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Two human homeobox genes, *c1* and *c8*: Structure analysis and expression in embryonic development

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ABSTRACT **Two human cDNA clones (HHO.c1.95 and HHO.c8.5111) containing a homeobox region have been characterized, and the respective genomic regions have been partially analyzed. Expression of the corresponding genes, termed *c1* and *c8*, was evaluated in different organs and body parts during human embryonic/fetal development. HHO.c1.95 apparently encodes a 217-amino acid protein containing a class I homeodomain that shares 60 out of 61 amino acid residues with the Antennapedia homeodomain of *Drosophila melanogaster*. HHO.c8.5111 encodes a 153-amino acid protein containing a homeodomain identical to that of the frog *AC1* gene. Clones HHO.c1 and HHO.c8 detect by blot-hybridization one and two specific polyadenylylated transcripts, respectively. These are differentially expressed in spinal cord, backbone rudiments, limb buds (or limbs), heart, and skin of human embros and early fetuses in the 5- to 9-week postfertilization period, thus suggesting that the *c1* and *c8* genes play a key role in a variety of developmental processes. Together, the results of the embryonic/fetal expression of *c1* and *c8* and those of two previously analyzed genes (*c10* and *c13*) indicate a coherent pattern of expression of these genes in early human ontogeny.**

In the fruitfly *Drosophila melanogaster*, studies on developmental mutants have allowed the identification of a number of genes controlling morphogenesis, which are classified in three general groups: (*i*) maternal-effect genes, which specify the structure and spatial coordinates of the egg, (*ii*) segmentation genes, which determine the number and polarity of body segments, and (*iii*) homeotic genes, which specify the identity of each segment (reviewed in ref. 1). Most homeotic genes identified so far are clustered in the Antennapedia (*Antp-C*) and the bithorax (*bx-C*) complex (2, 3). Cloning of some of these genes has led to the identification of a 183-base-pair (bp) sequence, termed the homeobox, which is contained in most segmentation and homeotic genes and encodes a highly conserved protein domain (4, 5). Homeobox-containing genes have also been cloned from sea urchin (6), frog (7–9), mouse (10–20), and human (21, 22); however, direct evidence for their regulatory role in development is still unavailable (cf. ref. 23).

We have reported the isolation of human cDNA clones, termed HHO.c1, HHO.c8, HHO.c10, and HHO.c13, containing homeobox sequences that appear to represent transcripts from four different genes (22). HHO.c10 (24) and HHO.c13 (25) contain a conserved class I homeobox and detect by blot hybridization specific transcripts during human embryogenesis. We report here the characterization of clones HHO.c1.95 and HHO.c8.5111 and part of the corresponding genomic regions. HHO.c1 and HHO.c8 detect by blot hybridization one and two specific polyadenylylated transcripts, respectively, which are differentially expressed in a variety of organs or body parts at 5 to 9 weeks of embryonic/fetal development.

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METHODS

Human Embryos and Fetuses. Human embryos and early fetuses were obtained virtually intact from legal curettage abortions and were maintained in sterile culture medium at +4°C until processing (26). The age of embryos (5–8 weeks) was carefully established by morphologic staging according to multiple criteria (dating error was ± 2 days) (27, 28). Early fetuses (9 weeks) were staged on the basis of standard age/crown–rump length plots. Different organs or body parts were dissected under an inverted microscope and were stored in liquid nitrogen until analyzed.

cDNA Clones. Recombinant clones (8×10^5) of a cDNA library prepared from poly(A)$^+$ RNA of SV40-transformed human fibroblasts (29) were originally screened with a 600-bp *Bam*HI–*Pvu* II fragment of clone 903 (4, 30) containing the entire *Antp* homeobox region or a 1050-bp *Pvu* II fragment containing the homeobox and the 3′ region of the *ftz* gene (4), both labeled to a high specific activity by nick-translation (31). Clones (2.5×10^5) of the same cDNA library were subsequently screened with the insert of clone HHO.c1 or HHO.c8 under stringent conditions. Recombinant positive clones were isolated, and the longest inserts (HHO.c1.95, HHO.c8.5111) were characterized by restriction endonuclease mapping and were sequenced (32).

RNA and DNA Analysis. Total RNA was extracted from fresh or frozen samples by the guanidinium isothiocyanate technique and was selected for poly(A)$^+$ RNA by one passage on an oligo(dT)-cellulose column (33, 34). Ten micrograms of total RNA or 2–3 μg of poly(A)$^+$ RNA was electrophoresed on a 1.0% agarose/formaldehyde gel, blotted on nitrocellulose or nylon membranes, and hybridized to 10^7 cpm of DNA probe labeled by nick-translation to a specific activity of 3×10^8 to 8×10^8 dpm per μg (31). The blots were washed under stringent conditions (cf. ref. 25). Probes were then removed by boiling in a 0.1% $NaDodSO_4$ buffered solution, and the filters were rehybridized to a chicken β-actin probe for normalization (see refs. 24 and 25). Southern analysis was performed according to standard techniques (31).

RESULTS AND DISCUSSION

The nucleotide sequence of HHO.c1.95 is shown in Fig. 1*A*. Conceptual translation of its longest open reading frame, starting from the ATG codon at position 100, produces a peptide sequence of 217-amino acid residues that contains a class I homeodomain. The carboxyl-terminus of this protein lies 20-amino acid residues downstream from the homeodomain and ends in a row of six glutamic acid residues. A 609-bp 3′-untranslated region contains a polyadenylylation signal

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(AATAAA) 20 nucleotides upstream from the poly(A) tail. A putative glycosylation site (Asn-Xaa-Thr) is present at the 60th amino acid residue of the homeodomain (Fig. 1*A*), as is found in the *Antp* homeodomain (23). The original clone HHO.c1 is identical to HHO.c1.95 at positions 450–1359.

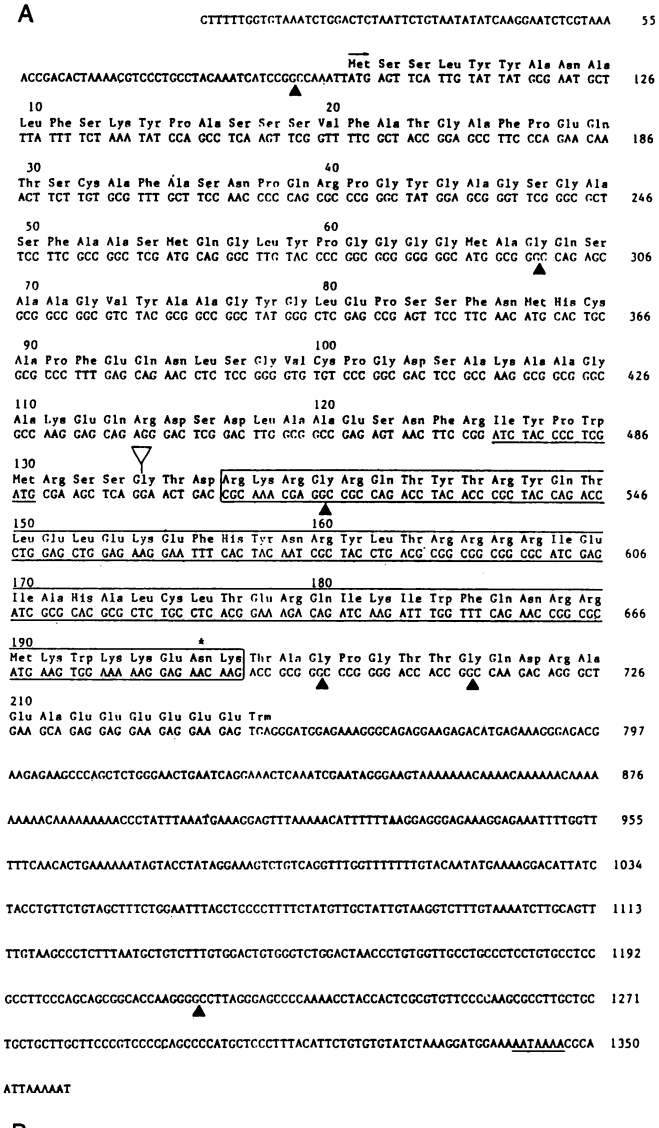

FIG. 1. (*A*) Nucleotide sequence of the human cDNA clone HHO.c1.95 and the conceptual translation of its longest open reading frame. The region encoding the homeodomain is boxed. The second conserved homeodomain and the polyadenylylation signal (AATAAA) preceding the poly(A) tail are underlined. →, Methionine (ATG) codon representing the putative translation start site; *, putative glycosylation site; Trm, translation termination codon; ▽, position of the intron; ▲, *Hae* III restriction sites used for subcloning HHO.c1.95. (*B*) Restriction map of the HHO.c1.95 transcription unit on two genomic clones (λ95.1 and λ125.3) as determined by Southern blot analysis. Open boxes indicate exons, and closed boxes indicate homeobox sequences. E, *Eco*RI; Bg, *Bgl* II; A, *Alu* I; H_3, *Hin*dIII; T, *Taq* I; S, *Sma* I.

HHO.c1.95 contains a second open reading frame, which might encode a 156-amino acid protein, starting from the methionine codon at position 116 through a TGA codon at position 584 (halfway through the homeobox). Further anal-

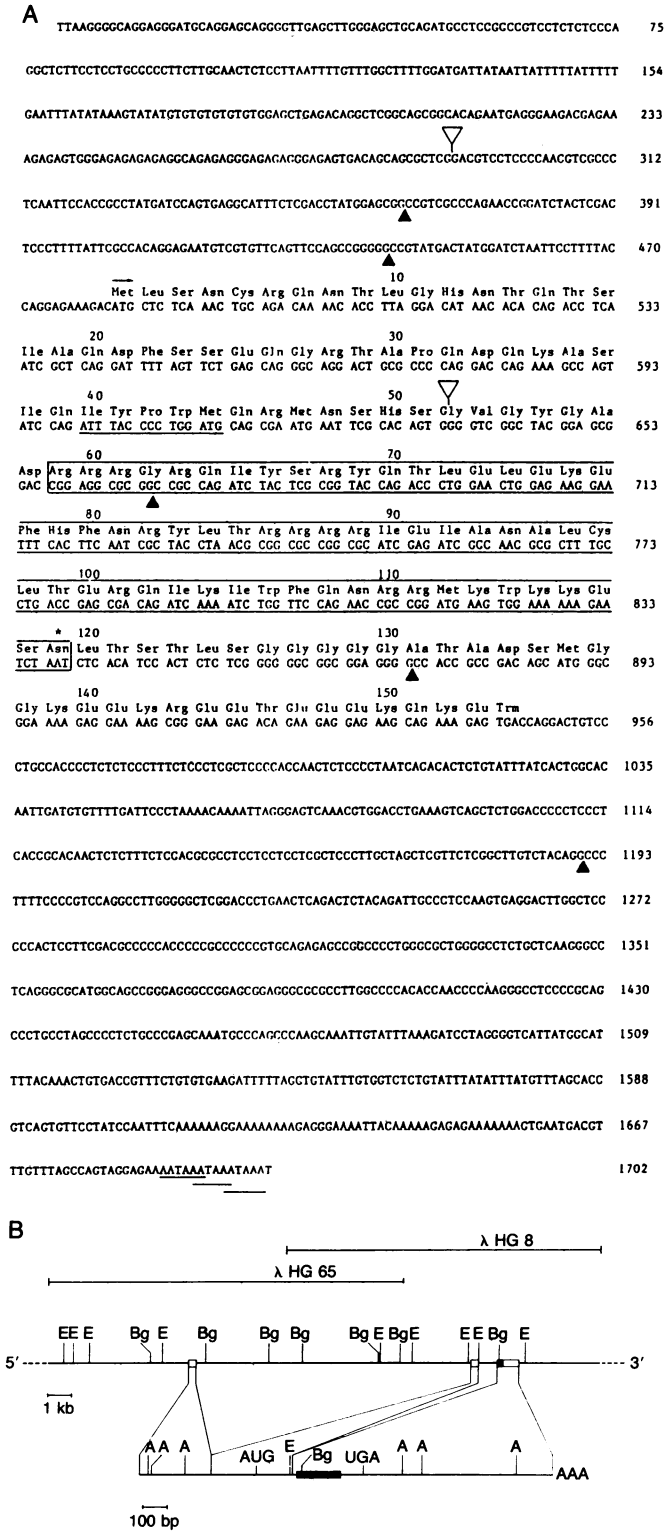

FIG. 2. (*A*) Nucleotide sequence of the human cDNA clone HHO.c8.5111 and the translation of its longest open reading frame. Symbols are as in Fig. 1*A*. (*B*) Restriction map of the genomic region corresponding to HHO.c8.5111, as determined by Southern blot analysis on two phage clones (λ8 and λ65). Open and closed boxes indicate exons and homeobox sequences, respectively. E, *Eco*RI; Bg, *Bgl* II; A, *Alu* I.

ysis is clearly necessary to assess the significance of this observation.

HHO.c1.95 was used as a hybridization probe to screen a human genomic library in λ Charon 28 by standard procedures. Two overlapping genomic clones, λ95.1 and λ125.3, which contain a region of ≈24 kilobases (kb) corresponding to HHO.c1.95, were characterized by restriction endonuclease mapping (Fig. 1*B*) and were partially sequenced (not shown). The HHO.c1.95 coding region is interrupted by an intron of ≈2400 bp located upstream from the homeobox (Fig. 1 *A* and *B*). Two additional homeobox sequences appear to be present in λ95.1 upstream from the *c1* gene (Fig. 1*B*).

Chromosome mapping by standard procedures (somatic cell hybrids and *in situ* hybridization) indicates that the *c1* gene is localized in the long arm of chromosome 17 (K. Hübner, personal communication), possibly within the gene cluster previously identified (13, 14) as containing *Hu1* (termed *c10* in refs. 24), *Hu2*, and *Hu5* (18). Preliminary restriction data on cosmid clones locate the *c1* gene in a position corresponding to *Hox2.3* within the *Hox2* murine cluster (17).

The nucleotide sequence of the HHO.c8.5111 insert is shown in Fig. 2*A*. Translation of the longest open reading frame starting from the ATG codon at position 483 produces a peptide of 153-amino acid residues containing a class I homeodomain followed by 34 amino acids before the termination codon. The 761-bp 3′-untranslated region contains a polyadenylylation signal 15 nucleotides upstream from the poly(A) tail, which is followed by two additional and partially overlapping AATAAA sequences. The original clone HHO.c8 is identical to HHO.c8.5111 from position 594 to the poly(A) tail.

The *c8* homeodomain is identical to that found in the cDNA clone AC1 from *Xenopus laevis* (7). The homology between the predicted peptide sequences derived from HHO.c8.5111 and AC1 is further extended outside the homeodomain (22). In particular, a potential glycosylation site is present at the 61st amino acid residue in both homeodomains (Fig. 2*A*; cf. ref. 7).

Fig. 2*B* shows the genomic region corresponding to HHO.c8.5111 obtained from two overlapping phage clones (λ8 and λ65). Restriction analysis and partial sequencing (not shown) indicate that the HHO.c8.5111 coding region is interrupted by an intron of ≈800 bp located upstream from the homeobox (Fig. 2 *A* and *B*). An intron of ≈11 kb is present further upstream in the 5′-untranslated region (Fig. 2 *A* and

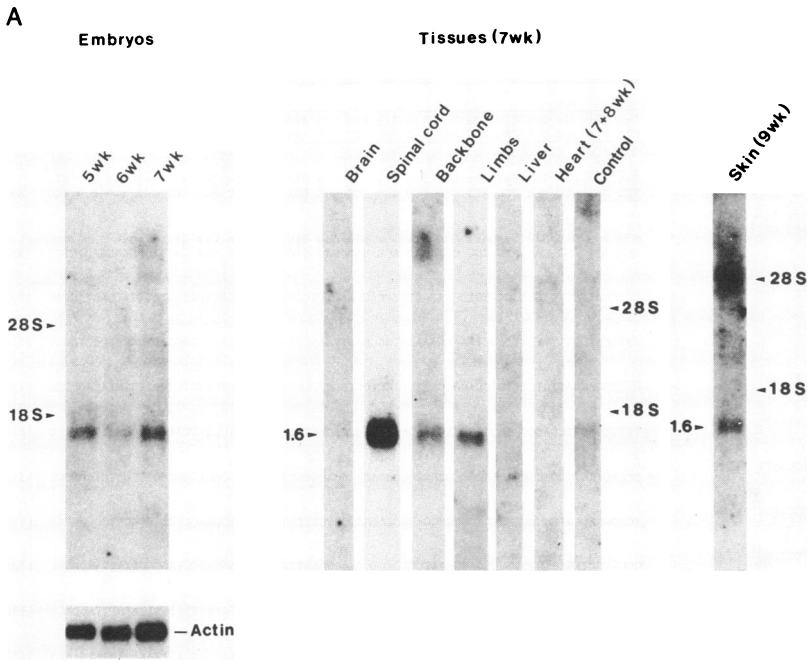

FIG. 3. (*A*) (*Left*) Blot-hybridization analysis of the expression of HHO.c1 in poly(A)$^+$ RNA from whole human embryos at 5, 6, and 7 weeks after fertilization. The relative mobilities of 28S and 18S rRNA are shown in size markers. Sizes are in kb. (*Right*) Expression of HHO.c1 in poly(A)$^+$ (2–3 μg per lane) and total skin (10 μg) RNA obtained from pools of organs or body parts dissected from human embryos at 7 weeks except for hearts, which were pooled from 7- to 8-week embryos, and skin, which was obtained at 9 weeks. Control, poly(A)$^+$ RNA from an embryo deprived of limbs and spinal column. (*B*) Expression of HHO.c1 transcripts in poly(A)$^+$ RNA (2 μg per lane) from spinal cord, backbone, and limbs and also in total RNA (10 μg per lane) from skin in human embryos from 6 to 9 weeks after fertilization, as analyzed by blot hybridization. Sizes are in kb. rRNA (28S and 18S) are shown as size markers.

B). The *c8* gene has been mapped on the long arm of human chromosome 12 (K. Hübner, personal communication), in a position unrelated to previously identified homeobox gene clusters on chromosomes 2 (ref. 25, and K. Hübner, personal communication), 7 (35), and 17 (ref. 14 and this paper).

The putative proteins of HHO.c1.95 and HHO.c8.5111 contain a conserved pentapeptide Ile-Tyr-Pro-Trp-Met that starts at amino acid position −11 and −19, respectively, from the homeodomain (25).

We have utilized HHO.c1 and HHO.c8 as cDNA probes to analyze the expression of the *c1* and *c8* genes in human embryonic development.

Blot-hybridization analysis of poly(A)$^+$ RNA obtained from whole embryos at 5, 6, and 7 weeks indicates that *c1* is expressed as a single major transcript of 1.6 kb at a roughly constant level throughout this period (Fig. 3*A*). No signal was detected by blot hybridization of the corresponding poly(A)$^-$ RNAs (not shown).

We have then analyzed the expression of *c1* in different organs and body parts of 7-week embryos. Samples from different specimens were pooled when necessary. Hearts from 7- and 8-week embryos were pooled. The central nervous system was obtained free of contaminating tissues; brain was dissected from spinal cord and medulla oblongata at the level of the pontine flexure. Skin was analyzed as total RNA. As shown in Fig. 3*A*, the expression of *c1* transcripts was tissue-specific in 7-week embryos: 1.6-kb messages were abundant in spinal cord and detectable in backbone rudiments and limbs. Virtually no hybridization signal was observed in brain, heart, and liver, whereas a barely detectable band was still present in a control embryo deprived of limbs and spinal column. A low signal at 1.6 kb was also detectable in total RNA from skin, together with a broad band of cross-hybridization to 28S rRNA.

The expression of *c1* in positive tissues was then analyzed at 6 to 9 weeks by blot-hybridization analysis of poly(A)$^+$ (spinal cord, backbone, and limbs) or total (skin) RNA (Fig. 3*B*). The abundance of 1.6-kb transcripts shows a 3- to 5-fold increase in spinal cord and backbone rudiments at 6 to 8 and 7 to 8 weeks, respectively; a nearly constant low level was observed in limbs and skin at 6 to 8 and 7 to 9 weeks.

The expression of *c8* gene was analyzed on the specimens described above. HHO.c8 detected two transcripts of 2.2 and 1.8 kb by blot hybridization of poly(A)$^+$ RNA from whole embryos at 5, 6, and 7 weeks at a roughly constant level (Fig. 4*A Left*). No signal was detected by blot hybridization of corresponding poly(A)$^-$ RNAs (not shown). The 2.2- and 1.8-kb transcripts are abundantly accumulated in spinal cord with a 1:2 ratio. The 1.8-kb RNA is predominantly expressed at a high level in limbs and backbone rudiments and at a lower

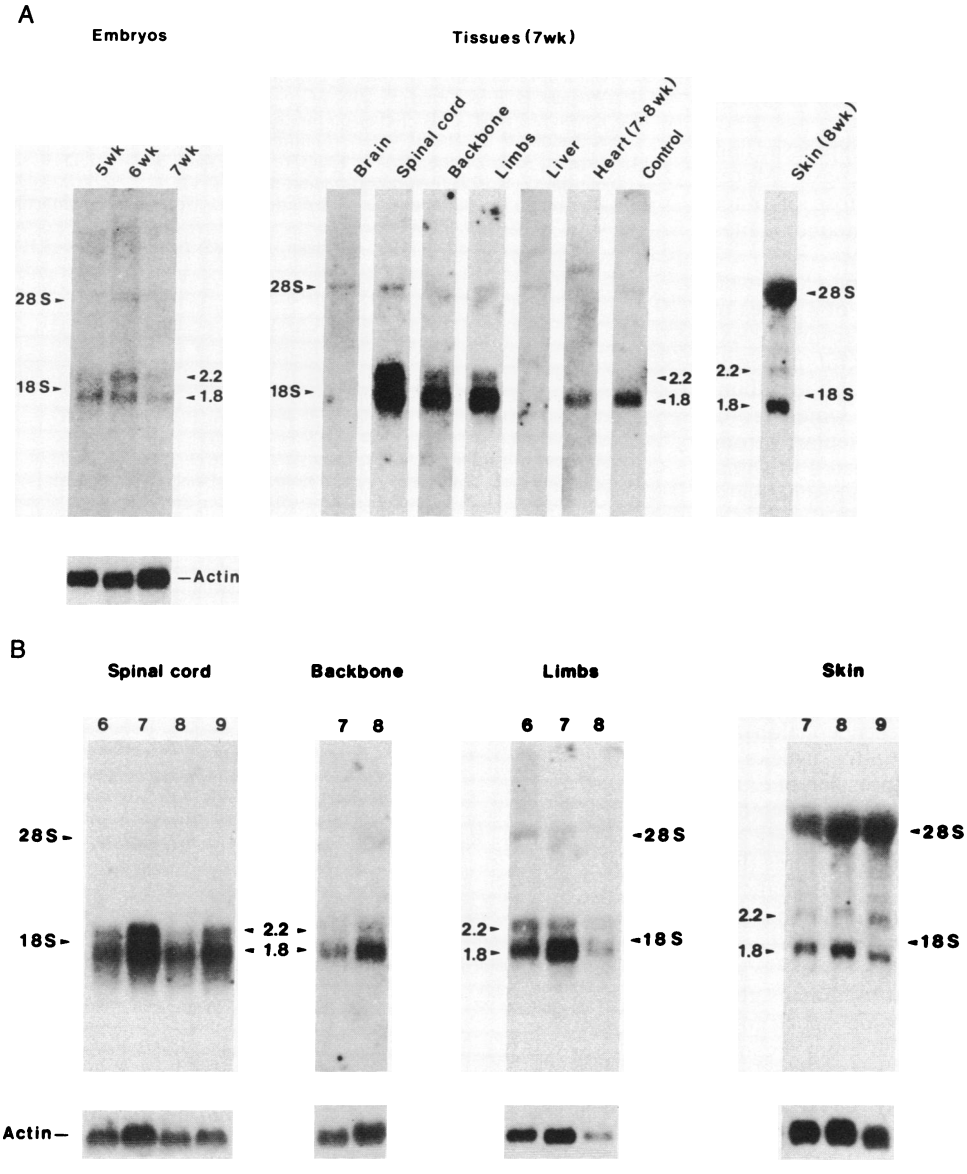

FIG. 4. (*A*) (*Left*) Blot-hybridization analysis of the expression of HHO.c8 in poly(A)$^+$ RNA (2–3 μg per lane) from whole human embryos at 5, 6, and 7 weeks after fertilization. Sizes are in kb. rRNA (28S and 18S) are shown as size markers. (*Right*) Expression of HHO.c8 in poly(A)$^+$ (2–3 μg) or total skin (10 μg) RNA from pools of organs or body parts dissected from human embryos at 7 weeks except for hearts, which were pooled from 7- to 8-week embryos, and skin, which was obtained at 8 weeks. Control, poly(A)$^+$ RNA from an embryo deprived of head, limbs, heart, liver, and spinal column. (*B*) Expression of HHO.c8 as analyzed by blot hybridization of poly(A)$^+$ RNA (2 μg per lane) from spinal cord, backbone rudiments, and limbs and also from total RNA (10 μg per lane) from skin obtained from human embryos from 6 to 9 weeks after conception. Sizes are in kb. rRNA (28S and 18S) are shown as size markers.

level in heart (Fig. 4*A Right*). No signal was observed in brain and liver. Both bands are clearly detected by blot hybridization of skin total RNA (together with a prominent band of cross-hybridization to 28S rRNA) at levels comparable to those observed in spinal cord or limbs.

A 1.8-kb band was still detected in poly(A)$^+$ RNA from a control embryo upon removal of head, limbs, spinal column, liver, and heart. Although at least part of this signal may be accounted for by the presence of skin, it cannot be ruled out that *c8* transcripts are expressed in other organs or tissues.

Analysis of *c8* expression at different stages of development indicated that the abundance of both 1.8- and 2.2-kb transcripts, as well as their relative ratio, is nearly constant from 6 to 9 weeks in spinal cord, 6 to 8 weeks in limbs, 7 to 8 weeks in backbone rudiments, and 7 to 9 weeks in skin (Fig. 4*B*).

In order to exclude artifacts due to cross-hybridization to transcripts derived from other homeobox genes, we have hybridized blots of spinal cord poly(A)$^+$ RNA to different subclones of both inserts. In particular, we utilized two noncontiguous *Hae* III fragments containing the homeobox and the 3′-translated and untranslated regions of HHO.c1.95, as well as two contiguous *Hae* III fragments containing the homeobox and the 3′-translated and untranslated regions of HHO.c8.5111 (see Figs. 1*A* and 2*A*). In both cases, hybridization to all subclones detected exactly the same bands (not shown). Furthermore, these data indicate that both the 2.2- and 1.8-kb transcripts detected by HHO.c8 have essentially the same structure in the region spanning the homeobox and the proximal 3′-untranslated sequences, thus suggesting that they might differ in their 5′ portions.

We conclude that *c1* and *c8* are differentially expressed and developmentally regulated in human embryogenesis. Moreover, their expression patterns differ from those of other human (24, 25) and mouse (12, 15–18, 20) homeobox genes reported so far.

The pattern of expression observed for *c1* and *c8*, as well as *c10* and *c13* (24, 25), allows the formulation of a series of general concepts: (*i*) The four genes are differentially expressed according to tissue- and/or stage-specific patterns. Most of them encode multiple transcripts. (*ii*) All are abundantly transcribed in embryonic spinal cord, which represents a typical metameric structure. In particular, strong heterogeneous 2.1- and 2.5-kb bands are detected in spinal cord by HHO.c10 (24) and HHO.c13 (25), respectively. Clone HHO.c1 detects 1.6-kb transcripts of the same size class, which are also present in other embryonic tissues in lower abundance. Clone HHO.c8 detects two bands of 2.2 and 1.8 kb with a specific intensity ratio different from that observed in other embryonic tissues. (*iii*) All genes, with the possible exception of *c10*, are transcribed in backbone rudiments. (*iv*) *c1*, *c8*, and *c10* do not detect any transcripts in embryonic brain throughout the analyzed period. *c13* is expressed as a single 4.2-kb band in mesencephalon and metencephalon (not in prosencephalon) at 6 to 7 weeks but not at 8 weeks or later. (*v*) Although the expression of *c1* and *c10* is, respectively, largely and exclusively restricted to spinal cord (and apparently backbone), *c8* and *c13* are also expressed in a large spectrum of embryonic structures (limb buds or limbs, heart, skin, etc.), which follow dramatically different patterns of morphogenesis. (*vi*) Homeobox transcripts have not been detected so far in a variety of organs or body parts (e.g., liver).

In conclusion, the concept emerges that in humans genes containing homeodomains may play a wide spectrum of control activities in early development of different organs or body parts. According to a unified model, the homeodomain might exert a general function (possibly DNA-binding), which is necessarily shared by all homeobox gene products; more specific functions may be exerted by the nonconserved parts of homeobox gene proteins, which diverged in various evolutionary lineages.

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