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A yeast artificial chromosome containing the mouse homeobox cluster *Hox-2*

(cloning/*Hox-2.8*/*Hox-2.9*/amino acid sequence comparison)

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ABSTRACT **We have isolated two genes, *Hox-2.8* and *Hox-2.9*, from the mouse homeobox cluster *Hox-2*, located on chromosome 11. A 120-kilobase yeast artificial chromosome (YAC) containing a large region of the murine *Hox-2* cluster, including 45 kilobases of sequence upstream of the most 5′ gene, was cloned. The DNA sequence of the YAC is unrearranged relative to the genomic map. We have subcloned from the YAC insert a homeobox gene, *Hox-2.8*, whose homeodomain is highly related to that of the *Drosophila* homeotic gene proboscopedia (*pb*). The expression pattern of *Hox-2.8* during embryogenesis extends the trend established by genes from *Hox-2.5* to *-2.7* of successively anterior domains of expression in the neural tube. We have also subcloned and sequenced from a cosmid the labial (*lab*)-related *Hox-2.9*, the most 3′ member of the cluster to date. These data lend further support to the idea of a common evolutionary origin of the mouse *Hox* and *Drosophila HOM* clusters. The YAC will enable us to construct modified forms of the *Hox-2* cluster in yeast and to identify their effect on the phenotype of the animal in transgenic mouse strains.**

Drosophila genes containing a homeobox have been implicated in the control of embryogenesis and establishment of the basic spatial organization of the body (1–4). The homeobox, a 180-base-pair (bp) sequence encoding a DNA-binding domain, is conserved in organisms as evolutionarily diverse as insects and mammals (5, 6). In *Drosophila*, homeobox genes in the Antennapedia complex (ANT-C) and Bithorax complex (BX-C) are arranged in clusters on chromosome 3 (7, 8). The physical order of the genes within each cluster corresponds to the order of their domains of expression along the anterio-posterior (A-P) axis of the embryo (1, 3, 9). Mutations in these genes affect specification of body parts corresponding to these domains (1, 9). In the mouse, four major *Antp*-like homeobox gene clusters (*Hox-1*, *-2*, *-3*, and *-5*) have been identified (10–19), and support for a functional role for these genes in mouse development is derived from their patterns of expression (20). The *Hox* genes are expressed in overlapping domains along the rostro-caudal axis of the embryo, at high levels in the central nervous system and somitic mesoderm. Genes in these complexes, like their *Drosophila* counterparts, show a correspondence between position in a cluster and anterior boundaries of expression along the A-P axis (18, 21–23). Genes of the *Hox-5* complex are also coordinately expressed in overlapping domains during formation of the limb (24). The mouse and *Drosophila* clusters appear to be evolutionarily related to a common ancestor, based on common aspects of their DNA sequence, organization of their component genes along the chromosome, and expression (18, 21, 25). Such large-scale conservation of structure could reflect selection on the distribution of regulatory elements as well as protein-coding sequence. A complex array of cis-acting regulatory elements distributed along the cluster has been postulated for the *Antp* complex (26).

The study of the function and coordinated expression of such large gene complexes, however, poses a technical challenge: the cloning and manipulation of intact clusters whose size far exceeds the capacity of cosmid and phage vectors. In contrast, yeast artificial chromosome (YAC) vectors, carrying a centromeric sequence, a replication origin, selectable markers, and two cassettes of telomeric sequence, allow the cloning and propagation of large mammalian DNA fragments of up to 1 megabase (27). Therefore, to extend our analysis of the *Hox-2* complex, we have used yeast artificial vectors to clone a large region of this gene complex, while maintaining the order and organization of the component genes.‡

MATERIALS AND METHODS

Yeast Strains and YAC Library Construction. Construction of the YAC library in *Saccharomyces cerevisiae* strain AB1380 (MAT*a*, *ura3*, *trp1*, *ade2-1*, *can1-100*, *lys2-1*, *his5*) and yeast colony screening were as described (28). Preparation of chromosomes from *S. cerevisiae* strain YP148 and from YAC clones was as described (29), and separation was by contour-clamped homogeneous electric field gel electrophoresis (30). Switching times are as described in figure legends.

Southern and Northern Analyses. Probe 1 is a 13-kilobase (kbp) *Bam*HI fragment, probe 2 is a 7-kbp *Eco*RI fragment, probe 3 is a 1-kbp *Sac* I fragment, probe 4 is a 250-bp *Eco*RI–*Sal* I fragment, probe 5 is a 2.5-kb *Eco*RI–*Bam*HI fragment, probe 6 is a 2.0-kbp *Sac* I–*Kpn* I fragment, probe 7 is a 6-kbp *Bam*HI–*Not* I fragment, and probe 8 is a 900-bp *Eco*RI cDNA fragment (see Fig. 1). The degenerate oligonucleotide used covers amino acids 42–55 of the *Antp* homeobox. The 42-mer sequence, as presented, follows the Stanford ambiguity code: GAR MGV CAR RTS AAR ATY TGG TTY CAG AAY CGN MGV ATG AAG. Blotting, cross-linking of RNA to the filter, and washing conditions were exactly as described (13, 21, 31). Duplicate filters were hybridized with an antisense mouse β-actin RNA probe as a control to assess the relative loading and quality of RNA on the filters.

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Abbreviations: YAC, yeast artificial chromosome; ANT-C, Antennapedia complex; BX-C, Bithorax complex.

‡The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M34004 for *Hox-2.8* and M34005 for *Hox-2.9*).

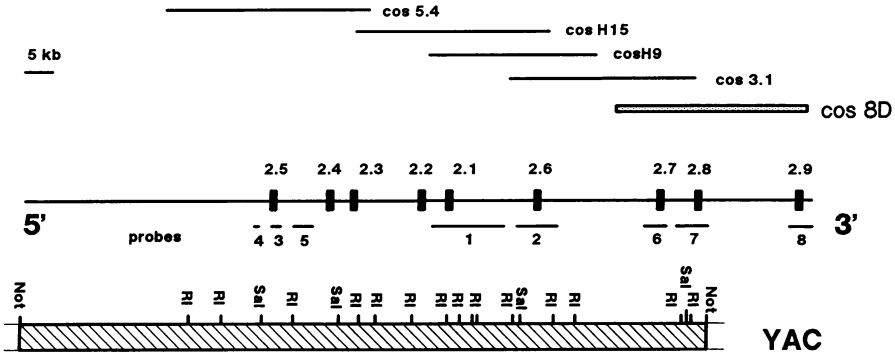

FIG. 1. Map of the *Hox-2* locus. Overlapping cosmids that span the cluster were isolated and characterized as in ref. 13. Also shown is the recently isolated cosmid 8D, containing *Hox-2.7*, *-2.8*, and *-2.9*. A detailed map of the cluster is shown along with the relative positions of probes used in hybridizations shown in Fig. 2. The extent of the YAC insert, a 120-kbp *Not* I fragment (as sized on pulsed-field gels), is illustrated below the map.

Sequencing of *Hox-2.8* and *-2.9* Homeodomains. The *Bam*HI–*Not* I 6-kbp YAC end fragment containing *Hox-2.8* was subcloned into pKS$^+$. The insert was sequenced by double-stranded sequencing using a Sequenase kit according to the manufacturer's instructions (United States Biochemical). *Hox-2.9* was isolated as a 900-bp cDNA fragment that was subcloned into pKS$^+$ and sequenced as in *Hox-2.8*.

RESULTS

Isolation and Characterization of YNot-Hox2. Seven contiguous genes of the *Hox-2* cluster have previously been cloned in overlapping cosmids spanning 80 kbp on mouse chromosome 11 (ref. 13 and Fig. 1). All seven genes have the same 5′ → 3′ orientation with respect to transcription (21). *Hox-2.5* is the most 5′ member and *Hox-2.7* is the most 3′ member present in cosmids. The mouse *Hox* clusters are thought to be related to each other by duplication and divergence (31). We therefore predicted that there would be at least one additional member of the *Hox-2* complex downstream of *Hox-2.7* (21, 22). To isolate the *Hox-2* region on a single piece of DNA, we screened a YAC library constructed using a complete *Not* I digest of mouse DNA ligated into the vector pYAC55. The library contained 9500 clones with an average insert size of 150 kbp. Four-thousand clones were screened, and of these, one YAC clone, YNot-Hox2 (≈120 kbp in size), hybridized with probes from *Hox-2.5* and *-2.7* (probes 3 and 7, Fig. 1) representing opposite ends of the cluster. This was therefore a good candidate for a clone spanning the *Hox-2* cluster.

We characterized the YNot-Hox2 clone to confirm that sequences along the YAC were a faithful copy of genomic DNA. We had previously generated a detailed map of the *Hox-2* cluster, with subclones from cosmids spanning the entire region between *Hox-2.5* and *-2.7* (≈80 kbp). We used part of this array of *Hox-2* clones (probes 1–6 in Fig. 1) and clones from the newly isolated cosmid (probes 7 and 8, Fig. 1) to characterize the YAC. Mouse genomic DNA, YNot-Hox2, and cosmid clones were digested with a variety of restriction enzymes (*Eco*RI, *Hin*dIII, *Sac* I, *Bam*HI, *Sal* I, *Not* I, *Kpn* I, and *Cla* I), and analyzed by Southern blotting. In all cases, the fragment sizes of the YAC DNA are identical to those of genomic DNA and of cosmid clones. Results from three such hybridizations comparing genomic DNA and the YNot-Hox2 clone are shown in Fig. 2. Though it is possible that very small alterations (<500 bp) may have been missed by this method, using this series of probes and enzymes we find no evidence for sequence rearrangements in the YAC DNA.

To assess the extent of the YAC clone, we mapped the ends of the insert relative to the known *Hox-2* map. Near the 5′ end of the *Hox-2.5* gene is a *Sal* I site and this region of the cluster is next to the short (4 kbp) arm of the YAC vector, which also contains a *Sal* I site. As seen in Fig. 3, hybridization of a *Sal*

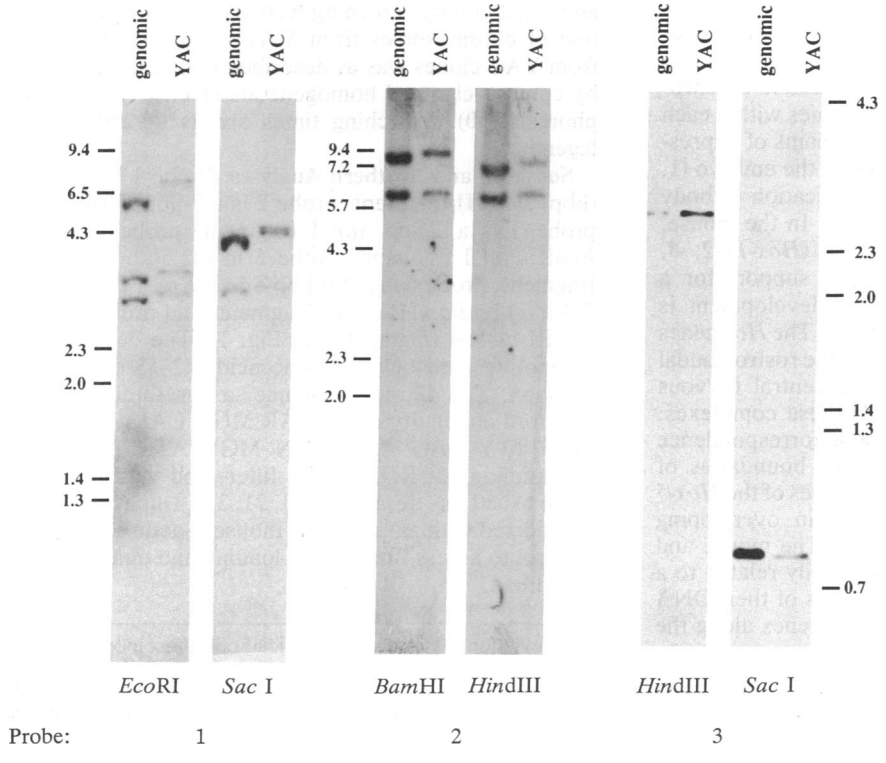

FIG. 2. Comparison of restriction fragment sizes in mouse genomic DNA and YAC clone YNot-Hox2. Shown here are pairs of selected digests from three agarose gels. DNA from these gels was blotted onto Hybond-N (Amersham) and hybridized to the three probes positioned in Fig. 1. Lanes contain equimolar amounts of DNA; the slight difference in mobility between genomic and yeast DNA fragments in lanes from the left and middle gel is due to the increased mobility (reduced retardation) of DNA fragments in lanes containing more DNA, in gels run in buffers containing ethidium bromide. Size markers, indicated in kbp, are shown for each gel.

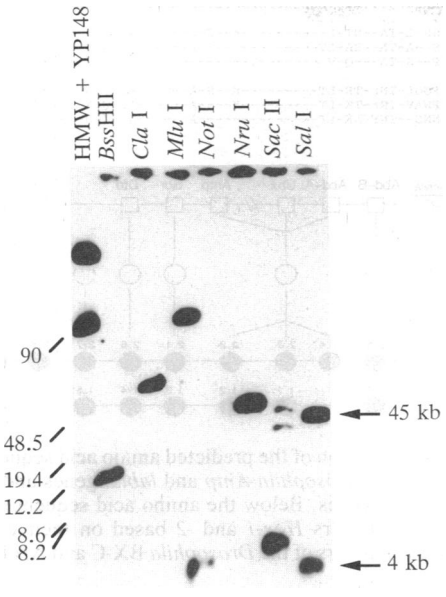

FIG. 3. Characterization of YNot-Hox2 DNA by rare-cutter restriction digests. YNot-Hox2 DNA was digested with a series of rare-cutter enzymes and separated by contour-clamped homogeneous electric field gel electrophoresis with a switching time of 8 sec. The DNA was transferred by alkali blotting to Hybond-N for 48 hr and hybridized to a 600-bp *Nru* I–*Sal* I fragment from pBR322 represented on the shorter, 4-kbp arm of the YAC vector. Arrows indicate the 4-kbp arm released upon *Sal* I digestion (also evident upon *Not* I digestion) as well as a 45-kbp fragment abutting the YAC vector fragment. The first lane contains high molecular weight size (HMW) markers (BRL) and yeast chromosome markers from YP148; sizes are in kbp.

I digest of YNot-Hox2 DNA with a probe specific for the small vector arm yields a band of 4 kbp corresponding to the arm and a band of ≈45 kbp representing the distance between the arm and the 5′ end of the *Hox-2.5* gene. An identical result was obtained using a probe (no. 2, Fig. 1) just upstream of the *Hox-2.5 Sal* I site (data not shown). To test for the presence of other homeobox-containing genes in this 45-kbp region, we hybridized DNA from YNot-Hox2 with a degenerate oligonucleotide that recognizes the highly conserved helix 3 region (amino acids 42–56) of the *Antp* class homeoboxes. This probe hybridizes to all other members of the *Hox* family genes but no additional homeobox sequences upstream of *Hox-2.5* were detected.

Sequence Analysis of the *Hox-2.8* and *-2.9* Homeodomain. We mapped the 3′ end of the YAC clone to be ≈9 kbp downstream of the *Hox-2.7* gene. Restriction enzyme and hybridization analysis identified a further homeobox gene near the 3′ terminus of the YAC insert. The ends of the YAC insert were subcloned and the homeobox, *Hox-2.8*, was sequenced. The nucleotide sequence and predicted homeodomain sequence of the *Hox-2.8* gene are shown in Fig. 4*a*. With the exception of the first amino acid in the homeodomain (serine instead of alanine), it is identical in protein-coding sequence to the human *K8* gene (32) and to the recently reported human *HOX2.8* gene (33, 34). The *Hox-2.8* sequence is not a member of the labial-related subfamily as previously predicted but, instead, shows an extremely high degree of identity (>95%) with the homeodomain of proboscopedia (*pb*) (D. Cribbs, M. Pultz, and T. Kaufman, personal communication; ref. 35). Although the four *Hox* clusters are derived from an ancestral cluster (36), no *Hox-2.8*-related homeobox gene has been found at a similar position in the *Hox-1* cluster nor has this region been cloned from the *Hox-3* or *Hox-5* complexes. Therefore, *Hox-2.8* is at present the sole murine member of the pb-subfamily.

Since the *Hox-1* cluster does contain a labial-related gene, *Hox-1.6*, we surmised that there could be an additional labial homologue in the *Hox-2* cluster, downstream of the *Hox-2.8* gene. The YAC insert terminates at the 3′ end of the *Hox-2.8* gene. Using a fragment subcloned from this end of the YAC as a probe, we have isolated a cosmid (cos 8D) that overlaps with the preexisting cosmid clones and extends 3′ of the YAC (see Fig. 1). This cosmid contains, in addition to *Hox-2.7* and *-2.8*, another homeobox gene, termed *Hox-2.9*, which we subcloned and sequenced. The nucleotide sequence and predicted amino acid sequence of the homeodomain are shown in Fig. 4*b*. The predicted amino acid sequence has the highest degree of identity with those of the labial and *Hox-1.6* homeodomains. Alignment of the sequences (see Fig. 6) shows changes in the amino acids of these three genes relative to the *Antp* gene and indicates that they form a labial subfamily.

Expression of the *Hox-2.8* and *-2.9* Genes During Mouse Embryogenesis. Fig. 5 shows a Northern blot of RNAs extracted from different fetal and adult tissues. The blot was hybridized under high stringency with a probe derived from the 3′ untranslated region of *Hox-2.8*, which does not cross-hybridize with other homeobox genes (13, 21, 36). The expression of *Hox-2.8* is not restricted to embryonic stages of development and can be seen in adult tissues. High levels of expression are detected in the fetal lung, and lower levels are observed in the adult lung. This supports our previous finding that genes located in the 3′ part of the *Hox*-2 cluster (*Hox-2.2*, *-2.1*, *-2.6* and *-2.7*) are expressed in tissues derived from anterior mesoderm (21). Although the level of the major transcript (1.8 kbp) remains unchanged in fetal and adult kidney, there are changes in the number and relative abundance of other transcripts. In adult kidney, a new, equally

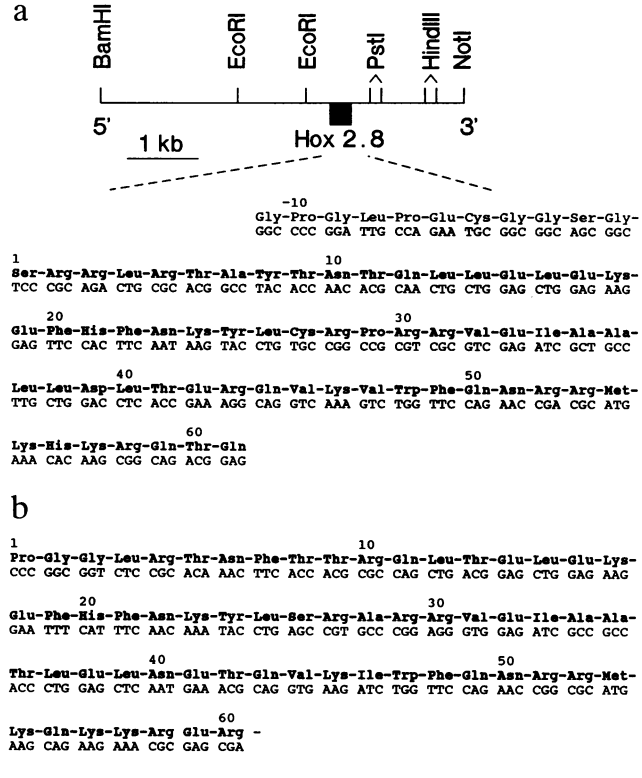

FIG. 4. (*a*) Nucleotide sequence and predicted amino acid sequence of the *Hox-2.8* homeodomain. YAC DNA was purified from a pulsed-field gel, digested with *Bam*HI and *Not* I, subcloned into pKS$^+$, and sequenced. (*b*) Nucleotide sequence and predicted amino acid sequence of the *Hox-2.9* homeodomain.

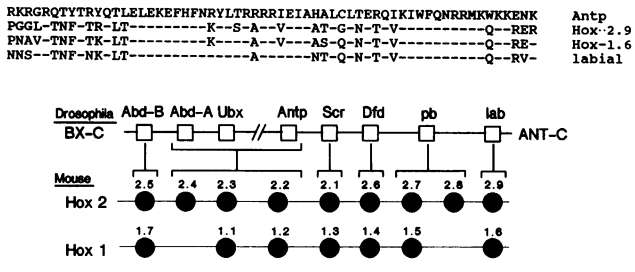

FIG. 5. Expression of *Hox-2.8* in poly(A)$^+$ RNA extracted from fetal and embryonic tissues, analyzed by Northern blotting. The probe is a single-stranded RNA probe derived from T7 transcription of the 1-kbp *Not* I–*Pst* I fragment from the subclone shown in Fig. 4*a*. Each lane contains 2 μg of poly(A)$^+$ RNA.

abundant 1.6-kbp band is observed, whereas the levels of the minor bands are greatly reduced. This complex transcription pattern is similar to that of *Hox-2.1*, *-2.6*, and *-2.7* (13, 21, 31). Multiple transcripts could arise from a combination of differential splicing and the utilization of multiple sites for initiation or polyadenylylation. *Hox-2.8* is expressed at low levels in the liver, unlike most other *Hox-2* genes. It is interesting to note that expression of the human *Hox-2.8* homologue, *K8*, is detected in the erythroid cell line K562 but not in other derivatives of the hematopoietic system (32). Therefore, the expression of *Hox-2.8* in liver may represent expression in erythroid cells. Finally, we have not detected expression of the gene in the midbrain or forebrain (not shown) but observed high levels of expression in spinal cord. This agrees with data from *in situ* hybridization experiments indicating that *Hox-2.8* is expressed in 8.0- to 9.5-day postcoitus (p.c.) mouse embryos at high levels in the developing neural tube (37). It shows a sharp limit of expression in the developing hindbrain that is more anterior than that of the adjacent *Hox-2.7* gene, extending the trend established by other *Hox-2* members of successively anterior domains of expression in the neural tube (21).

The same filter was stripped and rehybridized with a *Hox-2.9* probe. No expression of *Hox-2.9* was detected in RNA from these tissues at any of the stages. However, *in situ* hybridization has shown that the gene is expressed in the developing neural tube of 8.0- to 9.5-day p.c. mouse embryos in a segment-restricted manner (37). Expression of this gene is highest in early stages of embryogenesis and is down-regulated near the onset of organogenesis. This pattern is different from all of the other *Hox-2* genes, which are expressed throughout embryonic and adult stages. However, the temporal pattern is similar to that of *Hox-1.6*, which is not detected in embryos after 10.5 days p.c. (18). *Hox-1.6* is another member of the labial-related subfamily and this pattern may be a general property of the subfamily.

DISCUSSION

We have cloned on a single DNA fragment eight genes from the mouse homeobox complex *Hox-2*, including the gene *Hox-2.8*. We have used the end of this YAC insert to isolate a cosmid that extends the cloned region to *Hox-2.9*, the ninth member of the cluster, and the most 3′ member known to date. Sequence analysis of these genes reveals that *Hox-2.8* represents a part of a mouse subfamily highly related to *pb* and that *Hox-2.9* is related to the *Drosophila lab* gene.

The newly defined murine *Hox-2* complex comprises nine genes spanning 95 kbp, and all are organized in the same 5′ → 3′ direction with respect to transcription. The number of genes, sequence homology, spacing, and relative position of the homologues are highly conserved between the mouse and human *Hox-2* clusters (34). In Fig. 6, we have aligned the genes of *Hox-2* with those of *Hox-1* and their most closely

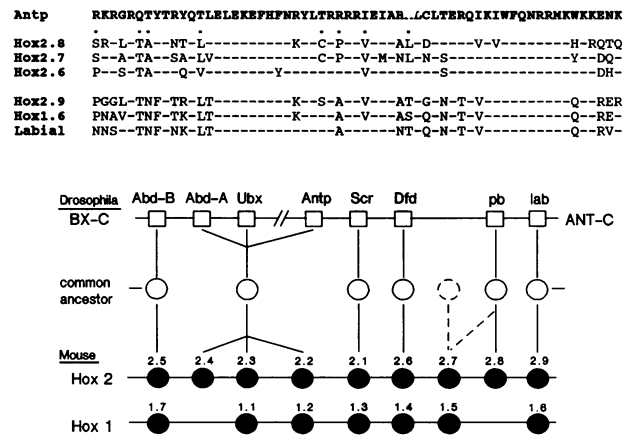

FIG. 6. Comparison of the predicted amino acid sequences of the homeodomains of *Drosophila Antp* and *labial* genes and the mouse *Hox-2.9* and *-1.6* genes. Below the amino acid sequences, we have aligned murine clusters *Hox-1* and *-2* based on their evolutionary relatedness to members of the *Drosophila* BX-C and ANT-C clusters (see text for details).

related *Drosophila* genes in the ANT-C and BX-C clusters to illustrate their evolutionary relationships and proposed derivation from a common ancestor (18, 21, 25). The homeodomain of *Hox-2.9* shows the greatest identity with those of *Hox-1.6* (eight amino acid changes, of which five are conservative substitutions) and labial (nine amino acid changes, of which six are conservative). Analysis of the partially predicted *Hox-2.9* protein from our clones reveals other regions, outside of the homeodomain, homologous with labial, suggesting that it is a member of the vertebrate *lab*-related subgroup.

In the *Hox-2* cluster there are two genes, *Hox-2.7* and *-2.8*, located between members related to the *Drosophila* genes *Dfd* and *lab* (see Fig. 6). Based on the relationship between the murine and *Drosophila* clusters we predict that one or both of these genes are related to the *Drosophila pb* family. In a preliminary analysis of *pb*, D. Cribbs, M. Pultz, and T. Kaufman (personal communication) have found that *pb* does contain a homeodomain that is very highly related to mouse *Hox-2.8* (four amino acid changes) and that there are conserved domains in other regions of the two proteins. *Hox-2.8*, therefore, appears to be the most closely related member of the mouse complex to *pb* and lends further support to the idea of a close evolutionary relationship between the *Hox* and *HOM* complexes. No mouse gene related to *Hox-2.8* has yet been found in the *Hox-1* cluster, and the corresponding region of the other clusters has not been cloned. However, the high degree of identity with *pb* suggests that it is not a recently evolved gene unique to the *Hox-2* complex but was part of the ancestral cluster that may have diverged or been deleted in the *Hox-1* cluster during vertebrate evolution.

The homology between *Hox-2.8* and *pb* raises the question of evolutionary origin of the subfamily that contains *Hox-2.7*. No related homeotic gene is found in the *Drosophila* ANT-C cluster at a similar position. Instead, there are two homeobox-containing genes involved in dorso-ventral patterning, *zen z1* and *z2* (38). Comparison of the *Hox-2.7* homeodomain, which has been cloned from vertebrates, including mouse (10, 13, 19) human (34), and chicken (A. Kirowa and P. Scotting, personal communication), with those of *Hox-2.6*, *Hox-2.8*, *zen z1*, and *pb*, shows that they all have a similar percentage of matching amino acids, with a range of 8–13 amino acid differences when conservative substitutions are considered (see Fig. 6). It is therefore unclear to which of these genes *Hox-2.7* is most highly related.

It is important to note that despite sequence homologies, *Hox* genes are not necessarily the true vertebrate homologues of the *Drosophila* homeotic genes. *Hox-2.7* could have arisen by one of the two mechanisms (21). The ancestral cluster could have contained a gene related to *Hox-2.7* and *zen*, which has diverged in arthropods but has been maintained in vertebrates. Alternatively, the *Hox-2.7* gene was not in the ancestral cluster but is unique to vertebrates, having recently evolved by duplication from the subfamily of which *Hox-2.8* is a member. Evidence for this phenomenon is derived from amino acid sequence comparisons of *Hox-2.7* and *-2.8*. *Hox-2.7* contains a specific grouping of amino acids, L-C-P-V-L at positions 13, 27, 29, 32, and 37 of the homeodomain, respectively (see dots in Fig. 6), which are conserved in *Hox-2.8* and characteristic of the *pb* subfamily. In addition, the relative size and position of the homeodomain in the predicted *Hox-2.7* and *Hox-2.8* proteins are very similar to each other and different from the other *Hox-2* genes (M. Sham and M.C., unpublished results). Analysis of homeobox clusters in other arthropods and invertebrate species will be required to determine the nature of the ancestral cluster and of subsequent species-specific alterations.

The size of the *Hox-2* cluster is still not precisely established. Although YNot-Hox2 has extended the cloned region surrounding *Hox-2*, no new homeobox-related sequences were found upstream of *Hox-2.5*. The human *HOX2* cluster was also devoid of homeoboxes in this region, whereas other human clusters contained several genes in the corresponding regions (34). We must attempt to isolate further upstream sequences, using chromosomal walking techniques, to establish whether *Hox-2.5* is the end gene of this complex. We think it probable that *Hox-2.9* is the downstream limit of the cluster because no homeobox genes downstream of the labial gene or its subfamily members has been found in *Drosophila* or in vertebrate clusters in which the region has been cloned.

The conserved organization of homeobox clusters during vertebrate evolution might reflect a conservation of controlling elements spread throughout the complex. These controlling sequences may be positioned a long distance from the transcription unit and may be present in flanking genes themselves. Preliminary experiments attempting to localize elements regulating temporal and spatial expression of the *Hox-2.1* gene support the idea of multiple distant control sequences. In addition, there is evidence from the human cluster *HOX3* that elements comprising transcription units themselves are dispersed over large distances (39). To reconstruct the normal patterns of expression for some genes in a *Hox* complex, it may be necessary to maintain intact the long-range organization of the cluster. This could be most easily accomplished using YACs. The DNA sequence of YNot-Hox2 is unrearranged relative to the genomic *Hox-2* cluster. This mini *Hox-2* locus offers the possibility of using homologous recombination within yeast to manipulate an intact murine homeobox gene complex and its regulatory regions. It will be a valuable tool for introducing modified versions of the *Hox-2* cluster into embryonic stem cells or fertilized embryos to directly test the relationship between the position of a gene in the complex and its specification of positional information along the rostro-caudal axis of the embryo.

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