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Biochemistry

Hox-1.11 and *Hox-4.9* homeobox genes

(*Hox-4.3*/*Hox-4.2*/homeobox nucleotide sequences)

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ABSTRACT **Mouse *Hox-1.11* and *Hox-4.9* genes were cloned, and the nucleotide sequences of the homeobox regions were determined. In addition, nucleotide sequence analysis of the homeobox regions of cloned *Hox-4.3* and *Hox-4.2* genomic DNA revealed some differences in nucleotide sequences and in the deduced homeodomain amino acid sequences compared with the sequences that have been reported.**

Homeobox genes code for proteins that bind to specific nucleotide sequences in DNA and either activate or inhibit the expression of the corresponding genes (for reviews, see refs. 1–5). Homeobox proteins are related to one another primarily in the sequence of the 60-amino acid residue DNA-binding-site portion of the protein, the homeodomain. The homeobox family of genes is large; more than 50 mouse homeobox genes or species of cDNA have been reported thus far, and additional homeobox genes undoubtedly will be found in the future. Many homeobox genes reside at neighboring sites in the chromosome in clusters of homeobox genes (5, 6). Whereas the *Drosophila* genome contains only one copy of the *Antennapedia (Antp)* and *Ultrabithorax (Ubx)* clusters of homeobox genes, mammalian genomes contain four copies of the combined *Antp–Ubx* cluster of homeobox genes, which presumably originated by successive duplications of an ancestral cluster of genes (7). The amino acid sequences of the homeodomains encoded by genes that originated as copies of the same ancestral gene, which are located in different clusters of genes, are more closely related to one another than the homeodomains encoded by other genes within the same cluster. Both the amino acid sequence of the homeodomain encoded by each gene and the order of the genes within the four mammalian *Antp–Ubx* clusters of genes have been highly conserved during evolution. Why the organization of genes within each cluster has been maintained during evolution is not known, but several clues have been found. There is considerable overlap in the expression of many of the homeobox genes in the *Antp–Ubx* clusters of genes along the anterior–posterior axis of the embryo, but the anterior border of gene expression is successively displaced towards the posterior, starting with the second gene from the 3′ end of the cluster and progressing toward the gene at the 5′ end of the cluster (8, 9). Thus, different combinations of homeobox genes are expressed in different regions along the anterior–posterior axis of the embryo (10, 11). In addition, treatment of cultured human embryonal carcinoma cells with retinoic acid results in the gradual, sequential activation of many homeobox genes in each cluster over a period of days, starting with the gene at the 3′ end of the cluster and proceeding towards the 5′ end of the cluster (6). These results suggest that the order of homeobox genes within each cluster may be involved in determining the topographic position and/or the developmental time of initiation of expression of these homeobox genes in the embryo.

In this report, the nucleotide sequences of the homeobox regions of *Hox-1.11* and *Hox-4.9* genes are described.†

METHODS AND MATERIALS

Clones of PCR-Amplified Mouse Genomic DNA. The homeobox regions of many mouse homeobox genes were amplified by PCR. Multiple species of oligodeoxynucleotides that correspond to highly conserved sequences in the homeoboxes of many mouse homeobox genes were synthesized with the aid of an Applied Biosystems DNA synthesizer model 380B and purified by OPC (Applied Biosystems) column chromatography. The (+)-oligodeoxynucleotide primers consisted of 64 species of oligodeoxynucleotides, each 28 nucleotide residues long, with a *Sac* I site near the 5′ terminus; the (−)-oligonucleotide PCR primers consisted of 48 species of oligodeoxynucleotides, 28 nucleotide residues long, with an *Eco*RI site near the 5′ terminus. (See Fig. 2 for the nucleotide sequences of the primers.)

A programmable DNA thermal cycler (Perkin–Elmer/Cetus) was used for the amplification of DNA. A typical 25-μl reaction mixture contained 1 μg of BALB/c mouse liver genomic DNA; 50 mM KCl; 10 mM Tris·HCl (pH 8.3); 1.5 mM $MgCl_2$; 0.01% gelatin; 15.6 nM of each species of (+)-oligodeoxynucleotide primer and 20.8 nM of each species of (−)-oligodeoxynucleotide primer; 1.0 mM each of dATP, dCTP, dGTP, and dTTP; and 2.5 units of *Taq* polymerase. Reaction mixtures were covered with 50 μl of mineral oil and were incubated for 35 PCR cycles; each cycle consisted of incubation for 1 min at 94°C, 2 min at 37°C, and 3 min at 65°C. After the last cycle, the reaction mixtures were incubated for an additional 10 min at 65°C. The DNA was precipitated with ethanol, incubated with *Eco*RI and *Sac* I, and subcloned in pBluescript II KS(+) (Stratagene).

RNA Probes. ^{32}P-labeled (+)-RNA probes were prepared by using a modification of the Stratagene RNA transcription protocol. A typical 10-μl reaction mixture contained 40 mM Tris·HCl (pH 8.0), 8 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl, 10 mM dithiothreitol, 10 μM [α-^{32}P]UTP (800 Ci/mmol; 1 Ci = 37 GBq), 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 194 fmol of linear proteinase K-treated DNA, 10 units of RNase inhibitor, and 4 units of phage T7 RNA polymerase. Reaction mixtures were incubated at 37°C for 30 min; then RNA was precipitated with sodium acetate and ethanol.

Clones of Unamplified Homeobox Genomic DNA. A mouse genomic DNA library in λGEM-11 (Promega) was screened for some of the homeobox genes that had been found with PCR-amplified DNA. *E. coli* KW251 cells (2×10^9) infected

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†The sequences for *Hox-1.11*, *Hox-4.9*, *Hox-4.3*, and *Hox-4.2* have been deposited in the GenBank data base (accession nos. M87801–M87804, respectively).

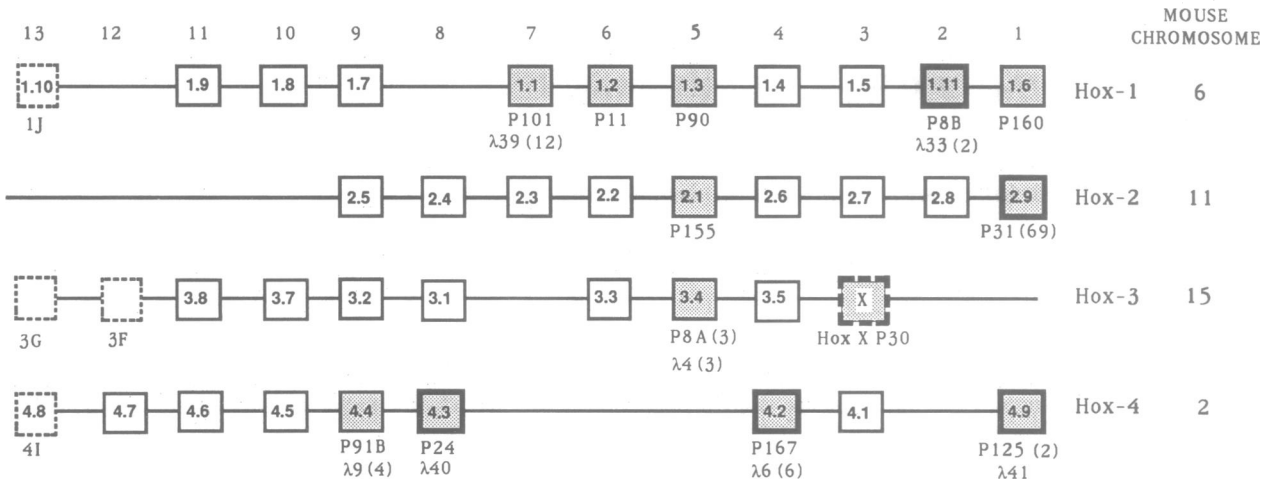

FIG. 1. Mouse homeobox gene clusters. Genes that code for proteins with similar homeodomain amino acid sequences that are thought to be copies of the same ancestral gene are aligned vertically. The numbers 1 through 13 (from right to left) at the top of the figure represent the vertical sets of related genes in different clusters. Clones of homeobox genes described in this report are shown with shaded backgrounds. Homeobox nucleotide sequences shown in this report are indicated by boxes drawn with thick solid or dashed lines. The chromosomal location of *Hox-X* is uncertain. The boxes drawn with thin dotted lines indicate that no mouse homeobox sequence has been reported; the names of the human *HOX* genes (6, 13) are shown beneath these boxes. Some DNA clones described in this report also are shown beneath the appropriate box; the number of DNA clones found is enclosed within parentheses. DNA clones that begin with P are clones of PCR-amplified mouse genomic DNA; clones prefaced by λ are clones of mouse genomic DNA that were not amplified prior to cloning.

with 25,000 recombinant phages were plated on each 150-mm Petri dish. Phage DNA adsorbed to replica nytran filters (GeneScreen*Plus*, DuPont) was hybridized overnight at 60°C with ^{32}P-labeled RNA (35 fmol/ml, 2×10^6 cpm/ml) synthesized from cloned PCR-amplified DNA. The hybridization buffer contained 1 M NaCl, 50 mM Tris·HCl (pH 7.6), 1% SDS, and 100 μg of yeast tRNA per ml. Filters were washed twice with 2× SSC (300 mM sodium chloride/30 mM sodium citrate, pH 7.0) at room temperature for 15 min (each wash), followed by two washes in 2× SSC/1% SDS at 60°C for 60 min (each wash) and finally by one wash in 0.1× SSC at 24°C for 30 min. Filters then were exposed to x-ray film in cassettes at −70°C. Recombinant phage with matching positive signals on autoradiograms of replica filters were cloned. DNA inserts were excised with *Sac* I and cleaved with various restriction enzymes; some DNA fragments were subcloned into pBluescript II SK(+).

DNA Sequencing. Both strands of cloned DNA fragments were sequenced manually by using Sequenase 2.0 (United States Biochemical) with universal phage M13 primers or specific primers by the dideoxynucleotide chain-termination method (12), and also by using an automated DNA sequencer (Applied Biosystems Model 373A) with *Taq* DNA polymerase at 70°C, dITP instead of dGTP, dideoxynucleotides or primers labeled with fluorescent dyes, double- or single-stranded DNA preparations, and other components according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Clones of PCR-Amplified Homeobox DNA. Small DNA fragments that correspond to part of the homeobox of many mouse homeobox genes were amplified from mouse genomic DNA with the use of sets of primers, each set consisting of multiple species of oligodeoxynucleotides that correspond to conserved nucleotide sequences within the homeobox (nucleotide residues 43–68 and 142–162). The amplified DNA was subcloned, and the chain lengths of the DNA inserts from

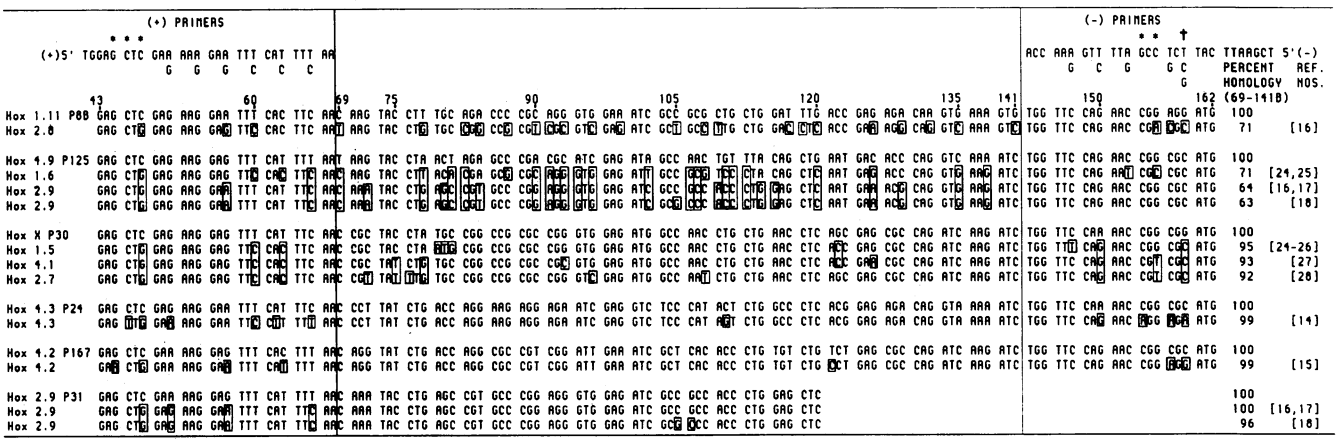

FIG. 2. The nucleotide sequences of six homeobox DNA clones, obtained by PCR amplification of mouse genomic DNA, are shown and are compared with the sequences of the most closely related mouse homeobox genes. The numbers at the top correspond to homeobox nucleotide residues. The sequences of oligodeoxynucleotide primers for PCR amplification of DNA are shown at the top. The asterisks above the primers indicate that only one nucleotide residue is present at the position indicated; thus oligodeoxynucleotides will base-pair correctly if the codon sequence in DNA is complimentary to that of the oligodeoxynucleotide but not if the DNA contains other synonym codons for the same amino acid. The symbol † indicates that (−)-oligodeoxynucleotide primers do not contain A at this position; hence, correct base pairs can form if the DNA contains five of the six arginine codons but not if the DNA contains the arginine codon CGT.

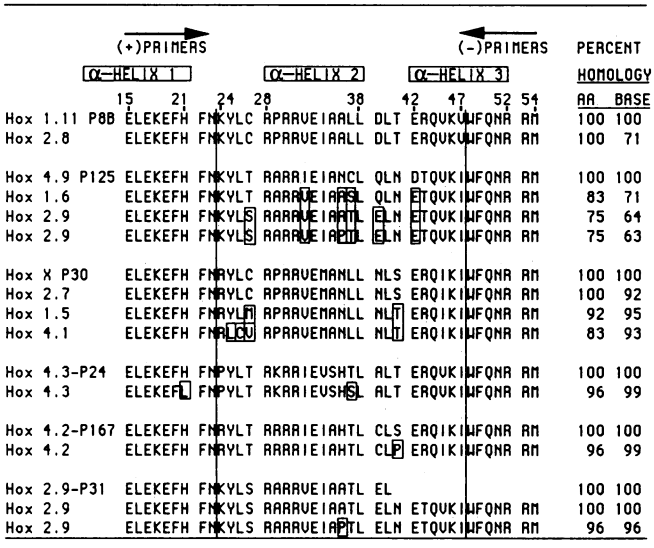

FIG. 3. The amino acid sequences deduced from the nucleotide sequences of PCR-amplified cloned mouse genomic DNA (Fig. 2) are shown and are compared with the most closely related mouse homeodomain amino acid sequences. The percent homology between related amino acid sequences for the central region between the PCR primers (amino acid residues 24–47) and the percent homology between nucleotide sequences shown in Fig. 2 (nucleotide residues 69–141) are shown. The numbers at the top refer to homeodomain amino acid residues. The positions of α-helices 1–3 in the *Antennapedia* homeodomain (1) of *Drosophila* also are shown.

93 clones were determined. The nucleotide sequences of some of the DNA inserts also were determined. Of 93 clones examined, 85 were identified as homeobox genes. Two clones, P8 and P91 with different DNA inserts, consist of two homeobox DNA fragments amplified from separate genes that were joined by ligation and cloned. The DNA clones found correspond to 13 mouse homeobox genes as shown in Fig. 1.

The nucleotide sequences of six amplified homeobox genomic DNA clones are shown in Fig. 2 and are compared with the sequences of the most closely related mouse homeobox genes. The percent homology also is shown between the central region of each cloned DNA insert without the primer sequences (homeobox residues 69–141) and the corresponding sequences of the most closely related mouse homeobox genes. The percent homology was calculated for only the central region of the cloned DNA between the primers because oligodeoxynucleotides that hybridize to DNA with some incorrectly paired bases can serve as PCR primers.

Clone *Hox-1.11* P8B corresponds to a novel mouse homeobox gene of the *Drosophila proboscipedia* homeobox class. The nucleotide sequence of clone *Hox-1.11* P8B is most closely related to that of the mouse *Hox-2.8* gene, but only 71% of the nucleotide residues compared are identical.

Clone *Hox-4.9* P125 is a member of the *labial* class of homeobox genes, probably the first gene in the *Hox-4* cluster of homeobox genes (see Fig. 1). The amino acid sequence of the Hox-4.9 homeodomain was reported recently (11), but the nucleotide sequence has not been described. The nucleotide sequence of *Hox-4.9* P125 differs considerably from the sequences of other labial class mouse homeobox genes, *Hox-1.6* (71% homology) and *Hox-2.9* (63–64% homology).

Clone *Hox-X* P30 may correspond to an unreported murine homeobox gene, probably the third gene in the *Hox-3* cluster of homeobox genes (Fig. 1). The nucleotide sequence of the *Hox-X* P30 homeobox clone is most closely related to that of *Hox-1.5* (95% homology).

The nucleotide sequence of clone *Hox-4.3* P24 differs from that of *Hox-4.3* (14) by only 1 of the 73 residues in the central region between the (+)- and (−)-oligonucleotide homeobox primers, which suggests that clone *Hox-4.3* P24 corresponds to the *Hox-4.3* gene. The nucleotide sequence of clone *Hox-4.2* P167 differs from the sequence reported for *Hox-4.2* (15) by only 1 nucleotide residue.

Of the 85 homeobox clones obtained, 69 were found to be only 78 nucleotide residues long because of the presence of a *Sac* I site within the homeobox. Sequence analysis of 9 of the 69 clones revealed one kind of DNA insert with *Sac* I sites at both the 5′ and 3′ termini. The nucleotide sequence of a representative clone, *Hox-2.9* P31, shown in Fig. 2, is identical to the sequence of the *Hox-2.9* gene reported by Rubock *et al.* (16) and Frohman *et al.* (17) and differs by only 2 nucleotide residues from the *Hox-2.9* sequence reported by Murphy and Hill (18).

The amino acid sequences deduced from the nucleotide sequences of PCR-amplified and cloned DNA are shown in Fig. 3 and are compared with the most closely related mouse homeodomain amino acid sequences. The amino acid sequence of clone *Hox-1.11* P8B is the same as that of the *Hox-2.8* gene, but the nucleotide sequences differ markedly (71% homology). Clone *Hox-4.9* P125 is a *labial* class homeobox gene with an amino acid sequence identical to the sequence recently reported for the *Hox-4.9* gene (11). Hox-4.9 P125 differs from the other *labial* class homeobox genes, *Hox-1.6* and *Hox-2.9*, in both nucleotide and amino acid sequences. The amino acid sequence of the homeodomain of clone Hox-4.3 P24 (residues 24–47) differs from that of the *Hox-4.3* gene by 1 amino acid residue. Since the corresponding nucleotide sequences differ by only 1 residue, Hox-4.3

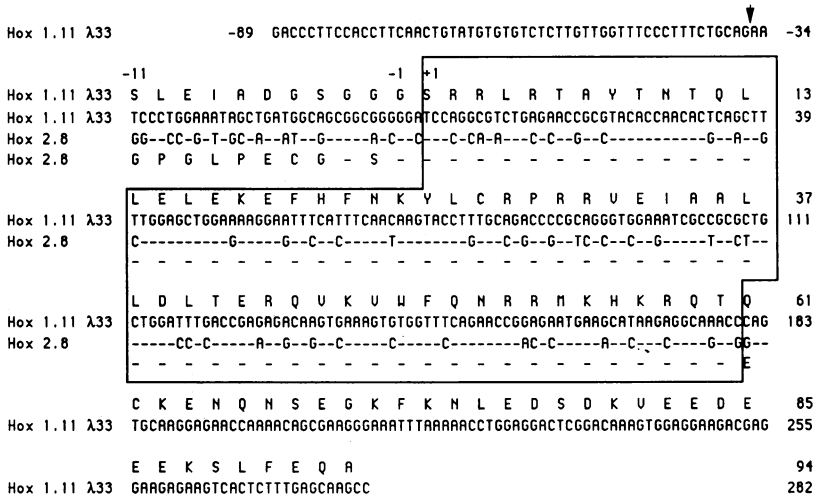

FIG. 4. The nucleotide sequence and deduced amino acid sequence of the *Hox-1.11* (clone λ33) homeobox and flanking regions are shown and are compared with the sequences of the most closely related mouse homeobox gene, *Hox 2.8* (16). Dashes in the nucleotide sequence or amino acid sequence represent a *Hox-2.8* nucleotide or amino acid residue that is identical to the corresponding nucleotide or residue shown for *Hox-1.11*. The homeobox sequence is enclosed within a box. The arrowhead represents an intron–exon junction.

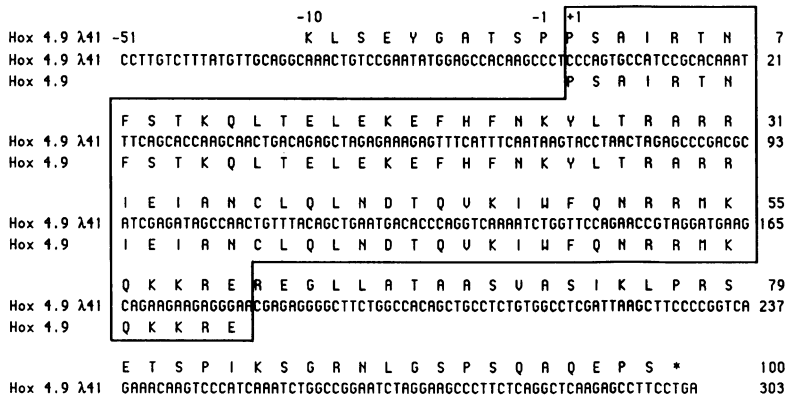

FIG. 5. The nucleotide sequence and deduced amino acid sequence of the homeobox region of the *Hox-4.9* gene (clone λ41) are shown. The amino acid sequence of Hox-4.9 λ41 is compared to the recently reported (11) Hox-4.9 amino acid sequence. The dashes represent Hox-4.9 amino acid residues that are identical to those of Hox-4.9 λ41. The homeobox region is enclosed within a box.

P24 DNA probably corresponds to the *Hox-4.3* gene. Similarly, the nucleotide and deduced amino acid sequences of clone *Hox-4.2* P167 differ from those of the *Hox-4.2* gene by only 1 nucleotide residue and 1 amino acid residue, which suggests that clone *Hox-4.2* P167 corresponds to *Hox-4.2*. The nucleotide and amino acid sequences of clone *Hox-2.9* P31 are the same as those of the *Hox-2.9* gene (16, 17).

Clones of PCR-amplified DNA also were obtained and sequenced that correspond to *Hox-1.1*, *Hox-1.2*, *Hox-1.3*, *Hox-1.6*, *Hox-2.1*, *Hox-3.4*, and *Hox-4.4* (data not shown).

Homeobox Genomic DNA Clones in λGEM-11. A mouse genomic DNA library in λGEM-11 with 15-kilobase (kb) DNA inserts (average size) that were not amplified prior to cloning was screened for homeobox genes with a mixture of ^{32}P-labeled RNA probes synthesized from PCR-amplified, cloned DNA that correspond to *Hox-1.11*, *Hox-4.9*, *Hox-4.3*, *Hox-4.2*, *Hox-3.4*, *Hox-2.9*, *Hox-1.2*, and *Hox-1.1*. Two million recombinants were screened, and 29 clones of homeobox genomic DNA were obtained. Restriction site analysis revealed seven kinds of DNA inserts that were shown by nucleotide sequence analysis to correspond to seven homeobox genes (*Hox-1.11*, *Hox-4.9*, *Hox-4.4*, *Hox-4.3*, *Hox-4.2*, *Hox-3.4*, and *Hox-1.1*).

Hox-1.11. Two genomic DNA clones, λ16 and λ33, were found that correspond to *Hox-1.11*. The nucleotide sequence and deduced amino acid sequence of the *Hox-1.11* λ33 homeobox and flanking regions are shown in Fig. 4 and are compared with homeobox sequences of the most closely related homeobox gene, *Hox-2.8*. The nucleotide sequence of *Hox-1.11* λ33 DNA, which was not amplified prior to cloning, was identical to that found with *Hox-1.11* P8B cloned from PCR-amplified mouse genomic DNA (nucleotide residues 69–141) shown in Fig. 2. Although only 74% of the *Hox-1.11* λ33 and *Hox-2.8* homeobox nucleotide residues are the same, the amino acid sequences of the homeodomains of Hox-1.11 and Hox-2.8 are identical. However, 9 of the 11 Hox-1.11 λ33 deduced amino acid residues that precede the homeodomain and 1 amino acid residue after the homeodomain differ from those of Hox-2.8. These results show that *Hox-1.11* and *Hox-2.8* are separate genes. The intron–exon junction shown in Fig. 4 at nucleotide residue −36 was identified by comparing the nucleotide sequences of *Hox-1.11* genomic DNA and cDNA, which will be described elsewhere (D. Tan, J. Ferrante, A.N., C. Kozak, V. Guo, and M.N., unpublished data); elsewhere we also show that the *Hox-1.11* gene resides in mouse chromosome 6, which suggests that the *Hox-1.11* gene is a member of the *Hox-1* cluster of genes.

The amino acid sequences of the Hox-1.11 and Hox-2.8 homeodomains are identical, which suggests that both species of homeobox proteins may bind to the same or similar nucleotide sequences in DNA. Another pair of homeobox proteins, Hox-1.3 (19, 20) and Hox-2.1 (21–23) also have identical homeodomains.

Hox-4.9 λ41. The nucleotide sequence and deduced amino acid sequence of the homeobox and flanking regions of clone *Hox-4.9* λ41 mouse genomic DNA are shown in Fig. 5. The nucleotide sequence of *Hox-4.9* has not been reported previously; however, the deduced amino acid sequence (λ41) is the same as the recently reported Hox-4.9 homeodomain amino acid sequence (11), which suggests that λ41 DNA is a *Hox-4.9* genomic DNA clone. The nucleotide sequence of *Hox-4.9* λ41 mouse genomic DNA (cloned from DNA that was not amplified) is identical to that found with PCR-amplified mouse genomic DNA (clone *Hox-4.9* P125 homeobox nucleotide residues 69–141 shown in Fig. 2).

Hox-4.3 λ40. The nucleotide sequence and deduced amino acid sequence of the homeobox and surrounding regions of *Hox-4.3* λ40 DNA are shown in Fig. 6 and are compared with the nucleotide and amino acid sequences of the most closely related homeobox gene, *Hox-4.3* (14). The nucleotide sequence of the λ40 mouse genomic DNA fragment, cloned from DNA that was not amplified, was found to be identical to that of *Hox-4.3* P24, derived from PCR-amplified mouse genomic DNA shown in Fig. 2 (homeobox nucleotide residues 69–141). Only 4 of the 209 λ40 nucleotide residues compared differ from those reported for *Hox-4.3* (14); however, three of the homeodomain amino acid residues differ from those reported for Hox-4.3. The high nucleotide se-

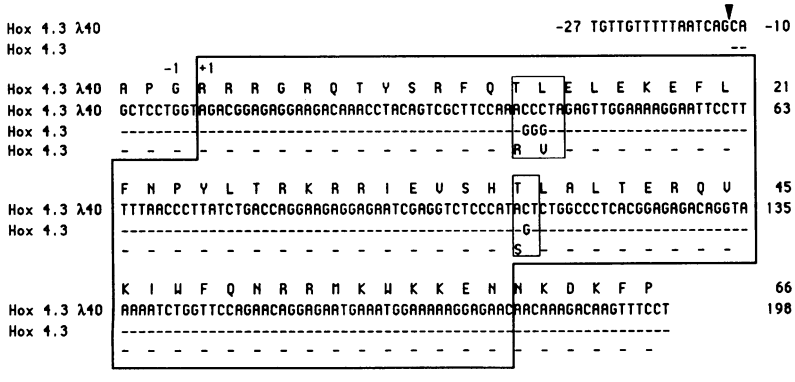

FIG. 6. The nucleotide sequence and deduced amino acid sequence of the homeobox and surrounding regions of *Hox-4.3* λ40 mouse genomic DNA are shown and are compared with the *Hox-4.3* nucleotide and amino acid sequences reported (14). Only nucleotide and amino acid residues of *Hox 4.3* that differ from those of clone λ40 are shown; residues that are the same are indicated by dashes. The homeobox region is enclosed within a large box. Nucleotide and amino acid residues that differ are enclosed within small boxes. The arrowhead represents an intron–exon junction reported for *Hox-4.3* (14).

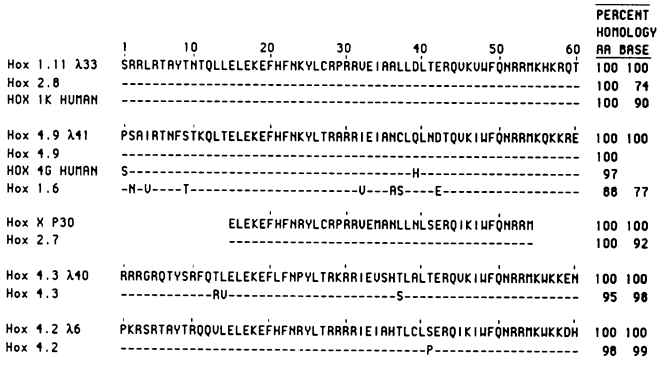

FIG. 7. The amino acid sequences of the homeodomains encoded by five mouse homeobox genes deduced from the nucleotide sequences of cloned DNA are shown and are compared with the amino acid sequences of the most closely related mouse or human homeodomains. The percent homology between the amino acid sequences of related homeodomains and between the corresponding homeobox nucleotide sequences also are shown. Only differences in amino acid sequence are shown. Dashes represent identical amino acid residues.

quence homology between λ40 and *Hox-4.3* suggests that clone λ40 DNA corresponds to the *Hox-4.3* gene. However, the possibility that clone λ40 DNA corresponds to a novel homeobox gene, *Hox-1.12*, the eighth gene in the *Hox-1* cluster of homeobox genes shown in Fig. 1, is not ruled out.

A summary of results is shown in Fig. 7. Homeodomain amino acid sequences deduced from the nucleotide sequences of cloned mouse genomic DNA are shown and are compared with the most closely related sequences reported for mouse or human homeobox proteins. The amino acid and nucleotide sequence homologies also are shown. The amino acid sequence of the Hox-1.11 homeodomain is identical to that of Hox-2.8; however, 9 of the 11 amino acid residues before the homeodomain and 1 amino acid residue after the homeodomain differ from those of Hox-2.8. Many differences also were observed in the nucleotide sequences of the *Hox-1.11* and *Hox-2.8* homeobox regions. The mouse Hox-1.11 homeodomain is the equivalent of the recently reported human HOX-1K homeodomain (6).

The amino acid sequence of the Hox-4.9 λ41 homeodomain is identical to the recently reported Hox-4.9 homeodomain amino acid sequence (11). The mouse Hox-4.9 homeodomain is the equivalent of the human HOX-4G homeodomain (13).

Six of the 73 *Hox-X* P30 nucleotide residues differ from the corresponding sequence of the most closely related homeobox gene, *Hox-2.7*; however, the deduced amino acid sequence of Hox-X P30 is the same as that of Hox-2.7. The cumulative error due to misincorporation of bases during DNA amplification was estimated by comparing the DNA sequences of 10 clones of mouse genomic DNA subjected to 35 cycles of DNA amplification (730 nucleotide residues compared) with mouse genomic DNA sequences that were not amplified prior to cloning, which correspond to *Hox-1.11, -4.9, -1.1, -3.4, -4.2, -4.3*, and *-4.4*. No DNA amplification errors were detected. Comparison of the nucleotide sequences of 13 additional clones of PCR-amplified DNA that correspond to *Hox-1.2, -1.3, -1.6, -2.1*, and *-2.9* with published nucleotide sequences revealed only 2 residues that differ (906 nucleotide residues compared). Hence, no more than 2 misincorporated nucleotide residues were found per 1636 residues sequenced in DNA molecules that were cloned after 35 cycles of DNA amplification; that is, the error due to PCR amplification of DNA is no more than 1 residue per 818 nucleotide residues of cloned DNA. Thus, we think it more likely that *Hox-X* P30 DNA corresponds to a homeobox gene that has not been reported previously, such as the third gene of the *Hox-3* cluster of homeobox genes shown in Fig. 1, rather than a DNA clone with an erroneous sequence due to misincorporation of 6 nucleotide residues during DNA amplification. However, the nucleotide sequence of a *Hox-X* mouse genomic DNA clone that was not amplified prior to cloning is needed to confirm the nucleotide sequence of *Hox-X* P30.

Also, as shown in Fig. 7, the deduced amino acid sequences of the Hox-4.3 λ40 and Hox-4.2 λ6 homeodomains were found to differ from the reported sequences (14, 15) by 3 and 1 amino acid residues, respectively.

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