Structural analysis of the *Hox-3.1* transcription unit and the *Hox-3.2–Hox-3.1* intergenic region

(murine/homeobox genes/transcription start sites/CT repeats/cis-regulatory elements)

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ABSTRACT The mouse Hox gene family is a set of mammalian homeobox genes that may represent developmental control genes. Complete information about the primary structure of these genes is a prerequisite for a systematic analysis of the mechanisms that determine their complex tempero-spatial expression patterns. In this report we describe the complete sequence of the Hox-3.1 locus and provide evidence for several closely spaced transcriptional start sites. Sequence analysis of the 5' region of the Hox-3.1 gene extending to its nearest upstream neighbor, Hox-3.2, allowed us to identify sequences known to be capable of interactions with transcription factors. Several of these sequence motifs are similar to cis-regulatory elements found in the regulatory regions of other known developmentally regulated genes.

The identification of controller genes has been a significant recent finding in developmental biology. Homeotic genes are an important class of controller genes, first described in Drosophila, where they serve to orchestrate expression of the genome during ontogeny (reviewed in ref. 1). Genes homologous to A-type homeotic loci, so named after the Drosophila gene Antennapedia (Antp), which serves as a paradigm of this group, have been discovered in other organisms and show considerable similarity in terms of structure, organization, and possibly function (reviewed in refs. 2-5). Several regions in the A-type homeoproteins are highly conserved both within and across species. Prominent among these is the homeodomain that is encoded by a 180-base-pair (bp) DNA element termed the homeobox. The homeobox specifies a helix-turn-helix motif of 60 residues with known sequence-specific DNA binding properties (4). Several lines of evidence suggest that homeodomain proteins function as transcription factors mediating developmental control during ontogeny (1). The A-type homeobox gene family in the mouse is arranged in four clusters, each containing five to nine genes. The clusters Hox-1, -2, -3, and -4 map to four different chromosomes-namely, 6, 11, 15, and 2, respectively (refs. 6-8 and references therein). Based on sequence comparisons, we have proposed that the Hox gene family has arisen by gene duplication (9-11). This formulation is strengthened by additional similarities between the insect and vertebrate clusters such as colinear correspondence between the arrangement of the genes within the clusters and their expression along the anteroposterior fetal axis (6, 7). In addition to this, all known mammalian A-type genes are transcribed in the same direction, suggesting that a clustered organization may have functional significance. To test this hypothesis, we have begun to accumulate DNA sequence information within the gene clusters that help us to

address several questions such as (i) do common mechanisms of transcriptional regulation exist among Hox genes, (ii) can binding sites for transcriptional control factors be detected and are these organized into particular patterns, and (iii) can unique sequence features be identified?

The Hox-3.1 gene is one of the most extensively characterized homeobox genes of the mouse (refs. 12–16 and references therein). In this report, we provide nucleotide sequence information¶ of the genomic region encompassing the entire Hox-3.1 gene and the homeobox of its nearest upstream neighbor, Hox-3.2 (15). Moreover, we characterize a region of transcriptional initiation of the Hox-3.1 gene and identify putative cis-regulatory elements.

MATERIALS AND METHODS

Construction and Screening of Libraries. Mouse (CD-1) adult spinal cord cDNA was synthesized (17) by using about $1 \mu g$ of poly(A)⁺ RNA and oligo(dT) primers in a commercial cDNA synthesis system (Amersham, RPN-1256). Approximately $4 \times 10^5 \lambda g t10$ phage clones were screened with the ³²P-labeled pMoEA insert (12), which resulted in the identification of about 30 positive clones. cDNA inserts were subcloned into pBluescript (Stratagene) or M13 mp18/19 vectors for further analyses. A mouse genomic library was constructed from DNA of LTK⁻ cells (18) in pJEB cosmid vector (19) and screened with the pMoEA insert, which resulted in the isolation of the cosMoEA clone. cosMoEA contains an insert of about 40 kilobases (kb) including the *Hox-3.1* and *Hox-3.2* homeoboxes (12, 15).

DNA Sequence Analysis. Sets of deletions using the exonuclease III/mung bean nuclease system (Stratagene) were produced in the two largest cDNA clones, c235 and c210, and the nucleotide sequences of both clones were determined (20). Genomic DNA fragments overlapping and flanking the c210 and c235 sequences were derived from cosMoEA, subcloned into M13 mp18/19 vectors, and sequenced by using Sequenase (United States Biochemical) and sets of custom-made oligonucleotides as primers. Sequence data were analyzed with software provided by DNAStar (Madison, WI) and the University of Wisconsin Genetics Computer Group (Madison, WI) (21).

RNase Protection and Northern Blot Analyses. RNase protection analysis was carried out as described (22). Total RNA (10 μ g) was hybridized overnight at 60°C to 1 × 10⁵ cpm of RNA probe (~1 ng) generated by SP6 polymerase with pHE400 as a template. Plasmid pHE400 contains a 395-bp

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Abbreviations: p.c., postcoitum; nt, nucleotide; TRE, thyroid hormone response element.

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[&]quot;The sequence reported in this paper has been deposited in the GenBank data base (accession no. M35603).

HincII–EcoRI fragment, extending from positions 6213–6608 in the *Hox-3.1* upstream region (see Fig. 2).

Poly(A)⁺ RNAs from 12.5-day postcoitum (p.c.) mouse embryos and adult spinal cord (7.5 μ g each) were analyzed by Northern blot hybridization following standard procedures (23) with formaldehyde-containing gels and ³²P-labeled c210B2 fragment (Fig. 1A) as a probe (5 × 10⁸ cpm/ μ g), with the only exception of adding 10% dextran sulfate to the 50% formamide-containing hybridization solution.

RESULTS AND DISCUSSION

Characterization of cDNAs. A partial restriction map of the genomic cosMoEA segment analyzed and the structures of the two largest cDNAs, c235 (2.35 kb) and c210 (2.1 kb), are shown in Fig. 1A. Both cDNAs contain the complete Hox-3.1 protein-coding region of 726 bp, consistent with earlier reports (14, 15). In c235, however, this coding sequence is interrupted by an intervening sequence of about 1350 bp. The presence of 5' and 3' splice consensus sequences (25) and the perfect alignment of the c235 restriction map with that one of the corresponding genomic DNA segment suggest that c235 has been generated from an unspliced precursor RNA (Fig. 1A). The intronic nature of this region was also supported by its failure to hybridize with poly(A)⁺ RNAs isolated from embryos and adult spinal cord (data not shown). c210 contains two perfect poly(A) signals (Fig. 2). A third poly(A) signal was revealed by sequence analyses of genomic DNA about 160 bp downstream of the c210 3' terminus (Fig. 2). The third poly(A) signal may be used in transcript processing as suggested by (i) the presence of a G+T-rich sequence element known to be important for efficient poly(A) addition (26) that is located 17 bp downstream of this signal and absent in the vicinity of the other two poly(A) signals (Fig. 2), and (ii) the detection of a Hox-3.1-specific, 2.7-kb transcript by Northern blot analysis of embryonic and adult spinal cord RNA with a probe specific for the third poly(A) signal (data not shown).



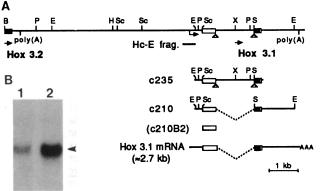


FIG. 1. Structure of Hox-3.1 locus. (A) A partial restriction map of the genomic region extending from the Hox-3.2 homeobox to the EcoRI site downstream of the third poly(A) signal of the Hox-3.1 gene is shown. The approximate region of transcriptional initiation of the Hox-3.1 gene is marked by an angled arrow, protein coding regions are boxed with the homeobox in black, splice sites are marked by triangles, and the directions of transcription are indicated by straight arrows. The schematic structures of the c235 and c210 cDNAs and of the c210B2 subclone, aligned to the corresponding genomic regions, are shown below. A diagram of the predicted Hox 3.1 mRNA with a presumed standard poly(A) tail of 260-300 bp (24) is shown at the bottom. Restriction endonuclease sites: B = BamHI, E = EcoRI, H = HindIII, Hc = HincII, P = Pst I, Sc = Sac I, S =Sal I, X = Xho I. (B) Northern blot hybridization of poly(A)⁺ RNAs (7.5 μ g each) from 12.5-day p.c. mouse embryos (lane 1) and adult spinal cord (lane 2) with the ³²P-labeled c210B2 probe. The size of the Hox-3.1 transcript is about 2.7 kb as indicated by the arrowhead.

Northern blot hybridizations using the c210B2 cDNA subfragment (Fig. 1A) detected a single major hybridizing transcript of about 2.7 kb in $poly(A)^+$ RNA samples from 12.5-day p.c. embryos and adult spinal cord (Fig. 1B). The additional 2.1-kb transcript previously detected in the adult spinal cord (12) could not be detected under the conditions used here, suggesting that earlier results were due to cross-hybridization to a related RNA species.

Determination of Transcriptional Start Regions. Northern blot hybridizations using probes derived from the upstream region of Hox-3.1 provided preliminary evidence for the presence of a major Hox-3.1 transcription start site in the vicinity of the proximal dinucleotide CT repeat (data not shown). To obtain further evidence for this, we carried out RNase protection analyses using RNA probes derived from a 395-bp HincII-EcoRI fragment (Figs. 1A and 2) subcloned into pGEM-2. Transcription with SP6 RNA polymerase resulted in two distinct RNAs of approximately 472 and 435 nucleotides (nt), distinguishable as a major and minor band, respectively, after PAGE (Fig. 3, lane 3). These probes were hybridized to total RNA samples from 12.5-day p.c. embryos. Liver RNA from adult mice was used as a negative control. After RNase treatment and PAGE, three major fragments of about 215, 190, and 180 nt were detected with the embryonic RNA. These data suggest the presence of several closely spaced transcription start sites in the vicinity of the 5' end of the proximal CT repeat (Fig. 2). A less-welldefined signal in the range of about 135-145 nt may indicate that some transcripts are initiated within the CT repeat. Two considerably less abundant fragments of about 403 and 365 nt could correspond to full-length protection of both forms of the probe, taking into account the length of the transcribed vector and polylinker regions. Consistent with this interpretation, hybridization of the gel-purified larger probe to embryonic RNA resulted in the disappearance of the 365-nt protected fragment (data not shown). This may suggest that in 12.5-day p.c. embryos, a smaller fraction of the Hox-3.1 mRNAs may initiate from another promoter located upstream of the HincII site. Hybridization of the same probes to RNA samples from other tissues known to express Hox-3.1, including adult spinal cord and kidney, yielded essentially the same pattern of protected fragments (data not shown).

Primer extension analysis using several unique primers downstream of the CT repeat failed to yield discernible specific products in a standard primer extension assay. To determine if specific cDNA products were generated in the primer extension analyses at levels below the sensitivity of the assay, we performed polymerase chain reaction amplification, followed by cloning and sequencing of the cDNA products (27). Sequence analysis of the 20 largest clones indicated that the 5' ends of the cDNA products were either within or near the 5' end of the proximal CT repeat, which is in good agreement with the results obtained from RNase protection assays. These results indicate that transcriptional initiation of the Hox-3.1 gene takes place in the vicinity of an extended CT repeat. No typical TATA boxes are found in this region. These results are consistent with the observation that a number of genes that lack TATA boxes contain a homopurine/homopyrimidine-rich region whose deletion leads to marked loss of transcriptional activity (24, 28-30).

Taken together, our data suggest a general structure of *Hox-3.1*-encoded mRNA prevalent in 12.5-day p.c. embryos and in adult spinal cord as shown in Fig. 1A. The predicted mRNAs contain a 5' untranslated leader region ranging from about 420 to 455 nt depending upon the transcriptional start site used, a protein coding sequence of 726 nt and a 3' untranslated trailer sequence of about 1270 nt with a standard poly(A) tail of 260-300 nt (26). The resulting lengths of these mRNAs would range between 2675 and 2750 nt, which is in

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FIG. 2. Nucleotide sequence ranging from the BamHI site 5' of the Hox-3.2 homeobox to the third poly(A) signal found in the Hox-3.1 transcription unit. The first nucleotide shown is the adenosine located in the incompletely shown BamHI site. The predicted amino acid sequences of the C terminus of the Hox-3.2 protein and of the complete Hox-3.1 protein are shown below the corresponding open reading frames. Boxed sequences delimit the Hox-3.2 and Hox-3.1 homeoboxes and the AATAAA poly(A) signals. Triangles indicate the positions of Hox-3.1 splice junctions. Repeat sequence (heavy lines) as well as the restriction endonuclease sites (thin lines) corresponding to the ones shown in Fig. 1A are underlined. The HincII site at position 6211 used for preparing the HE400SP6 probe for the RNase protection assays is also shown. Similarities to the consensus sequences of cis-regulatory elements shown in Table 1 are underlined by striped bars. The open bar 3' of the last poly(A) signal underlines a G+T-rich region. The open circle indicates the 3' end of the c235 cDNA. The region of transcriptional initiation containing several closely spaced transcription start sites ranges roughly from position 6405, just 15 nucleotides upstream of the CT-repeat, to position 6440 within that repeat.

good agreement with the transcript size of *Hox-3.1* of 2.7 kb as determined by Northern blot hybridizations.

Identification of Putative cis-Regulatory Elements. The establishment of the complex tempero-spatial expression pattern of the Hox-3.1 gene is likely to require cis-regulatory regions containing multiple target sequences for numerous transcription factors. As a first step in testing this hypothesis, we have analyzed the nucleotide sequence of the Hox-3.1 transcription unit and its 5' region extending to the neighboring Hox-3.2 homeobox for similarities to selected eukaryotic regulatory elements (Table 1). There are several potential sequences for interactions with homeodomain and Zn^{2+} finger proteins, which include a perfect match to the consensus sequence recognized by the Hox-1.3 protein (31). Several scattered G+C-boxes representing potential binding sites for the Sp1 transcription factor have also been found (36). It is interesting that a G+C-rich domain containing several Sp1 binding sites has been found in the upstream region of the Hox-1.4 transcription start site (40), and a similar region is located upstream of the Hox-3.1 translation start site. Sequence motifs resembling hormone response elements (reviewed in ref. 38), including a glucocorticoid response element (GRE) located upstream of the transcription start region and a thyroid hormone response element (TRE) in the intron, have been identified. Thyroid hormone receptor also binds to synthetic variants of the TRE where the two half sites are spaced by up to 9 bp (41). A sequence similarity to such a TRE variant containing a 7-bp spacer is present about 220 bp upstream of the transcription start region at position 6201 (GGTAACCTGAGTTGACC) (Fig. 2). Such TRE-like sequences may mediate responses to the morphogen retinoic acid (42). In this respect it is important to note that expression of the Hox-3.1 gene is inducible by retinoic acid in F9 embryonic carcinoma cells (13). In addition, sequence motifs with somewhat lesser degrees of homology to the estrogen response element (ERE) have been found in the 5' region at positions 1725, 5175, and 5935 (not shown in Table 1). Another response element pertinent to developmentally regulated gene expression is the heat shock element (HSE; reviewed in ref. 39).

Besides the regulatory elements discussed above, the sequence analyses showed the presence of several nucleotide repeat motifs (Fig. 2), some of which may be involved in the

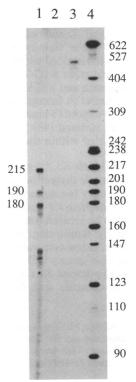


FIG. 3. RNase protection analysis of transcriptional start region. Total RNA samples (10 μ g each) from 12.5day p.c. mouse embryos (lane 1) and adult liver (lane 2) were hybridized to 3²P-labeled RNA probe derived from the *HincII-Eco*RI fragment described (see Fig. 1A and text). Subsequent to RNase treatment, protected fragments were separated by PAGE. Lanes 3 and 4 contain undigested probe and endlabeled *Msp* I-digested pBR322 plasmid used as a size marker, respectively.

Table 1.	Potential	transcription	factor-binding	sites in the
Hox-3.1 lo	ocus			

Factor/ response element	Binding site	Position*	Ref.
Hox-1.3	CYYNATTAKY [†]	1624, 2210	31
Antp	ANNNNCATTA	901, 1262, 1272, 1614, 4771, 8204	32
ftz, en, eve	ΤCAATTAAAT	4883	33, 34
Kr	AAAAGGGTTAA	7536	35
Sp1	GGGCGG	4018, 4616, 5577, 6806, 7994, 8590	36
SV40 enhancer	GTGGWWWG [‡]	1313, 1754, 4112, 6550, 7507	37
GRE	GAACANNNTGTTC	5429	38
TRE	GGTCATGACC	8036	38
HSE	CNNGAANNTTCNNG	1898, 3172, 4662, 8977, 9560	39

Sequences representing a complete match or a single nucleotide mismatch to the consensus binding sequence are reported in the references shown. Binding sequences are found in either orientation on the complementary strands of genomic DNA as shown in Fig. 2. GRE, glucocorticoid response element; HSE, heat shock element. *Position refers to the first nucleotide of the consensus sequence. $^{\dagger}Y = T \text{ or } C \text{ and } K = T \text{ or } G.$

 $^{\ddagger}W = T \text{ or } A.$

formation of altered chromatin structure and/or binding of regulatory proteins (ref. 43 and references therein). Good examples appear to be regions of alternating C and T residues found in the promoter regions of the Drosophila heat shock genes hsp 70 and hsp 26, and the histone genes his 3 and his 4 (44). The protein that binds to CT repeats, termed the GAGA factor, has been purified (44, 45) and shown to modulate the in vitro transcription of the Drosophila homeotic gene Ultrabithorax (Ubx). We have identified two extended CT repeats, a proximal repeat of perfect mirror symmetrical structure, (TC)19TCCCTCTCCC-T(CT)₁₈ located in the Hox-3.1 transcription start region and a distal repeat, (CT)₃₂(CCCTCTCT)₆(CT)₄ about 60 bp downstream of the putative Hox-3.2 poly(A) signal. Dinucleotide CT sequences have also been recognized in the vicinity of other Hox genes (46). Additional repeat motifs noticed within 2.5 kb upstream of the proximal CT repeat were a (G-T)₂₂ sequence motif, preceded by a (T)₂₆ sequence and a GATA repeat (47). Tracts of dA·dT have been found in intergenic regions of many genomes and an oligo(dA)·oligo(dT)-binding protein from yeast termed datin has been isolated (48). An additional repeat sequence, (CA)₁₉, is located within the 3' untranslated region of Hox-3.1.

The extended structural information of the Hox-3.1 region will provide an important basis for a systematic dissection of cis-regulatory elements. Certain cis-regulatory functions essential for the region-specific expression of Hox-3.1 during embryogenesis have been demonstrated to be located within the Hox-3.2-Hox-3.1 intergenic region by monitoring the expression of Hox-3.1-lacZ reporter gene constructs in transgenic embryos (49). Combining such in vivo assays with in vitro DNA binding studies will allow us to pinpoint Hox-3.1 regulatory elements, some of which may coincide with the sequence motifs discussed above.

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