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# At least three human homeoboxes on chromosome 12 belong to the same transcription unit

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ABSTRACT

Mammalian homeoboxes show a clustered chromosomal organization. In the mouse, at least seven homeoboxes on chromosome 6 and at least six on chromosome 11 identify the murine Hox-1 and Hox-2 loci, respectively. A number of homeoboxes on chromosome 7 define the human HOX-1 locus and homeoboxes on chromosome 17 define the human HOX-2 locus. We studied the genomic organization of three homeobox sequences of the HOX-3 locus on chromosome 12 and analyzed transcripts from this region. Structural characterization and sequencing of several cDNA clones reveal that the three homeobox sequences present in this chromosomal region identify a single transcription unit. Primary transcripts are alternatively processed to give mature messengers with a common 5' noncoding exon encoding different proteins containing one of the three homeodomains.

## INTRODUCTION

The homeobox is a DNA sequence conserved in several genes involved in the determination of Drosophila body segment (1). Homeobox-containing genes have been identified in a wide range of species and a number of such genes have been isolated from sea urchin (2), frog (3-8), mouse (9-23) and man (24-28). The high degree of conservation of the homeodomains identified in these species suggests that vertebrate and fly homeobox genes may perform corresponding functions in development (29). Consistent with the hypothesis of a common role in the control of development, Drosophila and vertebrate homeobox genes share some features. Firstly, most mammalian homeobox sequences show a clustered chromosomal organization. At least 13 murine homeoboxes are clustered in two 60 kb regions on chromosomes 6 and 11 (30). The seven boxes present on chromosome 6 define collectively the murine Hox-1 locus, whereas the six on chromosome 11 define the murine Hox-2 locus. Two other class I homeobox sequences have been mapped on chromosomes 15 and 12, which should define murine Hox-3 and Hox-4 loci, respectively. We have isolated human homeoboxes (26-28) representing the human cognates of murine homeoboxes in the HOX-1, HOX-2 and HOX-3 loci (31). Secondly, structural analysis of several Xenopus, murine and human cDNA clones revealed that in common with most Drosophila homeobox genes the homeodomain

lies in the last 3' exon with an intron just upstream from it. Finally, most Drosophila and vertebrate homeobox genes are expressed in multiple transcripts with a complex transcriptional organization.

We have determined the chromosomal organization of three human homeobox sequences of the HOX-3 locus on chromosome 12. Analysis of cDNA clones representing transcripts from this region reveals that these three homeoboxes are transcribed in a single primary transcript, alternatively spliced to give different mature messengers containing different homeoboxes in their 3' exons.

## MATERIALS AND METHODS

### cDNA and genomic clones

Three cDNA libraries were prepared from poly(A)+ RNA of full-term placenta in λgt11 (32). A genomic library in pcos2EMBL cosmid vector (33) was kindly provided by Anna-Maria Frischauf. These libraries were screened according to standard procedures (34). DNA fragments of interest were subcloned in pEMBL8 (35). DNA sequencing was performed according to Sanger et al. (36) and Maxam and Gilbert (37).

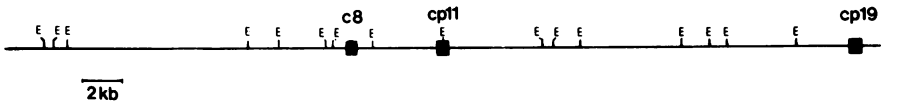
### RNA isolation and analysis

Eight-week human embryos were obtained virtually intact by legal curettage abortions (26). Total RNA was extracted from embryos and term placenta by the guanidinium thiocyanate technique (38) and poly(A)+-selected by one passage on oligo (dT)-cellulose columns. Poly(A)+ RNA was run on 1.0% agarose-formaldehyde gels, transferred to nitrocellulose (Schleicher & Schuell, BA-85) or nylon (Amersham, Hybond N) membranes by Northern capillary blotting and hybridized to $10^7$ c.p.m. of DNA probe labelled by nick translation to a specific activity of 3-8 x $10^8$ c.p.m. per μg. Pre-hybridization and hybridization were carried out as described (26). After washing under stringent conditions (15 mM NaCl/1.5 mM sodium citrate/0.1% SDS at 65°C), the blots were exposed for 1-7 days at -70°C to Kodak XR-5 films in X-omatic intensifying screen cassette. Probes were then removed by boiling in 0.1% SDS buffered solution, and the filters rehybridized to a human actin probe for normalization. Probes used in Northern blot experiments shown in Fig. 6 are as follows: a) probe labelled "5' exon" contains nucleotides 400-635 of the first exon of Fig. 2; b) probe "3' cp25" contains nucleotides 660-865 of the last exon of Fig. 2: c) probe "3' cp19" contains nucleotides 297-493 of the last exon of Fig. 3; d) probe "cp8 specific" contains nucleotides 437-664 of the second exon of Fig. 3.

## RESULTS

We have reported the isolation of several human cDNA clones containing homeobox sequences, which represent transcripts from four different genomic sequences (25). Representative clones of the four groups were termed c1 (=HOX-2.3), c10 (=HOX-2.1), c8 and c13.

In order to study the chromosomal organization of human homeobox genes we isolated genomic sequences present in overlapping cosmid clones using our cDNA clones as probes. A 45 kb DNA region of the human HOX-3 locus on

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                                        10                                  20
Antp    ArgLysArgGlyArgGlnThrTyrThrArgTyrGlnThrLeuGluLeuGluLysGluPhe
        CGCAAACGCGGAAGGCAGACATACACCCGGTACCAGACTCTAGAGCTAGAGAAGGAGTTT

c8      ArgArgArgGlyArgGlnIleTyrSerArgTyrGlnThrLeuGluLeuGluLysGluPhe
        CGGAGGCGCGGCCGCCAGATCTACTCGCGGTACCAGACCCTGGAACTGGAGAAGGAATTT

cp11    GlyLysArgSerArgThrSerTyrThrArgTyrGlnThrLeuGluLeuGluLysGluPhe
        GGCAAGCGGTCCCGAACCAGTTACACGCGCTACCAGACTCTGGAACTCGAGAAAGAATTC

cp19    ProLysArgSerArgAlaAlaTyrThrArgGlnGlnValLeuGluLeuGluLysGluPhe
        CCCAAGCGCTCGAGGGCAGCCTATACCCGGCAGCAAGTCCTGGAATTAGAGAAAGAGTTT


                                        30                                  40
Antp    HisPheAsnArgTyrLeuThrArgArgArgArgIleGluIleAlaHisAlaLeuCysLeu
        CACTTCAATCGCTACTTGACCCGTCGGCGAAGGATCGAGATCGCCCACGCCCTGTGCCTC

c8      HisPheAsnArgTyrLeuThrArgArgArgArgIleGluIleAlaAsnAlaLeuCysLeu
        CACTTCAATCGCTACCTAACGCGGCGCCGGCGCATCGAGATCGCCAACGCGCTTTGCCTG

cp11    HisPheAsnArgTyrLeuThrArgArgArgArgIleGluIleAlaAsnAsnLeuCysLeu
        CACTTTAACCGCTACCTCACTCGCCGCAGGCGCATAGAGATCGCCAACAACTTGTGTCTC

cp19    HisTyrAsnArgTyrLeuThrArgArgArgArgIleGluIleAlaHisSerLeuCysLeu
        CATTACAACCGCTACCTGACCCGAAGGAGAAGGATCGAGATCGCCCACTCGCTGTGCCTC


                                        50                                  60
Antp    ThrGluArgGlnIleLysIleTrpPheGlnAsnArgArgMetLysTrpLysLysGluAsnLys
        ACGGAGCGCCAGATAAAGATTTGGTTCCAGAATCGGCGCATGAAGTGGAAGAAGGAGAACAAG

c8      ThrGluArgGlnIleLysIleTrpPheGlnAsnArgArgMetLysTrpLysLysGluSerAsn
        ACCGAGCGACAGATCAAAATCTGGTTCCAGAACCGCCGGATGAAGTGGAAAAAAGAATCTAAT

cp11    AsnGluArgGlnIleLysIleTrpPheGlnAsnArgArgMetLysTrpLysLysAspSerLys
        AATGAGAGACAGATCAAGATCTGGTTCCAGAACCGACGGATGAAGTGGAAGAAAGATTCCAAA

cp19    SerGluArgGlnIleLysIleTrpPheGlnAsnArgArgMetLysTrpLysLysAspHisArg
        TCTGAGAGGCAGATCAAAATCTGGTTCCAAAACCGTCGCATGAAATGGAAGAAGGACCACCGA
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Figure 1: Genomic map of a region of the human HOX-3 locus containing three homeobox sequences (filled boxes). E=EcoRI. Below the map the nucleotide sequence of the three homeoboxes is shown together with the conceptual translation. The aminoacid changes relative to a consensus sequence, represented by the Drosophila Antennapedia (Antp) homeodomain, are underscored.

chromosome 12 was isolated using clone c8 as probe. Fig. 1 shows the map of this chromosomal region. Two new homeobox sequences have been mapped in this region downstream from the c8 homeobox and provisionally termed cp11 and cp19. Their sequence is also shown in Fig. 1.

We screened three different cDNA libraries prepared from human placenta with genomic probes containing these three HOX-3 homeoboxes. Several cDNA clones were isolated belonging to at least four classes. We report here the structure of representatives of three classes. Fig. 2 shows the nucleotide sequence of clone cp25, a placental messenger containing the c8 homeobox.

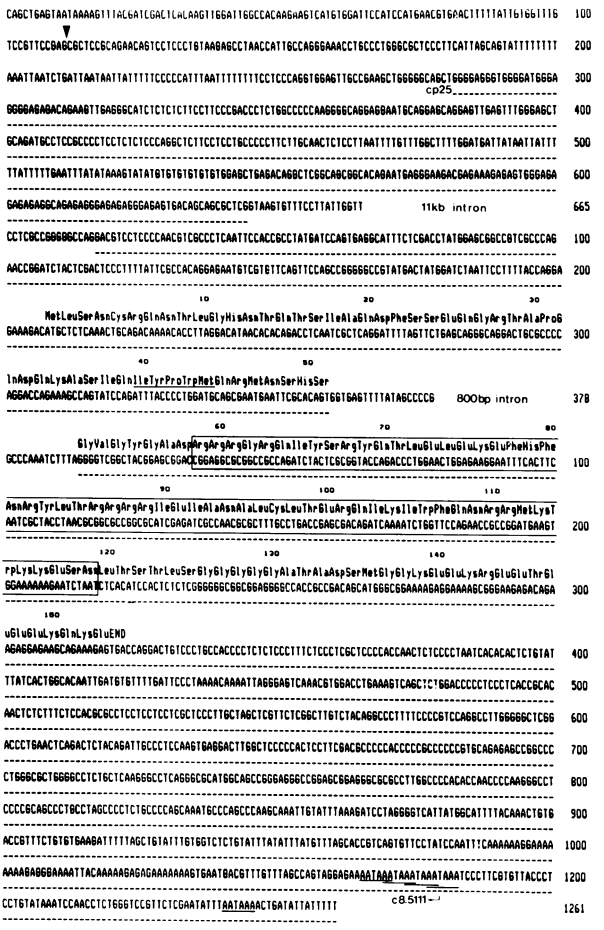


Figure 2: Nucleotide sequence of genomic regions corresponding to exons of the cDNA clone cp25. An arrowhead marks the 5' terminus of the first exon as determined by RNase protection experiments. Dashes indicate nucleotide identity between genomic and cDNA sequences. The conserved pentapeptide Ile-Tyr-Pro-Trp-Met is underlined as are polyadenylation signals. The homeodomain is boxed. The 3' terminus of clone c8.5111 (28) is indicated.

It has a 3' untranslated (3'-UT) region 82 bp longer than clone c8.5111 (28), isolated from adult fibroblasts, because it uses an alternative polyadenylation signal. It consists of 3 exons with a first long intron of about 11 kb and a second of 800 bp just upstream from the homeobox as is the case for c8.5111 (28). The coding region of predicted 153 aminoacid residues initiates in the second exon, which contains the conserved pre-box pentapeptide (27), and terminates in the 3' exon 34 aminoacid residues

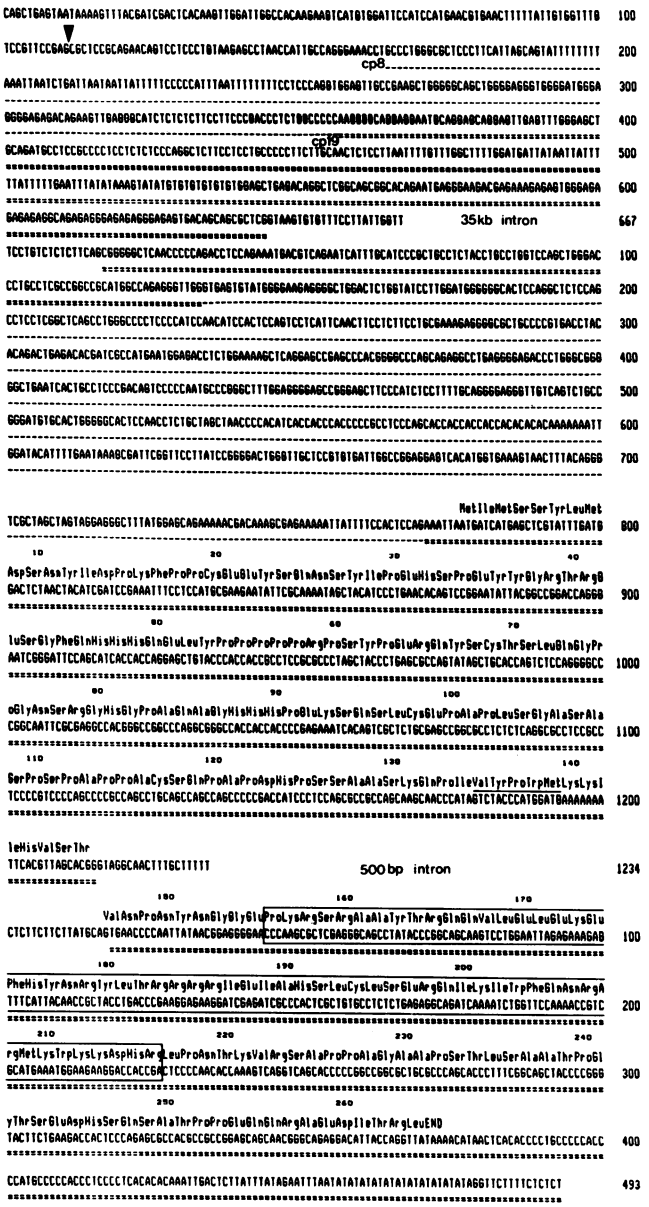


Figure 3: Nucleotide sequence of genomic regions corresponding to exons of the cDNA clone cp8. An arrowhead marks the 5' terminus of the first exon. Dashes indicate nucleotide identity between genomic and cDNA sequences (cp8 and cp19, upper and lower lines, respectively). The conserved Val-Tyr-Pro-Trp-Met pentapeptide is underlined. The homeodomain is boxed. Sequenced region does not include a polyadenylation signal.

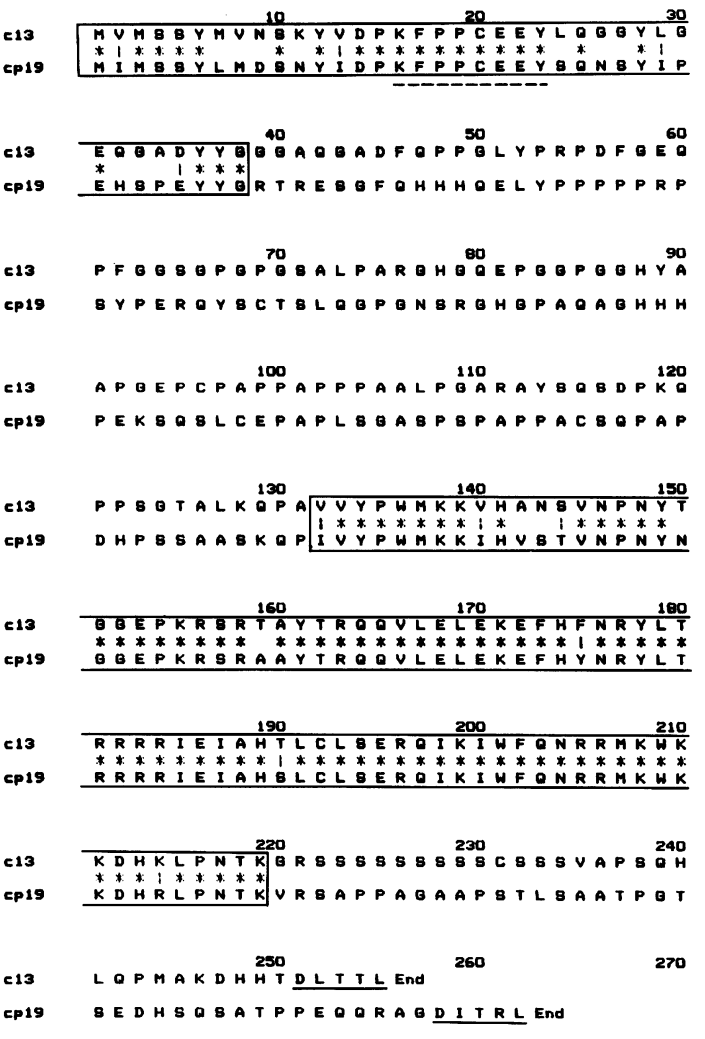


Figure 4: Alignment of predicted protein products of c13 (27) and cp19 cDNA clones. The aminoterminal domain is boxed as is a domain including the conserved pentapeptide, the homeodomain and five aminoacid residues downstream from the latter. Asterisks indicate aminoacid identity and a vertical bar aminoacid conservation. The carboxyl terminus is underlined. The Drosophila Dfd product carboxyl terminus is DLTAL (39). Dashed lines indicate a potential recognition sequence for tyrosine protein kinase (44) conserved in the Dfd product as well (39).

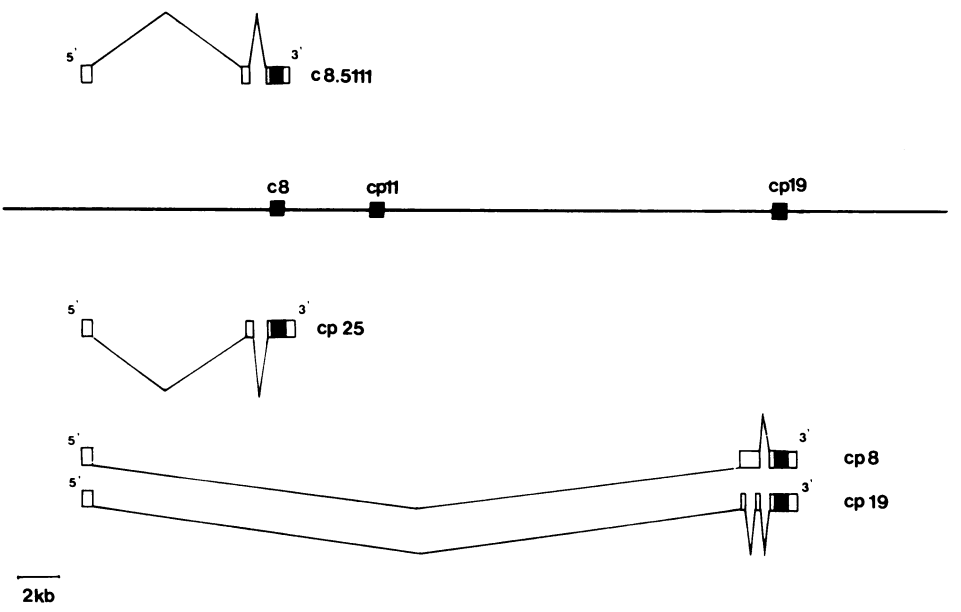


Figure 5: Structure of three cDNA clones isolated from placenta. Above the genomic map the stucture of clone c8.5111 (28) isolated from fibroblasts is shown. Boxes represent exons and a filled box indicates the homeobox. Clone cp25 differs from c8.5111 only in the terminal 3'-UT region. These clones use two alternative polyadenylation signals 76 bp apart.

downstream from the homeodomain. By means of RNase protection experiments we determined the 5' terminus of the first exon, as shown in Fig. 2.

Fig. 3 shows the structure of clones cp19 and cp8 containing the cp19 homeobox. Clone cp19 is composed of 4 exons with a first intron of about 35 kb, a second of 600 bp and a third of 500 bp upstream from the homeobox. The coding region of predicted 262 aminoacid residues initiates in the third exon and terminates 43 aminoacid residues downstream from the homeodomain. Thee 3'-UT region is not present in full in this clone. The predicted protein product encoded by clone cp19 appears to be homologous to the c13 product (27). The amino terminal domain and a second region centered around the homeodomain are particularly conserved (Fig. 4). The cp19 homeodomain itself and five aminoacids downstream from it are very similar to the murine Hox-1.4 (21), the human HOX-1.4, HOX-2.6 (unpublished) and c13 (27) and frog Xhox-1A (5) homeodomains, related in turn to the Drosophila Deformed (Dfd) homeodomain.

Clone cp8, bearing the same coding region of clone cp19, differs from it only because it contains, unspliced, the second intron of cp19 (Fig. 3). Northern blot experiments show that it is not a cloning artefact (see

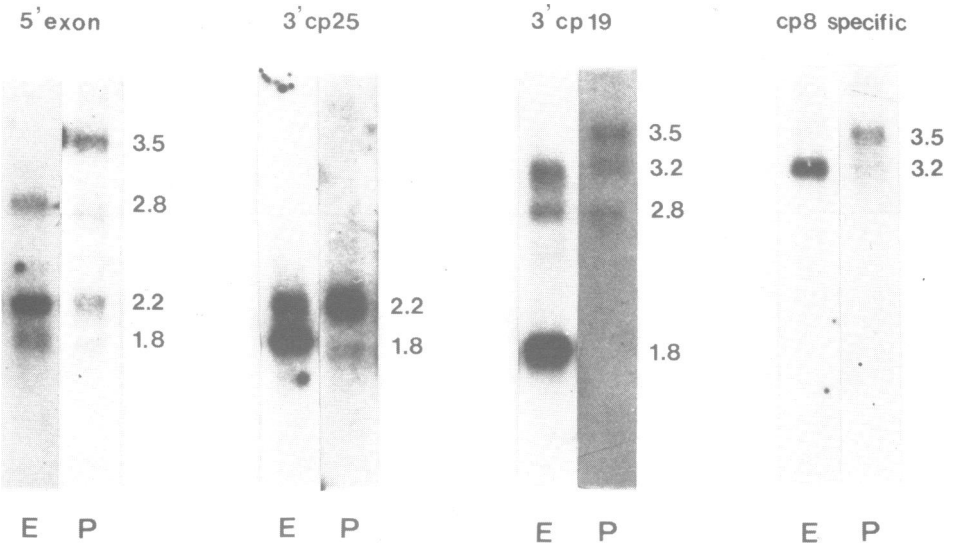


Figure 6: Northern blot analysis of polyadenylated RNA (3 μg) from 8-week embryos (E) and placenta (P). P lanes are overexposed. Probes used are about 200 bp DNA fragments from the common 5' exon, the 3'-UT region of cp25, the 3'-UT region of cp19 and the region of the second exon of cp8 not present in cp19 (Figs. 3 and 6). Transcript sizes are given in kb.

below). Strikingly, both cp8 and cp19 clones initiate within the genomic region representing the first exon of cp25. There is only one genomic region corresponding to this 533 bp 5' exon, located 13 kb upstream from the c8 homeobox region (Fig. 5). The three cDNA clones share a common 5' exon spliced, at the same 5' splice site, to different exons to give mature mRNAs encoding two different proteins with alternative homeodomains.

Northern blot experiments with placenta polyadenylated RNA (Fig. 6) reveal an intricate expression pattern. The 5' exon detects 3 major transcript classes of 3.5 kb, 2.2 kb and 1.8-1.7 kb. The 3.5 kb transcript class contains the 3' untranslated region of cp19, whereas the 2.2 kb size class contains the 3'-UT region of cp25. The cp25 3'-UT region probe detects in placenta a fainter 1.8 kb band representing transcripts possibly lacking the 5' exon. The cp19 3'-UT region probe detects two additional 3.2 kb and 2.8 kb bands. A probe derived from the second intron of cp19, termed "cp8 specific" probe in Fig. 6, detects the 3.5 kb band representing possibly the mRNA from which clone cp8 derives and the 3.2 kb band. cp25, cp19 and cp8 expression in 8-week total human embryos is also shown.

## DISCUSSION

Murine Hox-1 and Hox-2 homeoboxes show a clustered genomic organization with at least six homeoboxes per locus, whereas no Hox-3 homeobox has been

mapped other than Hox-3.1 (18). The human HOX-3 locus on chromosome 12 contains clustered homeoboxes as is the case of the human HOX-1 and HOX-2 loci and of a locus mapped on chromosome 2 (unpublished). We have identified so far 18 homeoboxes in these four loci (our unpublished results) with the same 5'-3' orientation in each locus. It is interesting to note that in all four human loci there is a Dfd-like homeobox: HOX-1.4, HOX-2.6, cp19 and c13. We have shown that the entire cp19 predicted protein bears similarity to the c13 protein (Fig. 4) and both, in turn, to the Dfd protein, as already noted (39). The same is true for the HOX-2.6 protein (unpublished). Therefore, there are at least four human protein products corresponding to Dfd. The significance of this redundancy is elusive and requires further investigation. Alignment of the four human loci in such a way that HOX-1.4, HOX-2.6, cp19 and c13 homeoboxes occupy corresponding positions leads to interesting conclusions on the organization and origin of various loci. For example, the triplet c8, cp11, cp19 should correspond to human (or murine) 1.2, 1.3, 1.4 and 2.2, 2.1, 2.6, suggesting a large-scale duplication of an ancestral Hox region, followed by dispersion in various chromosomes.

Drosophila homeotic and segmentation genes belonging to the bithorax- and Antennapedia-complex have separate promoters and often different orientation. The human HOX-3 region we have studied seems to exhibit a different transcriptional organization. cDNA clones encoding proteins with different homeodomains share a common 5' noncoding exon. We have reported (40) preliminary characterization of a HOX-3 cDNA clone, cp11, encoding a protein with a third homeodomain, namely the cp11 homeodomain, and sharing the same 5' noncoding exon. Therefore, the whole genomic region from the common 5' exon to the last cp19 exon appears to be transcribed in a single primary transcript, alternatively spliced to give the mature mRNAs from which the cDNA clones derive. Primer extension experiments (not shown) and an estimate of transcript length from Northern blot analysis suggest that one or more short exons may in turn precede the common 5' exon. The finding that at least in one tissue the three homeoboxes belong to the same transcription unit and do not represent three separate genes is bound to influence considerably our view on homeobox genes. For Drosophila homeotic and segmentation genes nothing similar has been reported so far. Several different Ultrabithorax (Ubx) proteins have been reported (41) to originate from alternative splicing. Different messengers with common 5' and 3' exons differ from each other for three internal short exonic sequences alternatively spliced. A similar, albeit simpler, organization may be present in Antennapedia (Antp) transcripts (41). In all these instances the different protein products contain the same homeodomain. In human placenta different proteins might be encoded in alternative mature transcripts obtained by means of an alternate use of homeobox regions. This should limit the number of different human homeobox genes, while increasing the complexity of their transcriptional organization. We are not yet in a position to assess how general this phenomenon is and, in particular, how many HOX-3 homeoboxes belong to the same transcription unit.

An alternative explanation for the origin of the cDNA clones studied

can be considered. In fact, exons specific for the three cDNA clones could be transcribed from three different promoters and the common 5' exon could be spliced post-transcriptionally with a trans-splicing mechanism as that found operating in trypanosomes (42). Although for the time being the explanation cannot be ruled out, we think this unlikely. First, the genomic region corresponding to the common 5' exon lies in cis, upstream from the 3' exons. Second, nuclear run-on transcription assays (43) show that the whole region is transcribed (not shown). Finally, we found several alternative cDNA clones containing various exons between the 5' and 3' exons. It appears likely that a considerable proportion of the genomic region under scrutiny will turn out to be present in mature transcripts. Northern blot analysis supports this picture and suggests that clone cp25 represents a placental messenger 2.2 kb long, that clone cp19 derives from a transcript of 2.8 kb and clone cp8 from a transcript of 3.5 kb. Additional placental mature transcripts map in this region and await a characterization.

Because we found this transcriptional organization in placental tissues, it seemed appropriate to wonder whether the observed mature messengers are predominant in these tissues or specific for them. Northern blot analysis of embryonic tissues reveals common mature transcripts and some differences. In particular, the embryonic spinal cord shows a complex expression pattern including all transcripts detected in placenta (not shown).

In summary, analysis of cDNA clones and polyadenylated RNAs from the HOX-3 locus suggests that transcription might start from a major promoter upstream from homeodomain-containing exons. Several polyadenylation signals are recognized giving rise to a number of polyadenylated RNAs, in turn spliced to generate mature messengers RNAs. We do not know whether this organization is unique to the HOX-3 locus or can be generalized to other homeobox loci. This transcription organization could readily account for the same 5'-3' orientation of all mammalian homeoboxes isolated so far and for the unique degree of conservation of the whole homeobox chromosomal region in mice and men (14). The presence of a major upstream promoter does not exclude that additional specific promoters in front of single homeobox regions exist in placenta or other tissues. Transcription from the upstream promoter might provide the substrate for a concerted fine tuning of the expression of homeobox messengers whereas transcription from specific secondary promoters might serve specific purposes.

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