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Remarkable Intron and Exon Sequence Conservation in Human and Mouse Homeobox *Hox 1.3* Genes

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A high degree of conservation exists between the *Hox 1.3* homeobox genes of mice and humans. The two genes occupy the same relative positions in their respective *Hox 1* gene clusters, they show extensive sequence similarities in their coding and noncoding portions, and both are transcribed into multiple transcripts of similar sizes. The predicted human Hox 1.3 protein differs from its murine counterpart in only 7 of 270 amino acids. The sequence similarity in the 250 base pairs upstream of the initiation codon is 98%, the similarity between the two introns, both 960 base pairs long, is 72%, and the similarity in the 3′ noncoding region from termination codon to polyadenylation signal is 90%. Both mouse and human *Hox 1.3* introns contain a sequence with homology to a mating-type-controlled *cis* element of the yeast Ty1 transposon. DNA-binding studies with a recombinant mouse Hox 1.3 protein identified two binding sites in the intron, both of which were within the region of shared homology with this Ty1 *cis* element.

The development of a fertilized egg into a complex multicellular organism is an intricate process requiring cell division, cell migration, and differentiation. Our insights into these mechanisms operating during embryogenesis, particularly during *Drosophila* body pattern formation, have advanced enormously in the past few years (for a review, see reference 25). Genetic analyses of *Drosophila* mutants have defined three classes of genes that play important roles in providing positional information: the maternal effect genes, the segmentation genes, and the homeotic genes. Many but not all of these genes contain a highly conserved sequence of 180 base pairs (bp), the homeobox (17, 24), which is known to encode a DNA-binding domain (for review, see reference 10).

In an earlier communication, we described the organization, sequence, and expression of the murine *Hox 1.3* homeobox gene, a member of the *Hox 1* cluster located on mouse chromosome 6 (21). This gene has two exons separated by a 960-bp intron and encodes a 270-amino-acid homeodomain protein. Recently, we have shown that the Hox 1.3 protein is phosphorylated and that it binds to DNA in a sequence-specific manner (20). The consensus binding motif is CPyPyNATTAT/GPy (Py = pyrimidine).

In this communication, we describe the sequence and organization of the human *Hox 1.3* gene and its location within the human *Hox 1* cluster. A high level of homology is observed between the human and mouse *Hox 1.3* genes. The nucleotide sequence analysis predicts that the human and the mouse proteins both have 270 amino acids and differ from each other at only seven positions. The homology is also very high in the region 5′ to the predicted cap site, the 3′ noncoding region, and, most surprisingly, the intron. We show here that a conserved region in the human and murine *Hox 1.3* introns contains two binding sites for the Hox 1.3 protein and shares homology with the central epsilon region of the yeast Ty1 transposon.

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Organization of the human *Hox 1* cluster. A human genomic λ EMBL 3 library (a gift from J. Weiss, Harvard University, Cambridge, Mass.) was screened under stringent conditions with a cDNA fragment (5′ probe) encoding the amino-terminal portion of the murine Hox 1.3 protein but not the homeodomain. Two λ clones (λ26 and λ32) were identified and further characterized by Southern blot analysis, using the 5′ probe and two additional probes which represented the homeobox region and the 3′ untranslated regions of the gene (designated Hox 1.3 box probe and 3′ probe). A 3.5-kilobase-pair (kbp) (*Sal*I-*Eco*RI) DNA fragment from λ32 hybridized with the 5′, 3′, and homeobox probes. A 6-kbp *Sal*I DNA fragment from λ26 hybridized with the 5′ probe and the *Hox 1.3* box probe but not with the 3′ probe, which suggested that this fragment did not contain the entire *Hox 1.3* gene. Both fragments were subcloned into the Bluescribe plasmid vector (Stratagene), and the relevant portions of each clone were sequenced on both strands by the dideoxy-chain termination sequencing technique (23). The two λ clones overlap by 400 bp in the 5′ protein-encoding region of the *Hox 1.3* gene. DNA from these two clones was prepared by standard procedures (14); Southern analysis, using a *Hox 1.3* homeobox probe, identified a total of four homeobox homologies in these overlapping clones. Two of them were located in λ26, and the other two were in λ32. To further characterize this homeobox cluster, we probed Southern transfers with specific oligonucleotide probes containing sequences immediately flanking the 3′ end of the homeoboxes of the murine genes *Hox 1.1*, *Hox 1.2*, *Hox 1.3*, and *Hox 1.4* (in the antisense orientation). The sequences of these oligonucleotide probes were, respectively:

Oligo 1.1 AACGGAGGGCACCGCGTCTTCCGGGGCTGCAG TGGGAGC
Oligo 1.2 TTTGGCCTCAGAGTCTTCCCCGCTGGCCTGCGT
Oligo 1.3 TCCTGCCGCGGCCATGCTCATGCTTT
Oligo 1.4 CGAGGCAGTGTTGGAAGATCGCATCTT

We also used the *Hox 1.1* pm6 probe, corresponding to the *Pvu*II-*Sac*I DNA fragment from clone pm6 (4) which con-

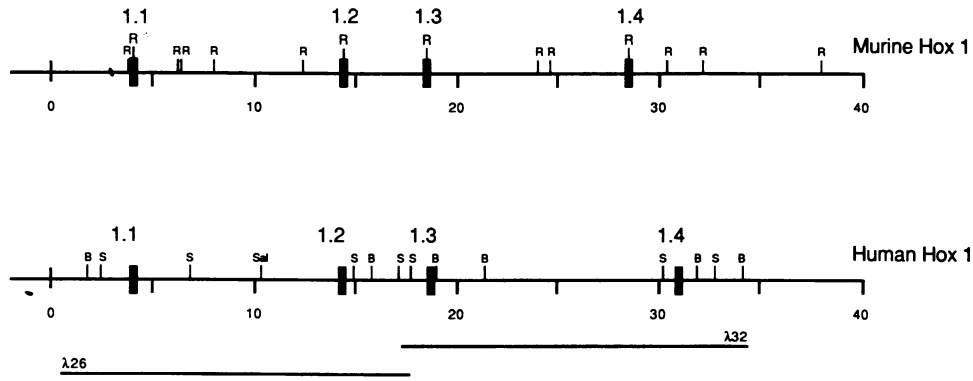

FIG. 1. Comparison of the human *Hox 1* cluster contained in the genomic clones λ26 and λ32 with the mouse cluster. The *Eco*RI restriction map of the murine cluster was compiled from references 4, 6, 15, and 21. The human restriction map of the *Hox 1* cluster was compiled by using the *Bam*HI (B), *Sac*I (S), and *Sal*I (Sal) restriction enzymes. Black boxes represent homeoboxes. The two overlapping recombinant phage inserts (λ26 and λ32) are shown below the human *Hox 1* cluster restriction map.

tains sequences downstream of the *Hox 1.1* homeobox. These probes were chosen because of their uniqueness, and none contained homeobox sequences.

When using the oligonucleotide probes oligo 1.1, 1.2, 1.3, and 1.4, hybridizations were performed overnight at 55°C in 2× SSC (SSC is 0.15 NaCl plus 0.015 sodium citrate)–10× Denhardt solution–1% sodium dodecyl sulfate. Blots were washed at 55°C in 1× SSC–0.1% sodium dodecyl sulfate until the background was sufficiently reduced (15 to 30 min). For the Hox 1.1 pm6 probe, hybridization was performed at 37°C in 43% formamide containing SSC, 1× Denhardt solution, and 1% sodium dodecyl sulfate. The blots were washed at 55°C in 0.2× SSC. Under these conditions, we detected two different restriction endonuclease fragments of λ26 that hybridized strongly, one with the *Hox 1.1* and the other with the *Hox 1.2* probe. Two restriction endonuclease fragments from λ32 gave a strong hybridization signal with the *Hox 1.3* homeobox probe, and one of them also hybridized with the Hox 1.3 oligonucleotide probe (data not shown). The other fragment, which was positive with the homeobox probe, did not give a strong signal when probed with the Hox 1.4 oligonucleotide, presumably because of sequence differences between the human and murine genes in the region 3′ of the *Hox 1.4* homeobox.

From restriction mapping and Southern analyses of the λ26 and λ32 clones, we deduced the genomic map shown in Fig. 1, in which the human cluster is compared with the murine cluster. The murine *Hox 1.3* gene is a member of a cluster containing at least six homeobox genes on chromosome 6 (21). Here we show that the human *Hox 1* cluster, mapped by Rabin et al. (22) to human chromosome 7 (7p14 to p21), is organized in a very similar way, with cognate homeoboxes occupying the same positions relative to one another. Similarly, the *Hox 2* clusters of mice and humans also display conserved organization (1, 11). This conserved clustered organization is intriguing. It is possible that the order and spacing of homeobox genes in a cluster are conserved to maintain the complex transcriptional pattern of these genes. Northern (RNA) blot analysis of mRNA (prepared as described by Maniatis et al. [14]) from either cultured nonconfluent human embryonic foreskin fibroblasts or frozen autopsy specimens from a 56-year-old woman showed that the human gene is expressed in transcripts of several sizes (Fig. 2). We have previously shown that the murine *Hox 1.3* sequence is transcribed into at least four different polyadenylated transcripts of approximately 1.85, 4, 8, and 9 kbp. The abundance of these transcripts relative to one another varies from tissue to tissue (21). The human *Hox 1.3* gene is expressed in transcripts of equivalent sizes and, as in mice, most of the adult human tissues examined do not contain all four transcripts (Fig. 2). For instance, kidney contains a 1.85-kbp and a 4-kbp mRNA, whereas lung contains almost exclusively a 1.85-kbp mRNA. Interestingly, in human embryonic fibroblasts we cannot detect the 1.85- and 4-kbp transcripts but find the 8- and 9-kbp transcripts. We have also found that the 5′ end of the murine 4-kbp transcript overlaps by over 1,100 bp with the 3′ end of

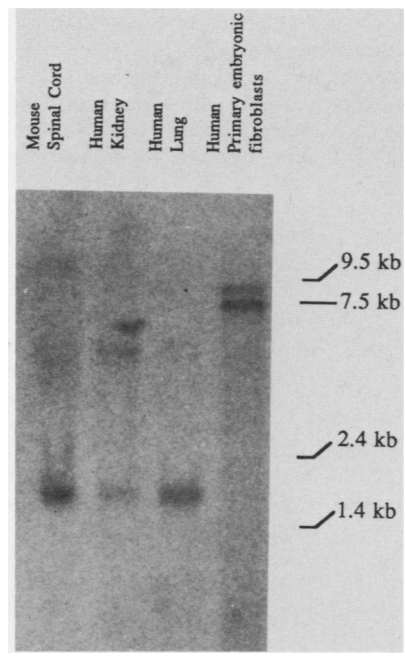

FIG. 2. Northern blot analysis of mouse and human *Hox 1.3* transcripts. Autoradiographic pattern of 5 μg of total RNA from day 15 embryonic mouse spinal cord and 2 μg of poly(A)$^+$ selected mRNA from lung and kidney of a 56-year-old woman or from primary human embryonic fibroblasts. A *Sal*I-*Bgl*II restriction fragment (positions 116 to 524) from the human *Hox 1.3* gene, devoid of any homeobox sequence, was labeled and used as a probe. Positions of the bands from an RNA ladder (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) are indicated on the right. Autoradiography exposure time was 10 days.

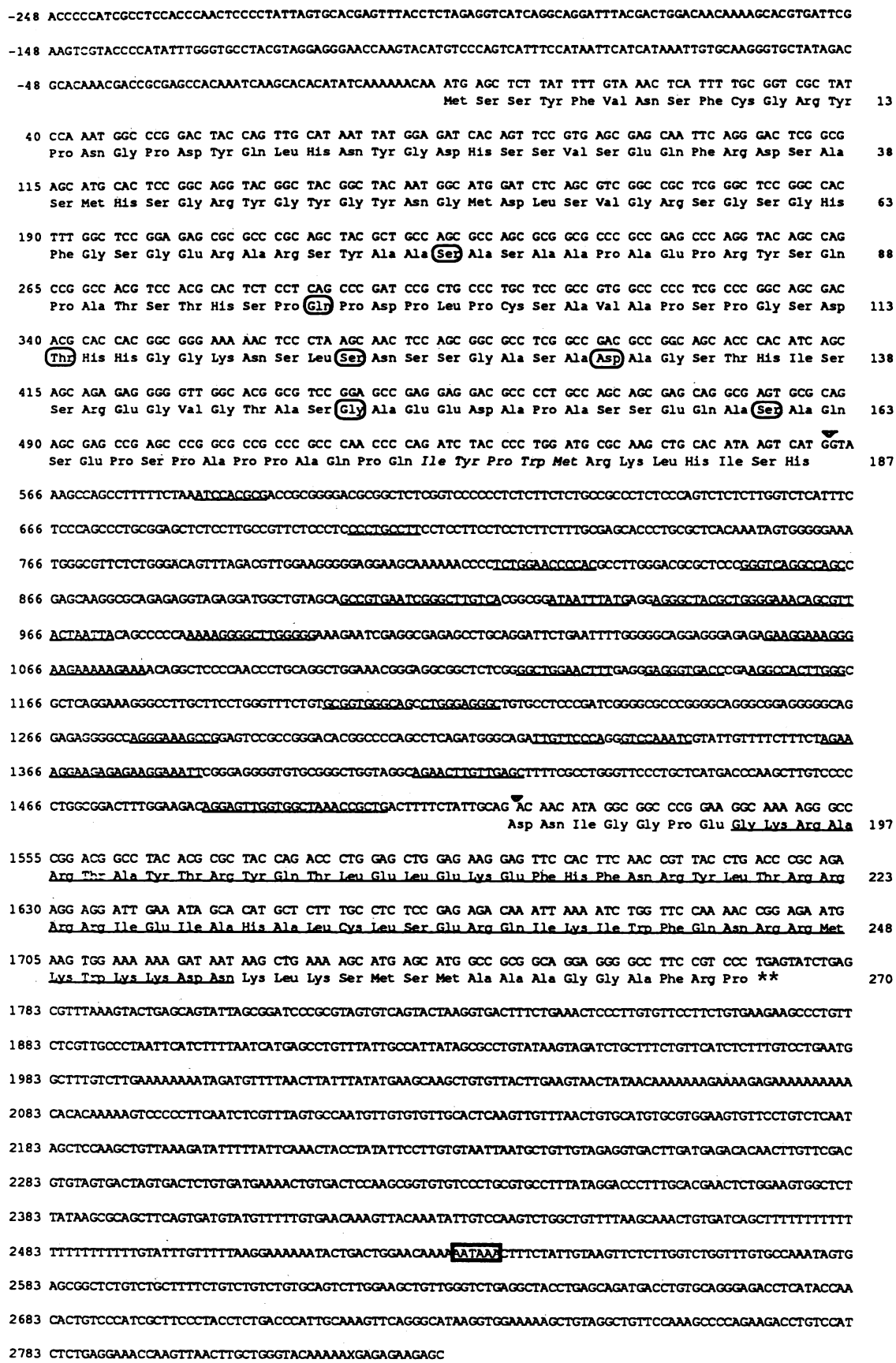

FIG. 3. Primary structure of the human *Hox 1.3* gene. Numbering begins at the predicted translation start codon (nucleotide numbering is on the left; amino acid numbering is on the right). The seven differences in the predicted amino acid sequence between the human and the murine genes are circled. Boundaries of the predicted intron (defined by identity with the murine gene) are indicated by arrowheads. Stretches of 9 bp or longer that are identical in the two introns are underlined. The homeobox is underlined with a solid line; a consensus polyadenylation signal is boxed.

the *Hox 1.2* transcript (J. Garbern, unpublished data). The similarity in size and sequence of human *Hox 1.2* and *1.3* transcripts suggests that a similar situation exists in humans as well.

Comparison of the human and murine *Hox 1.3* primary structures. Sequences of the murine and human *Hox 1.3* genes were analyzed by using the software package of the University of Wisconsin Genetics Computer Group (developed by J. Devereux et al.) on a VAX computer. The sequence of the human *Hox 1.3* gene (Fig. 3) is very similar to the murine *Hox 1.3* sequence (9, 21). This similarity allowed us to assign the putative exon boundaries and to predict that the human *Hox 1.3*, like its murine counterpart, is organized into two exons. The degrees of similarity between the two genes are 98% in the 250 bp upstream the start codon, 94% in the coding region, 72% in the intron, and 90% in the 3′ untranslated region. The predicted proteins encoded by the murine and human genes differ in only 7 of the 270 residues. These seven residues lie in the central region of the protein and are encoded by exon 1 (Fig. 3). A similar degree of conservation has been seen between the human and mouse *Hox 2.3* genes (18, 26) and the human and mouse *Hox 5.1* genes (8, 16). Thus, it appears that the cognate proteins are highly similar not only in the homeodomain but also in the regions outside this domain. In contrast, related proteins of the *Hox 1* and *Hox 2* clusters from the same species do not show such a high degree of similarity.

The high degree of homology between the coding regions of the murine and the human *Hox 1.3* gene extends to the 5′ noncoding regions, where the two genes are 98% identical in the 250 bp upstream of the predicted initiation codon. The murine *Hox 1.3* gene has a potential 5′ extension of the reading frame of 333 bp (Garbern et al., unpublished data). We have not fully sequenced the human gene across the entire corresponding region, but there are only five nucleotide differences within the first 250 bp upstream of the designated initiation codon, and the reading frame remains open. The five nucleotide changes occur at positions −243, −242, −241, −221, and −107 and would result in four amino acid changes as compared with the murine sequence. Thus, in both human and murine sequences, an open reading frame extends upstream of the first ATG and the 5′ end of the major 1.85-kbp murine transcript. This highly conserved region is transcribed as part of a 4-kbp transcript in mouse spinal cord and may encode another form of the Hox 1.3 protein.

Conservation has also been observed in the 5′ noncoding regions of the murine *Hox 2.3* gene and its human counterpart, where the 100 bp upstream of the initiation codon are 97% identical (18). However, these upstream regions do not have extended open reading frames. Alternatively the 5′ noncoding regions in the human and murine *Hox 1.3* genes may have been conserved because they are *cis* regulatory elements for the 1.85-kbp transcript expressed in lung and other tissues. Indications for such a role come from the following observations. DNase I protection and electrophoretic mobility shift assays experiments (preparation of the Hox 1.3 protein and the method used for gel shift are described below) with the recombinant *Hox 1.3* protein identify a protected region between positions −157 and −142, which lie upstream from the cap site of the 1.85-kbp transcript. Thirty-base-pair oligonucleotides containing this region or its human counterpart bind to the Hox 1.3 protein (20). The homologous region in the human cognate is identical to the murine sequence except for a T instead of a C at position −221 (Fig. 3). This single base-pair change does not severely influence binding to the Hox 1.3 protein. It is possible that these binding sites are necessary *cis* elements for regulation of the *Hox 1.3* gene by the Hox 1.3 protein. Such autoregulation has been shown to occur in a *Drosophila* homeobox gene, *fushi tarazu*, whose protein product is required for the functioning of its own transcriptional enhancer (12).

The 3′ untranslated sequences retain 90% similarity with the corresponding murine sequence. Differences between the sequences of the two species are due to short insertions or deletions between long stretches of perfectly conserved sequences. The high similarity observed in the 3′ untranslated regions of the human and murine *Hox 1.3* genes is unusual. The *Hox 2.3* murine gene and its human counterpart, both highly homologous in coding sequences and 5′ noncoding regions, display only 67% homology in the 3′ untranslated regions. The high degree of similarity between the human and murine *Hox 1.3* cognates in the 3′ regions drops to 35% 30 bp downstream to the polyadenylation signal (data not shown). This suggests that the region between translation termination and the polyadenylation signal is functionally important. Such functional importance of the 3′ untranslated regions, particularly in influencing mRNA half-lives, has recently been demonstrated for other messages (for review, see reference 2). The 3′ untranslated sequences retains 90% similarity with the corresponding murine sequence. Differences between the sequences of the two species are due to short insertions or deletions between long stretches of perfectly conserved sequences.

Comparison of the two introns. The similarity between the human and murine *Hox 1.3* introns is highlighted in Fig. 3 and 4. The human gene contains consensus splice donor and acceptor sites at the same positions as in the mouse gene. Both introns are predicted to be 960 bp. Although the intron sequences of the murine and human *Hox 1.3* genes are more divergent than the coding sequences, the similarity is still 72%, and there are many regions of perfect conservation. In Fig. 3, we have underlined stretches of nine bases or more that are identical to the murine sequences. One conserved sequence is 111 bp long (bp 888 to 998; Fig. 3) and displays 90% similarity between the two species. It contains two consensus binding motifs for the Hox 1.3 protein. In addition, this region contains a sequence with 59% similarity to positions 1179 to 1227 of the epsilon region of the yeast Ty1 transposon (7; Fig. 5).

To examine whether these intron sequences could bind the Hox 1.3 protein, we used a mobility shift assay with nuclear extracts containing recombinant Hox 1.3 protein. To obtain sufficient amounts of the Hox 1.3 protein, we have used the baculovirus expression system which, in addition to supporting high levels of protein expression, has been shown to perform correct posttranslational modifications of nuclear proteins (19, 20). The Hox 1.3 murine protein-encoding cDNA sequence (present in the major 1.85-kbp transcript) was inserted behind the polyhedrin promoter of the recombinant baculovirus and used to infect *Spodoptera frugiperda* cells (for details concerning shuttle vector construction and expression, see reference 20). Nuclear extracts were prepared according to the protocol of Dignam et al. (5) from Hox 1.3 recombinant virus-infected cells. The mobility shift assay was performed as follows. Four sets of 30-bp oligonucleotides, two corresponding to the human potential binding sites (Hu intron 1 and Hu intron 2) and two corresponding to the murine sites (Mu intron 1 and Mu intron 2) were prepared. Short duplex DNA fragments with 5′ overhanging

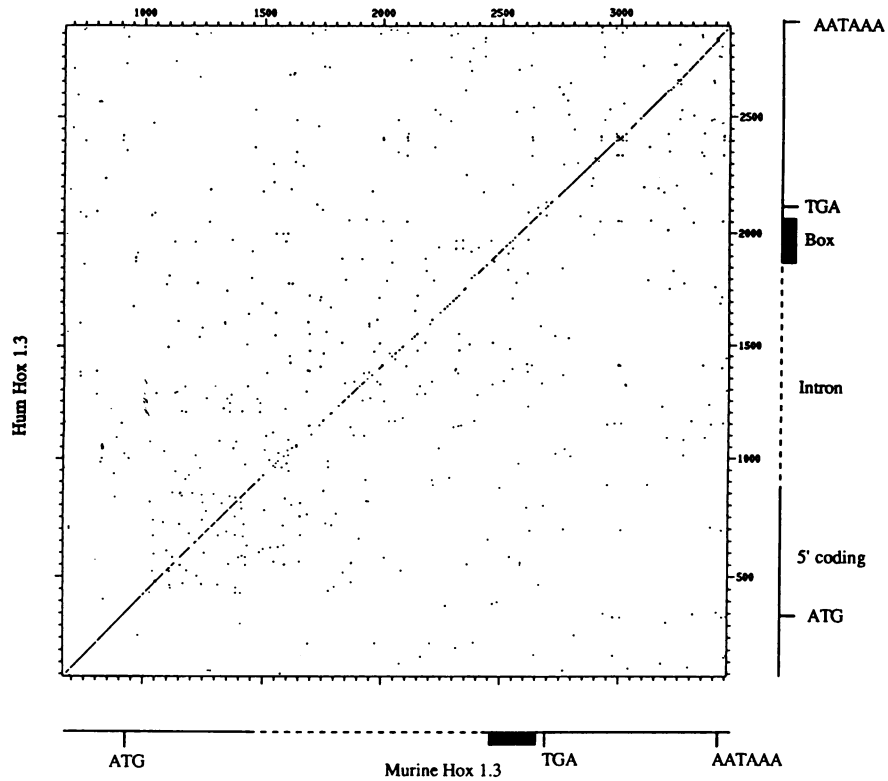

FIG. 4. Dot matrix comparison of the human *Hox 1.3* gene and its murine counterpart. The comparison was performed by using a k-tup (number of nucleotides compared at a time) of 7. The predicted translation initiation codons, termination codons, and polyadenylation signals are indicated as ATG, TGA, and AATAAA, respectively. Black boxes represent homeoboxes; broken lines indicate the respective introns.

ends were prepared by hybridizing two 27-base-long complementary oligonucleotides. The DNA fragments were end labeled by filling in the 5′ protruding ends with Klenow enzyme to create 30-bp blunt-ended double-stranded fragments containing three ^{32}P-labeled dCTP nucleotides. The plus-sense sequences of the DNA fragments used in this study were

Mu *Hox 1.3* TGCCAACTCCCCCATTAGTGCACGAGACCT
Hu intron 1 TTGTCACGGCGGATAATTTATGAGGAGCCA
Mu intron 1 TTGTCAGGGGAGATAATTTATGGGAAGCCA
Hu intron 2 AGGACAGCGTTACTAATTACAGCCCCCACT
Mu intron 2 AGGACAGCGTTACTAATTAGAGCCCCCACT

Mu *Hox 1.3* contains the *Hox 1.3* binding site located in the murine *Hox 1.3* promoter region (20). Hu intron 1 and Hu intron 2 contain the two potential *Hox 1.3* binding sites within the human *Hox 1.3* gene. Mu intron 1 and Mu intron 2 are almost identical to Hu intron 1 and Hu intron 2, respectively, and contain homologous sequences in the murine intron. Protein-DNA complexes were resolved in low-ionic-strength 9.5% polyacrylamide gels according to Carthew et al. (3), with the following modifications. For binding reactions, labeled fragments (20,000 cpm) were incubated for 2 h at 4°C with 2 μl of Hox 1.3 protein-containing extracts (~4 μg of total protein) and 4 μg of poly(dI-dC) in 200 mM KCl binding buffer (final volume, 20 μl). The reaction mixtures were loaded on a 9.5% polyacrylamide gel in 1× TAE (0.04 M Tris acetate plus 0.002 M EDTA) buffer and electrophoresed at 15 V/cm for 240 min. The gel was dried, and radioactivity was visualized by autoradiography.

All four oligonucleotides were able to bind the Hox 1.3 protein (Fig. 6). For both the human and the mouse sequences, the site containing the TAATTA sequence showed stronger binding. Multiple Hox 1.3 protein-DNA complexes could be resolved in 9.5% native polyacrylamide gels (Fig. 6), as we had previously observed with the putative murine Hox 1.3-binding site. These multiple complexes may be due to binding of the multiple forms of the Hox 1.3 protein (20). The highly conserved sequences in the noncoding regions may be critical to various transcriptional or posttranscriptional processes common to the human and murine genes. In this regard, interesting conservation of noncoding sequences has also been observed between the *engrailed* genes of two *Drosophila* species, *melanogaster* and *virilis*, which diverged about 60 million years ago, at about the time of the rodent-primate divergence. Kassis et al. (13) have found blocks of sequence homology in several areas, including the introns, which flank the exons of the *engrailed* genes of these two species. Recently, J. A. Kassis (personal communication) has shown that the 5′ untranscribed sequences alone are not sufficient for directing reporter gene expression in embryos in an *engrailed*-like pattern. However, the 5′ region together with sequences from the *engrailed* first intron of *D. virilis* does direct reporter gene expression in *engrailed*-like

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 955  GAAACAGCGTTACTAATTACAGCC   978 Hu Hox 1.3
1206  ********************G****  1229 Mu Hox 1.3
1204  ********AAAC**********GA  1227 Ty1
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FIG. 5. Homology of the human and murine *Hox 1.3* introns with the Ty1 activation region. Sequences between the Ty1 activation region (positions 1204 to 1227; 7) and the human and mouse *Hox 1.3* introns are aligned. *, Nucleotide identity. DNA core recognition for the Hox 1.3 protein is underlined.

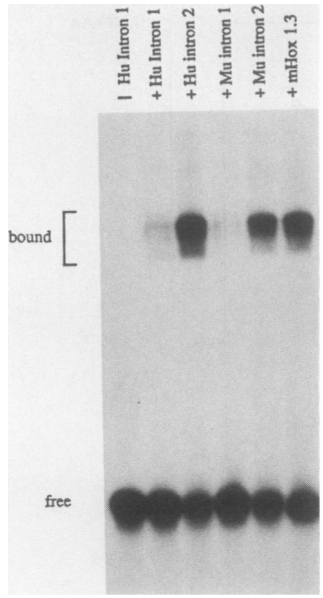

FIG. 6. *Hox 1.3* binding sites within the intron of the human and murine *Hox 1.3* genes. Shown are results of a gel retardation assay of Hox 1.3 protein-DNA complexes. Double-stranded end-labeled oligonucleotide probes, each 30 bp long and corresponding to sequences located within the promoter region (mHox 1.3) or within the murine and human *Hox 1.3* introns, were incubated without (−) or with (+) 4 μg of nuclear extract containing Hox 1.3 protein. The complexes were resolved on a 9.5% nondenaturing polyacrylamide gel and visualized by autoradiography of the dried gel.

fashion in *D. melanogaster*. This implies that intron sequences may influence the tissue-specific expression of the *engrailed* gene and that these *cis*-active sequences are conserved between distantly related species. The murine and human *Hox 1.3* introns are even more closely related than are the *engrailed* introns of the two *Drosophila* species, tempting speculation that regulatory sequences also exist in the *Hox 1.3* intron.

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