1 and msh-2 (Drosophila - Holland, 1991; Bodmer et al., 1990), Hox7.1 and Hox8.1 (Hill et al., 1989; Monaghan et al., 1991). (C) Genomic structure of Mox-1 as determined by comparison of cDNA and genomic clones (introns are not drawn to scale). Mox-1 and Mox-2 have identical genomic structures, except that a single extra amino acid residue is present in the second exon of Mox-2 compared to Mox-1 (not shown). The protein-coding region is shown as a shaded box, with the genomic structure of Mox-1 as a black box within it. The positions of individual clones over the Mox-1 locus are shown above the diagram.
BALB/cJ and CHO genomic DNA digested with BglI, MspI, PvuII and SstI. The 5.8 kb SstI variant in BALB/cJ was used for typing the hybrid panel (Fig. 2A). This series of hybrids does not contain mouse chromosome 11 and the SstI variant could not be detected, consistent with Mox-1 residing on chromosome 11. Previously, mouse-specific fragments that were absent from this panel were confirmed to lie on chromosome 11 by linkage analysis (Nadeau et al., 1989, 1990).

To identify a restriction fragment variant for mapping Mox-1 in interspecific backcrosses, genomic DNAs from C57BL/6J and M. spretus progenitors were digested with endonucleases. Variants were found for BglII, EcoRI, PvuII, SstI and XbaI. C57BL/6J DNA contained a 4.7 kb EcoRI fragment, while this fragment was 5.8 kb in M. spretus. These variants were used to follow the segregation of Mox-1 in the interspecific mapping panel.

A total of sixty-six interspecific backcross progeny were typed for Mox-1. These results were compared to results for genes previously scored in this mapping panel (Nadeau et al., 1989, 1990; Ambrose et al., 1991). Complete cosegregation was found between Mox-1 and Hox-2 (homeobox-2 complex), Re (rex) and Krt-1 (type I keratin gene family), which all reside in the same region of chromosome 11. No crossovers were detected among the 48 progeny typed for both Mox-1 and Hox-2 (upper 95% confidence limit for the recombination frequency was 0.061). Similarly, no crossovers were found among 60 progeny typed for Mox-1 and the Re - Krt-1 complex (upper 95% confidence limit for recombination frequency was 0.049).

The results of both the hybrid panel and interspecific backcross analyses demonstrate that Mox-1 lies on chromosome 11, closely linked to three families of genes: the Hox-2 complex, the keratin type 1 genes and three loci affecting skin and hair development (rex, bareskin and denuded). Mox-1 does not appear to be allelic with any mouse mutation distinguished by gross defects in the formation of the mesoderm.

Mox-2
Genomic DNA from the progenitors of recombinant inbred strains was digested with eight different restriction enzymes. Hybridization of a Mox-2 cDNA fragment to StuI digested DNA (see Methods) resulted in variants for AKR/J and C57BL/6J (21 kb), and DBA/2J and C3H/HeJ (19 kb) strains. These variants were used to follow the segregation of Mox-2 in a panel of 63 recombinant inbred crosses, as presented in Table 1. Mox-2 is linked to two loci on chromosome 12: aromatic hydrocarbon responsiveness (Ah; upper 95% confidence limit for recombination frequency was 0.109) and lymphocyte antigen-18 (Ly-18; upper 95% confidence limit for recombination frequency was 0.034). Mox-2 is therefore not linked to any of the four major Hox clusters and, as with Mox-1, is not allelic with mouse mutations associated with somitic or sclerotomal defects.

Expression of Mox-1 and Mox-2 during gastrulation and neurulation

Mox-1
Having characterized the Mox-1 gene, we first used developmental northern blots to determine temporal expression...
patterns (Fig. 2B). A single 2.3 kb transcript is detected. This band is present in 7.0-7.5 d.p.c. RNA and high steady-state levels of Mox-1 RNA are observed at 8.5 and 9.0 d.p.c. Although not photographically reproduced in Fig. 2B, close examination of the autoradiogram reveals a weak 2.3 kb band slightly over background in RNA extracted from pooled 6.5-7.0 d.p.c. litters. This indicates that the beginning of Mox-1 expression occurs at, or shortly after, the formation of the mesodermal germ layer.

A detailed spatial-temporal analysis of Mox-1 expression patterns was obtained by in situ hybridization on entire serially sectioned embryos. Although earlier stages were analyzed, we first detected expression of Mox-1 during the gastrulation stage (7.0-7.5 d.p.c.) of mouse embryogenesis, where it is found in the mesoderm, possibly including the primitive streak (e.g. Fig. 3A). Neuroectoderm and endoderm do not hybridize with the probe. The Mox-1 expression domain extends into the posteriormost regions,

### Table 1. Recombinant inbred strain panels to map Mox-2

| Locus | 1 | 2 | 3 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 |
| Ly-18 | D | D | A | D | A | D | A | D | A | A | D | A | D | A | A | A | A | A | A | A | D | D | A | A | A | A |
| Mox-2 | D | D | A | D | A | D | A | D | A | A | D | A | D | A | A | A | A | A | A | D | D | A | A | A | A |

Inheritance of Mox-2 and lymphocyte antigen-18 (Ly-18) in 25 crosses between AKR/J (A) and DBA/2J (D) recombinant inbred mice. Mox-2 shows complete segregation with Ly-18.

(B) Inheritance of Mox-2 and other chromosome 12 loci in BXH recombinant inbred strains

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Inheritance of Mox-2, Ly-18 and aromatic hydrocarbon responsiveness (Ah) in 12 crosses between C57BL/6J (B) and C3H/HeJ (H).

(C) Inheritance of Mox-2 in BXD recombinant inbred strains

| Locus | 1 | 2 | 5 | 6 | 8 | 9 | 11 | 12 | 13 | 14 | 15 | 16 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 27 | 28 | 29 | 30 | 31 | 32 |

Inheritance of Mox-2 and Ah in 26 crosses between C57BL/6J (B) and DBA/2J (D). For details see Results text.

Fig. 3. Spatial distribution of Mox-1 transcripts during gastrulation. Sagittal section of 7.0-7.5 d.p.c. mouse embryo hybridized with Mox-1 probe. In this and all subsequent Figures, panels are arranged in brightfield/darkfield pairs (e.g. A/A'; B/B'; etc.). Mox-1 transcripts are not found in head process mesoderm (arrowhead) at this stage. Other sections where the endoderm has separated from the mesoderm clearly show absence of signal from endoderm (not shown). Anterior is to the left. Abbreviations: al, allantois; am, amnion; e, endoderm; m, mesoderm; n, ectoderm. With similar emulsion exposure times, no signal above background was observed in control hybridizations with sense strand probes (see Methods).
Fig. 4. Mox-1 expression postgastrulation and during early organogenesis. (A) Mox-1 expression at 8.5 d.p.c., a,a′ and b,b′ are two different transverse sections through an 8.5 d.p.c. embryo showing the strong Mox-1 signal in somitic, intermediate and lateral plate mesoderm, and its absence from neural tissue, cardiac tissue and head mesenchyme. (B) Mox-1 expression at 9.5 d.p.c. (a,a′) Transverse section at the level of the heart. Mox-1 signal is restricted to the somitic (dermamyotome and sclerotome), intermediate and lateral plate mesoderm. (b,b′) Parasagittal section through the dorsal region of an embryo (dorsal to the left, anterior uppermost). The section is slightly skewed so that the anterior most somites are mostly sectioned through the sclerotome. Both dermamyotome and sclerotome display a strong signal. The apparently lower grain density in the dermamyotome is a photographic artefact caused by overexposure with toluidine blue which obscures grains that are clearly visible at high magnification under brightfield illumination (compare to transverse sections in panel above). A weaker signal is also seen in the undifferentiated mesenchyme surrounding the pronephric tubules. Anterior or dorsal is uppermost in all panels. Abbreviations: dm, dermamyotome; h, heart; he, heart; l, lateral plate mesoderm; nt, neural tube; pn, pronephros; s, somites; sc, sclerotome.
but an anterior border appears to correspond to the demarcation between the future cranial/cardiac mesoderm and the posterior mesoderm that will contribute to the rest of the embryo. Mox-1 seems to be expressed in all posterior mesodermal cells, including the presumptive notochord. At later stages, in contrast, the definitive notochord does not express Mox-1.

At neurulation stages (8.0-9.5 d.p.c.), Mox-1 transcripts are detected in somitic, intermediate and lateral plate mesoderm (Fig. 4A,B), in a posterior domain consistent with the earlier expression pattern. While in their initial epithelial state, the entire somite expresses Mox-1. Mox-1 is also expressed in presomitic mesoderm lying posterior to the youngest somites, in a fairly broad band equivalent to several somites in length (data not shown, see Discussion). As the somites lose their epithelial character and begin to differentiate, Mox-1 signal localizes to the dermomyotome, myotome and sclerotome (Fig. 4; data not shown). The Mox-1 signal in the lateral plate mesoderm is lower than in somitic mesoderm. There is no detectable Mox-1 mRNA in the primordial heart at this stage.

Mox-2
Probably because of a lower abundance of transcripts, we have not yet detected Mox-2 by northern blot analysis. The paucity of Mox-2 transcripts relative to Mox-1 is also reflected in the longer exposure times and the weaker signals, in Mox-2 in situ analyses when compared to Mox-1 (compare, for example, Figs 4, 5).

With the preceding caveat and at the level of sensitivity of our in situ analyses, Mox-2 is not expressed before approximately 8.0 d.p.c. (data not shown). However, during neurulation, a Mox-2-specific signal is detected over a broad anteroposterior mesodermal domain, quite similar to that of Mox-1. At 8.0-8.5 d.p.c., the Mox-2 probe decorates the entire epithelium of the somite (Fig. 5 A,B). At 9.5 d.p.c., when the somite has clearly differentiated into dermomyotome and sclerotome, Mox-2 expression becomes restricted to the sclerotome (Fig. 5C,D). This should be contrasted with the expression of Mox-1 at the same stage in all three compartments of the somite (Fig. 3C,D). We do not detect Mox-2 expression in neuroderm, endoderm or extraembryonic tissues.

Expression of Mox-1 and Mox-2 during organogenesis
Mox-1
The number of tissues showing Mox-1 signal progressively decreases during organogenesis, until only relatively small regions of expression are present in 14.5-15.5 d.p.c. embryos (Figs 6, 7). Organogenesis in the mouse is essentially completed by 15.5 d.p.c. and we find no specific hybridization signal in 16.5 d.p.c. embryos.

The overall conclusions regarding Mox-1 at these later stages are that it is expressed transiently in mesenchymal cells that are (i) associated with sites of skeletal muscle assembly, including both mesodermal (axial, appendicular) and neural crest-derived (craniofacial) populations, (ii) adjacent to the adenohypophysis, (iii) in the stroma of the developing nephrogenic tissues and (iv) in specialized regions of the heart (cushions, truncus arteriosus), where endocardial cells undergo epithelial-mesenchymal transfor-

mation. The major expression domains of Mox-1 are described in more detail below.

Lateral plate derivatives
The gut mesenchyme (splanchnopleure) of early embryos is labelled above background by the Mox-1 probe but, in later embryos, the signal disappears as this mesenchyme differentiates (data not shown). The body wall (somaticopleure) associated with thoracic and abdominal regions is positive (e.g. Fig. 7). The crura, but not the central tendon, of the immature diaphragm shows Mox-1 expression (Fig. 7C).

Heart
As shown in Fig. 3, Mox-1 is not expressed at 7.0-7.5 d.p.c. in anterior cardiac/head mesoderm or in cardiac mesoderm of 9.5 d.p.c. embryos (Fig. 4B,a). Mox-1 hybridization signal is first found in localized regions of the heart at 10.5 d.p.c., when this organ undergoes complex growth and rearrangement. As shown in Fig. 6, these include the truncus arteriosus, which is the precursor of the aorta and pulmonary artery, and the atroventricular cushions, which form the septa and valves (see, for example, Anderson, 1991). In both cases, the endocardial endothelium does not express Mox-1. Rather, the signal is localized to the mesenchyme of the outflow tract, including precursors of the spiral septum, and to the mesenchymal cells surrounding and partitioning the atroventricular canal, both of which form by epithelial-mesenchymal transformation from the endothelial layer. These include precursors of the atroventricular cushions and valvular septa (Fig. 7). Mox-1 hybridization signal is not seen in the fully formed heart of late gestation embryos.

Kidney
Mox-1 is expressed in the mesenchyme surrounding the epithelial tubules of the pronephros (Fig. 4B, panel b) and in the 10.5 d.p.c. mesonephros (data not shown). At 12.5 d.p.c., Mox-1 transcripts are found in undifferentiated mesenchyme of the metanephros with a higher signal in the cortical region. It is possible that all undifferentiated kidney mesenchyme expresses Mox-1, with the medulla appearing to contain less Mox-1 signal because it is quite densely packed with ureteric tubules and/or buds, which do not express Mox-1 (Fig. 6C). No signal is detected over the adrenal glands, or male or female gonads (data not shown). At 14.5 d.p.c., the Mox-1 signal in the kidney is only slightly above background. Higher resolution studies of developing and older kidneys with Mox-1 antibodies will be useful in determining whether Mox-1 expression is associated with non-terminally differentiated mesenchymal cells.

Loose connective tissue
The most widespread expression of Mox-1 is in loose mesenchymal tissues at sites of bone formation and skeletal muscle-connective tissue apposition. In the trunk, Mox-1 is expressed around vertebral bodies (Figs 6, 7) and surrounding transverse processes, sternum and ribs (Figs 6, 7), and as multiple foci adjacent to the scapula and humerus.
Fig. 5. Mox-2 expression during neurulation. (A,A′ and B,B′) Expression of Mox-2 at 8.5 d.p.c. A, A′ represent a bright-field/ dark-field pair of a parasagittal section, while B,B′ show a transverse and frontal section of an embryo midway through the rotation that the embryo undergoes at 8.5-9.0 d.p.c. Mox-2 signal is found in the entire somite but is absent from neural tissue, presomitic and cardiac mesoderm. Anterior is to the right. (C,C′ and D,D′) Expression of Mox-2 at 9.5 d.p.c. C,C′ is a transverse section and D,D′ is a parasagittal section. Note that Mox-2 expression has become restricted to the sclerotome and is now absent from the dermamyotome. Anterior is uppermost in C,C′ and to the right in D,D′. Abbreviations: da, dorsal aorta; de, deciduum; dm, dermamyotome; he, heart; lb, limb bud; psm, presomitic mesoderm; nt, neural tube; s, somites; sc, sclerotome.
Fig. 6. Mox-1 expression at 12.5 d.p.c. (A, A') Parasagittal section of 12.5 d.p.c. mouse embryo. The areas boxed as B and C are shown at higher magnification in panels B,B' and C,C', respectively. Arrowheads indicate Mox-1 expression corresponding to jaw adductor (j) and extraocular muscles (e). Expression in the region labelled scp includes the scapula and adjacent shoulder structures. (B,B') In the region of the developing ribs and vertebrae, Mox-1 is expressed in the loosely packed mesenchyme surrounding the perichondral region. (C,C') Expression in the kidney and in the area of the pelvic/urogenital system. The streaks of negative tissue (around the 'us' label) posterior of the kidney probably represent pelvic nerves. Note the absence of Mox-1 signal from the ureteric epithelium. Other abbreviations: bv, blood vessel; he, heart; k, kidney; li, liver; pc, perichondral region; rc, rib cartilage; scp, scapular region; sto, stomach; us, urogenital system; v, vertebrae.
The pattern of expression in the head/neck region is more complex, but consistent with sites of myogenesis. Areas within the tongue are circumscribed with Mox-1-positive cells, corresponding to junctional zones of intrinsic and extrinsic (e.g. genioglossus and hyoglossal - Fig. 7B/B′ and C/C′) tongue muscle assembly. Bands of Mox-1 expression radiating rostrally and caudally from the basihyoid correspond to future hyoglossal, geniohyoid and sternohyoid muscle formation sites. Similarly, distinct foci of Mox-1 expression are found where jaw adductor muscle attachments will later develop, even though the specific bones have not yet formed at these stages examined. Multiple foci surrounding the optic stalk and posterior eye correspond to regions of future eye muscle attachment. Mox-1 is expressed in association with hypaxial, epaxial, intercostal, appendicular, tongue, jaw and extraocular muscles, whose connective tissues are derived from paraxial and lateral mesoderm as well as the neural crest.

Notably, two small areas of condensed, Mox-1-positive, mesenchyme are present beside the rostral and caudal sur-
faces of the developing adenohypophysis. The fate of this neural crest-derived tissue is not known, though it is unrelated to muscle assembly.

**Mox-2**

Using several different Mox-2 in situ probes, we have been unable to localize Mox-2 transcripts beyond 10.5 d.p.c. At 10.5 d.p.c., Mox-2 probe labels the sclerotomally derived cells, including the vertebral and costal precursors, but not cells derived from dermatome or myotome (data not shown). We cannot detect a specific signal in any other tissues during organogenesis stages, perhaps because Mox-2 transcripts are less abundant during embryogenesis (see Materials and methods). Mox-2 antibodies, which are currently being generated, should be useful in this respect.

**Discussion**

We describe the isolation and characterization of a new homeobox gene subfamily with expression restricted to the mesoderm of early embryos. To our knowledge, such mesoderm-specific expression is unique among the homeobox genes that have so far been reported. Later in development, Mox-1 is also expressed in neural-crest-derived mesenchyme. This subfamily has thus been given the name Mox (mesoderm/mesenchyme specific) to reflect this expression.

As homeodomain proteins, Mox-1 and Mox-2 are inferred to function as transcription factors. The observed distributions of Mox-1 and Mox-2 strongly suggest roles in regionalizing the early mesoderm, rather than as cell-type-specific regulators, which is another function of diverged members of the homeobox gene family (e.g. Rosenfeld, 1991; Li et al., 1990, Guazzi et al., 1990).

The Mox-1 expression domain at 7.0-7.5 d.p.c. (which we have recently confirmed with antibody staining; data not shown) can be compared to the patterns of expression of the Hox cluster members. Around this stage, the Hox cluster genes are just beginning their sequential deployment, starting with the 3′most genes. Thus, for example, at 7.5 d.p.c., Hox-1.6 and Hox-2.9 are expressed over most of the primitive streak region, in both neuroectoderm and mesoderm (Murphy and Hill, 1991; Sundin et al., 1990; Frohman et al., 1990). From our present in situ data, the Mox-1 mesodermal expression domain seems to be established at 7.0-7.5 d.p.c. (and maybe slightly before - see Results) and this is therefore very early in the Hox gene deployment program. Because positional identities are being rapidly established during these early stages of mouse development, more analysis will be required to identify the extent to which mesoderm-specific patterning involving Mox-1 preempts, or is concomitant with, patterning carried out by the Hox clusters. In any case, Mox-1 and Mox-2 could provide useful mesoderm-specific markers for studies of early anteroposterior differences in mesodermal identity during gastrulation and neurulation.

As described above, Mox-1 and Mox-2 are related to each other, implying duplication and divergence from an ancestral precursor, so that Mox-1 and Mox-2 carry out overlapping functions in early vertebrate development. Support for this argument is provided by the similar, but distinct, spatial-temporal expression profiles of the two genes during somitic differentiation. For example, Mox-2 expression is first expressed a little later than Mox-1, but in a similar spatial domain, i.e. in the postcranial/cardiac mesoderm. A little later, in paraxial mesoderm, both genes are expressed over the whole epithelial somite. When the somites differentiate, Mox-1 is expressed in dermatome, myotome and sclerotome, while Mox-2 becomes rapidly restricted to the sclerotomally derived cells. Speculatively, Mox-1 and Mox-2 could carry out redundant functions in sclerotomally derived cells. Conversely, Mox-2 may have diverged to carry out specific roles during migration and resegmentation of the sclerotome into vertebral and costal segments.

Although they are also expressed in other parts of the embryo (see below and Results), during early somitic differentiation, the spatiotemporal expression patterns of Mox-1 and Mox-2 are therefore suggestive of roles in specification and/or differentiation of dermatome, myotome and sclerotome, and add to the growing list of other regulatory molecules co-expressed in these tissues, e.g. Pax-1 (Gruss and Walther, 1992), myogenin and myoD1 (Sassoon et al., 1989), and PDGF α-receptor (Orr-Urtreger et al., 1992; Schatteman et al., 1992).

Another mouse homeobox gene, S8, is expressed predominantly in the mesenchyme of mid- to late gestation embryos (Ospelten et al., 1991; S8 transcript distributions in earlier (6.5-9.0 d.p.c.) embryos were not reported in that study), but there are notable differences in the expression pattern compared to Mox-1 and Mox-2. For example, S8 levels in somites are apparently higher in the loosely packed sclerotomal mesenchyme than in the dermatomyotome. This is similar to Mox-2 (although Mox-2 transcripts are absent from the dermamyotome), whereas a strong Mox-1 hybridization signal is seen over all somitic compartments. In addition, S8 is expressed in the allantois, large regions of head mesenchyme, the entire heart and, later, in otic epithelium, meningeal primordia, dental papillae, and whisker follicle mesenchyme. Mox-1 (and Mox-2) is either not expressed in any of these tissues, or is only expressed in specific substructures (e.g. cushions of the heart). S8 signal is noticeably absent from the kidney, where Mox-1 is clearly expressed.

Although Mox-1 cosegregates in our mapping crosses with the Hox-2 region of chromosome 11, it is probably not a Hox-2 cluster member. First, no recombination events separate several genes in this area that in actuality could be very well separated physically (Nadeau et al., 1992). Second, Mox-2 maps to chromosome 12, detached from the duplicated Hox-1, Hox-3, or Hox-4 clusters. So far, we have failed to find, but are continuing to search for, Mox-1 in contiguous YAC clones spanning and flanking the Hox-2 cluster (data not shown).

The large intron within the homeobox and the highly diverged amino-terminal sequence of the homeodomain are probably why Mox-1 and Mox-2 have not been detected previously in searches of genomic DNA utilizing PCR amplification and degenerate primers designed against conserved 5′ and 3′ homeobox sequences. We have not yet found other Mox genes in mouse, and have only isolated Xenopus homologs of Mox-1 and Mox-2 by low-stringency cross species library and Mox-specific PCR screening.
Although Mox-1 transcripts were not detected in neural or endodermal tissue at any stage studied, certain areas of neural crest-derived mesenchyme are Mox-1-positive in mid-gestation embryos (e.g. mandibular cartilage; Fig. 6B,C). This is in keeping with the notion that homeobox genes can have several different roles during embryogenesis, e.g. Drosophila Pit-1 and Oct-2 (Krause et al., 1988; He et al., 1989) The most parsimonious explanation of Mox-1 expression in the neural crest mesenchyme is that it corresponds to loose connective tissues associated with sites of muscle assembly and attachments. It is known that interactions between developing myoblasts, primary myocytes and adjacent connective tissue precursors are necessary determinants of the spatial assembly of muscles, including sites of myoblast movement arrest, orientation of primary myocyte elongation and segregation of individual muscles from common precursor pools (McClearn and Noden, 1988; Noden, 1991). Possibly Mox-1 expression reflects one element of the genetic control of these spatial interactions.

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References


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