Sequence and embryonic expression of the murine Hox-3.5 gene

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Summary

The murine Hox-3.5 gene has been mapped and linked genomically to a position 18 kb 3′ of its most 5′ locus neighbour, Hox-3.4, on chromosome 15. The sequence of the Hox-3.5 cDNA, together with the position of the gene within the locus, show it to be a paralogue of Hox-2.6, Hox-1.4 and Hox-4.2. The patterns of embryonic expression for the Hox-3.5 gene are examined in terms of three rules, proposed to relate a Hox gene’s expression pattern to its position within the locus. The anterior boundaries of Hox-3.5 expression in the hindbrain and prevertebral column lie anterior to those of Hox-3.4 and all other, more 5′-located Hox-3 genes. Within the hindbrain, the Hox-3.5 boundary is seen to lie posterior to that of its paralogue, Hox-2.6, by a distance equal to about the length of one rhombomere. Patterns of Hox-3.5 expression within the oesophagus and spinal cord, but not the testis, are similar to those of other Hox-3 genes, Hox-3.3 and Hox-3.4.

Key words: Hox, Hox-3.5, homeobox, rhombomeres, development.

Introduction

Based on comparisons of homeobox gene sequences, genomic organisation and spatial domains of embryonic expression, both mammalian and Drosophila class I homeobox genes are believed to be descendants of a single homeo-gene cluster that was present in a common, ancient ancestor (Duboule and Dollé, 1989; Graham et al., 1989). Mouse class I, or Hox, genes (38 described so far) are organised as four clusters of between nine and eleven genes on four different chromosomal locations. Hox-1 on chromosome 6, Hox-2 on chromosome 11, Hox-3 on chromosome 15 and Hox-4 on chromosome 2 (Hart et al., 1985; Bucan et al., 1986; Breier et al., 1988; Featherstone et al., 1988). The spacing of genes in each cluster and their homeobox sequence shows that genes occupying the same relative position in each locus also have similar sequence characteristics, showing them to belong to the same sub-family or paralogous group.

All Hox genes are transcribed in the same direction, and the more 3′ a gene the more anterior the embryonic expression (Gaunt et al., 1988; Duboule and Dollé, 1989; Graham et al., 1988). Paralogous genes, especially those 3′ in location, often display similar anterior boundaries of expression in the spinal cord and prevertebral column, and individual genes within each cluster often show similar tissue specificities in expression (reviewed by Gaunt, 1991). The complex anterior-posterior and dorsal-ventral array of Hox gene expression patterns produced in the early embryo can provide the basis of specifying positional co-ordinates to cells, possibly by a Hox code (Kessel and Gruss, 1991). Evidence for the developmental importance of Hox genes and confirmation of the significance of the anterior boundaries of expression has come from targeted gene deletion studies with the Hox-1.5 (Chisaka and Capecchi, 1991) and Hox-1.6 (Lufkin et al., 1991; Chisaka et al., 1992) genes.

The expression boundaries of the more anteriorly expressed genes have attracted much attention, since the boundaries correspond to the segmental boundaries of rhombomeres in the hindbrain. Paralogues Hox-2.6, Hox-1.4 and Hox-4.2 all have anterior limits of expression at the rhombomere 6/7 boundary (Hunt et al., 1991). Hox-2.7, Hox-1.5 and Hox-4.1 all have boundaries at rhombomere 4/5 (Hunt et al., 1991). Hox-2.8 and Hox-1.11 have boundaries at rhombomere 2/3. Hox-1.6 and Hox-2.9 have boundaries at rhombomere 3/4, although Hox-2.9 is expressed exclusively in rhombomere 4 (Murphy and Hill, 1991). The significance of these rhombomere expression patterns is not yet clear, but the effects of the deletion of Hox-1.5 (Chisaka and Capecchi, 1991) and Hox-1.6 (Lufkin et al., 1991; Chisaka et al., 1992) suggest that the Hox genes may specify different sets of neural crest cells emanating at different rhombomeric levels (Hunt and Krumlauf, 1991).

Here we describe the identification of the Hox-3.5 gene as the next homeobox gene 3′ of Hox-3.4. Location and homeobox sequence show Hox-3.5 to be a member of the Hox-2.6, Hox-1.4 and Hox-4.2 subfamily. Hox-3.5 appears to mark the 3′ extent of the Hox-3 locus since chromosome
walking more 3' in both mouse and humans has, so far, failed to identify other Hox-3 members (E. Boncinelli, personal communication). The expression boundary of Hox-3.5 in the hindbrain appears to be more posterior than that of its paralogues.

**Materials and methods**

*Isolation of Hox 3.5 genomic and cDNA clones*

A chromosome walk was carried out on a mouse genomic lambda (Charon 35) and cosmid libraries by screening with a Hox-3.4 (EcoRI-XbaI fragment) probe (Gaunt et al., 1990). Clones were subsequently mapped and flanking regions were used for further screening. The 5' region of Hox-3.5 was identified by hybridization to a human HOX3E (cp19) probe (Simeone et al., 1988) at high stringency. The cDNA was isolated by screening a day-8.5 mouse embryonic cDNA library with the HOX3E and genomic Hox-3.5 probes at high stringency.

*DNA sequencing*

The BamHI-EcoRI fragment of genomic clone 31-1 was subcloned into pSP72 (Promega) and sequenced in both directions using a T7 DNA polymerase sequencing system (Promega). Restriction fragments of the cDNA clone 4-1 were subcloned into pSP72 and sequenced as above, using the dideoxy method described by Sanger et al. (1977).

*RNAase protection assay*

The BamHI-EcoRI fragment of clone 31-1 (Fig. 4B) was subcloned into pSP72. 32P-labelled antisense RNA probes were generated using the riboprobe system (Promega) and T7 RNA polymerase. Protection assays were performed as described by Zinn et al. (1983) using 40 µg of day-11.5 embryo total RNA and tRNA (Boehringer) as a negative control. Total RNA was prepared using the guanidinium method of Sambrook et al. (1989). The assay was run alongside a sequencing reaction as a marker.

*Primer extension assay*

One picomole of the 30 mer oligonucleotide 5'-TAGCGACCCCT-GTAAAGTTACTTCCCATG-3' complementary to the sequence +86 to +116 was end-labelled with [32P]ATP and hybridized with 50 µg of liver RNA, kidney RNA and total mouse 13.5-day embryonic RNA. The primer extension assay was performed according to the method of Sambrook et al. (1989). E. coli tRNA (50 µg, Boehringer) was used as a negative control.

*In situ hybridization*

In situ hybridization to sections of mouse embryos was carried out as previously described by Sharpe et al. (1988). The full length Hox-3.5 cDNA clone 4-1 was subcloned into pSP72 and cut with XhoI (nucleotide position 655 in Fig. 2) prior to preparation of antisense 35S-labelled riboprobes.

**Results**

*Genomic location and organisation of Hox-3.5*

A genomic clone containing the Hox-3.3 and Hox-3.4 transcription units (Sharpe et al., 1988) was used as the start of a chromosomal walk in a Charon 35 genomic library. A homeobox-containing clone was identified by hybridisation and the homeobox mapped to 18 kb 3' of Hox-3.4. Initial sequencing of the hybridising fragment confirmed that it

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**Fig. 1.** Map of Hox-3 cluster from Hox-3.1 to Hox-3.5 and clones isolated during walks. Clone Cos3 was isolated from a cosmid library (data not shown). Black boxes represent positions of the homeoboxes. Clone 59-2 was only mapped with BamHI.
Fig. 2. DNA sequence and predicted protein sequence of *Hox-3.5*. The pentapeptide and the homeodomain are boxed. The position of the intron, derived from genomic clones is marked by an arrowhead. The 3′ untranslated sequence does not contain any obvious polyadenylation signals and is therefore probably not complete. Sequence complementary to the oligonucleotide used for primer extension assay is underlined. Transcription start sites are shown by arrows. Numbering begins at the first transcription start site. Sequence in lower case denotes untranscribed DNA. Amino acids in lower case denote amino acids coded for by the first initiation codon which are not included by the second.
contained a homeobox and was named Hox-3.5. Overlapping phage and cosmid clones 5′ to Hox-3.3 were also isolated to physically link Hox-3.3, Hox-3.4 and Hox-3.5 to Hox-3.1 (Fig. 1).

Hox-3.5 sequence

cDNAs containing the Hox-3.5 homeobox were isolated by screening a day-8.5 embryonic cDNA library with a probe from the human cp19 gene (HOX3E, Simeone et al., 1988) and from a Hox-3.5 homeobox-containing genomic fragment. A 1.4 kb cDNA was isolated and sequenced in full (Fig. 2). The homeobox sequence of the cDNA was identical to the genomically derived sequence of the Hox-3.5 homeobox. The homeobox sequence was also identical to the MAB87 PCR product recently identified by Murtha et al. (1991) and assumed to be the Hox-3 Deformed (Dfd) cognate. Comparison of the full cDNA sequence with the genomic sequence showed the cDNA to be derived from two exons separated by a 500 bp intron with the splice site being situated 35 bp 5′ of the homeobox (Fig. 2). Assuming that the first inframe ATG is the initiator codon, the two exons code for an open reading frame of 264 amino acids to the inframe stop codon 47 amino acids downstream of the homeobox. Although in the majority of cases the first AUG is the initiation codon, in a few exceptions (Kozac, 1987) the first initiation codon may be ignored and translation initiated at the following AUG. The main requirement for this is the presence of an adenine nucleotide at

Fig. 3. (A) A comparison of the amino acid sequences of the Hox-3.5 homeobox and its paralogues, to the Drosophila Dfd and Antp genes, showing a similar pattern of differences between Hox-3.5, its paralogues and Drosophila Dfd in relation to the Drosophila Antp gene. (B) Comparison of the Hox-3.5 predicted protein sequence with its human homologue and its paralogues in the mouse. The pentapeptide and the homeobox regions are boxed. Hyphens represent identical amino acids and spaces have been inserted to produce best possible alignment. There are two main regions of conservation between the proteins: the first 22 amino acids of the protein; the pentapeptide region through to eight amino acids after the homeobox region.

Fig. 4. (A) RNAase protection assay of the BamHI-EcoRI fragment of clone 31-1. Lane 1, free probe. Lane 2, Control lane, probe hybridized to tRNA. Lane 3, probe hybridized to day-11.5 embryo total RNA showing a main protected fragment of 205 bp and a minor protected fragment of 224 bp. (B) Map of genomic clone 31-1, showing the adaptor sites (black boxes). The BamHI-EcoRI fragment of the clone is enlarged showing the positions of the transcription start sites and the location of the first codon. (C) Primer extension assay of the 5′ end of the Hox-3.5. The oligonucleotide complementary to the sequence +86 to +116 (Fig. 2) was end labelled and annealed to 50 µg of total embryo mRNA. After extension with reverse transcriptase, the extended products were separated by electrophoresis. A band of 115 bp was detected in the embryo and kidney RNA lanes. Lane 1, E. coli tRNA. Lane 2, Liver RNA. Lane 3, Kidney RNA. Lane 4, day-13.5 mouse embryo RNA.
position −3 in respect to the primary initiation codon. As can be seen from Fig. 2, there is an adenine −3 bp from the second initiation codon but not from the first codon. Initiation of translation from this second Met codon would make the first three amino acids of Hox-3.5, in common with many other homeobox genes, Met Ser Ser and therefore coding for a sequence of 262 amino acids. This putative Hox-3.5 protein product is, in common with many other homeobox-containing proteins, proline (13.2%) and serine (11.7%) rich.

The position of the Hox-3.5 gene in the Hox-3 cluster (Fig. 1) suggests that it should be a member of the Hox-1.4, Hox-2.6 and Hox-4.2 paralogue group. This prediction is confirmed by a comparison of the Hox-3.5 and the Antennapedia (Antp) homeodomains (Fig. 3A). The pattern of differences between these genes is characteristic of the Dfd-like Hox-1.4, Hox-2.6 and Hox-4.2 paralogue group (Scott et al., 1989).

The Hox-3.5 homeodomain shows greatest homology (98%) with the human HOX3E gene. Although amino acid sequence homology of Hox-3.5 to its human homologue HOX3E extends throughout the predicted protein sequence, this is not true of the paralogue members where amino acid conservation is restricted to two main areas (Fig. 3B). The first of these is the N terminus of the protein extending from three amino acids upstream of the translation start site to the first 22 amino acids after the homeodomain. The Hox-3.5 transcript has a large 3′ untranslated sequence of at least 626 bp. Since the cDNA did not contain any obvious polyadenilation signals, the full extent of the 3′ sequence is not determined.

The transcription start site was mapped by two separate methods. The first, RNAase protection assay identified two sites which were mapped approximately to positions 170 and 189 bp upstream of the translation start site (Fig. 4A). The second, primer extension assay, accurately mapped a single transcription start site at 197 bp upstream of the translation start site (Fig. 4C). This 197 bp start position probably corresponds to the 189 bp position mapped approximately by RNAase protection. The 170 bp RNAase protected fragment not detected by primer extension may be an alternative start site only detected with the more sensitive assay, or may be a degradation product. In common with many homeobox genes (Smale and Baltimore, 1989) no obvious TATA or CAAT boxes were identified 5′ of the start sites.

Embryonic expression of Hox-3.5

The expression pattern for Hox-3.5, detected by in situ hybridization, is considered in terms of three rules that have been formulated to relate a Hox gene’s expression to its cluster location (reviewed by Gaunt, 1991).

Consistent with Rule one (Hox genes and their expression domains are collinear), the anterior boundary of Hox-3.5 expression (Fig. 5C) was found to lie anterior to that of Hox-3.4 (Fig. 5B), both in the 12.5-day hindbrain and prevertebral column. Within the prevertebral column (Fig. 6), Hox-3.5 transcripts increased in abundance over prevertebrae 5-7 (pv 5-7), were most abundant over pv 7-13/14, and then declined more posteriorly. No evidence for Hox-3.5 labelling above background was detected in pv 1-3, but several sections showed low levels of transcripts apparently present in ventral parts of pv 4. The anterior boundary of Hox-3.5 expression was therefore located anterior, by one prevertebra to that found earlier for Hox-3.4 (Gaunt et al., 1990).

Regarding Rule two (anterior boundaries of expression may be conserved between paralogous genes, especially those 3′ in location), Fig. 7 compares, at 10.5 days, the
hindbrain boundary for Hox-3.5 with that of its parologue, Hox-2.6. The anterior boundary of Hox-3.5 expression was found to lie posterior to that of Hox-2.6. The Hox-2.6 boundary (Fig. 7F,H) clearly coincided with a rhombomere junction (the anterior boundary of rhombomere 7; Wilkinson et al., 1989). In contrast, the Hox-3.5 boundary (Fig. 7B,D) did not coincide with a morphologically visible rhombomere junction, but it was located posterior to the position of the Hox-2.6 boundary by a distance equal to about the length of one rhombomere. An apparently similar result for the position of the Hox-3.5 boundary in the hindbrain was also observed at 9.5 days (Fig. 8).

Regarding Rule three (the tissue specificity in expression of a Hox gene may vary according to its chromosomal cluster), Hox-3.5 transcripts were, like Hox-3.4 (Gaunt et al., 1990), abundant in the 12.5-day oesophagus, and detected at only very low levels in the trachea and lung (Fig. 6). So far, expression in the oesophagus has been detected only for Hox-3.4 and Hox-3.5, and has not been observed for any Hox-1, Hox-2 or Hox-4 genes (Gaunt et al., 1988, 1989). Fig. 9 compares expression of Hox-3.5 with that of its paralogues, Hox-2.6 and Hox-1.4, as detected on nearby transverse sections through the 12.5-day cervical spinal cord. The patterns for Hox-2.6 and Hox-1.4 are as previously described (Gaunt et al., 1990), and appear to be characteristic for Hox-2 and Hox-1 genes, respectively. The pattern for Hox-3.5 most resembles that described earlier for Hox-3.4 and Hox-3.3 (Gaunt et al., 1990), with transcripts being abundant both ventrally and centrally within the mantle layer. Medially located transcripts were, however, found to be more abundant for Hox-3.5 (Fig. 8B) than have been noted earlier for other Hox-3 genes (Gaunt et al., 1990). Hox-3.5 transcripts were detected within the 12.5-day metanephric kidney but not within the adjacent testis (Fig. 10B). This differs from findings made for Hox-3.3 and Hox-3.4, whose transcripts are abundant within the testis, but is similar to results obtained for Hox-3.1 (Gaunt et al., 1990).

**Discussion**

The Hox-3.5 gene is located 18 kb 3' of Hox-3.4 on chromosome 15. Sequencing of genomic and cDNA fragments show that transcripts isolated from a day-8.5 embryonic cDNA library are derived from two exons separated by a 500 bp intron. No other Hox-3.5 transcripts were isolated from this library. Simeone et al. (1988) reported that the human homologue of Hox-3.5, HOX3E, is differentially spliced in placental tissue by insertion of a 5' exon located 12 kb 5' to HOX3C. Although we have observed differential splicing with this 5' exon in Hox-3.3 transcripts (unpublished data), we have repeatedly failed to detect similarly differentially spliced transcripts in embryos of either Hox-3.4 or Hox-3.5. In common with other class I homeobox genes, the 3' exon codes for the conserved 61 amino acid homeodomain which has been shown to have a helix-turn-helix structure (Qian et al., 1989) and which is able to bind to DNA (Mihara and Kaiser, 1988) and act as a transcription factor (Kuziora and McGinnis, 1991). The 5' exon contains two regions of conservation, one at either extreme seen in many Hox genes. The first spans the first 22 amino acids and contains the triplet Met Ser Ser which is a common translation start sequence in many homeobox-containing genes. The second region of conservation begins nine amino acids upstream of the splice site and contains another typical feature of these genes, the pentapeptide. The region between these two areas of conservation in the 5' exon is unconserved and is proline and serine rich. It has been shown that proline-rich areas are unable to produce a helical structure.

Hox genes are arranged in the mouse along four clusters Hox-1 (chromosome 6), Hox-2 (chromosome 11), Hox-3 (chromosome 15) and Hox-4 (chromosome 2) and it has been shown that highest homologies are to be found in genes having the same location along these clusters (i.e. paralogue groups). Hence, Hox-3.5 has highest homology to genes Hox-1.4 (Galliot et al., 1989), Hox-2.6 (Graham et al., 1988) and Hox-4.2 (Featherstone et al., 1988; Duboule et al., 1990). Hox-3.5 also has similar characteristic amino acid differences in the homeodomain from the archetypal Antennapedia sequence as Hox-1.4, Hox-2.6 and...
This conservation between paralogues appears to be due to a series of duplications and divergences of an ancestral homeobox cluster (Graham et al., 1989; Kappen et al., 1989). The *Drosophila* cognate of *Hox-3.5* and its paralogues is the *Deformed* (*Dfd*) gene, which also has a high degree of conservation in the homeodomain sequence, and hence the *Hox-3.5* paralogue group has also been named the *Deformed*-like subgroup. The human cognate of *Hox-3.5*, HOX3E (Simeone et al., 1988) is highly homologous in the entirety of its protein coding region, and this homology extends for approximately 200 bp in both 5′ and 3′ untranslated regions of the gene.

The transcription start site mapped, in common with many other homeobox genes (Smale and Baltimore, 1989), is not associated with a common basic promoter motif such as TATA or CAAG or a G/C rich area (G/C content in the preceding 400 bp is only 64%).

Fig. 8. Hox-3.5 transcript boundary located on a coronal section of the 9.5-day hindbrain. (A) Dark-field, (B) bright-field illumination. ov, otic vesicle; arrows, junctions between rhombomeres; r5, 6 and 7, rhombomeres 5, 6 and 7. Bar, 0.1 mm.
The distribution of Hox-3.5 transcripts, detected by in situ hybridization, was found to be generally in-keeping with three rules that have earlier been suggested (Gaunt, 1991) to relate a Hox gene’s expression pattern to its cluster location. However, two points, considered below, merit further discussion.

First, Hunt et al. (1991) recently assumed, though did not demonstrate, that Hox-3.5, like other paralogues in its subfamily (Hox-2.6, Hox-4.2 and Hox-1.4) has its anterior boundary of expression at the rostral limits of rhombomere 7. This assumption was based on a view that paralogous genes display identical boundaries of expression within the hindbrain. In apparent contradiction of this conclusion, we now show that the anterior boundary of Hox-3.5 transcripts is seen to lie posterior to that of Hox-2.6 by a distance equal to about the length of one rhombomere. This boundary remains constant between 9.5 and 10.5 days suggesting that Hox-3.5 expression does not start more anteriorly and regress as has been reported for Hox-1.6 (Murphy and Hill, 1991). As one possible explanation for this finding Hox-3.5 may have, during the course of evolution, acquired a more posterior position for its boundary than that observed by its three paralogues. It may be of significance in this respect that Hox-3.5 appears to mark the 3’-most extent of the Hox-3 cluster. Thus, more anterior genes, present in the other clusters, are not present in Hox-3 and have either failed to be duplicated, been lost, or been relocated during evolution. Whatever the case, Hox-3 genes do not contribute in the development of the hindbrain region to the same extent as the Hox-1, Hox-2 or Hox-4 genes. As a second possibility, however, Hox-3.5 expression may be down-regulated in rhombomere 7, and the reduced levels of Hox-3.5 tran-

Fig. 9. (B) Hox-3.5, (C) Hox-2.6, (D) Hox-1.4 expression detected on transverse sections through the mid-cervical region of the 12.5-day spinal cord. Transcripts from these three paralogous genes are compared here on adjacent sections. (A) Bright-field, (B-D) dark-field illumination. ep, ependymal layer; cm, vm, central and ventral regions of the mantle layer; cg, cervical ganglion. Bar, 0.1 mm.

Fig. 10. (B) Hox-3.5, and (C) Hox-4.2 expression within the 12.5-day testis and kidney. Transcripts from these two paralogous genes are compared on nearby sections from the same embryo. (A) Bright-field, (B,C) dark-field illumination. met, mes, metanephric and mesonephric kidneys; t, testis. Bar, 0.1 mm.
scripts within this rhombomere (as noted for Hox-1.4; Hunt et al., 1991) may not be detected in our in situ hybridizations.

Another unexpected finding was the absence of Hox-3.5 transcripts within the 12.5-day testis. This differs from findings made earlier for Hox-3.3 and Hox-3.4 (Gaunt et al., 1990), but is similar to the finding for Hox-3.1 (Gaunt et al., 1990). Expression in the testis is apparently limited only to Hox-3 and Hox-4 genes. It is of interest that Hox-3.5 and Hox-3.1 both have paralogues within the Hox-4 cluster that show strong expression within the testis (Izpisua-Belmonte et al., 1990). In contrast, Hox-3.3 and Hox-3.4 do not have corresponding paralogues within the Hox-4 cluster. These observations suggest the interesting possibility that paralogous genes from different clusters may be coordinately regulated in their patterns of tissue-specific expression.

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