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Hxt encodes a basic helix-loop-helix transcription factor that regulates trophoblast cell development

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SUMMARY

Trophoblast cells are the first lineage to form in the mammalian conceptus and mediate the process of implantation. We report the cloning of a basic helix-loop-helix (bHLH) transcription factor gene, *Hxt,* that is expressed in early trophoblast and in differentiated giant cells. A separate gene, *Hed,* encodes a related protein that is expressed in maternal deciduum surrounding the implantation site. Overexpression of *Hxt* in mouse blastomeres directed their development into trophoblast cells in blastocysts. In addition, overexpression of *Hxt* induced the differentiation of rat trophoblast (Rcho-1) stem cells as assayed by changes in cell adhesion and by activation of the placental lactogen-I gene promoter, a trophoblast giant cell-specific gene. In contrast, the negative HLH regulator, Id-1, inhibited Rcho-1 differentiation and placental lactogen-I transcription. These data demonstrate a role for HLH factors in regulating trophoblast development and indicate a positive role for *Hxt* in promoting the formation of trophoblast giant cells.

Key words: trophoblast, placenta, transcription, helix-loop-helix, *Hxt*, mouse, rat

INTRODUCTION

The first cell lineages to be established in the mammalian embryo contribute only to extraembryonic structures that form the placenta (see Cross et al., 1994). It is only after implantation that significant differentiation occurs in the embryonic ectoderm, cells that give rise to all the structures of the embryo proper. The placenta is formed from three extraembryonic lineages: trophoblasts that lie adjacent to maternal cells in the implantation site, endoderm that migrates across the inner surface of the trophoblast layer and mesoderm that gives rise to placental blood vessels. The development of these extraembryonic lineages has largely been ignored in the molecular analysis of development. Nonetheless, abnormalities in trophoblast development or in formation of the placenta account for the most common gestational abnormalities in humans and animals, including failures of implantation, intrauterine growth retardation and stillbirth. Human fecundity is only around 25%, owing partially to fertilization failure but more significantly to failures in early development. For example, even after careful selection of embryos, the success of embryo transfer in humans is only 20-25% (Wilcox et al., 1993) and approximately one quarter of human embryos that begin to implant die before the pregnancy is recognized clinically (Wilcox et al., 1988). In farm animals, failures in development at implantation account for almost 80% of the embryonic loss that occurs throughout the entire length of gestation (Roberts et al., 1990).

Factors that regulate the differentiation of placental cell lineages are largely unknown. The POU-domain transcription factor Oct-4 is expressed in undifferentiated cells during cleavage stage development and is downregulated as cells differentiate into trophoblast and endoderm (Palmieri et al., 1994). Although Oct-4 may play a direct role in preventing differentiation into the trophoblast lineage, other factors must play a positive role. Pem is a homeodomain transcription factor that is expressed in trophoblast and yolk sac in the mouse placenta (Wilkinson et al., 1990), but its expression peaks after implantation. GATA-3 is a zinc finger transcription factor that is expressed in the placenta and in trophoblast cell lines which may regulate placental lactogen-1 transcription (Ng et al., 1994). However, GATA-3 is also expressed in other cell types (Ko et al., 1991).

Genetic evidence indicates that basic-helix-loop-helix (bHLH) transcription factors function as cell-lineage determinants in skeletal muscle development in mammals (*MyoD, myogenin, mrf-4, myf-5*) and in mesoderm and neuronal cell differentiation in Drosophila (*achaete-scute*) (Olson, 1990, 1992; Jan and Jan, 1993). Cell-specific bHLH factors have also

been identified in other mammalian cell types, where it is likely they function as regulators of lineage commitment and differentiation. This suggested to us that such factors might also regulate the trophoblast cell lineage. Members of the bHLH transcription factor family function as heterodimers, typically between cell-specific factors and the widely expressed E factors, such as E12 and E47, which are products of the *E2A* gene (Murre et al., 1991), HEB (Hu et al., 1992), and ITF2 (Henthorn et al., 1990). To take advantage of the ability of cell-specific factors to heterodimerize with E factors, we used the HLH domain of E47 as protein probe to identify two novel bHLH factors, by means of the so-called interaction cloning procedure (Blanar and Rutter, 1992). One of these factors, *Hxt*, is specifically expressed in trophoblast cells. We present evidence that *Hxt* regulates trophoblast differentiation and suggest a model for the role of bHLH factors in trophoblast development.

MATERIALS AND METHODS

Interaction cloning and sequencing of *Hxt* and *Hed*

Phage expression libraries prepared from day 13 ovine conceptuses (Kramer et al., 1994) or differentiated mouse embryoid bodies (Robbins et al., 1990) were screened by interaction cloning (Blanar and Rutter, 1992). A detailed description of the mutant shPan-1 (E47; German et al., 1991) protein probe is provided elsewhere (Blanar et al., 1995). Briefly, the bHLH domain of E47 was cloned into an *E. coli* expression vector downstream of a sequence encoding the recognition sequence for cAMP-dependent protein kinase (heart muscle kinase). Basic residues in the bHLH domain were mutated to block its ability to bind DNA. This protein was labeled with heart muscle kinase (Sigma) and γ-[^{32}P]ATP. The original *Hxt* isolate from the ovine conceptus library (λ8.1) did not contain a full-length *Hxt* cDNA. A 5′ end fragment was used to probe the original library by using standard procedures (Sambrook et al., 1989). cDNAs cloned into pBluescript (Stratagene) were sequenced by using the dideoxy chain termination method.

Plasmids

A cytomegalovirus promoter-*Hxt* expression vector (pCMV*Hxt*) was prepared by ligating an *Xho*I-*Eco*RI fragment of the ovine *Hxt* cDNA into pcDNA-I (Invitrogen). The plasmid pCMV*Mash2* was constructed by ligating the rat *Mash2* cDNA (Johnson et al., 1990) into pcDNA-I. Id-1 sense (pMXIds) and antisense (pMXId1as) expression vectors were obtained from J. Campisi (Lawrence Berkeley Laboratory). The Id-1 cDNA was also ligated into the *Bam*HI site downstream of a promoter regulated by operator binding sites for a tetracycline-regulated transactivator (Gossen and Bujard, 1992), to produce pTTO*Id-1*. The plasmid pβactin*lacZ* was produced by inserting the *lacZ* gene from pNASSβ (Clontech) downstream of the human β-actin promoter (from K. Sturm). The IFN-τ promoter-luciferase construct (pBTP-450Luc) was prepared by ligating a *Hin*dIII-*Nco*I IFN-τ promoter fragment (Cross and Roberts, 1991) upstream of the luciferase gene in the plasmid pFOXLuc (from M. German, University of California, San Francisco). Mouse placental lactogen-I promoter-CAT constructs (Shida et al., 1993) were obtained from D. Linzer (Northwestern University).

Interspecific backcross mapping

Interspecific backcross progeny were generated by mating (C57BL/6J×*M. spretus*)F_1 females and C57BL/6J males as described (Copeland and Jenkins, 1991). A total of 205 H2 mice were used to map the *Hed* and *Hxt* loci using standard procedures (Jenkins et al., 1982). The original probe was a 220 bp PCR-derived fragment overlapping the bHLH domain (reaction is described below) that was generated from mouse genomic DNA and was labeled with α-[^{32}P]dCTP by random prime labeling. Washing was done to a final stringency of 1.0× SSCP (Sambrook et al., 1989), 0.1% sodium dodecyl sulfate (SDS) at 65°C. Major fragments of 20.5 and 7.8 kb were detected in *Bam*HI-digested C57BL/6J DNA compared to 11.0 and 9.6 kb in *M. spretus* DNA. The 11.0 kb *Bam*HI *M. spretus*-specific fragment defined the *Hxt* locus to chromosome 11, whereas the 9.6 kb *Bam*HI *M. spretus*-specific fragment defined the *Hed* locus to chromosome 8. These assignments were confirmed with 3′ noncoding region probes for the two genes.

The probes and RFLPs for the loci linked to *Hed*, including scavenger receptor (*Scvr*), jun D (*Jund*) and mitochondrial uncoupling protein (*Ucp*), have been reported previously (Freeman et al., 1990; Kuo et al., 1991). However, the lipoprotein lipase (*Lpl*) locus has not been reported for this interspecific cross. The *Lpl* probe was a 1.4 kb *Eco*RI fragment of mouse cDNA that detected 5.0, 4.4, 3.6, 0.7 and 0.54 kb fragments in C57BL/6J and 10.5, 5.0, 2.8, 0.8, 0.62 and 0.54 kb fragments in *M. spretus Taq*I-digested DNA. The 10.5, 2.8, 0.8 and 0.62 fragments co-segregated and were followed in backcross mice. The probes and RFLPs for the loci linked to *Hxt*, including adrenergic receptor, alpha 1 (*Adra1*), granulocyte-macrophage colony-stimulating factor (*Csfgm*) and skeletal myosin heavy chain (*Myhs*) have been reported (Buchberg et al., 1989; McKenzie et al., 1993). Recombination distances were calculated (Green, 1981) by using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

RNA analysis

Total RNA was prepared from cells and tissues by extraction in a guanidine-acid phenol solution (Biotecx), according to the manufacturer's recommendations. RNAs were electrophoresed in MOPS/formaldehyde gels (Sambrook et al., 1989), capillary blotted with 10× SSC (Sambrook et al., 1989), and UV-crosslinked onto nylon membranes (Duralon, Stratagene). A blot containing poly(A)+ RNA from several mouse tissues was obtained from Clontech Laboratories (mouse MTN blot). cDNA probes for *Hxt*, *Id-1/2* (Sun et al., 1991), *Mash-2* (Johnson et al., 1990), *Pem* (Wilkinson et al., 1990) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were labeled with α-[^{32}P]dCTP by random prime-labeling. Blots were hybridized for 3 hours in Quick-Hyb solution (Stratagene) and were washed in 0.1× SSC, 0.1% SDS at 58-63°C.

The presence of *Hxt* mRNA at preimplantation stages of development was assessed by using a PCR-based assay of plasmid cDNA libraries prepared from mouse eggs, 2-cell embryos and blastocysts (Rothstein et al., 1992). Dilutions of library or vector were subjected to 30 cycles of PCR (94°C, 55°C, 72°C; 1 minute each) by using *Hxt*-specific primers (5′-GCGGAATTCTCCTATGGTCCAGACGCCAG-3′ and 5′-GGCGAATTCGCGCAGAGTCTTGATCTTGG-3′). The 220 bp product was assessed by agarose gel electrophoresis.

In situ hybridization

In situ hybridization was performed by whole-mount or on sections of implantation sites as described (Wilkinson and Nieto, 1993), except that whole-mount conceptuses were incubated in RIPA buffer instead of proteinase K (Burdsal et al., 1993). Digoxigenin- or [^{35}S]UTP-labeled RNA probes were made by preparing sense and antisense strand transcripts, with a kit from Boehringer Mannheim, from the 3′ end of mouse *Hxt* cDNA that had been cloned into pBluescript (Stratagene). Some conceptuses stained by whole-mount were embedded in ethyl methacrylate JB-4 medium (Polaron) and sectioned at 8 μm.

Injection, culture and β-galactosidase staining of mouse embryos

2-cell mouse embryos obtained at day 1.5 of gestation from matings of CF-1 females and CD-1 males (Charles River) were injected using

standard procedures (Hogan et al., 1986). Briefly, pβactin*lacZ* and either pcDNA-I or pCMV*Hxt* (20 ng/μl each in 5 mM Tris-HCl, pH 7.2, and 0.1 mM EDTA) were injected into the nucleus of a single, randomly chosen cell. Embryos that survived injection were cultured until the blastocyst stage in TE medium (Spindle, 1980). Because development of injected embryos was not affected by pcDNA-I, compared to embryos that were injected with pβactin*lacZ* alone (data not shown), the data were pooled. β-galactosidase staining was done either with X-gal after fixation (Vernet et al., 1993) or in live blastocysts with a fluoresceinated substrate, Imagene Green (Molecular Probes).

Culture and transfection of Rcho-1 cells

Rcho-1 cells were maintained as described previously (Faria and Soares, 1991; Shida et al., 1993; Hamlin et al., 1994). For the isolation of homogeneous differentiated subpopulations of cells, trypsin-labile cells were removed from the cultures as described in the legend to Fig. 6. Differentiation of adherent cells was accelerated by switching medium from NCTC-135 (Sigma) supplemented with 20% fetal bovine serum to that supplemented with 10% horse serum. Cells were transfected by using Lipofectamine (Gibco-BRL), because this liposome was more efficient than Lipofectin, as was previously used (Shida et al., 1993). Transient transfections performed to measure promoter activities were performed as described in the Figure Legends. Reporter gene activities were normalized to control for transfection efficiency by co-transfection of pRSVβGal, and all experiments were repeated at least twice. Luciferase activity was measured using a kit from Promega. CAT enzyme activity was measured by extraction of reaction products with ethyl acetate (Sambrook et al., 1989).

Stable transfectants were produced in which *Id-1* was expressed under the control of a tetracycline-regulated transactivator (Gossen and Bujard, 1992). The plasmid pUHD15-1, encoding the transactivator, was co-transfected in the presence of tetracycline (1 μg/ml) to inhibit the transactivator, with vector alone or pTTO*Id-1* and transfectants were selected with G418 and hygromycin B. After die-off was complete, transfected cells were pooled, amplified for two passages, plated in the absence of tetracycline and analyzed as described in the legend to Fig. 6.

RESULTS

Interaction cloning identifies bHLH factors expressed in the peri-implantation conceptus

To identify bHLH factors expressed in early placental cell lineages, we used the HLH domain of E47 to screen cDNA libraries from early conceptus tissues. Approximately 600,000 plaques of an ovine elongated blastocyst cDNA library (Kramer et al., 1994) were screened. Three positive plaques were identified and the phages were found to contain identical 1.3 kb inserts, based on digestion with several restriction endonucleases. Longer clones, up to 1.8 kb (clone λ81.5), were obtained by using a 0.5 kb *Bam*HI fragment as a probe to rescreen the original library. Analysis of ovine, mouse and human cell and tissue RNAs revealed an mRNA size of approximately 1.8 kb (see below) indicating that the longest clone was probably full-length. The cDNA insert in λ81.5 had a potential translation start site at position 220 and an open reading frame of 202 codons. The amino acid sequence was unique but was similar to that of members of the bHLH family within a 55 amino acid region that represents the bHLH domain. Based on its mRNA expression pattern (see below), the gene was called *Hxt* (for HLH transcription factor expressed in extraembryonic mesoderm and trophoblast).

Screening of a mouse embryoid body cDNA library (Robbins et al., 1990) with the E47 protein probe yielded single clones for *Id-1* and *Mash-1*, five clones of the mouse *Hxt*, and two clones of a novel gene that was called *Hed* (for HLH transcription factor expressed in embryo and deciduum). The mouse *Hxt* cDNA encoded a protein of 216 amino acids (Fig. 1A). Although Hed was unique, its deduced amino acid sequence was 87% identical within the bHLH domain to that of mouse Hxt (Fig. 1B). For this reason, all probes that were used in subsequent studies represented the 3′ noncoding regions of *Hxt* and *Hed*, because probes that encompass the bHLH domain of *Hxt* hybridized to both *Hxt* and *Hed*.

Hxt and *Hed* encode related bHLH factors with distinct chromosomal locations

The bHLH domains of the Hxt and Hed proteins most closely resemble (43-47% amino acid identity) that of HEN1, which is expressed in the central nervous system (Brown and Baer, 1994), Scl (Begley et al., 1991) and Lyl-1 (Kuo et al., 1991) which are expressed in hematopoietic cells, and Twist (Wolf et al., 1991) and Meso1 (Blanar et al., 1995) that are expressed in developing mesoderm. Similarity to other members of the bHLH family, such as E12/47, MyoD, myogenin, c-myc and Max, is restricted to those residues that represent the bHLH consensus (Garrell and Campuzano, 1991).

The chromosomal locations of the *Hxt* and *Hed* genes were determined by interspecific backcross analysis of matings of [(C57BL/6J×*Mus spretus*)F_1 × C57BL/6J] mice (Copeland and Jenkins, 1991). The results indicated that *Hxt* is present on mouse chromosome 11 (Fig. 1C). The most likely gene order and the fraction of the total number of mice exhibiting recombinant chromosomes among the total number of mice analyzed are: centromere-*Adra1 (*8/123)-*Csfgm* (4/131)-*Hxt* (4/133)-*Myhs*. Conversely, *Hed* is located in the central region of mouse chromosome 8. The most likely gene order and the ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci are: centromere-*Scvr* (15/153)-*Hed* (5/154)-*Lpl* (0/154)-*Jund* (6/119)-*Ucp*. No recombinants were detected between *Lpl* and *Jund* in 150 animals typed in common, suggesting that the two loci are within 2.0 cM of each other. Comparison of the interspecific maps of chromosomes 8 and 11 with composite linkage maps that report the locations of several uncloned mouse mutations (compiled by M. T. Davisson, T. H. Roderick, A. L. Hillyard and D. P. Doolittle and obtained from The Jackson Laboratory, Bar Harbor, ME) indicated that *Hed* and *Hxt* map to regions that lack mutations with expected phenotypes, given their expression patterns.

Hxt is expressed in the placenta, whereas *Hed* is expressed in the deciduum and embryo proper

To compare mRNA expression patterns, we prepared blots with RNA isolated from several mouse tissues. The mRNAs for *Hxt* and *Hed* were undetectable in RNA from undifferentiated embryonic stem cells but present in differentiated cells (data not shown), consistent with the cloning of *Hxt* and *Hed* from an embryoid body cDNA library. Neither *Hxt* nor *Hed* mRNAs were detected in undifferentiated F9 embryonal carcinoma cells or in F9 cells that had been treated with

A.

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  1   MNLVGRYAHH HHHHHSHPHH PMLHEPFLFG PASRCHQERP YFQSWLLSPA DAAPDFPAGG
 61   PPPTTAVAAA AYGPDARPSQ SPGRLEALGS RLPKRKGSGP KKERRRTESI NSAFAELREC
121   IPNVPADTKL PKIKTLRLAT SYIAYLMDVL AKDAQAGDPE GFKGELKKTD GGRESKRKRE
181   LPQQPEGFPP ASGPGEKRIK GRTGWPQKVW ALELNQ
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B.

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                                                                   % Identity
oHxt      RRKGSGPKKERRRTESINSAF.AELRECIPNVPADTKLSK.IKTLRLATSYIAYLMD       98
mHxt      KRKGSGPKKERRRTESINSAF.AELRECIPNVPADTKLPK.IKTLRLATSYIAYLMD      100

mHed      KRRGTANRKERRRTQSINSAF.AELRECIPNVPADTKLSK.IKTLRLATSYIAYLMD       87

mHEN1A    KYRTAHATRERIRVEAFNLAF.AELRKLLPTLPPDKKLSK.IEILRLAICYISYLNH       47
mSCL      VRRIFTNSRERWRQQNYNGAF.AELRKLIPTHPPDKKLSK.NEILRLAMKYINFLAK       43
mLYL-1    ARRVFTNSRERWRQQHVNGAF.AELRKLLPTHPPDRKLSK.NEVLRLAMKYIGFLVR       43
mTWIST    TQRVMANVRERQRTQSLNEAF.AALRKIIPTLPSDK.LSK.IQTLKLAARYIDFLYQ       38
Meso1     RQRQAANARERDRIQSVNTAFTA.LRTLIPTEPVDRKLSK.IETLRLASSYIAHLAN       45
            Basic        Helix I               Helix II
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C.

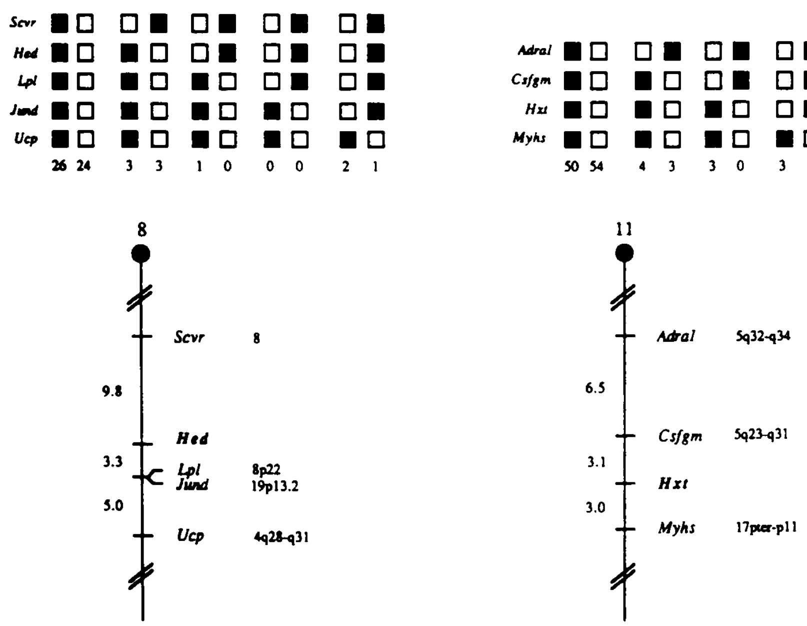

Fig. 1. Sequence and chromosomal mapping of the Hxt and Hed transcription factors. (A) The complete amino acid sequence deduced from the murine *Hxt* cDNA is shown (the bHLH domain is underlined). (B) Aligned amino acid sequences of the bHLH domains in the ovine (o) and murine (m) Hxt proteins, murine Hed protein, and the related murine bHLH factors HEN1A, Scl, Lyl-1, Twist and Meso1. Numbers indicate the per cent amino acid identity.
(C) Chromosomal locations of *Hed* and *Hxt* in the mouse genome were mapped by interspecific backcross analysis. The segregation patterns of these loci and flanking genes in backcross animals that were typed for all loci are shown above the chromosome maps. Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*)F_1 parent. The black boxes represent the presence of a C57BL/6J allele and the white boxes represent the presence of a *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. Partial chromosome linkage maps showing the location of *Hed* and *Hxt* in relation to linked genes are shown. Recombination distances between loci (in cM) are shown to the left of the chromosome and the positions of loci in human chromosomes, where known, are shown to the right. References for the map positions of loci mapped in human chromosomes can be obtained from the Genome Data Base, a computerized database of human linkage information maintained by the William H. Welsh Medical Library, Johns Hopkins University (Baltimore, MD).

retinoic acid to promote endoderm differentiation (Fig. 2A). *Hxt* and *Hed* had distinct mRNA expression patterns in mouse adult and embryonic tissues. *Hed* mRNA was detected as a single 2.5 kb band that was abundant in total RNA from decidual tissue, but that was detected only in poly(A)+ RNA in the adult, heart, liver and testis, and was undetectable in other tissues (Fig. 2). In situ hybridization with a *Hed*-specific probe failed to detect signal in either embryonic or extraembryonic tissue at day 7.5 (data not shown). However, *Hed* was expressed in the embryo proper at days 8, 9 and 10 (Fig. 3), although we have not localized its site of expression.

In contrast, the *Hxt*-specific probe detected an abundant 1.8 kb transcript in total RNA from day 7.5 conceptuses that contained both embryonic and placental tissue (Fig. 2A) but failed to detect *Hxt* mRNA in total RNA from other tissues. In poly(A)$^+$ RNA, however, 1.8 kb transcripts were identified in adult brain, muscle and testis (Fig. 2B). Consistent with expression in brain, we have detected *Hxt* mRNA in PC12 cells (data not shown). In addition, weakly hybridizing transcripts at 2.4 and 3.6 kb were detected in all samples. Their significance is unknown.

To identify the site of *Hxt* mRNA expression during embryonic development, we separated postimplantation conceptuses (day 7.5 to 10.5) into embryonic and placental tissues. Major *Hxt* expression was restricted to the placenta at all stages examined, although faint signals were present in the embryo (Fig. 3A). At day 9.5, we removed the chorioallantois from the trophoblast cell-rich spongiotrophoblast layer. *Hxt* mRNA was abundant in trophoblast, but was not detected in the chorioallantois (data not shown). Plasmid cDNA libraries made from mouse conceptuses at preimplantation stages of development (Rothstein et al., 1992) were used in polymerase chain reaction (PCR) analyses to determine when *Hxt* mRNA is first expressed. Although not quantitative, the analysis indicated that *Hxt* mRNA is present as a maternal transcript in the egg as well as during cleavage development before blastocyst

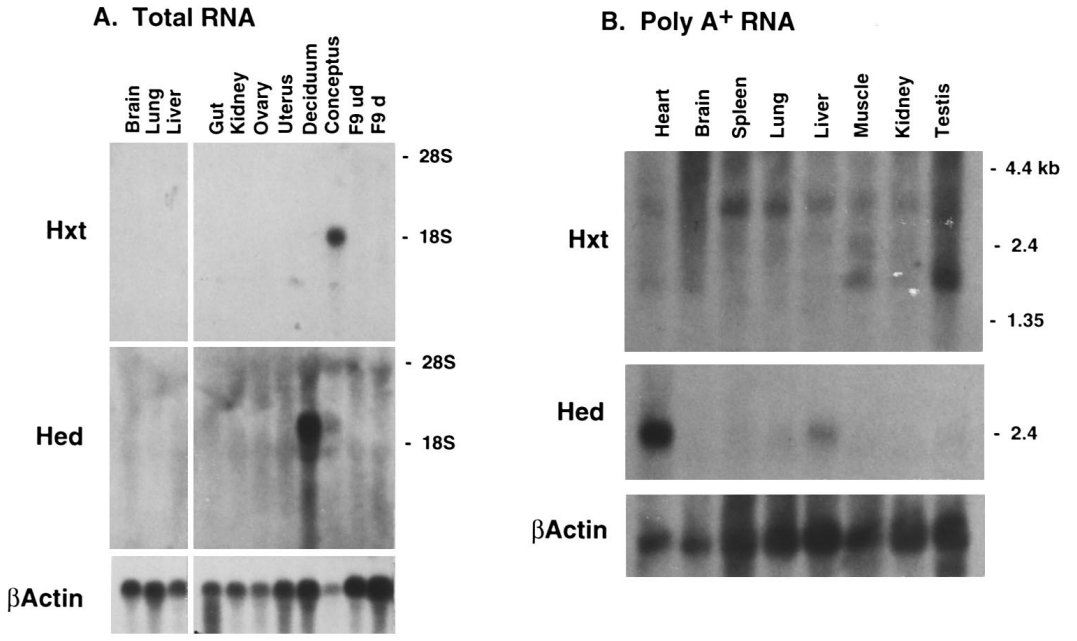

Fig. 2. Expression of *Hxt* and *Hed* mRNAs in mouse tissues. *Hxt*- and *Hed*-specific cDNA probes were used to probe total RNA (10 μg) (A) or poly(A)$^+$ RNA (2 μg) (B) RNAs were derived from mouse tissues as indicated, or from undifferentiated F9 embryonal carcinoma cells (F9 ud) or F9 cells induced to differentiate into endoderm-like cells by 4 days treatment with 10^{-8} M retinoic acid (F9 d). All tissues were from adult mice, except for decidual tissue and whole conceptus (embryo + placenta), which were obtained at day 7.5 of pregnancy. The *Hxt* mRNA appears at approximately 1.8 kb and the *Hed* mRNA appears at approximately 2.5 kb.

formation (Fig. 3B). Because placental structure differs widely among mammals (Cross et al., 1994), we tested whether *Hxt* is produced in the placenta in other species. *Hxt* mRNA was abundant in preimplantation bovine conceptuses (day 18) as well as in trophoblast-rich, cotyledon throughout pregnancy (Fig. 3C). *Hxt* mRNA was also detected in human (JEG-3 and JAR; data not shown) and rat (Rcho-1; see below) trophoblast cell lines.

Hxt is expressed in trophoblast and transiently in mesoderm of the placenta

To identify the site of *Hxt* expression in peri-implantation conceptuses, we cultured mouse blastocysts for 3 days in serum to promote attachment and outgrowth of trophoblast. In situ hybridization analysis localized *Hxt* mRNA exclusively to trophoblast cells in the outgrowth (E. Newman, and Z. W., unpublished data). We analyzed postimplantation stages of development for *Hxt* expression by whole-mount in situ hybridization. At day 7.5 of gestation, the *Hxt* antisense probe hybridized strongly to trophoblast cells in the ectoplacental cone (Fig. 4). Sections of these embryos revealed that *Hxt* mRNA was abundant in the more differentiated cells on the outside of the ectoplacental cone. Hybridization was not detected in chorionic ectoderm, cells that derive from polar trophectoderm (trophoblast) cells (Cross et al., 1994). Because the parietal yolk sac (including trophoblast giant cells) is tightly adherent

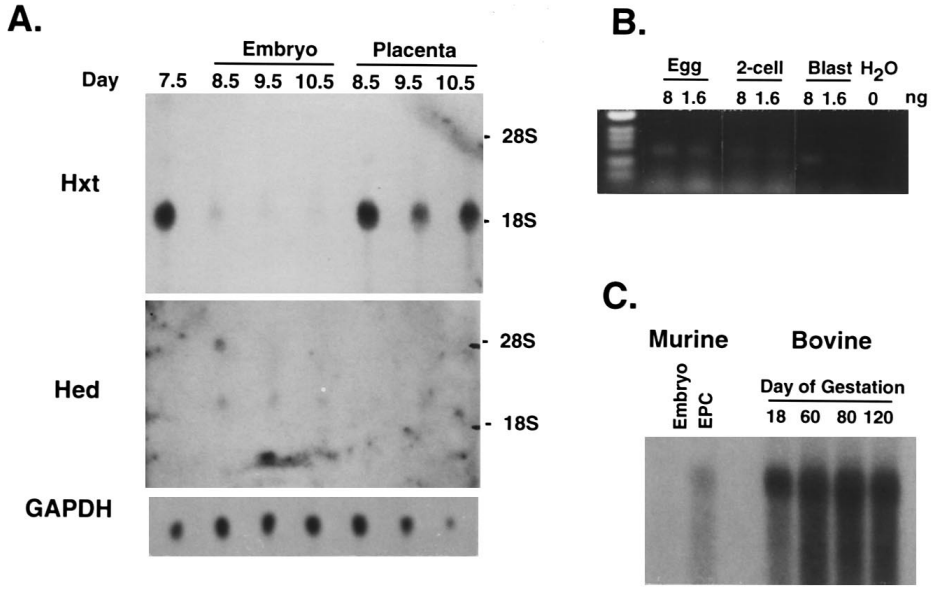

Fig. 3. *Hxt* mRNA expression in the placenta. (A) RNA blot of separated embryo and placental tissues. Total RNA (10 μg) prepared from whole day 7.5 mouse conceptus or separated embryo and placental fractions (including the ectoplacental cone, amnion and chorioallantois) from days 8.5, 9.5 and 10.5 of pregnancy was used to prepare an RNA blot that was sequentially probed with *Hxt*, *Hed*, and *GAPDH* probes. (B) PCR analysis for the presence of *Hxt* in mouse egg, 2-cell embryo and blastocyst cDNA libraries (Rothstein et al., 1992). Decreasing amounts of plasmid DNA were subjected to PCR. A *Hxt*-specific 220 bp product was observed when using plasmid aliquots from all libraries but not in the blank reaction. (C) RNA blot analysis of *Hxt* expression the placenta of ruminants. Bovine day 16, preimplantation conceptus and cotyledonary total RNA (10 μg) from days 60, 80 and 120 of gestation were probed with the ovine *Hxt* cDNA. Mouse embryo and ectoplacental cone tissue (day 9.5) were used a negative and positive controls, respectively.

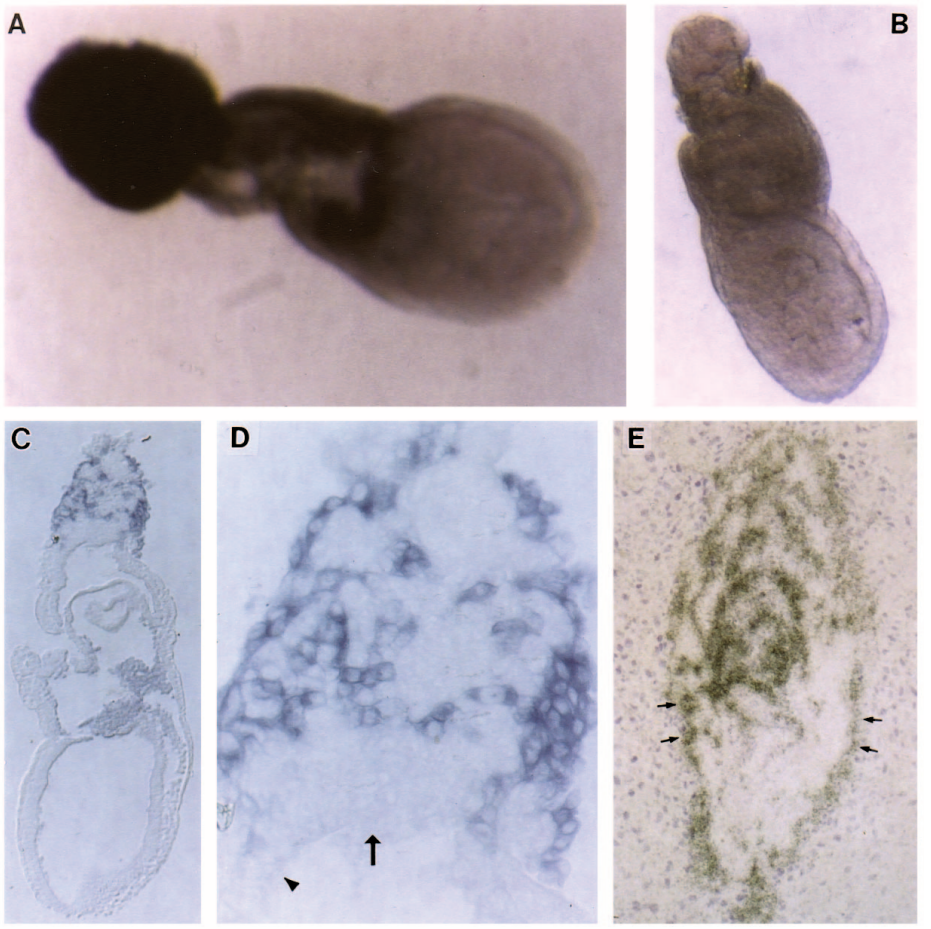

Fig. 4. In situ hybridization of *Hxt* mRNA in day 7.5 mouse conceptuses. (A-D) Whole-mount in situ hybridization was performed with antisense (A) or sense (B) strand *Hxt*– probes. Embryos were then sectioned (C) to highlight staining of extraembryonic mesoderm cells and trophoblast cells in the ectoplacental cone (D). Note that staining was not detected in the chorionic ectoderm (D, arrowhead) and the core of the ectoplacental cone (D, arrow). (E) In situ hybridization performed with an antisense Hxt probe on sections of implanted day 7 mouse conceptuses. Silver grains were abundant over the ectoplacental cone and trophoblast giant cells (arrows).

to the decidua, we also performed in situ hybridization on sections of day 7.5 conceptuses left within decidua. This analysis demonstrated that *Hxt* RNA was abundant in trophoblast giant cells in the parietal yolk sac (Fig. 4).

We also observed weaker *Hxt* hybridization in extraembryonic mesoderm cells that form the allantois and line the amnion and chorionic ectoderm (Fig. 4). Hybridization was observed over mesoderm exclusively at the posterior end of the primitive streak. Cell lineage analysis showed that cells in this region contribute exclusively to extraembryonic structures (Lawson et al., 1991). In contrast to trophoblast, expression of *Hxt* in extraembryonic mesoderm was transient; *Hxt* transcripts were not detected in the chorioallantois at day 9.5 either by in situ hybridization or by RNA blot analysis (data not shown). *Hxt*-specific hybridization was not detected in the embryo either at day 7.5 (Fig. 4) or at day 9 by whole mount (data not shown), consistent with RNA blot analysis (Fig. 3A).

Hxt limits differentiation of blastomeres to trophectoderm in preimplantation mouse embryos

Since *Hxt* expression was abundant in trophoblast, we tested whether Hxt could induce uncommitted cells into the trophoblast lineage. Embryonic stem cells were transfected with a *Hxt* expression vector (pCMV*Hxt*) but they grew extremely slowly and could not be cloned (data not shown). This suggested that *Hxt* overexpression arrested the growth of embryonic stem cells. Because *Hxt* is expressed during cleavage-stage development, albeit at low levels, and is down-regulated in the ICM after blastocyst formation, we tested whether continuous expression of *Hxt* would limit differentiation of blastomeres. A *Hxt* expression plasmid was co-injected with a construct containing the β-actin promoter fused to the *lacZ* gene into the nucleus of a single cell in mouse 2-cell embryos. The developmental fate of the injected cell was followed by histochemical staining. Embryos cultured to form blastocyst outgrowths were found to no longer express β-galactosidase activity and, therefore, embryos were assessed at the blastocyst stage. Cells injected with a control vector contributed equally to ICM and trophectoderm in blastocysts (Fig. 5; Table 1), as expected. In contrast, cells injected with the *Hxt* expression vector were biased towards the trophectoderm (Fig. 5; Table 1). In several cases, these cells were larger than normal, suggesting that they had differentiated earlier during cleavage stage development, or that their division rate was slower than that of their uninjected cousins. Consistent with these possibilities, *Hxt*-injected blastocysts had significantly fewer cells than those injected with control vector (Table 1). These large cells had intact nuclei based on DNA staining (data not shown) and were considered trophoblast based on their flattened morphology and integration into the trophoblast layer. In contrast, non-viable cells are excluded from the blastocyst during development. *Hxt*-injected embryos also formed significantly more trophoblastic vesicles which resembled blastocysts but lacked an ICM (based on nuclear staining). Such structures can form by premature differentiation or removal of cells, resulting in an embryo in which all of the blastomeres

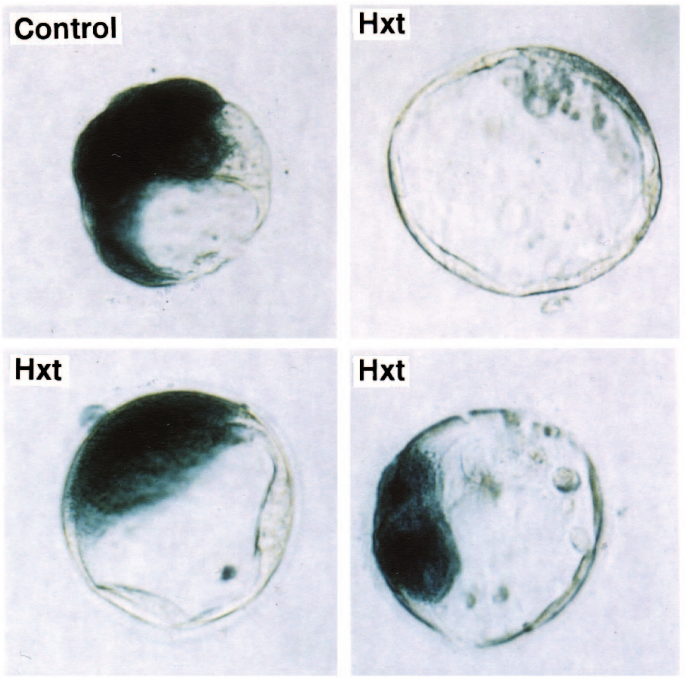

Fig. 5. Effect of *Hxt* overexpression in cleavage-stage mouse embryos. The nucleus of a single cell in 2-cell embryos was injected with pCMV*Hxt* or vector alone, and pβactin*lacZ*. Embryos were cultured for 2-3 days to the blastocyst stage and then stained for β-galactosidase activity, and visualized with X-gal or fluoresceinated substrate. The figure shows control or *Hxt*-injected embryos visualized folowing X-gal staining.

are used to produce trophectoderm, leaving insufficient cells to produce an ICM (Spindle, 1982). Trophoblastic vesicles resulting from *Hxt* injection had approximately one-half the number of cells found in blastocysts (Table 1). These experiments suggested that Hxt arrest cell growth and has trophoblast-inducing activity.

Hxt is induced in Rcho-1 trophoblast cells during giant cell transformation

Expression of *Hxt* in trophoblast giant cells and the apparent effect of *Hxt* overexpression on arresting embryonic stem cell and blastomere growth suggested that Hxt might regulate trophoblast giant cell transformation. This hypothesis was tested in Rcho-1 (rat choriocarcinoma) cells which differentiate in vitro into cells that are typical of trophoblast giant cells in vivo (Faria and Soares, 1991; Hamlin et al., 1994). However, the Rcho-1 culture system initially described produces mixed populations with both differentiated giant cells and proliferating stem cells (Faria and Soares, 1991; Shida et al., 1993; Hamlin et al., 1994). In such mixed cultures, most of the cells are small and angular in shape, rapidly growing and easily removed by brief trypsinization. After plating these trypsin-sensitive cells for 12-24 hours, we found that a small fraction (about 5%) of them differentiated into larger cells that were trypsin-resistant. This change in cell morphology and adhesion was an early event in Rcho-1 differentiation and was irreversible; the adherent cells stopped proliferating, underwent morphological giant cell transformation and induced the expression of trophoblast giant cell markers (data not shown).

Table 1. Distribution of cells between the ICM and trophectoderm at the blastocyst stage after injection with control or *Hxt*-expression vector

	% of embryos	
	pcDNA-I	pCMV*Hxt*
(A) Blastocysts		
ICM	17	0
ICM + trophectoderm	42	3
Trophectoderm	12	28
(B) Trophoblastic vesicles		
Trophectoderm	29	69
n=	24	29

*A single blastomere in 2-cell embryos was co-injected with pβactin*lacZ* and control or *Hxt*-expression plasmid. 50-70 embryos were injected per group. Embryos that developed into blastocyst structures were stained for β-galactosidase activity and the location of positive cells was noted. Cell numbers were assessed by staining nuclei with Hoechst 33258 and were significantly lower ($P<0.05$) in blastocysts (29.6±2.1) and trophoblastic vesicles (17.2±1.2) derived from *Hxt*-injected embryos than in those derived from control blastocysts (36.7±2.9). The experiment was repeated four times with similar results.

Based on this behavior, we produced purified populations of early differentiated cells devoid of stem cells. Rcho-1 stem cells (trypsin-sensitive) were plated for 2 days, then trypsinized to select for early differentiated cells (day 0), which were cultured further to promote differentiation. To determine whether *Hxt* expression is regulated during Rcho-1 differentiation, we isolated RNA from stem cells and purified adherent cells allowed to differentiate for various times (day of trypsinization = day 0). *Hxt* mRNA levels were equivalent in stem cells and adherent cells on the day of trypsinization, but increased during differentiation, reaching a peak at day 4 that was approximately 5 times greater than the levels in stem cells (Fig. 6).

The activity of bHLH transcription factors is regulated by the negative HLH factors Id-1 and Id-2 (Sun et al., 1991). Because they lack basic domains, Id-1 and Id-2 cannot bind DNA but inhibit the DNA-binding activity of those bHLH factors that do bind DNA. *Id-1* and *Id-2* are expressed at higher levels in growing cells and are down-regulated during differentiation (Sun et al., 1991; Barone et al., 1994). This is also true of trophoblast cells, because both *Id-1* and *Id-2* mRNAs were abundant in Rcho-1 stem cells, whereas they were essentially undetectable in early differentiated cells (Fig. 6). During differentiation, *Id-1* and *Id-2* mRNA levels remained low.

We also compared the expression of *Hxt* with that of *Mash-2* (Johnson et al., 1990) and *Pem* (Wilkinson et al., 1990). *Mash-2* was originally identified in proneuronal cells, but is also expressed in proliferative mouse trophoblast cells (Guillemot et al., 1994). Two *Mash-2* transcripts were detectable in Rcho-1 stem cells, which decreased during early differentiation, but levels were similar at later stages of differentiation (Fig. 6). Levels of *Pem* mRNA were slightly higher in Rcho-1 stem cells compared to differentiated giant cells (Fig. 6). Therefore, *Hxt* is the only factor that is preferentially expressed in trophoblast giant cells.

Hxt promotes and *Id-1* inhibits Rcho-1 trophoblast cell differentiation

The expression patterns of *Id-1/2* and *Hxt* suggested that they

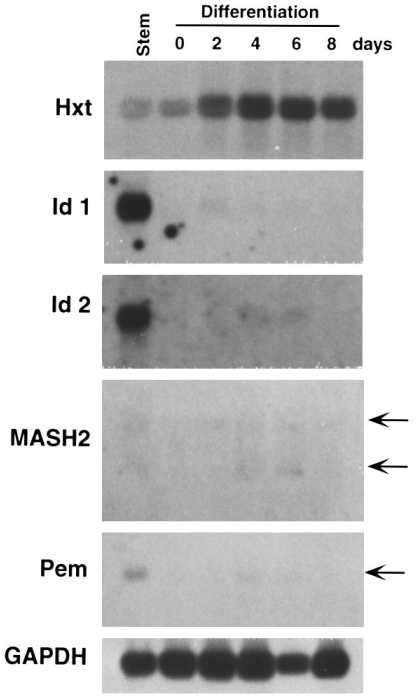

Fig. 6. Expression of transcription factors during Rcho-1 cell differentiation. RNA blot analysis was performed on total RNA (10 μg) isolated from Rcho-1 stem cells, or adherent cells allowed to differentiate for up to 8 days after removal of stem cells (day 0). Trypsin-labile Rcho-1 stem cells were plated and grown in 20% fetal bovine serum for 2 days until the cells reached confluence. The majority of cells in these cultures were small, angular cells typical of proliferative stem cells. After brief trypsinization (0.25% trypsin, 2 minutes), these stem cells were washed away, leaving small islands of larger, flat cells (day 0). During continued culture in 10% horse serum, the cells transformed into trophoblast giant cells. RNAs were probed with cDNA probes for *Hxt, Id-1, Id-2, Mash-2, Pem* and *GAPDH* (control).

might play opposite roles in regulating trophoblast differentiation. To test this hypothesis, we overexpressed the HLH factors, and counted the number of Rcho-1 cells that underwent differentiation as assessed by trypsin sensitivity. *Id-1* dramatically reduced the differentiation of stably transfected Rcho-1 cells (Fig. 7A), indicating that Id factors repress early events in trophoblast development. However, similar to the results with embryonic stem cells, Rcho-1 cells stably transfected with *Hxt* grew slowly and morphologically appeared differentiated (data not shown). To overcome this problem, Rcho-1 stem cells were electroporated with an *Hxt* expression vector and the number of cells differentiating in 24 hours were counted. In control transfections, 5.9±0.5% of Rcho-1 stem cells differentiated into trypsin-resistant cells (Fig. 7B). Whereas overexpression of *Mash-2* decreased or had no effect on differentiation, overexpression of *Hxt* in Rcho-1 stem cells for 24 hours significantly increased the number of adherent cells ($P<0.05$; Fig. 7B). Cells transfected in parallel with pβactin*lacZ* and stained for β-galactosidase activity indicated that the transfection efficiency in these experiments was 2.9%. Based on this transfection efficiency, we estimate that the increase in the number of cells differentiating following transfection with *Hxt* can be acounted for if 99±24% of transfected cells had differentiated.

Molecular markers that are specific to early stages of trophoblast differentiation do not exist in the mouse. Trophoblast interferon (IFN-τ) is expressed exclusively in trophoblast cells, but only in sheep and cow conceptuses (Roberts et al., 1992), and is probably the earliest trophoblast-specific gene identified to date. Therefore, to confirm that *Hxt* is able to promote early trophoblast differentiation, we transfected Rcho-1 cells with a luciferase gene controlled by a bovine IFN-τ promoter (Cross and Roberts, 1991). IFN-τ promoter activity was stimulated two- to three-fold by co-transfection of *Hxt* (control: 5,603±1,113 light units; *Hxt*: 13,962±877 light units; $P<0.05$). *Hxt* overexpression had a similar effect on the human chorionic gonadotrophin-α promoter, whereas it had no effect on the activities of the mouse syndecan-1 or human β-actin promoters (data not shown). The mouse placental lactogen-I (PL-I) gene is specifically expressed in trophoblast giant cells and the promoter is more active in diffentiated compared to proliferating Rcho-1 cells (Fig. 7D; Shida et al., 1993; Ng et al., 1994). *Hxt* overexpression in Rcho-1 stem cells stimulated the 'full-length' PL-I promoter (to position −2700 relative to the transcription start site) two- to three-fold in Rcho-1 stem cells ($P<0.05$), whereas there was no significant effect in differentiated cells (Fig. 7D). When the promoter was truncated to position −188, *Hxt* overexpression had no effect (Fig. 7D). Together these data suggest that *Hxt* promotes trophoblast cell differentiation. In contrast to the effect of *Hxt*, PL-I promoter activity was reduced by overexpression of Id-1 in transfected Rcho-1 stem cells, whereas the expression of antisense Id-1 transcripts increased promoter activity (Fig. 7C).

DISCUSSION

Hxt encodes a putative regulator of trophoblast cell development

Because bHLH transcription factors regulate the commitment and differentiation of other cell lineages (Olson, 1990, 1992; Jan and Jan, 1993), we sought to determine whether bHLH factors control trophoblast development. In the present study, we identified a gene encoding a bHLH transcription factor, *Hxt*, and we propose that it is an important regulator of placental development for several reasons. *Hxt* is expressed at high levels in trophoblast cells of the placenta. *Hxt* mRNA was detected in only a few adult tissues (brain, muscle and testis) but at much lower levels than in trophoblast. *Hxt* mRNA and protein were detected in trophoblast from the earliest stages of development. Finally, *Hxt* overexpression, both in committed but still undifferentiated trophoblast cells (Rcho-1) and in uncommitted cells in cleavage-stage mouse embryos, induced trophoblast cell differentiation. Together these data imply that Hxt may regulate both trophoblast cell commitment and subsequent differentiation. Whether it is essential for these functions in vivo requires formal testing. In addition to its specific pattern of expression in the mouse, *Hxt* mRNA was also detected in trophoblast cells from humans and rats (choriocarcinoma cell lines) and in ruminant species in which *Hxt* mRNA is abundant in both preimplantation stage conceptuses and in cotyledonary (placental) tissue from all stages of gestation. Because the placenta has vastly different forms among mammals (Cross et al., 1994), this finding is significant and suggests that Hxt may regulate trophoblast development in all mammals.

Hxt and *Hed* define a new family of bHLH transcription factors

The similarity of Hxt to other proteins was limited to members of the bHLH family and strictly within the bHLH domain. Amino acid sequence identity within this region was 43-47%

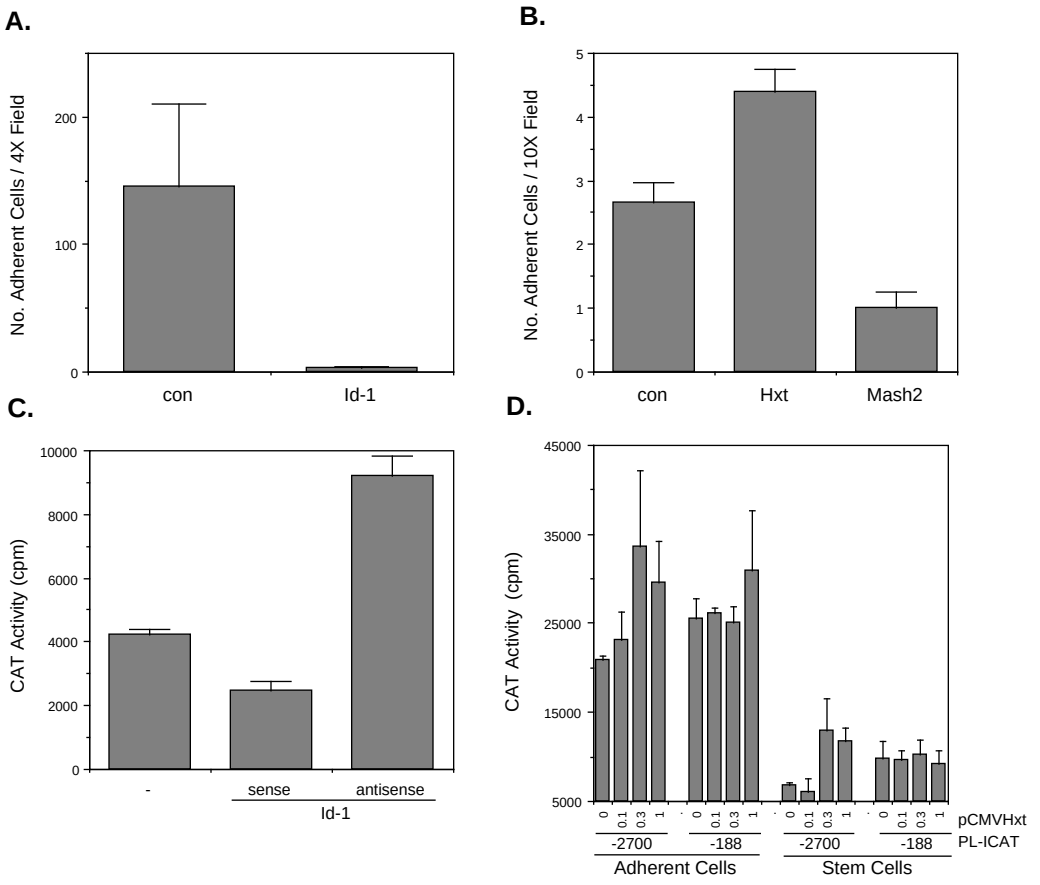

Fig. 7. Effect of *Id-1* and *Hxt* on Rcho-1 cell differentiation and PL-I promoter activity (Bars represent the mean ± s.e.m). (A) *Id-1* reduces Rcho-1 cell differentiation. Rcho-1 cells were transfected with a tetracycline-regulated expression construct for *Id-1*, or with a control construct expressing β-galactosidase. Stably transfected pools of trypsin-labile cells were plated at low density under inducing conditions and grown for 2 days until the cells reached confluence. Stem cells were removed by trypsinization and the adherent cells were counted. (B) *Hxt* induces Rcho-1 cell differentiation. Rcho-1 stem cells were electroporated with *Hxt* (pCMV*Hxt*), control (pcDNA-I) or *Mash-2* (pCMV*Mash2*) expression vectors. After 24 hours, stem cells were removed by trypsinization and the adherent cells were counted. See text for details. (C) *Id-1* regulates PL-I promoter activity. Rcho-1 stem cells were plated for 8 hours then transfected with pPL-I/-2700CAT (2 μg) in the absence (–) or presence of Id-1 sense or antisense expression vectors (300 ng) and harvested for CAT assay after 3 days. (D) *Hxt* stimulates PL-I promoter activity. Rcho-1 cells were plated for 2 days and then stem cells were separated from differentiated adherent cells by trypsinization and plated for 8 hours. The two cell populations were transfected with PL-I promoter/CAT constructs (2 μg) with control vector or increasing amounts of pCMV*Hxt* (0, 0.1, 0.3 or 1 μg). Cells were harvested for CAT assay 3 days later.

for the factors Hen1A (Brown and Baer, 1994), SCL (Begley et al., 1991), Lyl-1 (Kuo et al., 1991), Twist (Wolf et al., 1991) and Meso1 (Blanar et al., 1995). However, the bHLH domain in the Hxt protein was 87% identical to that in the Hed protein, a factor whose cDNA was cloned from an embryoid body library. Although the Hxt and Hed proteins are related, there is an interesting structural difference between them. Of the sequence mismatches between Hxt and Hed in the bHLH domain, five are clustered within the basic domain, although four of these differences are conservative substitutions. However, an asparagine residue that is highly conserved among bHLH factors is present only in the Hed protein and has been replaced by a proline in the Hxt protein basic domain (Pro104). In crystal structures of MyoD (Ma et al., 1994) and E47 (Ellenberger et al., 1994), this asparagine contacts the thymidine base within the consensus recognition site for these factors, the so-called E-box element (CANNTG). The asparagine-to-proline substitution also occurs in the *Drosophila* bHLH factors hairy (h) and enhancer-of-split [E(spl)] (Garrell and Campuzano, 1991). Although, E(spl) does not bind to consensus E-box elements, it binds to a related element called an N-box (CACNAG; Sasai et al., 1992; Tietze et al., 1992). These data suggest that the Hxt and Hed proteins have different DNA-binding specificities.

Role of several bHLH factors in trophoblast cell development

Trophoblast cell development includes a commitment step at the morula-to-blastocyst transition, and a balance between proliferation and trophoblast giant cell differentiation in later development (Cross et al., 1994). During trophoblast cell commitment, positional cues dictate that blastomeres found on the outside of the morula become trophoblast, whereas cells on the inside of the structure remain undifferentiated as part of the ICM. Trophoblast cells adjacent to the ICM (polar trophectoderm) continue to proliferate, presumably in response to a mitogenic signal produced by the ICM, whereas the trophoblast away from the ICM (mural trophectoderm) undergoes giant cell transformation. Similarly, during postimplantation development a fraction of cells derived from the polar trophectoderm differentiate into giant cells.

Mash-2 (Guillemot et al., 1994) and *Id-1* (Evans and O'Brien, 1993) mRNAs are expressed at the time of trophoblast cell commitment, similar to *Hxt*. What role these factors play in trophoblast cell commitment at the blastocyst stage is unclear, although trophoblast cells form normally in conceptuses that lack the *Mash-2* gene (Guillemot et al., 1994). However, differentiation of proliferative trophoblast cells into giant cells is accompanied by down-regulation of *Mash-2*,

based on its expression pattern in vivo (Guillemot et al., 1994), and of *Id-1*, based on its expression pattern in Rcho-1 cells. Both factors are probably important for maintaining the proliferative pool of cells, because this population is lost early in conceptuses homozygous for a targeted mutation in the *Mash-2* gene (Guillemot et al., 1994). Similarly, sustained expression of *Id-1* reduces the ability of Rcho-1 cells to differentiate. This is consistent with the expression pattern and activity of the Id factors in a variety of other cell systems (Sun et al., 1991; Barone et al., 1994). In contrast to *Mash-2* and *Id-1*, *Hxt* expression persists and increases in trophoblast during giant cell transformation. This pattern, and the ability of *Hxt* to promote trophoblast differentiation, is consistent with the hypothesis that *Hxt* regulates trophoblast giant cell transformation. The ability of *Hxt* to activate trophoblast-specific gene transcription suggests that these genes are downstream of *Hxt* in a developmental pathway. Future work is focussed on determining whether transcription of these genes is regulated directly by *Hxt* or whether Hxt affects their transcription indirectly by controlling other aspects of a differentiation program. The former possibility is reasonable since the region of the PL-I promoter upstream of position −188 that was responsive to activation by *Hxt* contains several sequences which resemble both E- and N-box elements (J. C. C., unpublished observations).

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