Isolation and characterization of a novel trophoblast-specific cDNA in the mouse

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A novel trophoblast-specific cDNA has been isolated by differential screening of a 13.5-day mouse placental cDNA library. The 755-bp cDNA, which is almost full length, encodes a polypeptide of 124 amino acids with an 18-amino-acid hydrophobic leader sequence. No significant homology with any known gene or protein has been detected. Northern blot analysis has shown that the gene is expressed abundantly in the placenta and is not detected in any other tissues. In situ hybridization revealed that the gene is expressed first in a subgroup of ectoplacental cone cells at 7.5 days of development and its later expression is confined to the spongiotrophoblast cells of the mature placenta. The role of the gene is not currently known, but the presence of a signal peptide suggests that it may be a secreted protein.

[*Key Words:* Trophoblast; placenta-specific DNA; in situ hybridization; mouse]

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The trophoblast cell lineage of the developing mammalian embryo (Fig. 1) produces tissues that are vital to the survival of the fetus. These tissues provide maintenance, nourishment, and protection of the fetus during development. The trophoblast is the first lineage to differentiate in the mammalian embryo, and is one of the most well-characterized cell lineages of mammalian development (Rossant 1986). It arises from the trophoctoderm of the blastocyst (Fig. 1, 4.5 days) and contributes predominantly to the fetal portion of the mature chorioallantoic placenta in later development.

In an effort to understand the genetic control of development and differentiation of the trophoblast lineage in the mouse, we have sought to identify genes whose expression is specific to this lineage. The results of such a search will provide useful molecular markers for experimental identification of cells of trophoblast origin, where few such markers are available in the mouse (Adamson 1986). In addition, further study should elucidate *cis*- and *trans*-acting elements required for gene regulation in the trophoblast lineage. Finally, characterization of trophoblast-specific gene products should aid in understanding the mechanisms by which this tissue carries out its functions.

We report here the isolation of a novel trophoblast-specific cDNA clone by differential screening of a λ gt10 cDNA library made using mRNA derived from 13.5-day mouse placentae. The nucleotide sequence of the clone and the deduced amino acid sequence of the polypeptide are presented. In situ hybridization reveals that expression of the gene is trophoblast-specific and delineates a subgroup of trophoblast cells present in the mouse embryo from 7.5 days until birth.

Results

Isolation of a placental-specific cDNA clone

A cDNA library was constructed in λ gt10 using poly(A)⁺ RNA isolated from CD-1 mouse placentae at 13.5 days of pregnancy. Following amplification, 10⁵ recombinants were screened differentially using ³²P-labeled cDNA derived from 13.5-day placenta and ³²P-labeled cDNA from 13.5-day embryo. Ten plaques which gave strong signals with the placental cDNA probe and not with the embryo cDNA probe were picked. Seven continued to hybridize solely to the placental probe when taken through two rounds of low-density screening. Of these seven cDNA clones, six were shown to cross-hybridize with each other. The seventh clone proved to be mouse placental lactogen II, as determined by hybridization to the mouse placental lactogen II cDNA clone (Jackson et al. 1986). The largest of the six still unidentified clones, clone 4311, was chosen for further characterization.

Placental specificity of cDNA clone 4311

Northern blot analysis was performed to confirm the placental specificity of the 4311 clone (Fig. 2). The clone hybridized to a single abundant mRNA species of ~750 nucleotides in the placenta, but no mRNA was detected in the yolk sac or in the tissues of the embryo proper. The mRNA was first detectable at 10.5 days of gestation, and persisted through all stages examined. No detectable mRNA was seen in 10 μ g of total RNA from adult kidney, salivary glands, heart, liver, testes, brain, spleen, lymph nodes, and lung tissues (data not shown), even after 2-week exposure periods. Equal amounts of RNA

Figure 1. Schematic representation of the trophoblast, primitive ectoderm, and primitive endoderm lineages during mouse embryonic development.

were loaded in each lane as assessed by signal intensity after hybridization of the blot with a β -actin probe (data not shown).

Figure 2. Placental specificity of 4311 mRNA. Total RNA was isolated from the embryo (lane E), placenta (lane PL) and visceral yolk sac (lane YS) from the embryos at various days of gestation as described in Materials and methods. Hybridization was first detected at 10.5 days of development exclusively in the placenta.

Sequence of mouse cDNA and genomic structure

Various restriction fragments of clone 4311 were subcloned into pUC118, (Vieira and Messing 1987), and single-stranded sequence analysis was performed according to the strategy outlined in Figure 3. The 755 bp for clone 4311 and its predicted amino acid sequence are shown in Figure 4.

Primer extension, using a single-stranded primer complementary to nucleotides 41–60 of clone 4311, indicated that this clone is missing 5–10 nucleotides from the 5' end of the mRNA, and is therefore nearly full length (data not shown). There is only one extensive open reading frame, from nucleotides 76 to 450, predicting a polypeptide of 124 amino acids. The ATG codon at position 76–78 is used most likely for initiation of translation since it is preceded by stop codons in the reading frame. In addition, this ATG has the most favorable context for initiation according to the analysis of Kozak (1987). The open reading frame ends with the translation termination codon TAA, followed by 257 bp of 3'-untranslated sequence and a poly(A) tract. A poly(A) addition site (Proudfoot and Brownlee 1974) is located 22 bp upstream of the poly(A) tract. Hydrophobicity analysis of the amino acid sequence revealed a hydrophobic region of 21 amino acids at the amino-terminal end of the protein, suggesting the presence of a

Figure 3. Restriction map and sequencing strategy of cDNA clone 4311. The restriction sites used in sequencing clone 4311 are diagrammed above the linear representation of the clone and the number of base pairs of the cDNA clone are marked below. The arrows indicate the direction of sequencing of the various restriction fragments.

signal peptide (von Heijne 1985). The putative site of cleavage was determined to follow the 18th amino acid according to the rules of von Heijne (1986). No trans-membrane domain was found. Nucleotide and protein sequence homology searches (GenBank and NBRF, Genetics Computer Group) failed to find any significant homologies to previously characterized genes or proteins.

Southern blot analysis of mouse genomic DNA using 4311 as a probe indicated that there is probably only one gene encoding 4311 (Fig. 5). Digestion with enzymes *Bam*HI and *Hinf*I, which have restriction sites within the known cDNA sequence, generated only two major bands in the Southern blot, as expected for a single-copy gene. Other enzymes such as *Eco*RI, *Hind*III, and *Pst*I, which do not cut in the cDNA sequence, also generated

Mouse trophoblast-specific cDNA

Figure 5. Southern blot of mouse genomic DNA probed with 4311. Lanes: (a) *Bam*HI; (b) *Eco*RI; (c) *Hind*III; (d) *Hinf*I; (e) *Pst*I.

two bands, suggesting that there is, in fact, an intron in the gene which contains restriction sites for these enzymes. Further resolution of the genomic structure awaits genomic cloning which is underway.

Pattern of expression of 4311 during embryogenesis

[³⁵S]UTP-labeled probes were transcribed from both the sense and antisense strands of the full 755 bp cDNA clone and were hybridized to 7-μm tissue sections of embryos at various stages of embryogenesis. No hybridization was observed with the sense probe at any stage of development. At 6.5 days only a few isolated cells in the ectoplacental cone region hybridized to the antisense probe (data not shown). By 7.5 days, hybridization was detected clearly in a subset of cells at the mesometrial end of the ectoplacental cone region (Fig. 6A,B), as well as in a few isolated cells in the maternal decidua (data not shown). At 8.5–10.5 days of gestation (Fig. 6C–F), mRNA transcripts were detected in a subset of cells in the ectoplacental cone, in a region just below the giant cell layer. In addition, a few isolated cells in the maternal decidua also hybridized to the probe. For stages from 13.5 to 18.5 days of gestation, the placentae were dissected out and hybridized separately from the embryo. No hybridization occurred to any tissues in the developing embryo. The 13.5-, 15.5-, and 18.5-days placentae all contained abundant 4311 transcripts in the spongiotrophoblast region (Fig. 7). The pattern of hybridization was patchy, consisting of dense and sparse areas of silver grains. This patchiness did not seem to reflect any underlying variation in tissue structure. No hybridization occurred to the giant cells nor to the labyrinthine layer, suggesting that 4311 is specific to cells in the spongiotrophoblast, and that it may mark the progenitors of these cells in the earlier stages.

There was also some hybridization to cells in the mesometrial decidual tissue outside the giant cell layer of the placenta, which increased in intensity from 13.5 to 18.5 days of gestation. Whether these cells were maternal or trophoblastic in origin was not clear. Thus, we

1	GATCTCCAAGGA																
13	CCTCTGAAGAGCTGAACCACTGGAGTCCGACGACAGCTTTGGACATCAGGTTACTTGAGAC																
76	ATG	ACT	CCT	ACA	ATC	TTC	CTA	GTC	ATC	CTA	TGC	CTG	GGA	GTG	GCC	TCA	
	MET	Thr	Pro	Thr	Ile	Phe	Leu	Val	Ile	Leu	Cys	Leu	Gly	Val	Ala	Ser	
124	GCT	GTC	ATA	GTC	CCT	GAA	GCG	CAG	TTG	GAT	GCT	GAA	CTG	CAA	GAG	CAG	
	Ala	Ala	Ile	Val	Pro	Glu	Ala	Gln	Leu	Asp	Ala	Glu	Leu	Gln	Glu	Gln	
172	AAG	GAT	AAA	GAA	GTT	CTC	ATA	AAA	GCA	GTG	TGG	AGT	AAG	TTT	ATG	AAG	
	Lys	Asp	Lys	Glu	Val	Leu	Ile	Lys	Ala	Val	Trp	Ser	Lys	Phe	Met	Lys	
220	ACT	AAC	AAA	CTT	CAC	AGT	AGC	GAA	AAT	GAC	CAG	GAG	ACG	GAA	GGC	TCC	
	Thr	Asn	Lys	Leu	His	Ser	Ser	Glu	Asn	Asp	Gln	Glu	Thr	Glu	Gly	Ser	
268	AAC	ATA	GAA	ATG	AGT	GCC	TCC	GGT	CAG	CTA	ACT	GAT	GAA	GAA	CTC	ATG	
	Asn	Ile	Glu	Met	Ser	Ala	Ser	Gly	Gln	Leu	Thr	Asp	Glu	Glu	Leu	Met	
316	AAA	ATA	ATG	ACT	ACT	GTT	TTA	CAC	CCA	ATG	TTT	GAA	GAG	GAG	GAA	AAC	
	Lys	Ile	Met	Thr	Thr	Val	Leu	His	Pro	Met	Phe	Glu	Glu	Glu	Glu	Asn	
364	AAA	CCA	CAG	CCA	GTT	GTT	GAT	GAC	CCT	GAA	TTT	GAG	GAT	TAC	ACA	CAG	
	Lys	Pro	Gln	Pro	Val	Val	Asp	Asp	Pro	Glu	Phe	Glu	Asp	Tyr	Thr	Glu	
412	AGT	GGC	GAT	GGG	TTT	TTT	GTA	CCA	AAT	CAG	CCA	CAG	TAA	CAT	GGACAGCA		
	Ser	Gly	Asp	Gly	Phe	Phe	Val	Pro	Asn	Gln	Pro	Gln	*				
462	GTATGAAGTTAGGCAACAGCGAAACAGCCACTGTGCCATTGTCTAAGAAATCTCTGCCCCAA																
525	CATATGAAGTCGCTGATGGTGAAAGAAAGACATCTGAACTGGGGCAAGGGCAAGCAAAAAACAGC																
588	AGGGACTTCCCAATTCCAGTATCCCTGACAAGTGGGATCCTGCACCTCAAGTCCCTTAAATAT																
651	ATACTATGATATGAATCCAAAGATGTTTCACACTAATAAACCTTACCATTACCTTAAAAAA																
714	AA																

Figure 4. Nucleotide and predicted amino acid sequences of cDNA clone 4311. Translation is assumed to begin at the first ATG of the nucleotide sequence (76–78) and continue until the codon TAA (*, 448–450). The polyadenylation signal is underlined and the predicted signal peptide is enclosed between parallel lines.

Figure 6. Localization of 4311 mRNA in the mouse embryo by in situ hybridization. (A,B) One-month exposures of 7.5-day ectoplacental cone photographed under bright-field (A) and dark-field (B) illuminations. The arrow in B denotes a subset of cells in the ectoplacental cone expressing the gene. (C) An 8.5-day embryo in the maternal decidua photographed under bright-field illumination and (D) a higher magnification of the boxed area photographed under dark-field illumination showing the outer ectoplacental cone cells expressing the gene (1-week exposure). (E,F) One-week exposures of 9.5-day (E) and 10.5-day (F) embryos in the maternal decidua showing intense hybridization in the outer regions of the ectoplacental cone (arrows). Grid bar represents 50 μ m (A,B,D) and 1 mm (C,E,F).

analyzed the genotypic composition of this layer after transfer of genetically marked Tg-M β G-1 embryos to CD-1 recipients (Lo 1986; Varmuza et al. 1988). Tg-M β G-1 is a transgenic line containing $\sim$ 1000 tandem copies of a plasmid containing the β -globin gene. The transgene is detectable by in situ DNA–DNA hybridization, which revealed that there were considerable numbers of Tg-M β G-1 cells in the maternal layers of the

15.5-day placenta outside of the dense spongiotrophoblast (Fig. 8). These cells must be derived from the trophoblast of the Tg-M β G-1 conceptus, since previous in situ studies have established that no ICM-derived cells are found in this region of the placenta (Rossant and Croy 1985). Thus, it seems likely that 4311 is expressed predominantly in spongiotrophoblast and outlying trophoblast cells in the mature placenta. The presence of

hybridization in isolated decidual cells earlier in gestation cannot be explained by outlying trophoblast cells, however, since DNA–DNA in situ analysis of Tg-M β G-1 embryos in CD-1 decida at 8.5 days revealed no labeled cells in this region (data not shown).

Discussion

A cDNA clone representing a novel placental-specific mRNA has been isolated by differential screening of a 13.5-day mouse placenta cDNA library in λ gt10. A single, highly abundant transcript of approximately 750 nucleotides was detected in placental mRNA by Northern blot and primer extension analysis. An almost full-length cDNA clone was sequenced in its entirety and shown to contain a single open reading frame encoding a polypeptide of 124 amino acids with a putative hydrophobic signal sequence of 18 amino acids. No transmembrane domain was found, indicating that the protein is probably processed in the endoplasmic reticulum and secreted from the cell. A search of the nucleotide and protein sequence databases (GenBank and NBRF) revealed no significant homologies to any listed gene or protein. This suggests that the cDNA clone represents a novel gene. Southern blot analysis of genomic DNA indicated that there was probably only one gene encoding 4311.

The tissue-specificity of expression of clone 4311 is regulated tightly in development. Using Northern blot analysis, we were unable to detect transcripts in any tissue analyzed other than the placenta. In situ hybridization revealed that expression was restricted further to a subgroup of fetally derived trophoblast cells within the placenta. Expression was first detected at 7.5 days in a subpopulation of trophoblast cells at the mesometrial end of the ectoplacental cone, and as development proceeded, expression of the gene continued to mark a group of cells in this region of the ectoplacental cone. By 13.5 days of gestation, only the spongiotrophoblast cells of the placenta were expressing the gene. No expression was detected in the trophoblast giant cells, the labyrinthine trophoblast, nor in any tissues of the embryo. Expression was observed in some cells outside the trophoblast giant cell layer in the putative maternal region of the placenta. However, this is not inconsistent with trophoblast-specific expression because some cells in this region were shown to be trophoblast in origin by in situ analysis of genetically marked embryos that had been transferred into unmarked foster mothers. This emphasizes the importance of a full knowledge of the derivatives of a cell lineage such as the trophoblast to make a valid assessment of tissue specificity of expression. The only cells other than trophoblasts that showed any evidence of expression of 4311 were a few isolated cells in the decida between days 7.5 and 10.5 of development. The significance of this low-level expression is not clear.

Expression of 4311 in a subgroup of trophoblast cells as early as 7.5 days of development suggests that there

Figure 7. Localization of 4311 mRNA in the mature placenta. Two-day exposures of 13.5-day placenta (A), 15.5-day placenta (B), and 18.5-day placenta (C) showing intense hybridization to the spongiotrophoblast and less intense hybridization to cells in the maternal layer. Patchy hybridization can be seen in the spongiotrophoblast of the 13.5-day (A) and 15.5-day (B) placentae. Bar, 1 mm.

may be early compartmentalization within the ectoplacental cone, with 4311 expression marking those cells that will later form the densely packed, hormone-producing spongiotrophoblast. The mechanism by which 4311 expression is regulated within the trophoblast lineage is unknown, but it is interesting to note that it is the ectoplacental cone cells furthest from the embryo that begin expression of 4311. In another area of tro-

Figure 8. DNA–DNA in situ hybridization of a 15.5-day placenta from the Tg-MβG-1 transgenic line transferred into a CD-1 foster mother showing labeled fetal cells in the maternal layer. (M) Maternal decidua; (GC) giant cell layer.

phoblast differentiation, namely trophoblast giant cell formation, it has been shown that the cells of the embryo exert some influence that keeps trophoblast cells in a proliferative state. Only those cells furthest from the influence of the embryo cease division and endoreduplicate (Gardner 1972; Ilgren 1981; Rossant and Tamura-Lis 1981). It is tempting to speculate that 4311 expression is also affected by the distance of trophoblast cells from embryonic derivatives, thus producing a gradient of trophoblast differentiation with giant cells at the outside, followed by 4311-expressing spongiotrophoblast precursors, and completed by labyrinthine precursors in proximity to the embryonic tissues. This hypothesis would predict that removal of ectoplacental cone cells from the influence of the embryo would extend the domain of expression of 4311, a concept testable in vitro. Alternatively, the 4311 gene may respond to signals emanating from the maternal uterine tissue. The spongiotrophoblast has been shown to express significant quantities of the colony-stimulating factor (CSF)-1 receptor, *c-fms* (Regenstreif and Rossant, in prep.), while the overlying maternal epithelium produces large amounts of CSF-1 (Pollard et al. 1987). If 4311 expression is controlled by CSF-1 or some other uterine signal, it may be possible to test this idea by cocultivation of the ectoplacental cone and the pregnant uterus.

A variety of other mouse placental-specific genes have been isolated by screening placental cDNA libraries, including placental lactogens I and II (Jackson et al. 1986; Colosi et al. 1987), proliferin and proliferin-related protein (Linzer and Nathans 1985; Linzer et al. 1985), and placental alkaline phosphatase (Terao and Mintz 1987). However, 4311 is not identical to any of these genes and its isolation by differential screening illustrates that new tissue-specific genes that have not been identified by other approaches can be isolated in this manner. The

possible role of 4311 in trophoblast development and differentiation is not yet clear. No similarities with identified proteins, including other secreted proteins (extracellular matrix proteins, proteases, growth factors, peptide hormones, etc.) were revealed by sequence comparisons. Because of its small size, presence of a putative signal peptide, and the abundance of the mRNA, we can speculate that it may be a growth factor/hormone, perhaps involved in interaction between the maternal and fetal systems in maintenance of pregnancy. Currently, we are attempting to raise antibodies to the putative protein to gain more insight into the localization and function of the protein.

Thus, differential screening of a placental cDNA library has allowed isolation of a new trophoblast-specific cDNA whose expression marks a subgroup of trophoblast cells presumably destined to become spongiotrophoblast. This gene is a useful addition to the limited array of mouse trophoblast-specific markers and it may play an important role in the specialized functions of trophoblast in late pregnancy. Early expression could not be detected either by Northern blot analysis or in situ hybridization, suggesting that 4311 may not be required for establishment of the trophoblast lineage, but is instead a marker of terminal differentiation of spongiotrophoblast. A search for genes involved in establishment of the trophoderm lineage at the blastocyst stage will require sensitive subtractive hybridization of cDNA libraries from earlier stages of development.

Materials and methods

RNA isolation

All cellular RNA was prepared as described by Maniatis et al. (1982), with the exception of that used for primer extension. In this case, total cellular RNA was prepared by homogenization

in 6 M urea/3 M LiCl with a Polytron homogenizer. After freezing for 18 hr at -20°C , the sample was centrifuged at 10,000 rpm for 30 min at 4°C . The pellet was resuspended in 7 M urea, 10 mM Tris-HCl (pH 8.0), 2 mM EDTA, 200 mM NaCl, and 1% SDS; centrifuged at 10,000 rpm for 10 min at room temperature; extracted twice with phenol/chloroform and twice with chloroform; and ethanol-precipitated with 0.3 M NaOAc at -70°C . Poly(A)⁺ RNA was isolated from oligo(dT) columns according to Maniatis et al. (1982).

Isolation of placental-specific cDNA clones

Pregnant CD-1 mice were sacrificed on day 13.5 of gestation (day of plug is day 0.5) and six placentae were dissected free of maternal and embryonic tissues, yielding 450 μg of total RNA, and 30 μg of poly(A)⁺ RNA. cDNA was synthesized from 1 μg of poly(A)⁺ RNA according to the method of Gubler and Hoffman (1983), and cloned into $\lambda\text{gt}10$ (Hyunh et al. 1985). The library, consisting of 10^5 clones, derived from 45 ng of cDNA, was amplified in C_{600} Hfl⁻ cells.

The library was screened by differential hybridization (Maniatis et al. 1982) using [³²P]dCTP-labeled cDNA probes that were made from oligo(dT)-primed poly(A)⁺ RNA from 13.5-day placentae and 13.5-day embryos. Signals that were positive with the placental probe and negative with the embryo probe were picked, and taken through two rounds of low-density screening. The plaques that continued to test positive only with the placental probe were picked and phage DNA was isolated according to Maniatis et al. (1982).

Northern and Southern blotting

Total cellular RNA from yolk sac, embryo, and placental tissues at various stages of embryogenesis was electrophoresed in a glyoxal agarose gel according to Maniatis et al. (1982), and transferred to GeneScreen Plus (NEN), as described by the manufacturer. Filters were prehybridized at 42°C for 1 hr in 50% formamide, 1% SDS, 10% dextran sulfate, and 1 M NaCl. Hybridization took place in the same buffer at 42°C for 18 hr with 100 $\mu\text{g}/\text{ml}$ denatured, sheared herring sperm DNA, and ³²P-labeled clone 4311 DNA, prepared by random priming (Feinberg and Vogelstein 1983). The filters were washed at 65°C in $2\times$ SSC and 1% SDS, followed by a more stringent wash at $0.2\times$ SSC and 0.1% SDS at 65°C .

Total cellular RNA prepared from various mouse adult tissues was electrophoresed on a formaldehyde gel and transferred to GeneScreen Plus (NEN) according to Joyner et al. (1985). The filter was prehybridized at 65°C for 30 min in 15% formamide, 0.5 M Na_2HPO_4 , 1 mM EDTA, 1% BSA, and 7% SDS. Hybridization took place in the same buffer for 16 hr at 65°C with random primed clone 4311 DNA (Feinberg and Vogelstein 1983). The filter was washed in 150 mM Na_2HPO_4 /1% SDS at room temperature and at 50°C , in 30 mM Na_2HPO_4 /0.1% SDS at 50°C , and finally in 15 mM Na_2HPO_4 /0.1% SDS at 65°C .

Genomic DNA prepared from CD-1 mouse spleens was digested with various restriction enzymes according to the manufacturer's directions. Sample aliquots of 5 μg were loaded in an 0.8% agarose gel and subject to electrophoresis. After transfer to GeneScreen Plus, blots were prehybridized for 4 hr at 65°C in 10% dextran sulfate, 1% SDS, 1 M NaCl, and 100 $\mu\text{g}/\text{ml}$ salmon sperm DNA. Random-primed 4311 cDNA probe was added at 4×10^6 cpm/ml and hybridization took place overnight at 65°C . All washes were at 65°C with the final wash containing $0.1\times$ SSC and 1% SDS.

Sequencing

Restriction fragments of cDNA clone 4311 were subcloned into pUC118 and single-stranded DNA was prepared as described by Vieira and Messing (1987). Both strands of the subclones were sequenced by the dideoxy chain-termination method using the Sequenase enzyme (U.S. Biochemical Corporation), according to the method described by the manufacturer.

In situ hybridizations

In situ hybridization was performed using the method as described by Davis et al. (1988). Briefly, embryos and placentae were dissected from the uterus between days 8.5 and 18.5 of development, and 7- μm frozen sections were collected and fixed in 4% paraformaldehyde as described (Davis et al. 1988). The [³⁵S]UTP-labeled single-stranded RNA probes were prepared from pGem1 and pGem2 (Promega) plasmids containing the full-length 4311 clone, thus transcribing both strands of the clone. Hybridizations, washes, autoradiography, and staining were performed according to Davis et al. (1988). Coverslips were mounted, and the slides were examined under transmitted light- and dark-field illumination. Some tissues were subject to in situ hybridization after ester wax embedding. Embryos of 6.5 and 7.5 days were dissected from the decidua, and 15.5-day placentae were fixed in 4% paraformaldehyde for 10 min, dehydrated in an ethanol series at 4°C , and embedded in ester wax (BDH 1940). Sections of 7 μm were collected on poly-L-lysine-coated slides and dried at 37°C . The slides were treated in xylene for 5 min to dissolve the wax, rehydrated in an ethanol series, acetylated in a freshly prepared solution of 0.25% acetic anhydride and 100 mM triethanolamine (pH 7.5), rinsed in $3\times$ PBS for 5 min and twice in $1\times$ PBS for 3 min, dehydrated in ethanol, and air-dried. Probe preparation, hybridizations, washes, and autoradiography were then performed as for cryostat sections (Davis et al. 1988).

In situ identification of embryo-derived cells in the placenta and decidua

Blastocysts were recovered from matings between mice homozygous for the transgenic marker, Tg-M β G-1 (Lo 1986; Varmuza et al. 1988), and transferred to the uteri of pseudopregnant CD-1 recipient females. Recipients were sacrificed at 8.5, 12.5, and 15.5 days of pregnancy and intact decidua containing the transferred embryos were fixed in acetic acid/ethanol (1:3), and processed for ester wax embedding (Rossant 1985). Sections of 7 μm were hybridized in situ with biotinylated pM β A2 DNA, which is homologous with the transgenic insert, and hybridization was detected by streptavidin-horseradish peroxidase binding as described previously (Varmuza et al. 1988). Nuclei containing positive hybridization signals identified cells of fetal origin in the developing placenta or decidua.

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