The regulation of the murine Hox-2.5 gene expression during cell differentiation

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ABSTRACT
The mouse Hox-2.5 gene containing a Drosophila Antennapedia-type homebox sequence is expressed in a spatially and temporally restricted manner during embryogenesis. We found that the mouse embryonal carcinoma cell line P19 expresses Hox-2.5 during differentiation by the treatment with retinoic acid (RA). Expression of the Hox-2.5 gene was not detected in undifferentiated P19 cells, but detected 72 hours after treatment with RA. In order to analyze this inductive response, we first identified the Hox-2.5 transcription initiation site and a possible promoter region. Subsequently, we prepared constructs containing various Hox-2.5 DNA fragments fused to a firefly luciferase reporter gene and transfected these into undifferentiated or differentiating P19 cells. These studies have demonstrated that a region −279 to +15 with respect to the transcription initiation site has a differentiation-responsive promoter activity. Deletion analysis suggests that the sequences responsible for this induction are located in several distinct domains within the 294 bp promoter region. Two of the possible differentiation-responsive elements were identified by analysis of DNA-protein interactions, and in vivo competition assays lend support to the notion that these regions are involved in the differential expression of Hox-2.5 promoter activity.

INTRODUCTION
A highly conserved sequence, homeobox, has been detected in a number of genes involved in the specification of positional information in Drosophila melanogaster (1). The homeobox encodes a protein domain, the homeodomain, that mediates the sequence-specific binding of homeobox-containing gene products to DNA (2). It is assumed that the homeobox gene products regulate transcription of genes. Homeobox sequences have also been found in other multicellular organisms including vertebrate (3).

Some of the Drosophila homeobox genes called as homeotic selector genes that determine the anterior-posterior specification of the body are clustered in adjacent complexes such as Antennapedia-complex (ANT-C) and Bithorax-complex (BX-C). The physical order of the homeotic genes within the clusters correlates with the order in which these genes are expressed along the anterior-posterior axis of the embryo body. As in the case of Drosophila, Antennapedia-type homeobox genes are also organized in clusters in other animals. In the mouse, they are segregated into four clusters that can be aligned with the Drosophila, ANT-C and BX-C clusters (4). Moreover, as in Drosophila, a strong correlation exists in the mouse between the position of a gene within a cluster and its expression pattern along the anterior-posterior axis (5). The more 3' a gene is located within a cluster, the more anteriorly it is expressed.

Elucidation of the mechanisms by which these genes are regulated is certainly important for understanding pattern formation during development. Very little is known about the regulation of the homeobox gene expression during mammalian development. Recently, however, several lines of research have opened up in which transgenic mice were used to study Hox-1.1 (6), Hox-1.3 (7) and Hox-2.3 (8) etc. gene expression.

We chose for our research one of the mouse Antp-type homeobox genes, Hox-2.5. Hox-2.5 is a member of the Hox-2 cluster on mouse chromosome 11 and belongs to the Abd-B subfamily (9). Since no homeobox sequence was found in the region upstream to Hox-2.5 within the Hox-2 cluster in either human or mouse, Hox-2.5 is probably the most upstream gene of the Hox-2 cluster (10). The study of the mechanisms by which expression control of this distal gene is controlled may yield important clues concerning the conservation of the order of genes within the cluster during evolution and the relation between the location of a specific gene within the cluster and its role in controlling expression pattern during embryogenesis.

In this study we present the results of the analysis of Hox-2.5 gene expression in the P19 mouse embryonal carcinoma (EC) cell line. P19 cells can be induced to differentiate into various cell types, including neuron-like cells by treatment with retinoic
acids (RA). As has been shown for the human EC cell line NT2/D1, P19 cells express various homeobox genes during differentiation (11). It is possible that the differential Hox gene regulation in observed in EC cells correlates with its regulation during embryogenesis. We investigated the regulation of the Hox-2.5 gene in order to identify the genetic elements involved in the regulating pattern of Hox-2.5 gene expression during embryogenesis.

**MATERIALS AND METHODS**

**Cell culture and DNA transfection**

P19 embryonal carcinoma cells were cultured in a-MEM medium supplemented with 10% fetal calf serum in a 5% CO2 incubator. In tissue culture dishes were induced to differentiate by treatment with 0.3 μM retinoic acid. Exogenous DNA was introduced into P19 stem and RA-treated cells using a modified calcium phosphate-DNA coprecipitation transfection method (12). The medium was changed after 18 hours of incubation and the cells were harvested 6 hours later. For each transfection, 18 μg of the luciferase construct and 2 μg of pCH110 harboring E. coli LacZ gene under the control of the SV40 early promoter was co-transfected as an internal control for transfection efficiency. Luciferase activities were measured as described by We et al. (13) using a TD-4000 lumiphotometer (Laboservice, Japan). LacZ activities were determined as described by Herbomel et al. (14). In vivo competition assays were performed by cotransfecting either 4.5 μg of the appropriate construct, 13.5 μg of pBluescript-DRE1 or -DRE2, pBluescript (Stratagene) harboring two copies of DRE oligonucleotide, and 2 μg of pCH110. As a control, 13.5 μg of pBluescript was co-transfected instead of pBluescript-DRE1. DRE oligonucleotide was:

5'-GATCTCGAGCTGATCATATCAATAAATCCATCCTGGA-3'  
3'-ACTCGAGTCATTTTGTGTTAGGTAGGACCTCTAG-5'.

And DRE2:

5'-GATCTCGAGCTGATCATATCAATAAATCCATCCTGGA-3'  
3'-AGCGGCGTAAACTAACATACGCTCGAGTCAT-5'.

**Northern hybridization, RNase protection mapping and primer extension**

Mouse embryonic and placental RNA was obtained from ICR mice. Total RNA was isolated by homogenizing cells and tissues in guanidium thiocyanate (16) and Poly (A)+ RNA was obtained after elution from oligo (dT)-latex columns (Oligotex-dT30, Takara shuzo, Japan). 10 μg of poly (A)+ RNA was separated on 1% agarose-formamide gel and transferred to a Genescreen plus (NEN) nylon membrane. Hybridization conditions were as described in Zeta-probe instruction manual (Biorad). 40 μg of Total RNA was used for RNase protection mapping carried out as described by Sambrook et al. (15). An RNA probe was prepared from pBluescript harboring the Smal I-Sal I fragment shown in Figure 1 by Transprobe T kit (Boehringer-Mannheim). 50 μg of mouse embryonic total RNA and 20 μg of P19 D+ total cell RNA were used for primer extension essentially according to McKnight and Kingsbury (17). The reaction mixture contained [α-32P]dCTP in place of dCTP. The primer sequence was:

5'-GCGTGGGATCCGCGGCAGCAAGC-3'.

**RESULTS**

The Hox-2.5 gene expression in P19 cells

A cosmid clone, cos19, containing a little more than 20 kb upstream region of the Hox-2.5 homeobox (9), was isolated from a mouse genomic library by screening with the Drosophila Antp and fushi tarazu homeobox sequences as probes (1). We isolated the 14 kb Eco RI fragment of cos19 containing the Hox-2.5 homeobox (Figure 1 A). The Hox-2.5 transcript was not detected in undifferentiated cells at 48 hours after treatment with RA, but the transcript of about 3 kb in size appeared at 72 hours in both Northern (Figure 1 B) and RNase protection assays (Figure 1 C). RNase protection mapping showed two major protected fragments containing 248 and 251 nucleotide residues, respectively. Primer extension assay (Figure 1 D) showed good agreement with the RNase protection mapping. These data indicate that transcription of the Hox-2.5 gene is initiated 15 bp or 18 bp upstream from Sac I site (Figure 1 E). The site of transcription initiation in embryo is identical to those in P19 cells (Figure 1 C and D). Figure 1 E shows the DNA sequence in the proximity of the Hox-2.5 transcription initiation site. Sp1 binding consensus sequences are located at -249, in reverse orientation, and at -193. No genuine TATA-box could be observed but a CCAAT
sequence is found at the transcription start site. Although three ATG codons can be observed downstream from the transcription initiation site, we predict that the initiation codon is the second ATG at +210, because this is in frame with the homeobox and the other two open reading frames have termination codons immediately downstream (Figure 1 E).

Identification of the Hox-2.5 gene promoter

Since the transcription start site was found 15 bp upstream from Sac I site (Figure 1 E), we ligated the Bgl II-Sac I fragment containing this transcription start site to a firefly luciferase gene as a transcriptional reporter gene (13), and constructed many derivatives of this segment containing deletions starting from the Bgl II site. Transient transfection assays were performed to test the promoter activity using differentiated P19 embryonal carcinoma cells at a stage when the endogenous Hox-2.5 transcript is expressed. In order to account for differences in efficiencies of transfection, the Hox-2.5/luciferase DNAs were cotransfected with pCH110 plasmids containing the β-galactosidase gene under control of a SV40 early gene promoter. Luciferase activities were normalized to the Lac Z activity in the same cell extract (Figure 2) (14). The construct B6C, containing only a 294 bp fragment immediately upstream from Sac I site, was found to confer levels of the luciferase activity comparable to the construct B1C containing the 2 kb Bgl II-Sac I fragment (Figure 2). Further deletion of sequences closer to the cap site lead to lower luciferase activities; 58 bp (the construct B10C), 144 bp (the construct B12), 195 bp (the construct B2C) deletion yielded 50, 15 and 5.5% of the original luciferase activity, respectively. The shortest construct still had some ability to promote the gene downstream, however. Since promoter activity was not observed using other Bgl II fragments within the 14 kb Eco RI fragment containing...
Figure 2. The promoter analysis of Hox-2.5 gene in differentiated P19 EC cells. Different length of the Hox-2.5 upstream sequences were inserted into the plasmid harboring promoterless luciferase gene (see Materials and methods). The construct B1C is the Bgl II-Sac I fragment in Figure 1 A. The right part of the figure represents the relative luciferase activity normalized by β-galactosidase activity measured in lysates from P19 cells. The relative luciferase activities in this figure are defined by the activity of the construct B6C as 100 and all data are indicated by the proportion to B6C. The activity of the promoter-less luciferase vector could not be detected (less than 0.02% of the B6C).

Table 1. Comparison of Hox-2.5 promoter activity in undifferentiated and differentiated P19 cells

<table>
<thead>
<tr>
<th>construct</th>
<th>relative luciferase activitya</th>
<th>undifferentiated</th>
<th>differentiated</th>
</tr>
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<tbody>
<tr>
<td>B1C</td>
<td>17 ± 3.2</td>
<td>90 ± 5.2 (5.3)b</td>
<td>90 ± 5.2 (5.3)b</td>
</tr>
<tr>
<td>B6C</td>
<td>20 ± 3.9</td>
<td>100 ± 13 (5.0)</td>
<td>100 ± 13 (5.0)</td>
</tr>
<tr>
<td>B10C</td>
<td>6.2 ± 0.20</td>
<td>50 ± 11 (8.1)</td>
<td>50 ± 11 (8.1)</td>
</tr>
<tr>
<td>B2C</td>
<td>0.74 ± 0.14</td>
<td>15 ± 2.5 (20)</td>
<td>15 ± 2.5 (20)</td>
</tr>
<tr>
<td>B8C</td>
<td>1.2 ± 0.22</td>
<td>5.5 ± 1.1 (4.6)</td>
<td>5.5 ± 1.1 (4.6)</td>
</tr>
</tbody>
</table>

a) Relative luciferase activities were determined as described in the legend of the Figure 2 and Materials and Methods. The values in parenthesis are the fold-induction observed in differentiated cells compared to that in undifferentiated cells.

the Hox-2.5 gene (Figure 1 A) (less than 0.1% activity of construct B6C), we concluded that the 294 bp fragment contains the Hox-2.5 promoter.

Induction property of the Hox-2.5 promoter during differentiation

Hox-2.5 gene expression is inducible. To test whether the promoter region described above is responsible for the induction by the treatment with RA, we compared the promoter activities in undifferentiated (D-) and differentiated (D+) P19 cells. The results of these experiments are summarized in Table 1. All these constructs showed an inductive response. The inducibilities of these constructs could even be greater than in these data, since the control SV40 early promoter was suggested to have a higher activity in differentiated EC cells than in the stem cells (20). The induction ratios increased from B6C to B2C. While B6C showed only a 5- to 6-fold increase in promoter activity following differentiation, B2C showed a 20 fold increase in D+ cells compared to D- cells. Moreover, while no difference was observed between the promoter activities of B2C and B8C in D- cells, the luciferase activity of B2C in D+ cells was 2.7 times higher than that of B8C. Thus, we could not separate completely the induction-specific enhancer activity from the basal promoter activity. These results suggest that at least two induction-responsive elements exist, one located in the segment from -130 to -80 and another in the segment from -79 to +15. In addition, there may be other regulatory elements between -275 and -131 that enhance promoter activity even in D- cells.

DNA-protein interaction in the Hox-2.5 promoter region

In order to further investigate the promoter functions of Hox-2.5 promoter, we analyzed DNA-protein interactions using the DNA fragments discussed above. DNase I footprinting analyses
revealed several differences between the protection patterns obtained with D- and D+ cell extracts (Figure 3 A). D+ cell extract yielded three protections, -126 to -96, -85 to -71 and -58 to -37. D- cell extract yielded overlapping but different protection patterns extending from -182 to -67 and from -62 to -45. Two of these footprints, -126 to -96 and -58 to -37, cover sequences that share homology with D1, a sequence in the Hox-2.3 promoter region that is specifically protected by nuclear extracts of differentiated C1003 cell (Figure 3 B) (21). Only one of the two Sp1 binding sites that is located at -249 with reverse orientation was protected in DNase I footprinting (data not shown).

For further investigation of the relationship between the protected DNA regions and DNA binding protein, we synthesized two double stranded oligonucleotides designated DRE1 and DRE2, corresponding to the sequence from -127 to -103 and from -58 to -37, respectively. DRE1 contains a large part of the footprint lying between -126 and -96. DRE2 contains the entire sequence covered by the footprint observed from -58 to -37. As shown in Figure 4 A, gel retardation assay using 32P-labeled DRE1 oligonucleotide, yielded one additional band with D- extracts in comparison with D+ extracts. This band may represent a negative regulatory factor since the loss of this band during RA-induced differentiation was associated with the increased promoter activity (Table 1). Since the shifted bands disappeared upon competition with the unlabeled DRE1 oligonucleotide, they must result from specific interaction between proteins and a sequence within this oligonucleotide (data not shown). DRE2 yielded only one retarded band with D+ nuclear extract and this DNA-protein complex disappeared upon competition with a 20-fold excess of unlabeled DRE2 oligonucleotide (Figure 4 B). Although the sequences of DRE1 and DRE2 are highly homologous (Figure 3 B), the band observed with labeled DRE2 was apparently different from both of those obtained with labeled DRE1 in view of their mobility and the lack of competition with each other (Figure 4 C).

Analysis of the differentiation responsive element
For more detailed analysis of DRE1 functions in the differentiation-responsive activity, the double stranded oligonucleotide containing this sequence was ligated to B8C and TK-LUC, a luciferase reporter construct containing an enhancerless thymidine kinase promoter. Figure 5 A shows that the induction ratio of the B8C derivative DRE1-8C was about...
only 2.5-fold, about one-half of the control with DRE1 and about the activation of B6C during differentiation. When a pBluescript derivative containing the DRE1 and DRE2 oligonucleotide the activity in P19 stem cells of control experiments is designated as 1. Black bars; luciferase activities in P19 D+ cells. White bars; luciferase activity in P19 D− cells.

10-times higher than background, but could not completely duplicate the activity of the B2C promoter. It is possible that the DRE1 sequence is not the only element present between −130 and −79. Alternatively, the correct spacing between DRE1 and some other element located within B8C region may be required for the full B2C promoter activity. Both DRE1-TK and ERD1-TK, in which the oligonucleotide is inserted in the original and reverse directions with respect to TK-LUC, showed about 10-fold increase in luciferase activity during differentiation, though the TK-LUC alone showed only a small induction response (less than a 2-fold increase) (Figure 5 B). DRE1 thus works as a differentiation-responsive enhancer independently of the kind of promoter with which it is paired. Furthermore, the lower luciferase activities of DRE1-TK and ERD1-TK compared to TK in D− cells (about one-half the activity of TK-LUC) supports the possibility that some negative regulator binds to this sequence in D− cells (Figure 5 B). If the DRE1 and DRE2 sequences bind differentiation-specific transacting factor(s), the cotransfection of an excess of these sequences would suppress the activation of B6C during differentiation. When a pBluescript derivative containing the DRE1 and DRE2 oligonucleotide sequences were co-transfected with B6C, a reduction in the inductive response was indeed observed; levels of induction were only 2.5-fold, about one-half of the control with DRE1 and about one third of the control with DRE2 (Figure 6). From these results, we conclude that the DRE1 and DRE2 sequences function as differentiation responsive elements in the Hox-2.5 gene.

DISCUSSION

Region-specific expression, a common character of homeobox genes, may be mediated by complex combinations of cis-regulatory elements capable of responding to position signals supplied by a gradient of diffusible substances or through cell-cell interactions. The activities of an endogenous diffusible morphogen can be mimicked by retinoic acid, which affects the determination of the anterior-posterior axis (22). The existence of exogenous retinoic acid in chicken limb bud causes the formation of extra-digits and the activation of Chox-4 genes (23, 24, 25). Moreover, retinoic acid induces Hox genes in embryonal carcinoma cells (10). Both in the anterior boundary of the expression in the early development of mouse hindbrain (26) and in the temporal order of the induction in human EC cell line (10), the expression of the Hox-2 complex genes correlates with their relative position in the cluster. It is expected that the mechanisms underlying homeobox gene induction in EC cells will, at least in part, correlate with the region specific expression in the embryo. Mouse EC cell line P19 expresses the Hox-2.5 gene when it is treated with retinoic acid. Therefore we have isolated here the Hox-2.5 promoter region and studied its inductive response to differentiation.

Activity and sequences of Hox-2.5 promoter

From the sequence analysis, we did not identify any typical TATA-box or CCAAT-box in the 5′ region of the Hox-2.5 gene. Furthermore, we could not identify binding consensus sequence for any known transcription factors, including a retinoic acid (RA) responsive element-like sequence (27). Although RA treatment induced Hox-2.5, it took a long time before the Hox-2.5 gene expression could be detected. Moreover we could not detect any significant enhancement of the luciferase activity when cells were treated with RA for 24 hours immediately after transfection (data not shown). These results suggest that the activation of the Hox-2.5 gene expression in response to RA may be indirect.

The transfection data suggested the existence of at least two differentiation-responsive elements between −130 and +15. DRE1 and DRE2 were identified by footprinting and other experiments. Gel retardation assays using a 32P-labeled oligonucleotide containing the DRE1 sequence suggested that two kinds of the DRE1 binding proteins exist in P19 cells, a D− cell-specific one and one observed in both D− and D+ cells (Figure 4 A). One possible induction model based upon these results is that a positive regulatory factor (faster moving band) and a negative regulatory factor (slower moving band) compete the same binding site in P19 D− cells. The reduction of the latter up-regulates the gene by decreased competition between two proteins (or protein complexes). The alternative explanation is also possible that a single binding protein could be in two states by modification, dimerization etc.; one state in D− P19 cells makes the low mobility complex which may be transcriptionally inactive and disappearance of this state by differentiation results in the presence of only the high mobility complex which may be transcriptionally active.

Both DRE1 and DRE2 share high sequence homology with the D1 footprint in the Hox-2.3 promoter (21). DRE2 showed a binding complex specifically with D+ nuclear extract in gel retardation assays. The mobility of the shifted oligonucleotide containing the DRE2 sequence is apparently distinct from that of the oligonucleotide containing the DRE1 sequence and both protein complexes did not show competition with the other oligonucleotide. It is possible that complexes shown in Figure 4 A bind to a sequence adjacent to this highly homologous sequence, for example ATCCA---TGGAT palindrome.

Possible role of the other elements

The observation that constructs lacking the region upstream from DRE1 showed reduced promoter activity suggests that there may be other elements important for the basal activation of the Hox-2.5 gene. It is also possible that this region cooperatively work with other elements for the induced as well as basal promoter activity.
There are no known DNA regulatory elements within this region except for Sp1 binding motifs, GGGCGG, like in other Hox genes whose promoter functions have been analyzed. However, the overlap for the Sp1 bind site with a Krox-20 binding site as found in the Hox-1.4 (28) and the Hox-2.3 (21) promoters was not observed in the Hox-2.5 promoter. Furthermore, removal of the Sp1 binding site at -249 resulted in only a partial reduction, in nonspecific enhancing activity (comparing B6C with B10C). One more Sp1 binding motif at -192 failed to produce DNase I footprint. A site defined by a footprint located just upstream from the second Sp1 site (data not shown) may not be involved in the promoter function, since absence of this footprint region did not result in a significant difference in promoter activity (B11C compared to B10C).

Some other elements or mechanisms might also be involved in the differential activation of the Hox-2.5 promoter, for example post-transcriptional regulation such as suggested by Colberg-Poley and his co-workers (29). We observed that the downstream region of the Hox-2.5 gene promoter had a silencing activity with respect to gene expression in D– cells. This DNA region contains a few TAAT motifs which are thought to be a consensus binding site for the homeobox proteins (data not shown). It is possible that this sequence confers hierarchical or autoregulatory activity. This regulation, whatever the mechanism, can be one of the controlling mechanisms of Hox-2.5 expression and may cooperatively work with other controlling systems.

Recently, transgenic studies have revealed that several elements which are dispersed in the long DNA region of Hox gene regulate the gene expression cooperatively (6, 30). Although we have shown here two elements that are likely to be involved in the regulation of Hox-2.5 expression, it is possible that there are more regulatory elements outside of the region we have examined.

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