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Hox-7, a mouse homeobox gene with a novel pattern of expression during embryogenesis

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A new mouse Hox locus, *Hox-7*, is defined on chromosome 5 by a gene homologous to the *Drosophila* gene *msh*, which contains a homeobox sequence distantly related to that of *Antennapedia*. By *in situ* hybridization, expression of *Hox-7* is detected in the neural fold of embryos, and also in cephalic neural crest. In addition, expression takes place in the developing valves of the embryonic heart. Mandibular and hyoid arches are strongly labelled, expression becoming restricted to the most distal part of mouth and face processes as development proceeds. Intense labelling is also observed in developing limb buds, in the distal region which has been shown to be essential for limb morphogenesis. The pronounced accumulation and regional localization of *Hox-7* transcripts in mandibular and limb processes point to a specific morphogenetic role for this mouse homeobox gene.

Key words: homeobox/mouse embryogenesis/pattern formation/mandibular and limb development

Introduction

One approach to the study of mammalian development has been to identify sequences sharing homology to developmentally important genes characterized in *Drosophila melanogaster*. In the fruit fly, a systematic analysis of mutations affecting normal development has led to the definition of segmentation genes (Nüsslein-Volhard and Wieschaus, 1980), that together with the homeotic genes are determinant in patterning the body plan (Gehring and Hiromi, 1986; Akam, 1987; Scott and Carroll, 1987). Genes from these two classes are expressed during *Drosophila* embryogenesis in a defined spatial pattern and temporal order. Many of these genes have a shared sequence of about 180 nucleotides, the homeobox, encoding the conserved homeodomain (reviewed by Gehring, 1987a). This sequence has homology with regulatory factors of the MAT locus in yeast, and with certain bacterial DNA binding proteins (Shepherd *et al.*, 1984; Laughon and Scott, 1984), suggesting that homeobox-containing genes may be directly implicated in gene regulation. Their involvement in the specification of the body plan has been directly demonstrated by ectopic expression of the homeotic *Antennapedia* (*Antp*) gene, which leads to the transformation of the head into a thoracic segment with legs (Schneuwly *et al.*, 1987). Recently, it has also been shown that homeobox-containing genes specify neuronal cell fate in the developing nervous system (Doe *et al.*, 1988). Many of these genes are clustered in the *Antennapedia* and *Bithorax* complexes. Genes within these two clusters contain closely related homeobox sequences. In contrast, genes located outside these complexes have more diverged homeobox sequences (Regulski *et al.*, 1985).

Homeobox-containing genes have been identified in a number of vertebrates. The potential role of these genes in controlling segmentation and morphogenesis in vertebrates was indicated recently by experiments where homeobox gene expression was manipulated in *Xenopus* embryos (Cho *et al.*, 1988; Harvey and Melton, 1988). In the mouse, distinct, but often overlapping patterns of expression are seen during embryogenesis, similar to the situation in *Drosophila*. For example, to date all mouse homeobox-containing genes which have been analysed are expressed in the neural tube, with distinct anterior and posterior boundaries (reviewed by Stern and Keynes, 1988). As in the fruit fly, most of these genes are clustered. At least 20 different homeobox sequences have been identified, in the mouse genome (for review see Dressler and Gruss, 1988). These sequences have all been isolated on the basis of cross-hybridization with homeoboxes of the *Antennapedia* type (*Ubx*, *Antp* or *ftz*), except the two mouse *engrailed* genes, which were isolated using the homologous *Drosophila* genes (Joyner *et al.*, 1985). It is therefore probable that genes which do not contain a homeobox of the *Antennapedia* type have been overlooked.

One of the most divergent homeoboxes described to date in *Drosophila* (with $<45\%$ homology relative to the *Antennapedia* homeobox at the amino acid level) is that of a gene originally called *99B* because of its location in this chromosomal band (see Gehring, 1987b). This gene was isolated on the basis of weak cross-hybridization with the *Ubx* homeobox. No mutation has yet been detected at this locus. *In situ* hybridization on *Drosophila* late embryos has shown expression of *msh* mainly in the central nervous system and the segmented striated muscles of the body wall (B.Jacq, A.Fjose and W.Gehring, in preparation). Therefore, it is now designated as *muscle segment homeobox* (*msh*).

We have used the *msh* homeobox sequence as a probe to look for a homologous sequence in the mouse genome. We have isolated a mouse gene containing a homeobox which is very similar to that of the *Drosophila msh* gene (92% homology at the amino acid level) and which is quite different from all previously described mouse homeoboxes. The gene, which we have designated *Hox-7* does not belong to any previously identified cluster but is located at a novel locus on mouse chromosome 5. Investigation of its expression by *in situ* hybridization shows accumulation of transcripts in the neural tube and also in the neural crest. Striking expression is seen both in the mandibular arch and later in the mouth structures which develop from it, and also in the distal portion of the developing limb bud. Transcripts in these

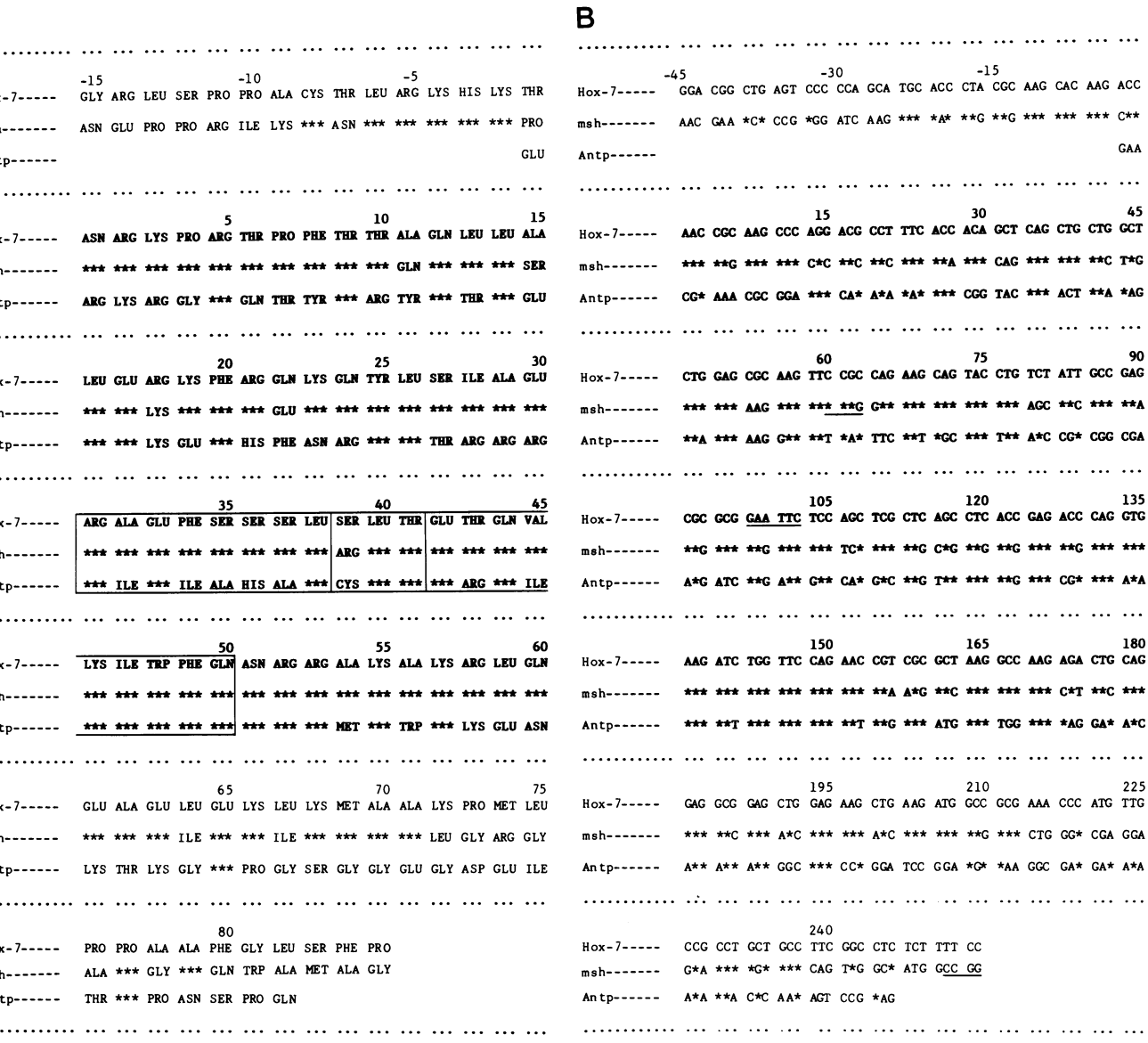

Fig. 1. Comparison of genomic sequences between *Hox-7*, *msh* and *Antennapedia* (*Antp*) homeoboxes at (**A**) amino-acid (as deduced from the nucleotide sequence) and (**B**) nucleotide levels. The region corresponding to the homeoboxes is printed in bold, and begins at position 1. The *Antennapedia* sequence begins at the first codon of exon 8 and ends at the stop codon of the gene (Schneuwly *et al.*, 1986). In (**A**) the putative helix-turn-helix forming regions are boxed. In (**B**) the *Hpa*II sites that delineate the *msh* probe and the *Eco*RI site bordering the 3′ end of the probe used for *in situ* hybridization are underlined. Asterisks indicate identity with the *Hox-7* sequence.

structures accumulate in zones of ectodermal/mesodermal cell contact. Expression in both mandibular and limb processes suggests a morphogenetic role for *Hox-7*, during mouse embryogenesis.

Results

Isolation of a mouse gene homologous to the Drosophila msh gene

In order to isolate a gene homologous to the *Drosophila msh*, we screened a cosmid library of mouse genomic DNA with a probe containing the 3′ two thirds of the *msh* homeobox plus a further seventy-five nucleotides 3′ to this, as defined in Figure 1B. Three independent clones, cos1, cos5a and cos6a were isolated, two of which (cos5a and cos6a) appeared to be identical cosmids according to their restriction patterns, while the third one, cos1, although different, shared many resriction fragments and was obviously derived from the same locus. Cos5a was selected for further identification.

The sequence of the mouse homeobox homologous to *msh* is shown in Figure 1. The conservation between the *Drosophila* and the mouse homeoboxes is remarkable, since 92% of the amino acids and 78.3% of the nucleotides are common to both. This conservation extends 3′ of the homeobox, where a further 11 amino acids are nearly perfectly conserved, the only changes being the substitution of two leucines by two isoleucines. On the 5′ side, the homology extends for eight more amino acids, six of which are identical between *Drosophila* and mouse. After this, there is a potential splice site at nucleotide −25 from the *Drosophila* homeobox (B.Jacq, unpublished results) and homology falls markedly.

Compared to the *Antennapedia* homeobox (Schneuwly *et al.*, 1986), *Hox-7* is significantly diverged, with only 42% homology at the amino acid level, and a little more (52%)

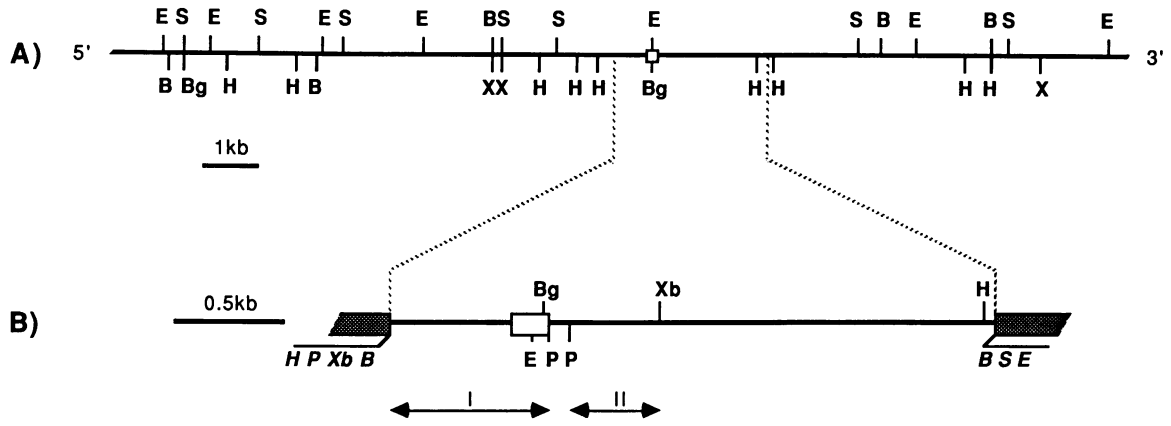

Fig. 2. (**A**) Restriction map of the cosmid cos5a around the *Hox-7* homeobox, and (**B**) restriction map of the insert in the *Hox-7*/B12 Bluescribe minus plasmid. The arrows under this map represent the probes used on Southern blots. The maps are orientated 5′ to 3′ relative to the sense strand of the homeobox. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I, S, *Sac*I; X, *Xho*I and Xb, *Xba*I. The sites in italics belong to the polylinker of the plasmid.

at the nucleotide level. Most of the conservation is seen in the putative helix-turn-helix forming region, which has been proposed to be the site for interaction with DNA (Laughon and Scott, 1984). The homology is particularly good in the second helix, and extends further towards the amino-terminus for seven amino acids. This region is probably responsible for the weak cross-hybridization between *Ubx* and *msh* homeoboxes. However, even within these conserved regions, there are substitutions which might be responsible for the different DNA binding properties of the two homeoboxes (M.Müller, M.Afolter and W.Gehring, unpublished observations). In this respect, the substitution at position 43 of the threonine for arginine is a very rare event, until now only encountered in the *Drosophila labial* (Hoey *et al.*, 1986; Mlodzik *et al.*, 1988) and in the mouse *Hox1-6* (Baron *et al.*, 1987) homeoboxes. The nine amino acids which have been found invariant in the homeo-domain (Gehring, 1987a) are also conserved in *Hox-7* (and in *msh*): Arg 5, Gln 12, Leu 16, Tyr 25, Leu 40, Trp 48, Phe 49, Asn 51 and Arg 53.

A restriction map was established around the homeobox (Figure 2A). The surrounding region was subcloned from a partial *Sau*3A digest into the *Bam*HI site of the Bluescribe plasmid (Figure 2B). Different fragments from this subclone were used to probe genomic blots of mouse 129 DNA (Figure 3). These show that the locus has the same structure in the cosmid and in the mouse genome. Furthermore, only weak hybridization is observed on blots with bands other than those belonging to the cloned locus, even under very low stringency conditions for both hybridization and washes (Figure 3, lane E). This cross-hybridization might occur between the *Hox-7* homeobox and homeoboxes of another type. However, considering that the 3′ end of the *Hox-7* homeobox, which is the most conserved region, constitutes only a small part of the *Pst*I probe used, it seems likely that the *Hox-7* gene belongs to a family of related sequences.

Hox-7 is located on chromosome 5

Genetic localization of *Hox-7* was achieved using a mouse interspecific back-cross constructed as described previously (Robert *et al.*, 1985). A restriction fragment length polymorphism (RFLP) was identified in the locus detected by the mouse homeobox probe (in this case, the 0.8-kb *Pst*I–*Pst*I fragment of plasmid Hox-7/B12 that contains the homeobox, defined as probe I in Figure 2B) between the

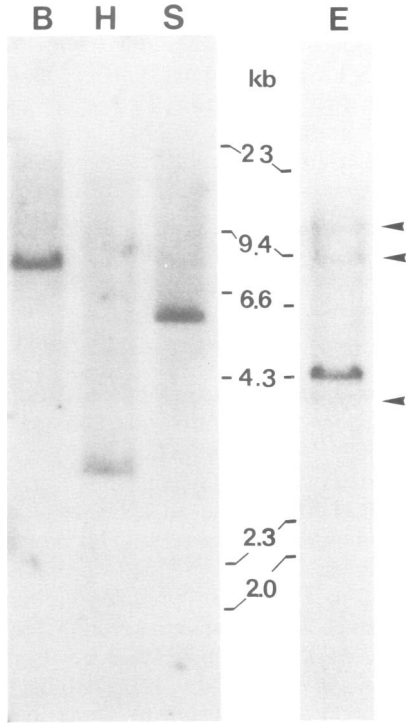

Fig. 3. A Southern blot analysis of the *Hox-7* locus in 129Sv mouse DNA. The *Bam*HI (**lane B**), *Hin*dIII (**lane H**) and *Sac*I (**lane S**) digests were separated on a 0.9% agarose gel and blotted as described in Materials and methods. The blot was probed with the *Pst*I–*Xba*I 0.4-kb fragment 3′ to the homeobox (probe II in Figure 2B), under standard stringency conditions for hybridization (42°C in 5 × SSC and 50% formamide) and washes (50°C in 0.1 × SSC). *Eco*RI digested sample (**lane E**) was probed with the 0.8-kb *Pst*I–*Pst*I fragment of plasmid *Hox-7*/B12 containing the homeobox (probe I in Figure 2B). Conditions of reduced stringency were used for hybridization (37°C, in 43% formamide and 5 × SSC) and washes (50°C in 2 × SSC). The arrowheads point to the bands which do not belong to the cloned locus.

DNAs of laboratory inbred strains derived from *Mus musculus* (C57BL/6 or BALB/c) and *Mus spretus* (SPE/Pas), upon *Taq*I digestion. This enzyme generates a 2.2-kb fragment in the DNA of *M.musculus* and a 4.6-kb fragment in that of *M.spretus*. The segregation of this RFLP was analysed in the 75 offspring of a back-cross (SPE/Pas ×

Table I. Analysis of the segregation of the *Hox-7*, *Pgm-1* and *Alb* loci in the back-cross (SPE/Pas × C57BL/6) × C57BL/6

Animal no.	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175
Hox-7	−	−	−	−	−	−	+	−	+	+	+	−	−	−	+	+	+	−	−	−	−	−	+	−	+
Pgm-1	−	−	−	−	−	−	−	−	+	−	+	−	+	−	+	+	+	−	−	−	−	+	+	+	+
Alb	+	−	−	−	−	−	−	+	+	−	+	−	+	−	+	+	−	−	−	−	+	+	+	+	+
Animal no.	177	178	180	180[b]	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	202
Hox-7	−	+	−	+	−	+	+	+	−	+	+	+	+	+	−	+	+	+	+	−	+	+	−	+	−
Pgm-1	−	+	−	−	−	+	+	−	−	+	+	+	+	−	−	+	+	−	+	−	+	+	−	+	+
Alb	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	+	+	−	+	−	+	+	−	−	
Animal no.	203	220	221	225	226	232	233	235	236	237	238	241	242	299	300	307	308	311	312	323	325	328	329	332	333
Hox-7	+	+	−	+	+	+	+	−	−	+	−	−	+	+	−	+	+	+	+	+	+	−	−	+	−
Pgm-1	+	−	−	−	+	+	+	−	−	+	−	−	+		−	+	+	+	+	−	−	+	−	+	−

Segregation pattern for these loci into the 75 offspring of the back-cross. Animals are scored either as homozygous for the *musculus* allele (−) or heterozygous (+).

C57BL/6) × C57BL/6. This was compared to the segregation patterns of 67 genes analysed in the same cross, which altogether cover about 80% of the mouse genetic map. The *Hox-7* gene shows 80% co-segregation with the *Pgm-1* (Green, 1981) locus on chromosome 5 (Tables I and II). The recombination frequency with the *Alb* (Green, 1981) locus, which is on the same chromosome, is 38%. This establishes the order of the three loci: *Hox-7*, *Pgm-1*, *Alb*, and places the *Hox-7* locus in the vicinity of *Emv-1*, the site of insertion of a retrovirus in certain mouse strains (Jenkins *et al.*, 1982). As expected, *Alb* and *Pgm-1* are shown to be linked by this analysis, although the recombination frequency in this experiment is rather higher than expected from the genetic map, on which they are only 12 cM apart.

Table II. Analysis of the segregation of the *Hox-7*, *Pgm-1* and *Alb* loci in the back-cross (SPE/Pas × C57BL/6) × C57BL/6

	Hox-7	*Alb*
Pgm-1	20.3 ± 4.7 (15/74)	20.4 ± 5.8 (10/49)
Alb	38.8 ± 7.0 (19/49)	

Percentage recombination between these three loci, together with the standard deviation. The actual figures are given in brackets in relation to the total number analysed. The linkage between *Hox-7* and *Pgm-1* is highly significant when examined by the χ^2 test ($\chi^2 = 25.33$, $P < 0.001$).

Expression of Hox-7 during mouse embryonic development

The expression of *Hox-7* was followed during mouse development using *in situ* hybridization on paraffin-embedded sections. A 150-nucleotide RNA probe, which contains the 5′ half of the *Hox-7* homeobox from the *Eco*RI site (see Figure 1B) together with an additional 50 nucleotides upstream of the homeobox, was used. This probe therefore contains only the less conserved region of the homeobox (see Figure 1B), and is unlikely to hybridize with any known homeobox sequence; for example, no cross-hybridization is seen with the *Hox-1.5* homeobox sequence, from plasmid Mo-10 (McGinnis *et al.*, 1984a), on DNA blots under low stringency conditions (data not shown). Cross-hybridization with transcripts from related genes cannot be totally excluded since minor bands are detected on Southern blots (Figure 3, lane E), although such a cross-hybridization is observed only under low stringency conditions and is unlikely to occur under the relatively stringent conditions used for *in situ* hybridization (see Materials and methods). It should be noted that the *Drosophila msh* probe, which is very similar to the *Hox-7* sequence, does not give a detectable signal with sections hybridized *in situ* (data not shown).

Early expression. The earliest stage at which we have detected *Hox-7* transcripts in the mouse is 6.5 days post coitum (p.c.) when expression is restricted to extra-embryonic tissue. Figure 4 shows a transverse section of a mouse embryo at 8 days p.c. *in utero* (see Rugh, 1968, for embryological identification). In addition to extra-embryonic tissue (amniotic membrane and ectoplacental cone), the mesenchymal tissue underlying the neural fold in the anterior portion of the embyro, corresponding to the rostral neural crest, is also labelled. In addition, adjacent lateral ectoderm and the tip of the neural fold, from which neural crest cells migrate (Nichols, 1981), show strong labelling.

By 9.5 days p.c. (24 somite stage), the embryo is labelled with the *Hox-7* probe in a number of distinct regions. On a sagittal section (Figure 5a), labelling is conspicuous in the neural tube, especially in the posterior neuropore and the region of the cephalic fold at the level of the fourth ventricle. This expression does not involve the whole neurectoderm in these regions, but is localized on the outer edge of the neural fold. At later stages of development (results not shown) labelling is still seen in restricted areas of the central nervous system. At day 11.5, it is still intense in the *tela choroidea*, the last region of fold fusion, and at day 15.5 in the posterior choroid plexus (results not shown). In the 9.5 day embryo, there is marked labelling in the subcranial mesenchyme which corresponds to the cephalic neural crest (Figure 5b and c). At this stage, there is some labelling of endothelial cells lining the lumen of the heart. There is also labelling of tissue of the first and second visceral arches, the mandibular and hyoid (not visible on this section) arches, which are regions colonized by neural crest cells. It is notable that with the *Hox-7* probe no labelling is detectable over the somites of the 9.5-day embryo (Figure 5); labelling is detected in the early limb bud (not shown). At this and subsequent stages of development, amniotic and allantoic derived structures continue to show *Hox-7* expression (see Figure 5a and d).

Hox-7 expression in the heart. Figure 6a represents a section showing the heart of a 10.5-day mouse embyro. Atrial and ventricular compartments can be distinguished. Hybridization with an actin probe corresponding to the 5′ non-coding region of the cardiac actin messenger RNA (Sassoon *et al.*,

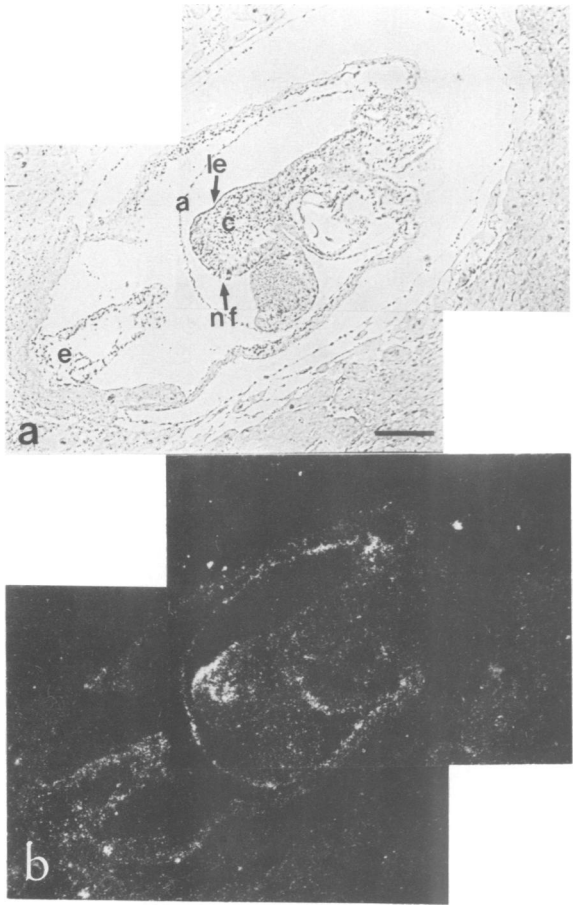

Fig. 4. Expression of *Hox-7* in 8-days p.c. mouse embryo. (**a**) Transverse section of embryo *in utero* viewed with phase optics. The section is at the level of the rostral portion of the embryo. (**b**) The amnion **a**, neural fold **nf**, neural crest **c**, ectoplacental cone **e**, and lateral ectoderm **le**, show accumulation of silver grains as viewed with darkfield optics. Scale bar = 100 μm.

1988) and therefore specific for this gene transcript serves as a positive control for the cardiac muscle tissue (Figure 6c). Hybridization with the *Hox-7* probe shows weaker labelling over the endocardial cushion tissue (Figure 6b), which is formed of mesenchymal cells which delaminate from the endothelial lining where label was observed in 9.5-day embryos (Figure 5c). The endocardial cushion tissue gives rise to the septum and valves of the heart (Patten, 1968; Kinsella and Fitzharris, 1980). In this figure labelled mandibular arch tissue is also visible.

Hox-7 expression in the mandibular arch. One of the most striking sites of *Hox-7* expression is in the mandibular arch which will contribute to formation of the mouth and facial structures. In Figure 5d, a sagittal section of a 11.5-day mouse embryo is shown. Labelling at this stage is concentrated in the distal mesenchyme. Adjacent epithelial cell layers are labelled at the most distal positions. There is no distinction yet between the region which will form the tongue and that which will form the mandible. In addition to labelling of the mandibular arch, strong labelling is seen in the immediately adjacent anterior meninges (Figure 5d). By day 15.5, labelling is strongest in the dental papilla, and is also present in surrounding soft mesenchymal tissue, which both are derived from neural crest. The tongue which is now mainly muscular is not labelled, nor is Meckel's cartilage and other regions of chondrogenesis. The lip furrow, lips and hair follicles, are also not labelled (data not shown).

Hox-7 expression in the developing limb bud. A second striking feature of *Hox-7* expression is its presence in the developing limb. Initially, at the 9.5-day stage, when the limb bud is just forming the entire bud is labelled. Subsequently the label is concentrated in the distal region. Thus in the 12.5-day embryo (Figure 7a) label is seen in the distal edge of the limb bud, in both the ectoderm which is thickened over the developing limb to form the apical ectodermal ridge (aer) and in the underlying mesoderm around the marginal blood sinus. Ectoderm outside this region of the embryo (lateral ectoderm) is not labelled. As digitation takes place (Figure 7b) labelling is now seen in the interdigital region, where it is distributed according to cell density and not associated specifically with necrosing cells present in these regions.

Discussion

We have isolated a novel homeobox gene in the mouse using the *Drosophila* gene *msh*. This mouse gene, *Hox-7*, shows very high homology with *msh* within the homeobox domain (92% amino acid conservation) and within an additional 11 amino acid residues on the C-terminal and 8 residues on the N-terminal side of the homeobox. Extensive homology between *Drosophila* and vertebrate homeobox genes has been observed for the *engrailed* homologues *En-1* and *En-2*, in the mouse (Joyner and Martin, 1987), and with homologues to the *Drosophila Dfd* gene in the mouse (*Hox-5.1*, Featherstone *et al.*, 1988; *Hox-2.6, Hox-1.4*, Graham *et al.*, 1988), in *Xenopus* (*XhoxlA*) and in human (*HHo.c13*) (Regulsky *et al.*, 1987). *Hox-7* and *msh* are both primarily transcribed in mesodermal tissues (unpublished observations and this report). The expression of *msh* in the striated muscle of *Drosophila* embryo, however, is not a characteristic shared by *Hox-7*. The relative divergence of *msh* from other well characterized homeobox genes in *Drosophila* is reflected in the *Hox-7* sequence which also shows considerable divergence from other mouse homeobox sequences described to date. We note, however, that the basic 'helix-turn-helix' motif as well as certain key residues common to homeodomain sequences (Gehring, 1987a) are conserved in both *msh* and *Hox-7*. *Hox-7* may belong to a family of related sequences, since it shows a weak cross-hybridization on genomic Southern blots with bands that do not belong to the cloned locus.

Hox-7 is located on mouse chromosome 5 where it defines a new homeobox gene locus. Only one other *Hox* sequence (*En-2*) maps to this chromosome and it is located in the same region (Joyner and Martin, 1987). However, *En-2* and *Hox-7* differ in sequence and they clearly represent different genes. We are currently determining the genetic distance between *En-2* and *Hox-7*. The chromosomal localization of *Hox-7* places the gene in a region where there are several developmental mutants: *luxate* (*lx*), *Hemimelic extra toe* (*Hx*), and *Hammer-toe* (*Hm*) (Green, 1981). These three mutant strains all involve changes in pattern formation in limbs. *Hm* homo- or heterozygous mice do not undergo complete regression of interdigital web tissue (Green, 1981). *Hx* heterozygotes show pre-axial polydactyly as well as malformations involving the tibia and radius (Knudsen and Kochhar, 1981). *lx* heterozygotes show pre-axial polydactyly

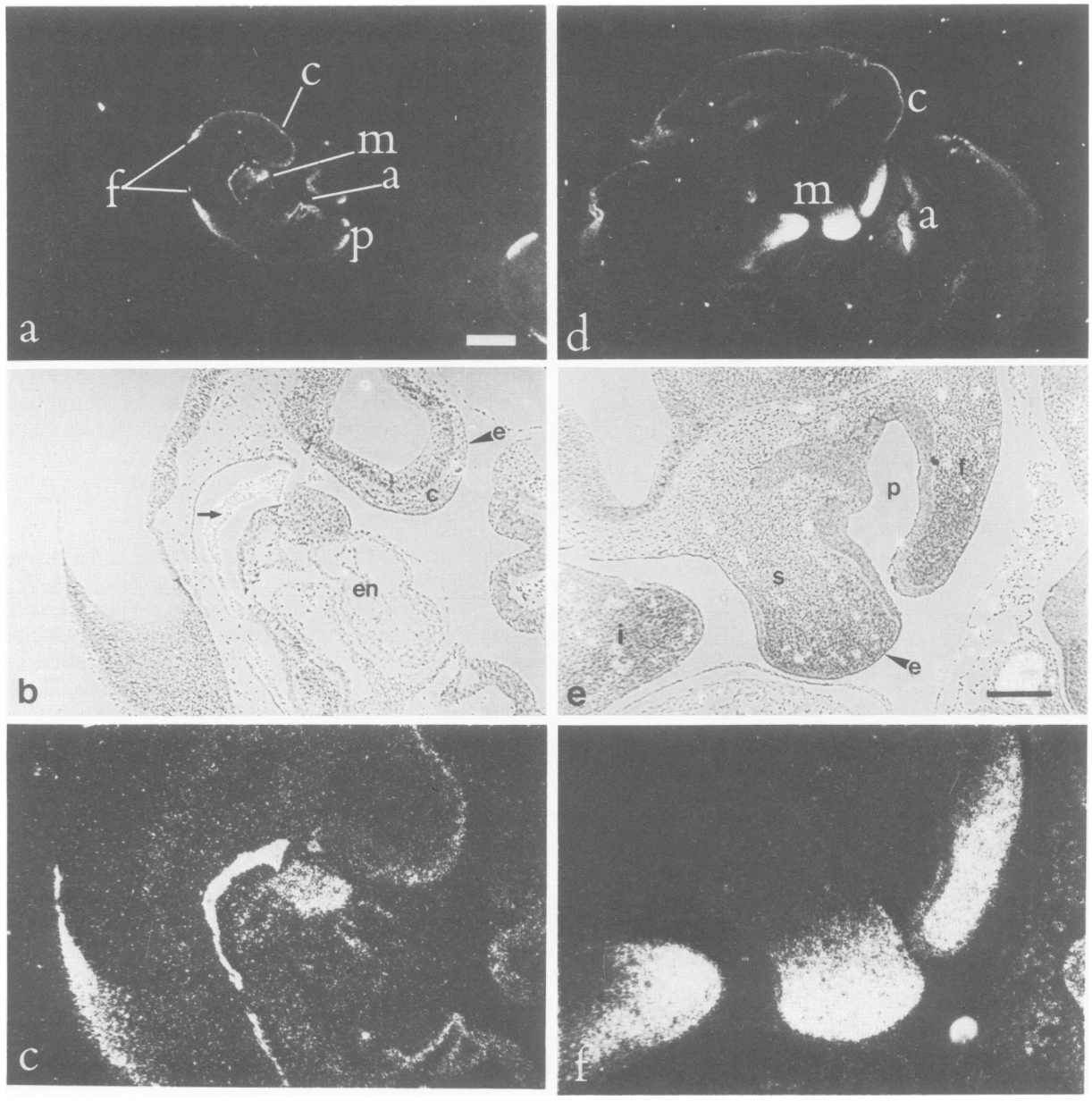

Fig. 5. Expression of *Hox-7* in 9.5-(**a-c**) and 11.5-(**d-f**) day p.c. mouse embryos. (**a**) Midsagittal section of a 9.5-day p.c. mouse embryo viewed with darkfield optics. At this plane of section the cephalic fold **f**, neural pore **p**, cephalic neural crest **c**, as well as mandibular arch **m** and amniotic structures **a** show strong labelling. (**b**) Higher magnification phase contrast micrograph of same embryo as in (**a**). Note that the cephalic neural crest **c** underlies the rostral ectoderm **e**. Also, at this level of magnification, the endothelial cells **en** of the heart lumen can be observed. (**c**) Darkfield image of previous panel (**b**), showing accumulation of silver grains in endothelial cells of the heart as well as structures identified in panel (**a**). Blood cells **arrow** are not labelled but display high birefringence with darkfield optics. (**d**) Midsagittal section of a 11.5-day p.c. mouse embryo viewed under darkfield optics. As seen in panel (**a**) for the 9.5-day p.c. embryo, cephalic neural crest cells **c** are labelled as well as amniotic structures **a**. At this section level, mandibular processes **m** show heavy labelling. (**e**) Higher magnification phase contrast image of the same embryo as in panel (**d**). Inferior **i** and superior **s** mandibular processes as well as fronto-nasal process **f** are labelled. Ectoderm **e** can be distinguished from the subjacent mesoderm. **p**, nasal pit. (**f**) Darkfield image of (**e**). Note that labelling of mesoderm is most intense in the distal portions of the mandibular processes and diminished proximally. The distal ectoderm is moderately labelled. Scale bars = 1 μm for **a** and **d**; 150 μm for **b**, **c**, **e** and **f**.

of the hind limb; in homozygotes, this is part of a more severe, pleiotropic syndrome (Carter, 1951). As one major site of *Hox-7* expression is the developing limb bud, it is tempting to speculate that one of these characterized mutants is allelic to it.

Using *in situ* hybridization, we have elucidated *Hox-7* expression during mouse embryonic development. In discussing the apparently significant features of *Hox-7* expression and thus its possible role, we make the assumption that the site of transcript accumulation is coincident with the site of expression of the gene, justified on the grounds that this appears to be the case for the *Drosophila* homeobox genes during embryogenesis (Gehring, 1987c). In keeping with the highly divergent nature of the *Hox-7* sequence, we find that the pattern of expression differs from that described for other *Hox* genes in the mouse. All homeobox-containing genes analysed in the mouse are expressed in the central nervous system with antero−posterior regional patterns characteristic for each (Stern and Keynes, 1988; Holland and Hogan, 1988a), and some of them are also expressed

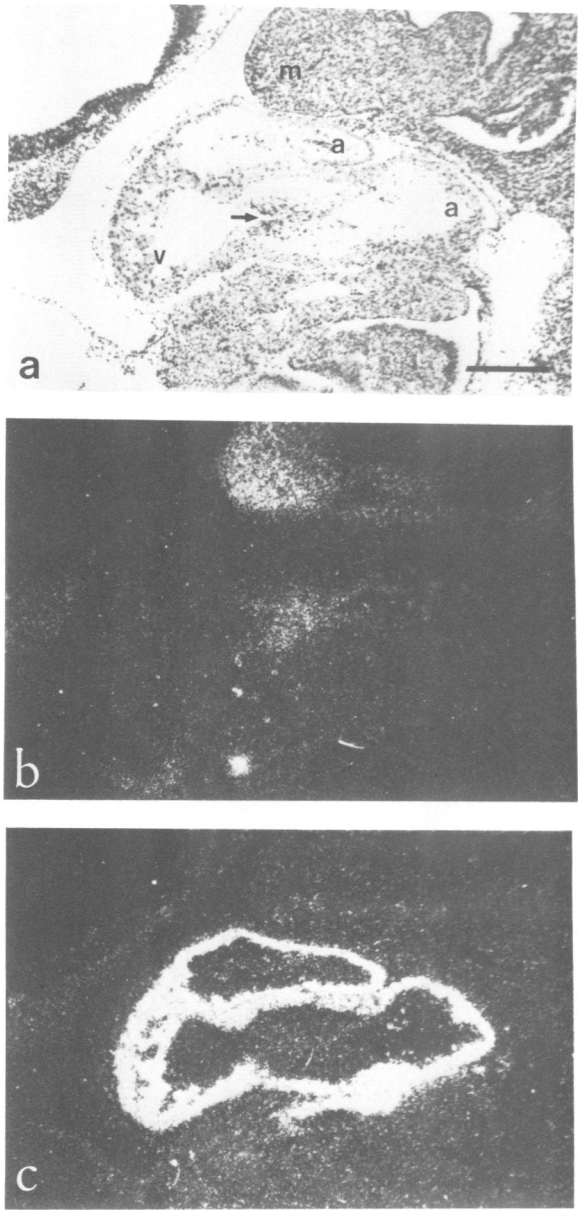

Fig. 6. Expression of *Hox-7* and α-cardiac actin in a 10.5-day p.c. mouse heart. (**a**) Phase contrast photomicrograph showing the presumptive ventricular **v** and atrial **a** compartments as well as the endocardial cushion **arrow** and mandibular arch **m**. (**b**) Darkfield of same section as in panel (**a**) showing detection of transcripts in the endocardial cushion and mandicular arch. (**c**) An adjacent section to (**b**) hybridized to a cRNA probe for α-cardiac actin, detecting transcripts in cardiac muscle tissue. Note that *Hox-7* and α-cardiac actin RNAs are not detected in the same tissues thereby demonstrating the specificity of *in situ* hybridization. Exposure times are identical for these two probes. Scale bar = 200 μm.

in adjacent somitic mesoderm (Dony and Gruss, 1987a; Gaunt, 1988). From these data, Holland and Hogan (1988b) and Gaunt (1988) have proposed that the co-expression of *Hox* genes in different antero−posterior delimited compartments of the central nervous system leads to unique segment identity along the antero−posterior embyronic axis in a manner similar to the *Drosophila Ubx* and *Antp* homeotic genes (Gehring, 1987a). *Hox-7* expression does not appear to be restricted to antero−posterior segments of the mouse, but rather, as shown clearly in the limb, obeys local boundaries which are proximo−distal. *Hox-7* is expressed in the neural tube of the 9.5-day p.c. embryo and subsequently becomes restricted to various central nervous system structures such as the neural folds (i.e. posterior neuropore, neural fold of ventricle IV, *tela choroidea*). The observation that expression is restricted primarily to the portion of neuroectoderm actively engaged in fusion may indicate that *Hox-7* plays a role in these morphogenetic processes.

We demonstrate that *Hox-7* transcripts accumulate in the rostral neural crest at 8 days p.c., and subsequently in the visceral arches. The mandibular and hyoid arches are colonized by neural crest cells as has been shown in amphibia, avia (reviewed in Le Douarin, 1982), and more recently in rodents (Tan and Morris-Kay, 1986). The expression of *Hox-7* in the mesenchyme of these visceral arches may reflect this embryonic lineage. In addition, we observe that *Hox-7* expression extends to the rostral end of the prosencephalic portion of the embryo. Nichols (1981), in a histological study, observed mesenchymal cells with neural crest characteristics in this region. While Tan and Morris-Kay (1986) could not demonstrate the existence of migrating neural crest cells in this region, they showed that midbrain neural crest cells could migrate rostrally around the prosencephalon under experimental conditions. Thus, the expression of *Hox-7* in the rostral position of the embryo may involve neural crest cells. Demonstration that the cells which are labelled in the visceral arches and rostral portion of the embryo are of neural crest origin awaits further analysis. We have reported here that the expression of *Hox-7* in the mandibular arch becomes restricted to the more distal portions of the mandibular structures as the embryo develops. Although *Hox-7* transcripts are mainly expressed in the mesenchymal tissue, the most distal portion of the mandibular ectoderm also shows high levels of *Hox-7* transcripts. This is similar to the situation in the developing limb bud where expression is also seen in distal mesenchyme and apical ectoderm (see Results and below). By 15.5 days p.c., expression in the mouth region becomes restricted primarily to mesenchymal tissue, with a more focalized expression in the dental papillae. The latter has been demonstrated to be a site of induction between cells derived from the neural crest and the epithelium, leading to the production of cells involved in tooth enamel formation (Le Douarin, 1982). Thus, it would appear that the expression of *Hox-7* in the rostral portion of the embryo is either concerned directly with cells of neural crest origin and/or cells that interact with neural crest cells.

Another major site of *Hox-7* expression is the developing limb bud. As described, the entire mesodermal compartment and apical ectoderm of the limb buds in the 9.5-day embryo show high levels of *Hox-7* transcripts. As limb outgrowth and morphogenesis progress, *Hox-7* transcripts become restricted to the most distal portion of the ectoderm and underlying mesenchyme. Although the function of the gene remains to be demonstrated, it is perhaps the fact that *Hox-7* transcripts accumulate in the limb bud that most compellingly suggests a morphogenetic role for this gene. The developing limb has been a subject of considerable research interest, in particular due to the accessibility of this tissue for experimental manipulation. The ectoderm located on the most distal aspect of the limb bud [apical ectodermal ridge (aer)] has an inductive role on the underlying mesoderm.

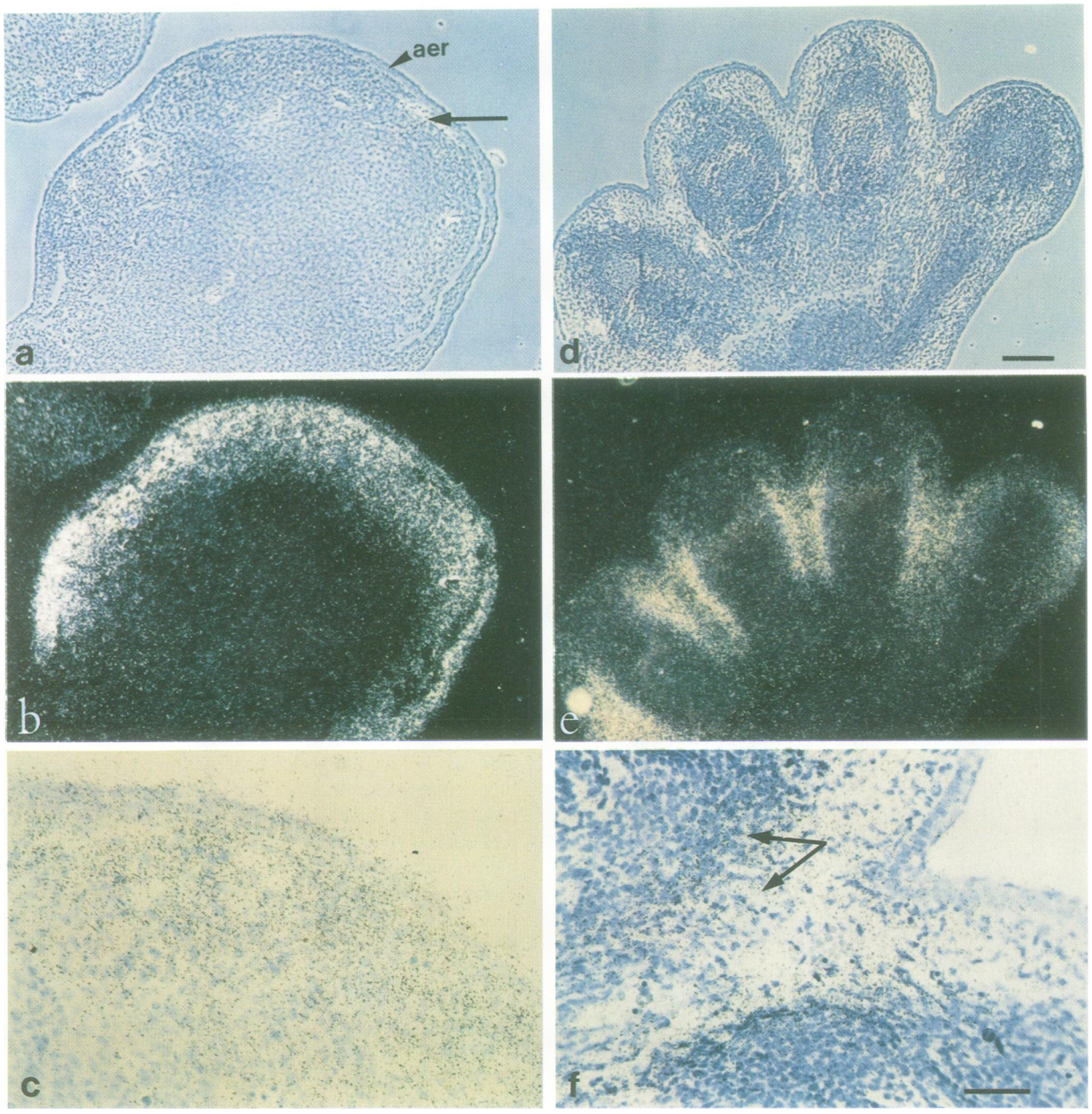

Fig. 7. Expression of *Hox-7* in developing limbs of mouse embryos. (**a**) Horizontal section of a right hind limb bud in a 12.5-day p.c. embryo. The position of the future digits can be discerned due to the condensation of pre-cartilage masses **c**. The apical ectodermal ridge **aer** and the underlying marginal blood sinus **arrow** are present at this plane of sectioning. (**b**) Same section as in (**a**) viewed under darkfield optics showing the distribution of *Hox-7* transcripts at both the level of the apical ectodermal ridge and the subjacent distal mesoderm. (**c**) High magnification of same section as (**a**) and (**b**) showing distribution of grains. (**d**) Horizontal section of the distal portion of a left hand limb from a 15.5-day p.c. mouse embryo. Cartilage has already formed and the digits are well developed. (**e**) Same section as in (**d**) viewed under darkfield optics. *Hox-7* transcripts are primarily concentrated interdigitally although some apical ectoderm and underlying mesoderm is labelled (see **arrow**). (**f**) High magnification of same section as in (**d**) and (**e**). The densest accumulation of grains corresponds to the perichondrial zone (bracketed by **arrows**). Scale bars = 100 μm for **a**, **b**, **d** and **e**; 35 μm for **c** and **f**.

In particular the aer, which appears as a thickening on the limb bud apical ectoderm, can induce ectopic or supernumerary limb structures when grafted onto non-limb mesoderm (reviewed in Zwilling, 1961; Saunders, 1977). Removal of the aer results in complete arrest of limb development. It has been proposed that the aer maintains subjacent mesenchymal cells in an undifferentiated and thus more 'labile' state (Summerbell *et al.*, 1973). The underlying mesenchyme, referred to by Wolpert and colleagues (Summerbell *et al.*, 1973) as the 'progress zone', has been shown to contain mostly dividing cells (Summerbell and Lewis, 1975). It has been proposed that they subsequently leave this zone and assume their positional value along the proximal–distal axis of the limb (Stark and Searls, 1973; Summerbell and Lewis, 1975). Although the situation has been most extensively studied in the chick, the data available for the mouse would appear to be consistent with these models (Milaire, 1987). Furthermore, rodent mesoderm or ectoderm can be substituted for chick tissues in grafting experiments (Jorquera and Pugin, 1971; Saunders, 1977). The notion that vertebrate homologues of *Drosophila* homeobox genes may play a morphogenetic role is supported

by the observations reported here, namely, that *Hox-7* transcripts accumulate in the apical ectoderm, an inductive tissue, and in the mesenchymal progress zone which has been experimentally demonstrated to be involved in pattern programming in the developing limb. Since the apical ectoderm is not as mitotically active as the subjacent mesenchyme (Hornbruch and Wolpert, 1970), it is unlikely that *Hox-7* merely plays a role in cell division but rather is associated with meso-ectodermal inductive interactions which result in pattern formation. It should be emphasized that the facial primordia depend for their pattern formation on epithelial/mesenchymal interactions, in much the same way as limb buds, and have very similar cellular responses to substances such as retinoic acid as the developing limb bud (Wedden, 1987; reviewed by Wedden *et al.*, 1988).

It has been suggested that positional values are a function of a variety of gene products (Gaunt, 1988; Holland and Hogan, 1988b). In keeping with this idea, we note that *Hox-7* expression during mouse embryogenesis has certain similarities with the pattern of TGF-β accumulation in the mouse embryo (Heine *et al.*, 1987). Overlap (i.e. co-expression) is observed in the developing limbs, mandible and atrio-ventricular valve. Other transcripts such as c-*fos* have also been reported to accumulate in the interdigital tissue of mouse limbs (Dony and Gruss, 1987b). It thus seems probable that *Hox-7* interacts with other types of cell regulatory factors to establish the pattern of various structures in the mouse embryo.

Materials and methods

Nomenclature

We proposed to call the gene that we have isolated *Hox-7*, in accordance with accepted nomenclature for homeobox-containing genes in the mouse (Martin *et al.*, 1987), and following the identification of *Hox-6* (Sharpe *et al.*, 1988). This designation has been agreed on by the International Committee on Standardized Genetic Nomenclature for Mice at their meeting in Cambridge, July 1988.

Cosmid library screening

A cosmid library of 129Sv mouse genomic DNA cloned into the pcos2EMBL (Poustka *et al.*, 1984; a kind gift from Dr A.M.Frischauf) was screened with a fragment of *Drosophila* DNA containing the 3′ two-thirds of the *msh* homeobox plus a further 75 nucleotides 3′ to this, as defined in Figure 1. The cosmid library was originally divided into five independent fractions, representing altogether 5–10 genome equivalents; DNA from three of these fractions exhibited the same strong restriction bands upon hybridization with the *Drosophila* probe under low stringency conditions (as defined under 'Southern blots'), while the remaining two did not show strongly hybridizing bands. Thus, 5×10^5 bacteria from a positive fraction were spread on filters, and replicates were hybridized under low stringency conditions with the *Drosophila* probe. Hybridizing clones were isolated by several rounds of cloning.

DNA sequencing

All sequences were determined by the dideoxy method (Sanger *et al.*, 1977; Biggin *et al.*, 1983). M13 recombinants were generated by subcloning total *Hae*III digests from cosmid Cos5a into the *Sma*I site of M13mp18; those hybridizing to the *msh* probe were selected for sequencing. A specific *Bgl*II–*Sac*I fragment from plasmid pHox7/B12 (Figure 2B) was integrated into the *Bam*HI–*Sac*I sites of M13mp18 to sequence the 3′ of the homeobox.

Southern blots

DNA was transferred from gels to nitrocellulose or Nytran nylon membranes (Schleicher and Schuell) in 10 × SSC according to Southern (1975). For low stringency conditions, hybridization was performed in 43% formamide, 5 × SSC, 5 × Denhardt's, 50 mM $NaPO_4$ pH 7, 0.1% SDS, 250 μg/ml sonicated calf thymus DNA at 37°C for 15 h, followed by washes in 2 × SSC, 0.1% SDS at room temperature and a final wash at 50°C in 2 × SSC, 0.1% SDS, as described by McGinnis *et al.* (1984b). For normal stringency conditions, hybridization was performed in the same buffer containing 50% formamide, at 42°C, and the final wash was in 0.1 × SSC, 0.1% SDS at 50°C.

Genetic mapping

Two alleles of *Hox-7* were defined between inbred strains of *M.musculus* (C57BL/6 or BALB/c) and *M.spretus* (SPE/Pas) as a *Taq*I RFLP. Their segregation was analysed among the 75 offspring of an interspecific back-cross raised by J.L.Guénet (Institut Pasteur), as described previously (Robert *et al.*, 1985). In brief, a SPE/Pas male was mated with a C57BL/6 (or BALB/c) female. The F1 females were back-crossed to a C57BL/6 (or BALB/c, to produce the last 26 offspring) male. DNA was prepared from spleens of 75 offspring and the segregation of RFLPs analysed on Southern blots.

In situ hybridization

Inbred lines of C3H or BALB/c mice were mated and the morning of vaginal plug was counted as p.c. day 0.5. Embryos were removed from surrounding tissue in cold phosphate-buffered saline (PBS: 0.13 M NaCl, 7 mM Na_2PO_4, 3 mM $NaH_2PO_4 \cdot 2H_2O$, pH 7.4), somites were counted (in embryos from 8 days p.c.). Embryos were fixed in 4% paraformaldehyde in PBS, and embedded in paraffin as described by Sassoon *et al.* (1988). They were cut at 5–7 μm on a standard paraffin microtome and sections were mounted on 'subbed' slides (Gall and Pardue, 1971). The procedures used for section treatment, hybridization, and washings were based upon those used by Wilkinson *et al.* (1987) with modifications described in Sassoon *et al.* (1988). Hybridization was carried out at 50°C for ~16 h in 50% de-ionized formamide, 0.3 M NaCl, 20 mM Tris–HCl (pH 7.4), 5 mM EDTA, 10 mM $NaPO_4$ (pH 8), 10% dextran sulfate, 1 × Denhardt, 50 μg/ml yeast RNA, with 75 000 d.p.m./μl of a ^{35}S-labelled cRNA probe generated on a Bluescribe minus recombinant using T7 RNA polymerase and [α-^{35}S]UTP (>1000 Ci/mmol, New England Nuclear). Coverslips were then gently floated off in 5 × SSC (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate) 10 mM dithiothreitol (DDT) at 50°C and the tissue subjected to a stringent wash at 60°C in 50% formamide, 2 × SSC, 0.1 M DDT. Slides were then rinsed in a washing buffer (Wilkinson *et al.*, 1987) and treated with RNase A (20 μg/ml) and T1 (2 μg/ml) (Boehringer) in washing buffer for 45 min at 37°C. Following washes at room temperature in 2 × SSC (15 min) and 0.1 × SSC (15 min) respectively, slides were rapidly dehydrated, processed for standard autoradiography using Kodak NTB-2 nuclear track emulsion and exposed for 7–9 days. Analysis was carried out using both light and dark field optics with a Zeiss Axiophot microscope.

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References

Akam,M. (1987) *Development*, **101**, 1–22.
Baron,A., Featherstone,M.S., Hill,R.E., Hall,A., Galliot,B. and Duboule,D. (1987) *EMBO J.*, **6**, 2977–2986.
Biggin,M.D., Gibson,T.J. and Hong,G.F. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3963–3965.
Carter,T.C. (1951) *J. Genet.*, **50**, 277–299.
Cho,K.W.Y., Goetz,J., Wright,C.V.E., Fritz,A., Hardwicke,J. and De Robertis,E.M. (1988) *EMBO J.*, **7**, 2139–2149.
Doe,C.Q., Hiromi,Y., Gehring,W.J. and Goodman,C.S. (1988) *Science*, **239**, 170–175.
Dony,C. and Gruss,P. (1987a) *EMBO J.*, **6**, 2965–2975.
Dony,C. and Gruss,P. (1987b) *Nature*, **328**, 711–714.
Dressler,G.R. and Gruss,P. (1988) *Trends Genet.*, **4**, 214–219.
Featherstone,M.S., Baron,A., Gaunt,S.J., Mattei,M. and Duboule,D. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 4760–4764.
Gall,J.G. and Pardue,M.L. (1971) *Methods Enzymol.*, **21**, 470–480.
Gaunt,S.J. (1988) *Development*, **103**, 135–144.
Gehring,W.J. (1987a) *Science*, **236**, 1245–1252.

Gehring,W.J. (1987b) In Firtel,R.A. and Davidson,E.H. (eds), *Molecular Approaches to Developmental Biology*. A.R.Liss, Inc., New York, pp. 115–129.
Gehring,W.J. (1987c) *The Harvey Lectures*, **81**, 153–172.
Gehring,W.J. and Hiromi,Y. (1986) *Annu. Rev. Genet.*, **20**, 147–173.
Graham,A., Papalopulu,N., Lorimer,J., McVey,J.H., Tuddenham,E.G.D. and Krumlauf,R. (1988) *Genes Dev.*, in press.
Green,M.C. (1981) *Genetic variants and strains of the laboratory mouse*. Gustav Fischer Verlag, Stuttgart.
Harvey,R.P. and Melton,D.A. (1988) *Cell*, **53**, 687–697.
Heine,U.I., Munoz,E.F., Flanders,K.C., Ellingsworth,L.R., Lam,H.-Y.P., Thompson,N.L., Roberts,A.B. and Sporn,M.B. (1987) *J. Cell Biol.*, **105**, 2861–2876.
Hoey,T., Doyle,H.J., Harding,K., Wedeen,C. and Levine,M. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 4809–4813.
Holland,P.W.H. and Hogan,B.L.M. (1988a) *Genes Dev.*, **2**, 773–782.
Holland,P.W.H. and Hogan,B.L.M. (1988b) *Development*, **102**, 159–174.
Hornbruch,A. and Wolpert,L. (1970) *Nature*, **226**, 764–766.
Jenkins,N.A., Copeland,N.G., Taylor,B.A. and Lee,B.K. (1982) *J. Virol.*, **43**, 26–36.
Jorquera,B. and Pugin,E. (1971) *Crit. Rev. Acad. Sci. Paris*, **272D**, 1522–1525.
Joyner,A.L. and Martin,G.R. (1987) *Genes Dev.*, **1**, 29–38.
Joyner,A.L., Kornberg,T., Coleman,K.G., Cox,D.R. and Martin,G.R. (1985) *Cell*, **43**, 29–37.
Kinsella,M.G. and Fitzharris,T.P. (1980) *Science*, **207**, 1359–1360.
Knudsen,T.B. and Kochhar,D.M. (1981) *J. Embryol. Exp. Morph.*, **65**, (Suppl.), 289–307.
Laughon,A. and Scott,M.P. (1984) *Nature*, **310**, 25–31.
Le Douarin,N. (1982) *The Neural Crest*. Cambridge University Press, Cambridge.
McGinnis,W., Hart,C.P., Gehring,W.J. and Ruddle,F.H. (1984a) *Cell*, **38**, 675–680.
McGinnis,W., Garber,R.L., Wirz,J., Kuroiwa,A. and Gehring,W. (1984b) *Cell*, **37**, 403–408.
Martin,G.R., Boncinelli,E., Duboule,D., Gruss,P., Jackson,I., Krumlauf,R., Lonai,P., McGinnis,W., Ruddle,F. and Wolgemuth,D. (1987) *Nature*, **325**, 21–22.
Milaire,J. (1987) *Arch. Biol. (Bruxelles)*, **98**, 67–98.
Mlodzik,M., Fjose,A. and Gehring,W. (1988) *EMBO J.*, **7**, 2569–2578.
Nichols,D.H. (1981) *J. Embryol. Exp. Morph.*, **64**, 105–120.
Nüsslein-Volhard,C. and Wieschaus,E. (1980) *Nature*, **287**, 795–801.
Patten,B.M. (1968) The development of the heart. In Gould,S.E. (ed.), *Pathology of the Heart and Blood Vessels*. C.C.Thomas, Springfield, Illinois, pp. 20–90.
Poustka,A., Rackwith,H.-R., Frischauf,A.-M., Hohn,B. and Lehrach,H. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 4129–4133.
Regulski,M., Harding,K., Kostriken,R., Karch,F., Levine,M. and McGinnis,W. (1985) *Cell*, **43**, 71–80.
Regulski,M., McGinnis,N., Chadwick,R. and McGinnis,W. (1987) *EMBO J.*, **6**, 767–777.
Robert,B., Barton,P., Minty,A., Daubas,P., Weydert,A., Bonhomme,F., Catalan,J., Chazottes,D., Guénet,J.-L. and Buckingham,M. (1985) *Nature*, **314**, 181–183.
Rugh,R. (1968) *The Mouse: its Reproduction and Development*, Burgess Publishing Company, Minneapolis.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
Sassoon,D.A., Garner,I. and Buckingham,M. (1988) *Development*, **104**, 155–164.
Saunders,J.W.,Jr (1977) In Ede,D.A., Hinchliffe,J.R. and Balls,M. (eds), *Vertebrate Limb and Somite Morphognesis*. Cambridge University Press, Cambridge, pp. 1–24.
Schneuwly,S., Kuroiwa,A., Baumgartner,P. and Gehring,W.J. (1986) *EMBO J.*, **5**, 733–739.
Schneuwly,S., Klemenz,R. and Gehring,W.J. (1987) *Nature*, **325**, 816–818.
Scott,M.P. and Carroll,S.B. (1987) *Cell*, **51**, 689–698.
Sharpe,P.T., Miller,J.R., Evans,E.P., Burtenshaw,M.D. and Gaunt,S.J. (1988) *Development*, **102**, 397–407.
Shepherd,J.C.W., McGinnis,W., Carrasco,A.E., De Robertis,E.M. and Gehring,W.J. (1984) *Nature*, **310**, 70–71.
Southern,E.M. (1975) *J. Mol. Biol.*, **98**, 503–517.
Stark,R.J. and Searls,R.L. (1973) *Dev. Biol.*, **33**, 138–153.
Stern,C.D. and Keynes,R.J. (1988) *Trends Neurosci.*, **11**, 190–192.
Summerbell,D. and Lewis,J.H. (1975) *J. Embryol. Exp. Morph.*, **33**, 621–643.
Summerbell,D., Lewis,J.H. and Wolpert,L. (1973) *Nature*, **244**, 492–496.
Tan,S.S. and Morris-Kay,G.M. (1986) *J. Embryol. Exp. Morph.*, **98**, 21–58.
Wedden,S.E. (1987) *Development*, **99**, 341–351.
Wedden,S.E., Ralphs,J.R. and Tickle,C. (1988) *Development*, **103**, (Suppl.), 31–40.
Wilkinson,D.G., Bailes,J.A., Champion,J.E. and McMahon,A.P. (1987) *Development*, **99**, 493–500.
Zwilling,E. (1961) *Adv. Morphogen.*, **1**, 301–330.

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