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
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-[32P]ATP. The original Hxt isolate from the ovine conceptus library (K8.1) 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 plBluescript (Stratagene) were sequenced by using the dideoxy chain termination method.

Plasmids

A cytomegalovirus promoter-Hxt expression vector (pCMVHxt) was prepared by ligating an XhoI-EcoRI fragment of the ovine Hxt cdNA into pcDNA-1 (Invitrogen). The plasmid pCMVMash2 was constructed by ligating the rat Mash2 cDNA (Johnson et al., 1990) into pcDNA-1. Id-1 sense (pMXId1s) and antisense (pMXId1as) expression vectors were obtained from J. Campisi (Lawrence Berkeley Laboratories). The Id-1 cDNA was also ligated into the pCMV-β-actin promoter-β-lactamase (pBluescript (Stratagene)) were sequenced by using the dideoxy chain termination method.

Interspecific backcross mapping

Interspecific backcross mapping was performed by mating (C57BL/6j×M. spretus)F1 females and C57BL/6j males as described (Copeland and Jenkins, 1991). A total of 205 F2 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 over-lapping the bHLH domain (reaction is described below) that was generated from mouse genomic DNA and was labeled with α-[32P]dCTP by random prime labeling. Washing was done to a final stringency of 1.0x SSC (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 BamHI-digested C57BL/6j DNA compared to 11.0 and 9.6 kb in M. spretus DNA. The 11.0 kb BamHI M. spretus-specific fragment defined the Hxt locus to chromosome 11, whereas the 9.6 kb BamHI 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 (Scv,r), 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 EcoRI 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 Tagl-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 (Gsfgm) 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 MADDNESS. 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 according to the manufacturer’s recommendations. RNAs were electrophoresed in guanidine-acid phenol solution (Biotecx), according to the manufacturer’s recommendations.

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, pβactinlacZ and either pCDNA-I or pCMVHxt (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 pβactinlacZ 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 fluoresceinabeled 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 pTTOld-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 BamHI 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 Mash1, 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 decidua). 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), ScI (Begley et al., 1991) and Ly1-1 (Kuo et al., 1991) which are expressed in hematopoietic cells, and Twist (Wolf et al., 1991) and Mes01 (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 x Mus spretus]F1 x 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)-Myh5. 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 unclooned 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
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, 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...
2517Hxt is a bHLH regulator of trophoblast
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 choriocytic cells that derive from polar trophectoderm (trophoblast) cells (Cross et al., 1994). Because the parietal yolk sac (including trophoblast giant cells) is tightly adherent 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.

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**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 decidua 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.

**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 chorionicamnion) 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 in ruminants. Bovine day 16, preimplantation...
to the decidua, we also performed in situ hybridization on sections of day 7.5 conceptuses left within decidua. This analysis demonstrated that \textit{Hxt} RNA was abundant in trophoblast giant cells in the parietal yolk sac (Fig. 4). We also observed weaker \textit{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 \textit{Hxt} in extraembryonic mesoderm was transient; \textit{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). \textit{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).

\textbf{\textit{Hxt} limits differentiation of blastomeres to trophoblast in preimplantation mouse embryos}

Since \textit{Hxt} expression was abundant in trophoblast, we tested whether \textit{Hxt} could induce uncommitted cells into the trophoblast lineage. Embryonic stem cells were transfected with a \textit{Hxt} expression vector (pCMV\textit{Hxt}) but they grew extremely slowly and could not be cloned (data not shown). This suggested that \textit{Hxt} overexpression arrested the growth of embryonic stem cells. Because \textit{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 \textit{Hxt} would limit differentiation of blastomeres. A \textit{Hxt} expression plasmid was co-injected with a construct containing the β-actin promoter fused to the \textit{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 trophoblast in blastocysts (Fig. 5; Table 1), as expected. In contrast, cells injected with the \textit{Hxt} expression vector were biased towards the trophoblast (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, \textit{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. \textit{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

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{In situ hybridization of \textit{Hxt} mRNA in day 7.5 mouse conceptuses. (A-D) Whole-mount in situ hybridization was performed with antisense (A) or sense (B) strand \textit{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 \textit{Hxt} probe on sections of implanted day 7 mouse conceptuses. Silver grains were abundant over the ectoplacental cone and trophoblast giant cells (arrows).}
\end{figure}
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).

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 proneural cells, but is also expressed in proliferative mouse trophoblast cells (Guillermot 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 Id-1 inhibits Rcho-1 trophoblast cell differentiation**

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

**Fig. 5. Effect of Hxt overexpression in cleavage-stage mouse embryos.** The nucleus of a single cell in 2-cell embryos was injected with pCMVHxt or vector alone, and βactinlacZ. 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 following X-gal staining.

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

<table>
<thead>
<tr>
<th>% of embryos</th>
<th>pcDNA-I</th>
<th>pCMVHxt</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Blastocysts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICM</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>ICM + trophectoderm</td>
<td>42</td>
<td>3</td>
</tr>
<tr>
<td>Trophoderm</td>
<td>12</td>
<td>28</td>
</tr>
<tr>
<td>(B) Trophoblastic vesicles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trophoderm</td>
<td>29</td>
<td>69</td>
</tr>
</tbody>
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n= 24, 29

* A single blastomere in 2-cell embryos was co-injected with βactinlacZ 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.
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 48 hours significantly increased the number of adherent cells (P<0.05; Fig. 7B). Cells transfected in parallel with β-galactosidase 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 accounted 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-α) 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-α promoter (Cross and Roberts, 1991). IFN-α 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 differentiated 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%.
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 Mesol (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[split]) (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.

**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 (pCMVHxt), control (pcDNA-I) or Mash-2 (pCMVMash2) 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 pCMVHxt (0, 0.1, 0.3 or 1 μg). Cells were harvested for CAT assay 3 days later.

**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 trophoblast) continue to proliferate, presumably in response to a mitogenic signal produced by the ICM, whereas the trophoblast away from the ICM (mural trophoblast) undergoes giant cell transformation. Similarly, during postimplantation development a fraction of cells derived from the polar trophoblast develop 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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Hxt is a bHLH regulator of trophoblast


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