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Detection of homeobox genes in development and evolution

(polymerase chain reaction/telencephalon/cnidarians)

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ABSTRACT **The homeobox genes encode a family of DNA-binding regulatory proteins whose function and genomic organization make them an important model system for the study of development and differentiation. Oligonucleotide primers corresponding to highly conserved regions of Antennapedia-class homeodomains were designed to detect and identify homeobox sequences in populations of DNA or RNA by means of the polymerase chain reaction (PCR). Here we present a survey of sequences detected by PCR using an initial set of primers (HoxA and HoxB) based on an early nucleotide consensus for vertebrate Antennapedia-class homeodomains. Several novel sequences are reported from both mouse genomic DNA and RNA from the developing mouse telencephalon. Forebrain-derived clones are similar to the chicken *CHox7*, *Drosophila H2.0*, and mouse *Hlx* genes. PCR also proved to be a rapid method for identifying homeobox sequences from diverse metazoan species. Cloning of three Antennapedia-related sequences from cnidarians provides evidence of ancient roles for homeobox genes early in metazoan evolution.**

The homeodomain was first described as a polypeptide motif conserved among *Drosophila* homeotic genes (1–4). Hybridization studies (2, 5, 6) and the cloning of homeodomain genes from vertebrates (7–9) demonstrated the remarkable conservation of the homeodomain among the higher metazoans. The regulatory function of homeotic gene products originally implied by genetic analyses (10) has been extensively characterized by numerous studies that have included structural analyses revealing the helix–turn–helix conformation of the homeodomain, the demonstration of its DNA-binding capability, and *in vitro* expression assays designed to measure the effectiveness of homeodomain genes as transcription factors (for reviews see refs. 11–15). The presence of homeodomain binding sites in the promoters of homeobox genes suggests a complex network of autoregulatory and cross-regulatory interactions within the gene family. The conservation of the homeodomain and its proliferation among the metazoans emphasize the importance of the role of homeobox genes in development and differentiation.

Antennapedia (*Antp*)-class homeodomains may be defined not only as sequences sharing a high degree of homology with *Antp*, but also as those more diverged from *Antp* that are closely linked to *Antp*-like sequences within gene clusters. In insects, this includes homeodomains located within a single complex, which in *Drosophila* is split into two half-clusters (ANT-C and BX-C) (16). For the mammals, this includes all homeobox sequences within four clusters arising from comparatively recent duplication events (17–19). Weaker homology exists between the homeodomain and portions of various other DNA-binding regulatory proteins. The term homeodomain now applies to protein sequences that are predicted to form a helix–turn–helix structure and that contain particular conserved amino acid residues (i.e., POU genes, yeast mating-type genes, etc.; for review see ref. 12). These include many sequences that show variable levels of homology with *Antp*-class sequences but that generally do not show linkage with *Antp*-class clusters, leaving evolutionary relationships unclear.

The *Antp*-class homeobox genes are of particular interest because of their developmental expression patterns and their conservation of chromosomal organization (for reviews see refs. 15, 20–22). For the most part, genes of this type exhibit a conserved embryonic expression pattern along the anteroposterior axis that reflects their relative position within the cluster. Genes within a cluster are transcribed from the same strand of DNA, giving the entire cluster a 5′- to -3′ polarity. Genes at the 3′ end of a cluster have the anterior-most domain of expression during development, and likewise, those at the 5′ end are expressed most posteriorly. Conservation of both the orientation and the proximity of genes within homeobox clusters suggests that strict regulatory constraints act on these gene complexes.

Here we show that PCR can be used to rapidly and efficiently survey populations of DNA or RNA for the presence of homeobox sequences. Primers described here were designed from a compiled list of the earliest reported mammalian *Antp*-class homeobox sequences (17), which fails to include several sequences reported more recently and most notably those in the more posterior portions of the clusters (19, 22, 23). A survey of mouse genomic DNA with these primers reveals several novel sequences. We further show that some of these sequences are enriched in RNA isolated from the developing mouse telencephalon. A survey of a variety of genomes suggests that *Antp*-class sequences are present among all metazoans. We also describe three novel cnidarian homeobox sequences from the hydrozoan *Sarsia*, representing the most highly diverged metazoan phylum shown to contain *Antp*-class sequences.‡

MATERIALS AND METHODS

Primer Design. Degenerate primers were based on a consensus derived from an early compilation of vertebrate homeobox nucleotide sequences (17). The unusual length of the primers was intended to span the regions of best homology and to compensate for known and potential mismatches between the target sites and the primers. The primers are flanked by restriction sites to facilitate cloning. Coordinates referred to here define the first nucleotide of the first codon of the homeodomain as +1 [arginine codon in *Antp* (2)]. The amplification products [166 base pairs (bp)] give 77 bp of informative sequence spanning nucleotide positions 62–138 (amino acid positions 22–46) of the homeobox. Primer HoxA spans nucleotide positions 31–61 with flanking *Pst* I and *Bam*HI sites. 5′-ACTGCAGGATCCTACCAGAC(C/G)(C/

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‡The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M81658–M81666).

T)TGGA(A/G)CTGGAGAA(A/G)GA(A/G)TT(C/T)C-3'. HoxB spans the complement of positions 172–139 with flanking *Eco*RI and *Hin*dIII sites. 5'-AGAATTCAAGCTT(C/T)TTCCA(C/T)TTCAT(C/G)C(G/T)(A/C/G/T)CG(A/G)TTCTG(A/G)AACCAGAT-3'. Primers were synthesized on an Applied Biosystems model 381A DNA synthesizer. After cleavage and deprotection and two ethanol precipitations, the oligonucleotides were used without further purification.

Genomic DNA Extraction. Mouse genomic DNA was extracted from the spleens of male adults (*Mus musculus* strain C57B6). DNA from the ascidian *Ciona intestinalis* and the colonial hydroid *Hydractinia symbiolongicarpus* was isolated from sperm. For DNA isolation, tissues were ground to a powder in liquid N_2 and digested with proteinase K (100 ng/ml) in lysis buffer (100 mM NaCl/10 mM Tris·HCl, pH 8/25 mM EDTA/0.5% SDS) (24) overnight at 55°C. The solution was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol, 25:24:1 (vol/vol). The aqueous phase was transferred to a tube containing 0.5 volume of 7.5 M NH_4OAc and mixed gently. Two volumes of 100% ethanol was layered onto the solution and the DNA was spooled onto a sterile glass rod. The DNA was dissolved overnight in TE (10 mM Tris·HCl/1 mM EDTA, pH 8). Before use, the DNA solutions were extracted three more times with an equal volume of phenol/chloroform/isoamyl alcohol, 25:24:1, reprecipitated with ethanol, and again dissolved in TE.

RNA Isolation and RNA PCR. Telencephalons from eighty 13.5-day mouse embryos were dissected using the telencephalic envagination as a landmark and immediately stored at −80°C. Total RNA was isolated by a single-step method using acid/guanidinium thiocyanate/phenol/chloroform extraction (25). Poly(A)$^+$ RNA was purified by two cycles of oligo(dT)-cellulose column chromatography (Pharmacia, type 7). Single-stranded cDNA was prepared using an oligo(dT) primer and avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, FL) (26). Five microliters of the cDNA mixture was diluted into 100 μl PCR mixture and amplified as described below.

PCR Conditions. Routinely 100–500 ng of genomic DNA, or an appropriate fraction of cDNA, was diluted into a total of 100 μl of PCR mixture [10 mM Tris·HCl, pH 8.3/50 mM KCl/3 mM $MgCl_2$/0.01% gelatin/200 μM each dNTP/1 μM each primer (HoxA and HoxB)]. The mixture was boiled for 10 min, after which 1 unit of AmpliTaq polymerase (Cetus) was added. Mineral oil (50 μl) was added on top of the reaction mixture and amplification was achieved by incubation in a Perkin–Elmer/Cetus DNA thermal cycler at 95°C (1 min), 40°C (1 min), 70°C (30 sec) for 30 cycles (27). The DNA ends were "polished" by a final 10-min incubation at 70°C.

Cloning and Sequencing. The PCR products (166 bp) were separated by electrophoresis in a 2% regular agarose gel. For cloning, the PCR products were digested with appropriate restriction enzymes and the fragments were gel-purified by electroelution onto DEAE-cellulose. The PCR product inserts were cloned into a pGEM (Promega) derivative (a gift of L. Bogarad, our laboratory, Yale University). DNA minipreparations of individual clones, obtained by alkaline lysis (28), were assayed for appropriately sized inserts and characterized by double-strand dideoxy sequencing using T7 polymerase and reagents from Pharmacia.

RESULTS AND DISCUSSION

Survey of Mouse Genomic DNA by PCR. Mouse genomic DNA was surveyed by PCR to identify novel homeobox genes as well as to characterize the distribution of homeobox sequences detected by primers HoxA and HoxB under the specified PCR conditions (see *Materials and Methods*). A total of 100 individual clones were sequenced representing 26 different homeobox genes. Twenty-one previously reported mouse homeobox sequences were detected, as well as 5 novel mouse sequences, 3 of which are most likely homologs of sequences reported for the human. Fig. 1 shows a schematic representation of the chromosomal organization of the murine and human *Antp*-class sequences reported to date and the distribution of sequences detected by primers HoxA and HoxB. Sequences of homeobox genes located in cognate groups I–III have only recently been reported and are poor templates for primers HoxA and HoxB. Most of the sequences from the remaining 10 cognate groups were detected, including three novel clones, MAB26, MAB66, and MAB87

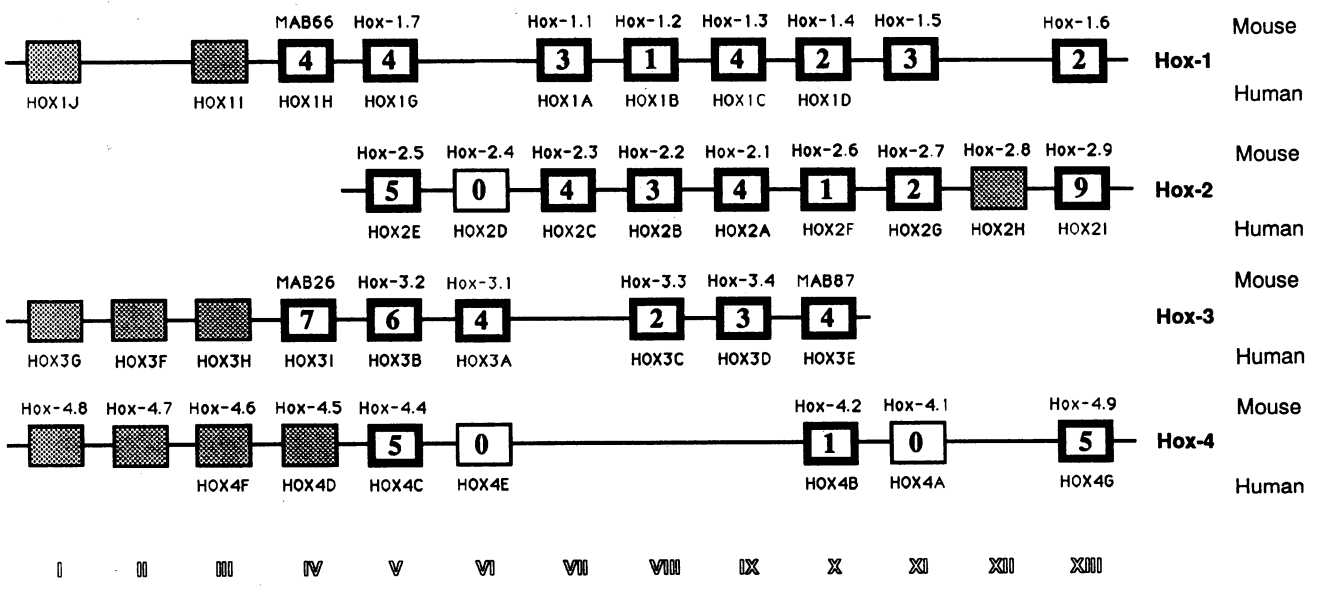

FIG. 1. Detection of murine homeobox sequences by PCR. Arrangement of the known murine and human *Antp*-class homeobox genes is shown. Heavy lined boxes are those detected by PCR in mouse genomic DNA with primers HoxA and HoxB. The numbers inside the boxes indicate the percentage of clones observed for each sequence. Shaded boxes indicate sequences that should not be detected by PCR with primers HoxA and HoxB. Boxes are arranged to reflect their relative position within each cluster. Boxes are also arranged vertically in cognate groups that reflect paralogous relationships. Clones MAB66, MAB26, and MAB87 are tentatively assigned as murine homologs of the human *HOX1H*, *HOX3I*, and *HOX3E* genes, respectively.

(Fig. 2), identical in derived amino acid sequence to the human *HOX3I*, *HOX1H*, and *HOX3E* genes, respectively (19, 23). Two sequences named MMoxA (4% of clones) and MMoxB (8% of clones) (Fig. 2) are identical in derived amino acid sequence to the chicken *CHox7* gene (29), and differ from one another by 13 silent nucleotide substitutions (data not shown). *Hox-2.8* and *Hox-4.5* are poor templates for primers HoxA and HoxB due to primer mismatches at the 3′ ends. Only three other known sequences have gone undetected; *Hox-2.4*, *Hox-4.1*, and the mouse homolog of human *HOX4E*. These genes appear to be good templates for primers HoxA and HoxB, and they should be detected as more clones are sequenced. Although the templates all differ in their homology to the primers, there appears to be no significant bias in the distribution of sequences detected, presumably due to the relatively low annealing temperatures used during amplification.

Detection of Homeobox Sequences in RNA Isolated from the Developing Mouse Telencephalon. To identify regulatory genes that might be involved in the development of the forebrain, PCR using primers HoxA and HoxB was performed to survey cDNA from 13.5-day mouse telencephalon for homeobox sequences. Among 25 individual clones tested, 5 different sequences were detected, including 3 previously unreported homeobox sequences. The frequency and distribution of these sequences are unlike those observed for genomic DNA, suggesting that telencephalic RNA is specifically enriched in these sequences. MMoxA was the most frequently observed sequence, present in 56% of the clones. Also detected are MMoxB (16%), *Hox-4.9* (12%), and a sequence named MMoxC (12%) (Fig. 2), related to the mouse *Hlx* (30) and *Drosophila H2.0* (31) genes. MMoxA and MMoxB are homologs of *CHox7* (Fig. 2), which is expressed during chicken embryogenesis (29). Only traces of the mouse *Hlx* gene are detected in the developing brain, which could be derived from expression in hematopoietic cells (30). The detection of these homeobox sequences in RNA isolated from the developing mouse telencephalon raises the possibility that they are involved in the specification and differentiation of the anterior central nervous system. It is not known whether the expression of the MMoxA, -B, and -C genes is restricted to the telencephalon or is more widespread throughout the embryo. In any case, the spatial expression patterns of these genes will be of interest, and they may prove to be regulatory markers for particular aspects of anterior development. Although the expression of *CHox7* and mouse *Hox-4.9* has not yet been observed in the telencephalon, contamination from neighboring tissues seems unlikely because we do not detect any of the several *Antp*-class sequences known to be expressed in the hindbrain (15, 21, 32). It remains possible that we are detecting low levels of these sequences beyond the sensitivity of *in situ* hybridization. In addition, one clone of *En-1* was observed, which is known to be expressed in the developing brain (33–35), even though it only has 58% homology with HoxA and 70% homology with HoxB.

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                AMINO ACIDS 22-46

 Antp:  FNRYLTRRRRIEIAHALCLTERQIK

MAB26:  --M----E--L--SKTIN--D--V-
HOX3I:  --M----E--L--SKTIN--D--V-

MAB66:  --M----E--L--SRSVH--D--V-
HOX1H:  --M----E--L--SRSVH--D--V-

MAB87:  Y--------------S---S-----
HOX3E:  Y--------------S---S-----

MMoxA:  CKK--SLTE-SQ-----K-S-V-V-
MMoxB:  CKK--SLTE-SQ-----K-S-V-V-
CHox7:  CKK--SLTE-SQ-----K-S-V-V-

MMoxC:  KQK-ISKPD-KKL-SK-G-KDS-V-
  Hlx:  IQK-V-KPD-KQL-AM-G--DA-V-
 H2.0:  QQK-I-KPD-RKL-AR-N--DA-V-
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FIG. 2. Murine homeobox sequences compared with their closest known homologs. Amino acid sequences are listed relative to *Antp* to emphasize homologies. Hyphens indicate identity with *Antp* at a given position. References are as follows: human *HOX3I* (23), human *HOX1H* (19), human *HOX3E* (19), chicken *CHox7* (29), mouse *Hlx* (30), and *Drosophila H2.0* (31).

Evolutionary Survey of *Antp*-Class Homeobox Sequences. The strict evolutionary conservation of homeobox sequences and their chromosomal organization suggests that the number and type of homeobox genes in a particular species, as well as the organization of those genes, may correlate with the level and complexity of their embryonic development. Such a correlation would make the homeobox gene system crucial to the understanding of evolutionary relationships among the metazoans. Genomic DNAs from selected metazoan species, whose presumed relationships are depicted in Fig. 3*a* (36), were surveyed by PCR with primers HoxA and HoxB. PCR products of the size expected from *Antp*-class homeobox sequences were observed in all species examined (Fig. 3*b*), including the hydrozoan *Sarsia* and the colonial hydroid *Hydractinia symbiolongicarpus*. To ensure that these products were not artifactual, several clones were characterized for *Sarsia*. Three sequences related to *Antp* were observed (Fig. 4). SAox1 and SAox2 are clearly related to the *Antp* class. SAox1 is most closely related to *Hox-1.6* and *lab* sequences, while SAox2 is more closely related to *Hox-2.8* and *pb* sequences. SAox3 is most likely an *Antp*-like homeobox sequence, but is relatively diverged and its relationship to known homeobox sequences is unclear. It shares 15 of 25 amino acids with *Ubx* but also shares 14 of 25 amino acids with several other types of *Antp*-class sequences (data not shown). We have also observed sequences similar to SAox2 and SAox3 in other cnidarians (41). In addition, the *Sarsia* sequences show a strong bias for A+T-rich codons, a characteristic observed for other cnidarian genes (H. Bode, personal communication). This bias may account for the previous inability to detect these sequences by more classical techniques (5, 6).

The presence of *Antp*-class homeobox sequences in cnidarians is of evolutionary significance. The striking conservation of *Antp*-class homeobox genes and their expression patterns among segmented organisms provided the first evidence of the important role of homeobox genes in developmental regulation. The continuing isolation of *Antp*-class sequences in nonsegmented organisms such as sea urchin (42–44), leech (45), and the nematode *Caenorhabditis elegans* (46–49) attests to the antiquity of the homeobox gene regulatory system. The recent discovery of homeobox genes in higher plants (50, 51), although they are quite different from *Antp*-class sequences, provides evidence that this DNA-binding motif is conserved outside the metazoan lineage. The identification of highly conserved cnidarian *Antp*-class homeobox sequences brings us even closer to understanding the origins and evolution of this ancient gene family. It is worth noting that the *Antp*-class sequences we have detected in cnidarians most closely resemble genes from the 3′, or anterior-most, portions of the clusters present in higher metazoans. This may suggest that a primitive regulatory system exploiting anteroposterior polarity evolved in the common ancestor of cnidarians and other metazoans. This might also suggest that duplications of the homeobox genes to form the clusters have tended to occur in one direction, towards the 5′, or posterior, portion of the complex. Subtle divergence of both the regulation and function of the newly duplicated homeobox genes could then have occurred in lineages giving rise to segmented animals.

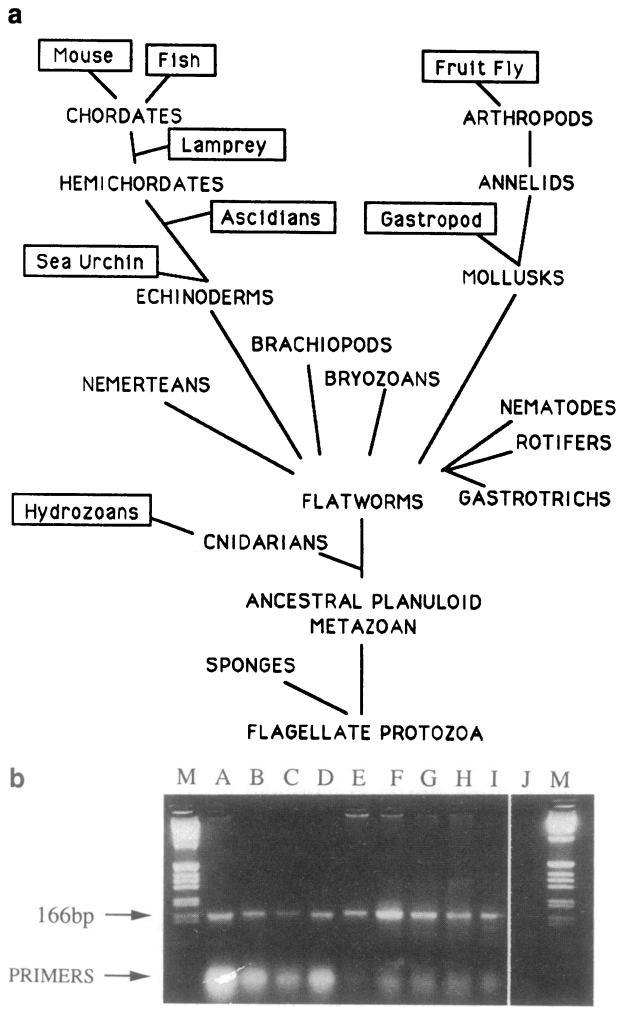

FIG. 3. (*a*) A possible phylogeny of the animal kingdom showing presumed relationships among species surveyed for homeobox sequences (outlined in boxes). (Modified from ref. 36.) (*b*) Evolutionary survey for *Antp*-class homeobox sequences by PCR. Genomic DNA (100–500 ng) from several metazoan species was amplified using primers HoxA and HoxB, and 10% of each reaction mixture was analyzed in a 2% agarose gel. A photograph of the ethidium bromide stained gel is shown, and the positions of the PCR product (166 bp) and oligonucleotide primers are indicated. Lanes: A, mouse (*Mus musculus*); B, fruit fly (*Drosophila melanogaster*); C, fish (swordtail); D, lamprey (*Petromyzon marinus*); E, ascidian (*Ciona intestinalis*); F, sea urchin (*Sphaerechinus granularis*); G, gastropod (*Patella coerula*); H, hydrozoan (*Sarsia* sp.); I, hydroid (*Hydractinia symbiolongicarpus*); J, no template; M, molecular-size markers [1-kb DNA ladder (BRL)].

CONCLUSIONS

PCR is a rapid and sensitive technique for the detection and characterization of homeobox sequences in populations of DNA or RNA. Our analysis allows a reliable estimate of the number and type of *Antp*-class homeobox genes expressed in a particular tissue or cell type from a minute amount of starting material, and this approach should greatly enhance existing methods used to study homeobox gene expression and function. Evolutionary relationships promise to be clarified and strengthened by comparing the type of homeobox sequences present in a particular species with the level and complexity of its development. The great sensitivity of our assay is demonstrated by the detection of *En-1* in the developing mouse telencephalon when better templates are scarce or unavailable. Singh *et al.* (52) have described some of the same homeobox sequences reported here, which they obtained by screening the mouse genome with a degenerate oligonucleotide based on one of the most conserved homeodomain motifs (KIWFQNRR). Several novel non-*Antp*-class sequences were also observed. This appears to be an excellent method for a more exhaustive search for homeobox genes in general. Our PCR method is better applied to a more rapid and less labor-intensive identification of specifically *Antp*-class sequences, as we now have primer pairs that detect all of the known mammalian *Antp*-class homeobox sequences (unpublished work). As reports of new and different types of homeobox sequences continue, batteries of different primers can be designed to expand the power of homeobox gene detection by PCR. The continuing application of PCR techniques will provide a powerful and efficient method for the study of the evolution and expression of the homeobox gene family.

SPECIES	SEQUENCE	AMINO ACIDS 22-46
	SAox1:	FNKYLTRARRVEIAQILKLTESQIK
Mouse	*Hox-1.6*:	----------AS-Q-N-T-V-
Mouse	*Hox-2.9*:	-----S--------AT-E-N-T-V-
Drosophila	*lab*:	--R-------I---NT-Q-N-T-V-
Mouse	*Hox-2.8*:	-----C-P------AL-D---R-V-
Drosophila	*pb*:	-----C-P--I---AS-D---R-V-
	SAox2:	NNRYLSRLRRIQIAAMLDLTEKQVK
Drosophila	*pb*:	F-K--C-P---E---S-----R---
Mouse	*Hox-2.8*:	F-K--C-P--VE---L-----R---
	SAox3:	SCHFLKKERRIELAKQLSLTERQIK
Drosophila	*Ubx*:	TN-Y-TRR----M-HA-C-------

FIG. 4. *Sarsia* homeobox sequences (amino acids 22–46) compared with the most similar known homeobox sequences. Hyphens indicate identity with the sequence at the top of each group. References are as follows: mouse *Hox-1.6* (37), mouse *Hox-2.8* (38), mouse *Hox-2.9* (39), *Drosophila* labial (*lab*) (40), *Drosophila* proboscipedia (*pb*) (T. Kaufman, personal communication), and *Drosophila Ubx* (4).

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