A new family of mouse homeo box-containing genes: molecular structure, chromosomal location, and developmental expression of *Hox-7.1*

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Two families of homeo box-containing genes have been identified in mammals to date, the *Antennapedia*- and *engrailed*-like homeo boxes, based on the sequence similarity to those from *Drosophila*. Here, we report the isolation of a homeo box-containing gene that belongs to a new family of which there are at least three related genes in the mouse genome. The homeo box of this new gene shows remarkable similarity to the *Drosophila Msh* homeo box that we designate as the prototype for this family. The gene maps to the proximal end of mouse chromosome 5 and does not cosegregate with any known homeo box-containing gene. We designate this locus *Hox-7.1*. In situ hybridizations to mouse embryos at different stages show a unique pattern of expression, as compared to other homeo box-containing genes described thus far. *Hox-7.1* transcripts are detected in 9.5-day-old embryos in the neural crest, developing limb bud, and visceral arches. Later, this gene is expressed in regions of the face that are derived from neural crest and in the interdigital mesenchymal tissues in both the fore- and hindlimbs.

[*Key Words*: Mouse homeo box; *Hox-7.1*; cDNA; sequence; expression; chromosomal location]

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A highly conserved sequence common to developmental genes in *Drosophila* has provided a means to identify genes that may be involved in controlling mammalian development. This conserved 180-bp stretch of DNA, the homeo box, was originally identified in *Drosophila* developmental genes involved in homeotic mutations (McGinnis et al. 1984a,b; Scott and Weiner 1984) and subsequently found in other developmental control genes such as segmentation genes (Scott and Weiner 1984; Poole et al. 1985; Macdonald et al. 1986) and maternal genes (Mlodzik et al. 1985; Frigerio et al. 1986). The interspecific conservation of this DNA domain is evidenced by its presence in a wide range of metazoans including vertebrates. *Drosophila* homeo box sequences have been used to isolate homeo box-containing genes from vertebrates, including *Xenopus* (Carrasco et al. 1984; Muller et al. 1984; Harvey et al. 1986), human (Levine et al. 1984; Boncinelli et al. 1985; Simeone et al. 1986; Mavilio et al. 1986), and mouse (see Holland and Hogan 1988).

Several lines of evidence suggest that the *Drosophila* homeo box genes encode regulatory proteins which, by sequence specific recognition, bind DNA. The homeo box domain of the protein product has structural similarity to the helix-turn-helix DNA-binding domain of some regulatory proteins in yeast and prokaryotes (Shepherd et al. 1984; Laughon et al. 1985). Homeo box-containing proteins have been localized intracellularly to the nucleus (White and Wilcox 1984; Beachey et al. 1985; Carroll and Scott 1985; DiNardo et al. 1985) and have been shown to exhibit DNA-binding activity in vitro (Desplan et al. 1985). Furthermore, genetic experiments in *Drosophila* suggest that some of these proteins exhibit a regulatory role by controlling the expression of other developmental genes (see Akam 1987; Ingham 1988).

The homeo boxes in *Drosophila* can be divided into three families based on sequence characteristics of which *Antennapedia* (*Antp*), *engrailed* (*en*), and *paired* are the prototypes (Gehring 1987a). Homeo box sequences can be similarly classified in mammals. The majority of mammalian genes containing homeo boxes are most closely related to the *Antp* family. These genes, of which >18 have been identified, are situated in three major clusters in the mouse genome localized on chromosome 6 (*Hox-1*) (Colberg-Poley et al. 1985; Bucan et

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al. 1986; Baron et al. 1987), 11 (*Hox-2*) (Hart et al. 1985; Joyner et al. 1985; Rabin et al. 1985; Munke et al. 1986; Meijlink et al. 1987), and 15 (*Hox-3*) (Awgulewitsch et al. 1986; Rabin et al. 1986; Breier et al. 1988), with several others scattered throughout the genome (*Hox-4*, *Hox-5*, and *Hox-6*) (Lonai et al. 1987; Featherstone et al. 1988; Sharpe et al. 1988). Two unlinked genes that map to chromosomes 1 and 5 in the mouse contain homeo boxes homologous to the *Drosophila en* family (Hill et al. 1987; Joyner and Martin 1987). These genes display additional sequence similarity to the *Drosophila* genes in adjacent regions outside the homeo box. There has been no report of any *paired*-type homeo box in mouse.

Another *Drosophila* homeo box that has diverged quite significantly from members of these three families has recently been isolated. This single gene has been termed *Msh* (for muscle segment homeo box) or 99B (its chromosomal mapping position) (Gehring 1987b; B. Jacq, pers. comm.). Here, we report the identification of a mouse gene that contains a homeo box most similar to that of *Msh*. There are at least three related genes in the mouse genome; therefore, these represent a fourth family of homeo boxes, which we have designated the *Msh* family. The mouse gene, which is termed *Hox-7.1*, maps to the proximal region of chromosome 5, 2–3 cM distal to the mouse *En-2* gene (*en*-like homeo box), but more closely linked (within 1 cM) to the mouse homolog of D4S43, a human probe closely linked to Huntington's disease. This establishes the mouse gene as a new homeo box locus. In situ analysis of expression shows that this gene has a unique pattern of expression among the homeo box genes studied thus far.

Results

Isolation of a homeo box-containing cDNA

We screened a λgt10 cDNA library prepared from mouse 8.5-day embryos (Fahrner et al. 1987) to isolate genes containing the homeo box domain. Hybridization conditions of reduced stringency were used in an attempt to select genes that have diverged significantly from the *Drosophila Antp* family of homeo boxes. Previously, using the *Drosophila ftz* gene (a member of the *Antp* family), we isolated a divergent cDNA that we showed was derived from the mouse *Hox-1.6* gene (Baron et al. 1987). This gene contains a homeo box that shows ~65% similarity to the *Antp* and *ftz* homeo boxes. In this study, we comparatively screened the above library with the *Hox-1.6* cDNA and the *ftz* gene and selected clones that hybridized with low intensity to *Hox-1.6* and undetectably to the *Drosophila ftz* gene. A λgt10 clone was isolated containing a 1.5-kb insert that was subcloned into a plasmid vector and is referred to as pMHλ2.

Northern analysis of RNAs from a series of embryonic stages showed that the transcript, detected by the pMHλ2 probe, was expressed in embryonic RNA isolated from the earliest stage of 9-day post coitum, with an apparent decrease at later stages (Fig. 1). A single mRNA species was detected that migrates at the position of the 18S rRNA, suggesting that the size of the mRNA is ~2–2.2 kb. To obtain a full-length cDNA, subcloned fragments from the 5′ end of the cDNA were used to rescreen the λgt10 cDNA library, and a 1.8-kb cDNA was isolated. Taking into account a 100- to 150-nucleotide contribution by the poly(A) region to the mRNA length, this cDNA may approximate the size of the full-length mRNA.

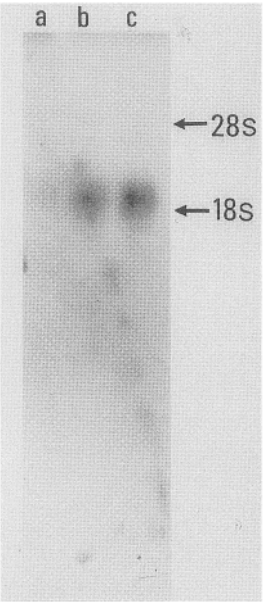

Figure 1. Northern blot analysis of *Hox-7.1* mRNA from mouse embryos at various stages. Total mRNA isolated from 14.5- (lane *a*), 11.5- (lane *b*), and 9-day (lane *c*) mouse embryos.

Primary structure of the homeo box-containing gene

The nucleotide sequence of the 1.8-kb cDNA is presented in Figure 2. A long open reading frame is encoded by this cDNA, which is in frame with the predicted homeo box domain. There are three methionine codons in this frame toward the 5′end of the cDNA, which we have considered as possible translation initiation sites. The first ATG (five codons upstream of the predicted open reading frame in Fig. 2) is in an unfavorable sequence context for a translational start site (Kozak 1987). The other two (underlined in Fig. 2) are in a more favorable context for the Kozak consensus sequence. In most cases, the first ATG that conforms to Kozak's rules is the predominant initiation site (Kozak 1987); therefore we have deduced the protein sequence from this site. One other ATG, found in the 5′-untranslated region of the cDNA (starting at nucleotide 74), that is not in frame with the homeo box would code for a short polypeptide of 24 amino acids. The significance of this short open reading frame is unknown; however, the presence of upstream ATG codons has been reported in other homeo box genes (Breier et al. 1988), and approximately two thirds of the known proto-oncogenes contain short 5′ open reading frames (Kozak 1987).

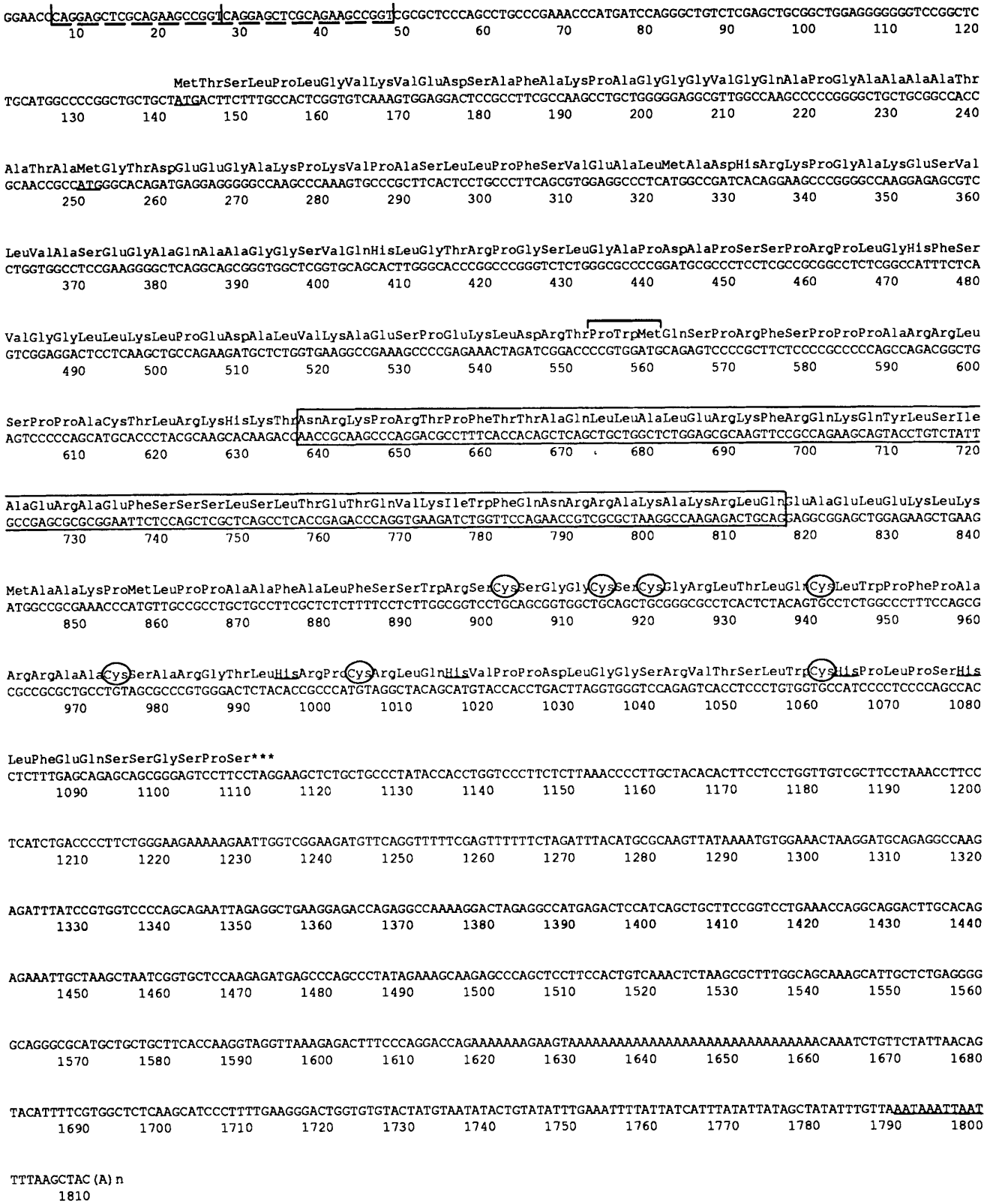

Figure 2. Nucleotide sequence of the *Hox-7.1* 1.8-kb cDNA and deduced protein structure. The boxed 180 nucleotides starting at position 637 represent the homeo box domain. Upstream ATG codons that are underlined best fit the translation start site concensus sequence (Kozak 1987), and the underlined amino acids are the only three that conform to the conserved hexapeptide found in most homeo box-containing genes. The direct perfect 21-bp repeat at the very 5′ end (starting at position 7) is indicated by dashed lines, and the putative overlapping poly(A) addition sites are underlined at the 3′ end of the cDNA.

The predicted protein will contain 324 amino acids (m.w. 34,500). The coding region of the putative homeo box domain starts at nucleotide 637 of the cDNA (boxed region of Fig. 2). The stretch of protein to the carboxy-terminal side of the homeo box is longer than that of other mammalian homeo box-containing genes reported to date. It is of particular interest that there is a high density of cysteine residues in this region of the protein (7 within a stretch of 54 amino acids), whereas only 1 other is found outside this region. This suggests that this region is constrained in a taut tertiary configuration involving disulfide bridges. Alternatively, these cysteine residues may be involved in intermolecular interactions with other proteins or in stabilizing binding to DNA. Histidines are also found in this region; however, this structure does not conform to the metal binding finger motif (Klug and Rhodes 1987; Evans and Hollenberg 1988).

Most homeo box-containing genes have a conserved hexapeptide sequence of Ile/Val-Tyr-Pro-Trp-Met-Arg, a short distance upstream of the homeo box (Mavilio et al. 1986; Kessel et al. 1987). However, this putative protein sequence contains only three of these amino acids (Pro-Trp-Met), starting 28 amino acids upstream of the homeo box (overlined in Fig. 2). Deviation from this consensus has also been reported for the *Hox-1.6* gene, in which only two of the hexapeptide amino acids are present (Baron et al. 1987).

Two other features in the cDNA are noted. In the 5′-untranslated region starting at position 7 (indicated in Fig. 2 by a dashed line) are two copies of a direct perfect 21-bp repeat. The function, if any, of these repeats may be of interest when determining the elements that regulate the expression of this gene. Using a matrix search program[1] (LUPES program made available by the SERC Protein Engineering Club), an 11-amino-acid sequence (starting with Pro at position 580) was found to have a high degree of resemblance (>80%) to a consensus matrix for a cAMP-dependent protein kinase phosphorylation site (S. Cox and A.R. Rees, unpubl.). This is particularly interesting in light of the data suggesting that *Drosophila en* is phosphorylated (Gay et al. 1988).

The putative homeo box sequence shows 57% nucleotide similarity to mouse *Hox-1.6* (the sequence used to isolate this gene), <50% nucleotide similarity to either *Antp* or *ftz*, and 55% similarity to the mouse and *Drosophila en*-like genes. Because this sequence differs considerably from other known homeo boxes and shows approximately the same low degree of similarity to both *Antp*-type and *en*-type homeo boxes this sequence cannot be considered to fit into either family. Recently, a new *Drosophila* homeo box sequence has been reported that is expressed in muscle segments and is located on *Drosophila* chromosome 3 near the tip of the right arm (at position 99B) (Gehring 1987b). This homeo box, termed *Msh*, shows 80% similarity to that of the mouse gene at the nucleotide level (data not shown) and 90% at the protein level (Fig. 3) (*Msh* protein sequence communicated by B. Jacq; also, B.D. Robert et al., in prep.). This homology extends outside the homeo box for 11 amino acids at the 3′ end. Unfortunately, very little sequence information is available for the region 5′ of the *Drosophila* homeo box. For the seven amino acids known, five are in common. Therefore, in view of the similarities between the mouse and *Drosophila* genes in and around the homeo box and the differences that exist with other known homeo box-containing genes, the *Drosophila Msh* and the mouse gene designate a new family of homeo box sequences. Because, as shown below, this gene is not closely linked to any of the known mouse homeo box-containing clusters (*Hox-1*, *Hox-2*, or *Hox-3*), this gene can be given a new *Hox* designation in agreement with previous mouse nomenclature (Martin et al. 1987). Therefore, we refer to this gene as *Hox-7.1*.

Multiple Msh*-like genes in the mouse genome*

In a rescreen of the 8.5-day embryonic λgt10 cDNA library, it became evident that there is at least one other related gene encoded by the mouse genome. Using the *Hox-7.1* clone, pMHλ2, as probe, a related but distinctly different cDNA was isolated. Preliminary analysis of this second *Msh*-like gene revealed a homeo box domain with ~85% nucleotide similarity to that of the *Hox-7.1* gene (R. Hill, unpubl.). To determine whether there are any other related mouse genes, we have screened total mouse DNA by Southern blotting. Southern analysis of mouse DNA with this *Hox-7.1*-related cDNA detects a single restriction fragment that hybridizes to yield a

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        -7                -1 +1

Hox-7   ThrLeuArgLysHisLysThrAsnArgLysProArgThrProPheThrThrAlaGlnLeuLeu
Msh     Asn - - - - - Pro - - - - - - - - - - - Gln - - -

        15
        AlaLeuGluArgLysPheArgGlnLysGlnTyrLeuSerIleAlaGluArgAlaGluPheSer
        Ser - - Lys - - - Glu - - - - - - - - - - - Thr -

        36
        SerSerLeuSerLeuThrGluThrGlnValLysIleTrpPheGlnAsnArgArgAlaLysAla
         - - - Arg - - - - - - - - - - - - - - - - - -

        57      60
        LysArgLeuGlnGluAlaGluLeuGluLysLeuLysMetAlaAlaLysProMetLeuProPro
         - - - -  - - - Ile - - Ile - - - - LeuGlyArgGlyAla -

        78
        AlaAlaPheAlaLeuPheSerSer...
        GlyAlaGlnTrpAlaMetAlaGly...
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Figure 3. Comparison of the homeo box and surrounding regions of the *Drosophila Msh* and the mouse *Hox-7.1* genes. The homeo box starts at position 1 and ends at 60. Only the amino acids of *Drosophila Msh* that differ from those of *Hox-7.1* are shown.

dark signal and several less intensely hybridizing fragments, depending on the restriction endonucleases used (Fig. 4). From cosmid analysis of this gene, we know that the three restriction endonucleases used in this analysis do not cleave within the hybridizing region of the *Hox-7.1*-related gene. These minor fragments are therefore interpreted as representing other related genes in the genome. The *Hox-7.1* homeo box-containing restriction fragments migrate with a molecular weight of ~7.2 kb (*Bam*HI digestion), 5.5 kb (*Pst*I digestion), and 0.25 kb (*Pvu*II digestion). The *Bam*HI (lanes 1–3) and *Pst*I (lanes 7–9) digestions each generate a minor fragment with a molecular weight similar to the *Hox-7.1* restriction fragment (indicated in Fig. 4) and at least one other. In this analysis, the 0.25-kb *Pvu*II fragment ran off the gel; however, this enzyme does generate one other detectable restriction fragment. These results imply that the mouse genome contains multiple related genes; the *Hox-7.1* gene, the gene identified using the *Hox-7.1*-related cDNA, and at least one other (indicated in Fig. 4).

Chromosomal location of Hox-7.1

The chromosomal location of *Hox-7.1* was determined using an interspecific mouse backcross mapping panel derived by crossing C57BL/6J and *Mus spretus*. This mapping panel has been typed for >220 distinct loci distributed over most mouse chromosomes. Digestion of C57BL/6J and *M. spretus* DNAs with *Pvu*II and hybridization with the *Hox-7.1* probe identified the restriction-fragment-length polymorphisms (RFLP) listed in Table 1. The RFLPs were used to follow the segregation of *Hox-7.1* in 204 interspecific backcross mice. The mapping results indicated that *Hox-7.1* is located on mouse chromosome 5 (Fig. 5). The relative gene order of *Hox-7.1*, with respect to six other chromosome 5 markers that have been typed in the interspecific backcross mice (Table 1), was determined by minimizing the number of double recombinants between loci (Fig. 5). *Gus* and *Alb* were used as anchor loci. The distance between loci and their relative order proceeding distally from the most proximal marker is as follows: *En-2*—2.5 ± 1.1 cM—*Il-6*—2.5 ± 1.1 cM—*D4S43*—1.0 ± 0.7 cM—*Hox-7.1*—19.1 ± 2.8 cM—*Kit*—5.9 ± 1.6 cM—*Alb*—20.6 ± 2.8 cM—*Gus*. The results of this analysis place *Hox-7.1* between *D4S43* and *Kit* on chromosome 5. *Hox-7.1* does not map to any other homeo box-containing locus already mapped in mouse chromosomes, indicating that it is not part of a previously defined homeo box gene cluster.

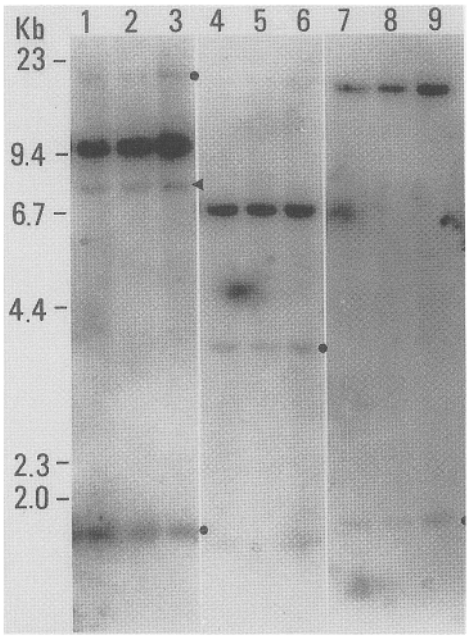

Figure 4. Southern analysis of the mouse genome using a *Hox-7.1*-related cDNA as probe. Mouse DNAs used were from C57BL/6 (lanes *1*, *4*, and *7*), an outbred strain of mice (lanes *3*, *6*, and *9*), and the F_1 progeny from the two strains (lanes *2*, *5*, and *8*). DNAs were digested with *Bam*HI (lanes *1–3*), *Pvu*II (lanes *4–6*), and *Pst*I (lanes *7–9*). The predicted positions of the *Hox-7.1* homeo box-containing restriction fragments are indicated by arrowheads, and the putative additional related genes are indicated by dots.

Distribution of Hox-7.1 *expression in the mouse embryo*

We have examined, by *in situ* hybridization, the expression of *Hox-7.1* in six mouse embryos between 9.5 and 13.5 days post coitum. Here, we describe the main features of the results. A more detailed analysis of the expression of this and other genes in this family will be published separately.

Sections of embryos at 9.5, 10.5, and 11.5 days of development were hybridized with probe 1 (the 3′ *Bgl*II fragment of pMHλ2; see Fig. 6). Labeling was observed in three main regions: the neural crests, a subset of tissues derived from the neural crests (visceral arches and craniofacial structures), and the developing limb buds. In each of these tissues, expression was restricted to specific regions that did not have any histologically detectable morphological boundaries. The developing neural tube showed intense expression in the neural crests before and after fusion; less intense labeling extended over the dorsal half of the closed tube (Fig. 7A,B). In 9.5-day embryos, the frontal mesenchyme of the head was intensely labeled (data not shown): by 11.5 days, labeling in this region was confined to a thin layer of cells lying on the dorsal surface of the forebrain. Isolated cells in the dorsal and lateral regions of the developing integument (both in the mesechyme and epithelium) showed expression. Restricted parts of the visceral arches were labeled (Fig. 7C,D). The developing facial connective tissue showed intense expression in the prospective maxillary region. A restricted pattern of labeling was also observed in the fore- and hindlimb buds, with peak expression in the distal and posterior mesenchyme (Fig. 7E,F). At the resolution afforded by ^{35}S, it was not possible to determine whether expression was confined to the mesenchyme or whether parts of the epithelium

Table 1. *Chromosome 5 loci mapped in interspecific backcross mice*

Locus	Gene name	Probe	Enzyme	C57BL/6J fragment sizes (kb)	*M. spretus* fragment sizes (kb)[a]	Reference
En-2	*Engrailed-2*	mp2	*Pvu*II	5.3, 1.5	4.5, 2.4	Joyner and Martin (1987); B.A. Mock et al. (in prep.)
Il-6	*Interleukin-6*	Il-6	*Bam*HI	10.2, 5.2	10.2, 4.9	B.A. Mock et al. (in prep.)
D4S43	a sequence tightly linked to Huntington's disease in humans	C9AHB1.4	*Pvu*II	19.0	13.2	Gilliam et al. (1987); S.V. Cheng et al. (in prep.)
Hox-7.1	*Homeo box locus-7.1*	pMHλ2	*Pvu*II	6.0, 0.6	6.5, 1.0	
Kit	*Kit* proto-oncogene	phckit-171	*Bam*HI	17.8, 2.0	16.9, 2.0	Yarden et al. (1987)
Alb	*Albumin*	AlbH	*Pst*I	5.1	9.4	B.A. Mock et al. (in prep.)
Gus	β-*glucuronidase*	pGus-1	*Bam*HI	16.5, 9.1, 1.9	22.1, 9.1, 3.7	Palmer et al. (1983); B.A. Mock et al. (in prep.)

[a] Underlined restriction fragment sizes indicate the restriction fragments typed in the analysis.

were also labeled. Where the thickened apical ectodermal ridge was observed, it was labeled to a markedly lesser degree than the adjacent mesenchyme. In the present analysis, we have observed no expression in other organs except in red blood cells (RBC). The latter labeling may, however, be artifactual because these cells also labeled with the control probe and because nonspecific binding of other probes has been described (Davidson et al. 1988).

Probe 1, which represents the 3′ half of the pMHλ2 cDNA, contains a short stretch of the homeo box sequence, 10% of the length of the probe (Fig. 6), and may therefore not be specific for the *Hox-7.1* gene product. This raises the possibility that part of the pattern of labeling may reflect the expression of one of the other members of this gene family. To avoid this ambiguity, we investigated the expression of *Hox-7.1* with a specific probe in a series of experiments on 13.5-day embryo. This probe (probe 2, see Fig. 6) comprises the 3′ *Bss*HII fragment and contains only material 3′ to the homeo box, 80% of which is in the 3′-untranslated region. The specificity of this probe is supported by Southern analysis and by the different patterns of labeling obtained with this probe and one derived from the *Hox-7.1*-related cDNA described above (R. Hill, unpubl.).

The results of our experiments using probe 2 are consistent with the patterns found earlier in development using probe 1 and show additional points of interest. The major areas of labeling were in craniofacial structures (the developing maxillary, mandibular, and nasal structures processes) and in the interdigital soft tissue in the fore- and hindlimbs (Fig. 8). We have not detected expression in any other organs (except in RBCs), though we have not examined transverse sections that might reveal limited regions of expression, e.g., in the neural tube. A control probe comprising the sense strand complementary to probe 1 showed no specific patterns of labeling in sections adjacent to those described, except in regions abundant in RBCs.

Discussion

We have described the isolation of a homeo box-containing gene in mouse that is a member of a new homeo

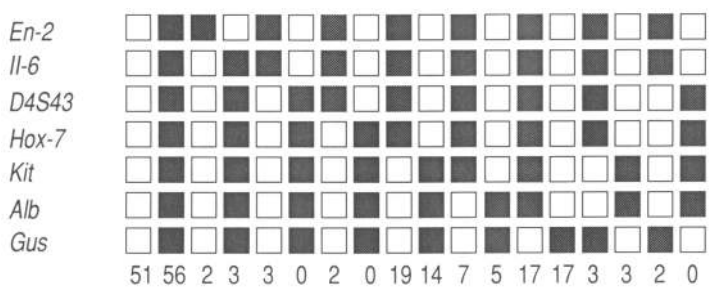

Figure 5. Summary of the results of the interspecific backcross analysis. (*Left*) Genes mapped in the analysis. Each column represents the chromosome identified in the N_2 progeny that was inherited from the (C57BL/6J × *M. spretus*) F_1 parent. The black boxes represent the presence of a C57BL/6J allele, and the white boxes represent the presence of a *M. spretus* allele. (*Bottom*) The number of each type of chromosome identified. The frequency of C57BL/6J and *M. spretus* alleles for all of the chromosome 5 loci examined in the N_2 progeny did not differ significantly from the 50 : 50 ratio expected if each allele was transmitted in a normal Mendelian fashion from the F_1 parents to the N_2 offspring. Eight double recombinants were observed, which is not significantly different from the expected number of double recombinants.

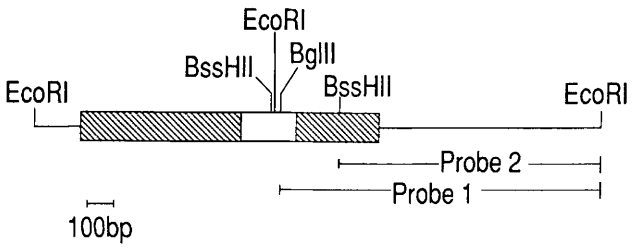

Figure 6. Diagram of the *Hox-7.1* (pMHλ2) cDNA and probes used for in situ hybridization. The hatched box represents the coding region, and the open box represents the homeo box. Regions of the cDNA used as probe for in situ are underlined.

box family. In this family, there are at least three related members in the mouse and one known in *Drosophila* called *Msh*. The comparison of the homeo box domain of the *Drosophila* gene and the newly isolated mouse gene called *Hox-7.1* shows that these two genes share 90% amino acid similarity. The homology also extends outside the homeo box, which is reminiscent of the comparison of the mouse and *Drosophila en*-like genes (Joyner and Martin 1987).

The degree of sequence similarity that exists between the *Hox-7.1* and *Drosophila Msh* genes indicates that the homeo box and surrounding regions existed before the branching of the evolutionary tree that resulted in the arthropod and vertebrate lineages. This interpretation poses the question, What has been responsible for the evolutionary conservation of this protein domain? In response to this question, we must consider that these evolutionary constraints reflect important functional properties of this protein domain. It has been argued that the predicted recognition helix of the helix-turn-helix domain (amino acids 42–50 in Fig. 3) determines the specificity of DNA binding in this domain (see Gehring 1987a). The fact that this region is highly conserved among the known homeo boxes (with the exception of

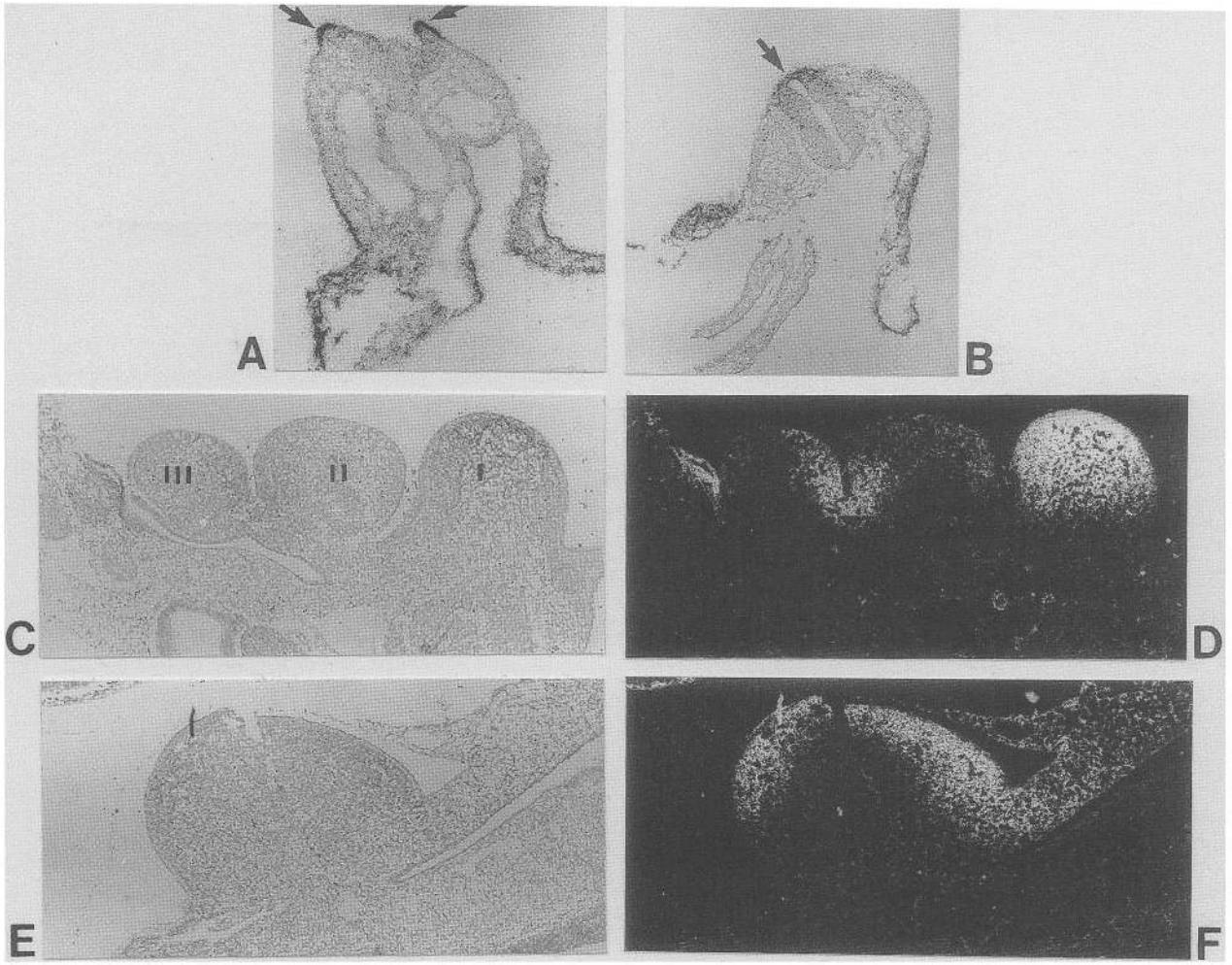

Figure 7. The pattern of expression of *Hox-7*$_{Msh}$ in 9.5-day mouse embryos. Transverse sections showing labeled neural crests, before (*A*) and after (*B*) closure of the neural tube (arrows). Note also labeling, ventrally, in the lateral mesoderm. Parasagittal section of visceral arches in bright-field (*C*) and dark-field (*D*) illumination showing mesenchyme of the visceral arches labeled in discrete regions (arch I, cleft between II and III, putative position of arch IV). Parasagittal section of forelimb bud in bright-field (*E*) and dark-field (*F*) illumination showing labeling in the distal and posterior parts of the limb bud and in the lateral mesoderm. Posterior is to the right. All sections treated with probe 1. Magnifications: (*A,B*) 80×; (*C–F*) 50×.

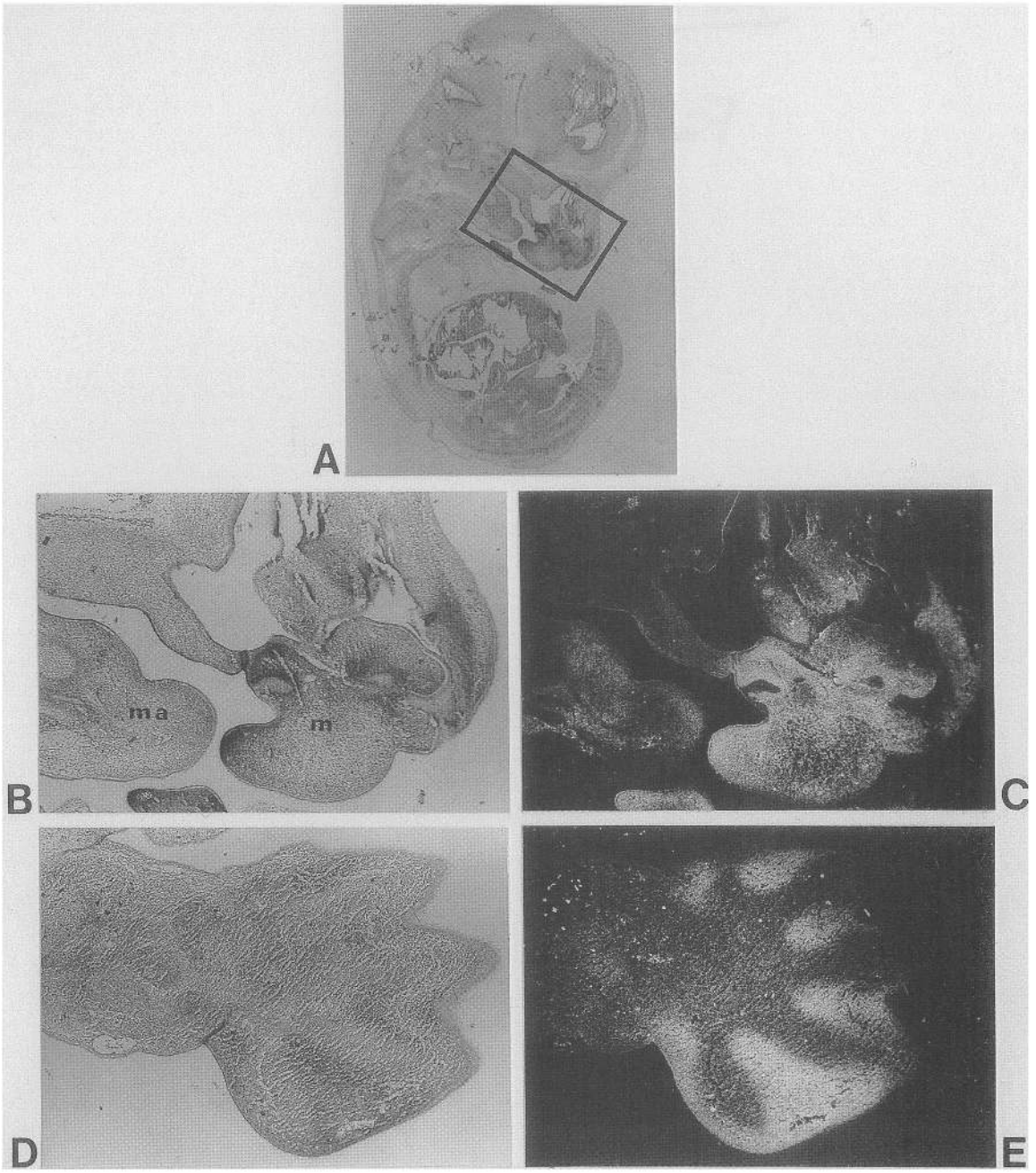

Figure 8. The pattern of expression of *Hox-7.1*$_{Msh}$ in parasagittal sections of 13.5-day mouse embryos. (*A*) Whole section showing the location of photographs in *B* and *C*. Midfacial region in bright-field (*B*) and dark-field (*C*) illumination, illustrating labeling in the position of the developing maxillary (m) and mandibular (ma) processes. Section though the developing foot plate of the hindlimb in bright-field (*D*) and dark-field (*E*) illumination, showing labeling in the interdigital soft mesenchymal tissue. All sections treated with probe 2. Magnifications: (*A*) 9×; (*B–E*) 32×.

the *paired* family) leads to the prediction that the recognition helix domains of the various genes bind to similar or identical sequences. However, at least in *Drosophila*, these genes perform highly specific functions during development. Therefore, it is possible that more than just the putative recognition helix is required in DNA binding and that the remainder of the domain may be involved in determining sequence binding specificity. This need not imply that the regions around the helix-turn-helix bind DNA directly but, rather, that the remainder of the homeo box may participate in protein–protein interactions that would provide the specificity of function needed during embryogenesis. On the basis of the conservation of the homeo box region, we can speculate that these proteins in mouse and *Drosophila*, at least, share some very similar properties inside the cell.

The *Hox-7.1* locus has been assigned to mouse chromosome 5. We have identified at least two other

members of this family. The genomic organization of these related genes may be similar to that found for the mouse *Antp*-like genes that are known to reside in several tightly linked multigenic clusters. Alternatively, these genes may be located at different positions, as are the two *en*-like genes (Joyner and Martin 1987). It is of interest that the mouse mutation luxate (*lx*) maps within the region of the *Hox-7.1* locus. The phenotype of this mutation involves polydactyly and oligodactyly of the hind feet, as well as other bone abnormalities of the hindquarters (Green 1981). The coincidence of the *lx* phenotype with our observations of *Hox-7.1* expression might suggest a direct relationship between this homeo box-containing gene and the mutation. This seems unlikely, because the *Hox-7.1* expression pattern is more extensive; however, further analysis of this relationship is warranted.

Hox-7.1 maps 1.0 cM distal to *D4S43* and 19 cM proximal to *Kit* on mouse chromosome 5. *D4S43* is a polymorphic locus identified in humans that is closely linked to the Huntington's disease gene (*HD*) on human chromosome 4p (Gilliam et al. 1987; S.V. Cheng et al., in prep.). *Kit* is a proto-oncogene that maps to human chromosome 4, band q11–q21 (Yarden et al. 1987). These mapping results suggest that *Hox-7.1* is part of a large conserved linkage group involving mouse chromosome 5 and human chromosome 4. In addition to *D4S43*, four other DNA markers located near *HD* on human chromosome 4p have mapped to mouse chromosome 5 (S.V. Cheng et al., in prep.). The relative order of these five loci is conserved in mouse and human chromosomes. *D4S43* is the most proximal marker of the five loci localized on mouse chromosome 5. These results strongly suggest that *Hox-7.1* is located on human chromosome 4p, just distal to *D4S43*. In humans, *D4S43* maps 1 cM proximal to *HD*. This localization would further suggest that *HD* and *Hox-7.1* lie on opposite sides of *D4S43*.

The putative DNA-binding function of homeo box gene products, their role in spatial patterning in *Drosophila*, and the evolutionary conservation of the homeo box motif suggest that these genes play key roles in controlling transcription during the development of complex tissue patterns in the mammalian embryo. In support of this view, homeo box gene expression in the mouse is generally restricted to specific domains in morphogenetically active tissues (for reviews, see Holland and Hogan 1988; Dressler and Gruss 1988). We have noted elsewhere that homeo box genes may play a role in establishing or maintaining the spatial integrity of tissues during complex cellular rearrangements, the vertebrate counterpart of early compartmentalization in the *Drosophila* embryo (Davidson et al. 1988). The patterns of expression of *Hox-7.1* in the limb and neural crest derivatives are consistent with this idea. More importantly, however, they may provide an opportunity to critically examine it. Unlike the sites of expression of other homeo box genes, the limb and neural crest have been used extensively to study fundamental questions of pattern formation by applying classical embryological techniques. Further studies of *Hox-7.1* expression in combination with classical experiments may thus provide particular insights into the roles of homeo box genes in mammalian development.

One opportunity for such an analysis is suggested by the pattern of *Hox-7.1* expression relatively late in the development of the limb. *Hox-7.1* activity is restricted to the interdigital soft tissue in a pattern that matches that of cell death expected in the mouse limb on the basis of comparison with the chick (Saunders and Fallon 1966; Fallon and Saunders 1968). In addition to its involvement in remodeling the foot plate, cell death occurs in the posterior part of the chick wing bud. Our analysis of the mouse forelimb bud suggests that *Hox-7.1* is also expressed in this region, though expression extends through the lateral mesoderm. Fallon and Saunders (1968) showed that death in this posterior necrotic zone is preprogrammed and occurs on schedule in tissue maintained in culture. Rescue can be effected by coculture with tissue from different parts of the wing bud. The experimental possibilities of this in vitro assay for cell programming could, perhaps, be exploited to investigate a possible causal relation between *Hox-7.1* expression and the maintainance of the program for cell death.

Our results suggest that earlier in limb development, the distal and posterior mesenchyme are sites of peak *Hox-7.1* expression. Both of these regions, the former in conjunction with the thickened ridge of apical ectoderm, have been shown to play crucial roles in controlling the patterns of cartilage elements during the development of the chick limb bud at stages similar to those examined here (Wolpert et al. 1975). Indeed, pattern-determining activity has been demonstrated in tissue from the posterior parts of the fore- and hindlimbs of 10-day-old mice grafted to the anterior region of chick wing buds (Tickle et al. 1976). It will be of particular interest to examine the possibility that *Hox-7.1* is involved in the specification or maintainence of positional information in the limb by investigating the pattern of expression of the gene at successive stages and the effects of classical limb bud deletions and heterotopic grafts on this pattern.

Our results indicate *Hox-7.1* activity in the neural crests in the posterior trunk and tail and in a subset of the midfacial and visceral arch structures that are derived from the cranial neural crests. Heterotopic transplantation experiments in amphibia and birds have shown that region-specific information, acquired by prospective cranial crest cells in the neural plate, later governs the shapes of the facial bones that they form (see Noden 1988). The pattern of *Hox-7.1* expression suggests that the gene could play a part in maintaining the regional specificity of spatial pattern information in the crest cells during their migration between the neural plate and the site of morphogenesis.

How might homeo box genes exert their putative influence on pattern formation? One intriguing possibility is suggested by the partial correlation between *Hox-7.1* expression and the distribution of the proto-oncogene *int-1* in the neural tube (Wilkinson et al. 1987) and the growth factor TGF-β_1 in the limb and in structures de-

rived from the cranial neural crest (Heine et al. 1987).

Although these results raise interesting possibilities, we have, at present, only circumstantial evidence for a causal relation between *Hox-7.1* expression and pattern formation. Morevover, we have assumed that mRNA concentration, as indicated by in situ hybridization, reflects gene function. Immunohistochemical assays for the protein product and functional assays, perhaps based on genetic manipulation of the *Hox-7.1* locus, will be required to test these assumptions.

Materials and methods

Screening of the cDNA library

Between 10^5 and 10^6 plaques from a λgt10 library prepared from 8.5-day mouse embryonic mRNA (Fahrner et al. 1987) were used for each screen. The standard Benton and Davis (1977) procedure was used to prepare nitrocellulose replicas from each plate. Hybridizations were performed in 5× SSC, 10% dextran sulfate, 4× Denhardt's solution, 0.2% SDS, and 0.2% sodium pyrophosphate. 'High' stringency hybridizations were at 68°C, and 'low' stringency hybridizations were at 60°C. Usually, dextran sulfate was omitted at low stringency to reduce background. All probes used for screening were isolated from the plasmid sequence by restriction digestion to release the insert, followed by extraction of the DNA fragment from agarose after gel electrophoresis by passage through Elu-tips (Schleicher and Schuell), according to the manufacturer's description. All probes used for screening were nick translated (Rigby et al. 1977). The *Drosophila ftz* cDNA was kindly provided by Dr. Walter Gehring.

DNA sequencing

The inserts from the isolated λgt10 clones of interest were subcloned into pTZ18U or pTZ19R (Mead et al. 1986). For sequencing, fragments were generated from isolated insert fragments (isolated as above) by restriction digestion or sonication and randomly subcloned into M13, mp18, or mp19 vectors. These single-stranded DNAs were sequenced by the dideoxy chain-termination method (Sanger et al. 1977) using Sequenase (USB, Inc.), according to the manufacturer's directions.

DNA and RNA extractions and blotting analysis

Extraction of RNA and Northern blot analysis were as described by Meehan et al. (1984). Extraction of DNA was from mouse liver. The extraction procedure and Southern blotting were as described by Hill et al. (1985).

Mapping in interspecific backcrosses

A total of 204 progeny from an interspecific backcross between (C57BL/6J × *M. spretus*) F_1 females and C57BL/6J males were analyzed for each of the seven probes described in Table 1. The backcross mice were generated at the NCI—Frederick Cancer Research Facility. High-molecular-weight genomic DNAs were prepared from frozen mouse tissue (Jenkins et al. 1982). Restriction endonuclease digestion, agarose gel electrophoresis, Southern blot transfer, and hybridizations were performed as described (Jenkins et al. 1982). The mp2, Il-6, AlbH, and pGus-1 probes were washed as described (B.A. Mock et al., in prep.). The C9AHB1.4, phckit-171, and pMHλ2 probes were washed three times in 1× SSCP and 0.1% SDS for 30 min per wash.

Statistical analysis of the recombination frequencies from the interspecific backcrosses was performed by calculating the maximum likelihood estimates of linkage parameters, as described by Green (1981), using the computer program 'Spretus Madness' [developed by D. Dave and A.M. Buchberg, NCI—Frederick Cancer Research Facility (Frederick, Maryland)].

In situ hybridization analysis

In situ hybridizations were performed on sections from mouse embryos derived from Swiss matings at 9.5, 10.5, 11.5, and 13.5 days. The cDNA probes were the restriction fragments of the pMHλ2 clone indicated in Figure 6, subcloned into the vector pTZ18U in front of the T_7 RNA promoter (Mead et al. 1986). Synthesis of the ^{35}S-labeled RNA probes and the in situ technique were performed as described previously (Davidson et al. 1988).

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