(Perlecan) in the Mouse Blastocyst Is Regulated during Normal and Delayed Implantation

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Previous studies have shown that expression of the heparan sulfate proteoglycan, perlecan, on the external trophectodermal cell surfaces of mouse blastocysts increases during acquisition of attachment competence. However, it is not clear if this change in perlecan protein expression also is reflected at the level of perlecan mRNA expression. In the present investigation, the spatial and temporal patterns of perlecan mRNA expression in the mouse embryo during the periimplantation period were examined by *in situ* hybridization and reverse transcriptase–polymerase chain reaction. In addition, a delayed implantation model was used to determine the expression of perlecan mRNA and protein in dormant and estrogen-activated hatched blastocysts. The results demonstrate that perlecan mRNA expression is low in morulae, but increases in Day 4 blastocysts, attaining maximal expression in Day 4.5 attachment-competent blastocysts. In contrast, perlecan mRNA is detected in both the dormant and estrogen-activated delayed blastocysts; however, within 12 hr of blastocyst activation by estrogen, both perlecan protein and heparan sulfate chain expression markedly increase. Taken together, these results suggest that during normal development perlecan mRNA expression increases with the acquisition of attachment competence. Moreover, perlecan protein expression also is attenuated during delayed implantation and appears to increase in response to nidatory estrogen, perhaps via the increased translation of preexisting perlecan mRNA.   © 1997 Academic Press

INTRODUCTION

Successful implantation is dependent upon an intimate "cross-talk" between the embryo and the uterus. Once the blastocyst hatches from the zona pellucida, its trophectoderm becomes attachment competent, i.e., gains the ability to attach to the uterine luminal epithelium. At the same time, the uterus must be in a "receptive" state for successful implantation to occur. The attainment of the receptive state of the uterus is primarily regulated by ovarian estrogen and progesterone (Psychoyos, 1986). A role for heparin/heparan sulfate proteoglycans (HSPGs) in mouse embryo attachment has been suggested (see review Carson *et al.,* 1994). Synthesis of HSPGs increases on the murine embryonic cell surface during the periimplantation period (Farach *et al.,* 1987). Inhibition of embryonic proteoglycan synthesis, specific removal of heparan sulfate (HS) from blastocyst cell surfaces,

or the presence of soluble HS competitors all delayed blastocyst attachment to a variety of matrices including laminin, fibronectin, and uterine epithelial cells (Farach *et al.,* 1987, 1988). It has also been demonstrated that mouse and human uterine epithelial cells express complementary HS binding proteins that may aid in blastocyst attachment (Wilson *et al.,* 1990; Raboudi *et al.,* 1992; Liu *et al.,* 1996; Rohde *et al.,* 1996). Recently, the HSPG, perlecan, has been identified at the sites of blastocyst apposition in the uterus *in vivo* (Carson *et al.,* 1993). Perlecan is a 400-kDa protein commonly found in the basal lamina underlying epithelial cells. Of the five domains in perlecan, the amino terminus is unique, including attachment sites for HS chains. In addition, there is no transmembrane domain (Noonan, *et al.,* 1991). Since perlecan expression on the cell surface of blastocysts increases during acquisition of attachment competence, it is possible that this proteoglycan is one of the HSPG mediators of trophectoderm–luminal epithelial cell attachment. However, it is not clear if the perlecan gene is expressed coordinately with the increase in perlecan protein

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under these conditions. In order to expand on previous studies, *in situ* hybridization was used to determine the spatial and temporal expression of perlecan mRNA in periimplantation embryos. In mice and other species, implantation may be delayed either naturally or experimentally (Yoshinaga and Adams, 1966). Therefore, it was of interest to determine if HSPG expression also is delayed in blastocysts under these conditions. Using an experimentally delayed implantation model, we examined the effects of nidatory estrogen on perlecan expression in activated blastocysts and dormant blastocysts. The results indicate that increased perlecan protein expression may result either from increased mRNA expression (normal implantation) or by increased translation of preexisting mRNA (delayed implantation). Collectively, these data further demonstrate that increased HSPG expression at multiple levels is tightly associated with acquisition of blastocyst attachment competence.

MATERIALS AND METHODS

Materials. CF-1 mice were obtained from Sasco (Omaha, NE). Tissue-Tek O.C.T. was purchased from Miles Inc. (Elkhart, IN). Rabbit antibody to human basic fibroblast growth factor (bFGF) was purchased from Collaborative Research, Inc. (Bedford, MA). Species-specific fluorescein-conjugated rhodamine-conjugated second antibodies to rabbit and rat IgG were obtained from Amersham Corporation (Arlington Heights, IL). Rat anti-heparan sulfate proteoglycan (perlecan) monoclonal antibody was purchased from Chemicon International Inc. (Temecula, CA). The mouse perlecan DNA, clone 5, was a generous gift from Dr. John Hassell (Eye and Ear Institute, Pittsburgh, PA). The *in vitro* transcription kit was obtained from Ambion (Austin, TX), and the $[\alpha$-^{35}S]UTP ($>$1000 Ci/mmole) from DuPont (Wilmington, DE). RNase A, restriction enzymes, and yeast RNA were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Ultrapure phenol was purchased from Gibco BRL (Gaithersburg, MD) and formamide from Fluka (Ronkonkoma, NY). All other reagents used in this study were of reagent grade or better.

Embryo collection. Female mice were injected intraperitoneally with 5 IU of pregnant mare serum gonadotropin and 48 hr later with 5 IU of human chorionic gonadotropin (hCG). Following hCG injection females were caged with males of proven fertility. Females were examined the following morning for the presence of a vaginal plug indicating Day 1 of pregnancy. It has been shown in previous studies (Carson *et al.,* 1993) that uteri collected from mice in our colony at 96 hr post-hCG injection and flushed with Hanks' balanced salt solution contain approximately 80% unhatched blastocysts. However, approximately 80% of the blastocysts are hatched when recovered 108 hr post-hCG injection. Using the gonadotropin injection regimen described above, embryos were flushed from the reproductive tract during three developmental time periods. Embryos were collected at 76 hr (Day 3 morula, unhatched), 96 hr (Day 4 blastocysts, unhatched), and 108 hr (Day 4.5 blastocysts, hatched) post-hCG injection. Zona pellucidae were removed from unhatched embryos with acid Tyrode's solution (Gordon, 1993).

Cytospin collection of embryos. Ten to twelve embryos from the time points stated above were transferred into a cytospin funnel preloaded with 200 μl of Hanks' balanced salt solution and centrifuged for 5 min at 2000 rpm in a Shandon Elliott tabletop centrifuge.

Slides were examined under a dissecting scope for the presence of embryos, allowed to air dry, and held on dry ice until all the embryos had been processed. Slides containing embryos were stored at $-70°$C until ready for use. For immunofluorescence or *in situ* hybridization, embryos were processed the same as tissue sections.

Delayed implantation. To determine whether perlecan mRNA is induced in response to nidatory estrogen, an experimentally delayed implantation model was used (Yoshinaga and Adams, 1966; Paria *et al.,* 1992). To maintain blastocysts in a state of delay, pregnant mice were ovariectomized on the morning of Day 4 (0800–0900 hr) and injected subcutaneously with progesterone (2 mg per mouse) on Days 5, 6, and 7. During the period of delay, blastocysts hatch, but remain unattached. To initiate blastocyst activation and implantation, mice were coinjected with estradiol-17β (25 ng per mouse) and progesterone on Day 7 of pregnancy (Huet-Hudson and Dey, 1990). Embryos were flushed from the uterus 12 and 24 hr post-estradiol injection, cytospun onto slides, and assessed for the presence of perlecan protein and mRNA as described below. Each experiment was performed at least two to three times on separate preparations of embryos (10–15 embryos per preparation).

Indirect immunofluorescence staining of implantation sites in utero. To assess the stage of embryonic development, one uterine horn was flushed and the other uterine horn was snap frozen in a dry ice/isopentane bath and stored at $-80°$C until sectioned. To establish whether embryos were hatched, 7-μm sections of uteri containing blastocysts were probed with antibodies to zona pellucida (ZP) proteins 2 and 3 and tenascin as previously described (Julian *et al.,* 1994). Detection of ZP2/ZP3 indicated that the blastocyst was still encapsulated by the ZP and unhatched. The absence of ZP2/ZP3 and the presence of tenascin, an extracellular matrix protein induced in subepithelial stroma, were used as markers for hatched, attachment-competent blastocysts (Julian *et al.,* 1994).

Indirect immunofluorescence localization of perlecan core protein and detection of heparan sulfate chains by bFGF binding. The procedure for the indirect immunofluorescence localization of perlecan core protein and detection of heparan sulfate chains was performed as previously described (Carson *et al.,* 1993). In the present study, a rat anti-perlecan monoclonal antibody was used. In agreement with earlier results, using the rabbit polyclonal antibody, the rat monoclonal antibody recognized perlecan in sections from Day 4.5 implantation sites in the basal lamina, in extracellular matrix surrounding uterine stroma, and between uterine epithelium and trophectoderm of hatched blastocysts (data not shown). These experiments were performed on at least 10 different implantation sites. For double labeling of cytospun embryos with both bFGF and anti-perlecan, embryos were fixed 10 min in 100% methanol and incubated with bFGF for 2 hr at 37°C. Upon application of the anti-bFGF, the anti-perlecan also was added. Embryos were processed as mentioned previously. FITC donkey anti-rabbit and RITC donkey anti-rat secondary antibodies were applied together.

Riboprobe preparation. Linearized DNA clone (clone 5 in Noonan *et al.,* 1988) for mouse perlecan in Bluescript (SK$^{+/-}$) vector (Stratagene) was used. Approximately 1 μg of linearized template DNA was used to generate sense and antisense riboprobes. The total reactivity incorporated into ^{35}S-labeled cRNA probes obtained using 1 μg of DNA as template along with RNA polymerase T3 or T7 was usually 0.3–1.4 $\times$ 10^8 dpm. The probes were purified by phenol:chloroform extraction and ethanol precipitation. To enhance probe penetration into the tissue, the pelleted probes were hydrolyzed to approximately 100 nucleotides in the presence of 60 mM Na$_2$CO$_3$ and 40 mM NaHCO$_3$, pH 10.2, as described by Cox *et al.* (1984). Riboprobes were recovered by ethanol precipitation and resuspended in 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, and 10 mM dithiothreitol and stored at $-70°$C until use.

In situ hybridization. Frozen serial sections of implantation sites and isolated embryos as described above were subjected to *in situ* hybridization using protocols described previously (De *et al.,* 1989; McMaster *et al.,* 1992). Briefly, slides were removed from the −70°C freezer, warmed to 37°C for 1 min, and fixed in 4% (w/v) paraformaldehyde in PBS for 15 min. Following prehybridization, 150 μl of hybridization solution containing 2×10^7 cpm/ml was added to each sample and covered with a silconized coverslip. Hybridization was carried out at 45°C for 4 hr. Unhybridized probe was digested with 20 μg/ml of RNase A at 37°C for 15 min. Hybridized probe was detected after 2 weeks of autoradiography using Kodak NTB-2 emulsion. Following development, slides were poststained with hematoxylin and eosin. At least 20 embryos at each developmental stage have been examined in these studies.

Isolation of total RNA. Total RNA was isolated from Day 4 and Day 4.5 mouse blastocysts as described by Andrews *et al.* (1991) with the following modifications: Blastocysts (80–100 per group) were collected as described above, quick frozen on dry ice, and stored at −70°C. Embryos were allowed to thaw on ice and resuspended in SDS buffer, phenol saturated with SDS buffer, and 20 μg yeast RNA. Included in the SDS buffer was 50,000 cpm of ^{35}S-labeled mouse β-actin RNA was used as a tracer to determine RNA recovery. Recovery of the tracer was determined by liquid scintillation counting of aliquots taken of the initial SDS buffer and the final RNA solution. This mixture was homogenized by repeated aspiration into a 27-gauge needle attached to a 1-cc syringe. To reduce sample loss, care was taken to avoid pulling the homogenate into the syringe. The sample was chilled at 4°C for 15 min and spun for 10 min at 4°C in an Eppendorf centrifuge. Following centrifugation, the organic layer was carefully removed and discarded. The aqueous layer was reextracted with an equal volume of phenol:chloroform:isoamyl alcohol (24:24:1) and centrifuged as described above. After centrifugation, the aqueous layer was transferred to a 1.5-ml Eppendorf tube and 3 vol of 4 M ammonium acetate and ethanol were added to precipitate RNA. RNA was allowed to precipitate overnight at −70°C. The RNA precipitate was collected by spinning in an Eppendorf centrifuge for 30 min at 4°C, and the pellet was washed with 80% ethanol, dried under vacuum for 2 min, and dissolved in 10 μl of DEPC water.

Reverse transcriptase–polymerase chain reaction. The reverse transcriptase–polymerase chain reaction (RT–PCR) procedure was performed with modification of the procedure described by Wegner *et al.* (1995). RNA from 80 blastocysts was reverse transcribed in a 20-μl volume containing 1× PCR buffer, 1 mM of each dNTP, 500 ng of poly(dT)$_{12-18}$, 4 mM MgCl$_2$, 20 units RNAsin RNA inhibitor, and 2.5 units of AMV reverse transcriptase. The reaction was incubated at 42°C for 75 min and was terminated by heating at 95°C for 5 min. The L19 primers were the same as those described by Wegner *et al.* (1995). The perlecan primers were: (forward) 5′-CCTACGATGGCCTTTCCCTC and (reverse) 5′-TGGCACTTG-CATCCTCCA and correspond to +83 to +102 and +299 to +316 of the mouse perlecan DNA sequence (Noonan *et al.,* 1988). The products obtained by use of the perlecan primers were of the expected size before and after restriction enzyme digestion. In addition, no products were observed if RNA or reverse transcriptase was omitted from the reaction, demonstrating that these products were not due to the presence of contaminating DNA. Partial sequencing of the PCR products also confirmed their identity. Ten microliters of PCR cocktail containing 1× PCR buffer, 60 pmole each of the perlecan and ribosomal protein L19 (internal control) oligonucleotide primers, 2 μCi [α-^{32}P]deoxy-CTP (3000 Ci/mmole), and 2.5 units of *Taq* DNA polymerase were added to the DNA generated above. The final volume of the reaction was adjusted to

100 μl with the addition of 70 μl of 1× PCR buffer and 2.5 mM MgCl$_2$ and overlayered with 100 μl of mineral oil. Following a 1-min incubation at 95°C, 35 repeated cycles were carried out with 30 sec at 95°C, 30 sec at 55°C, and 30 sec at 72°C followed by a 10-min final extension at 72°C. The PCR products were ethanol-precipitated, resuspended in tracking dye gel buffer, and loaded on a 5% nondenaturing polyacrylamide gel in 0.5× Tris–borate EDTA buffer. The gel was dried and exposed to X-ray film. The bands were quantified by scanning with a laser densitometer.

RESULTS

Analysis of perlecan mRNA in mouse embryos by in situ hybridization and RT–PCR during normal pregnancy. Previous *in vivo* and *in vitro* studies have demonstrated that perlecan protein is present on the exterior surface of the trophectoderm during the attachment phase of implantation (Carson *et al.,* 1993). Since perlecan protein is present after, but not before, the blastocyst hatches from the zona pellucida and becomes attachment competent, it was necessary to determine at which developmental stage mRNA encoding perlecan was first expressed. It was anticipated that perlecan mRNA could be expressed in the unhatched embryo prior to the time of attachment competence. Initially, unhatched (Day 4) and hatched (Day 4.5) embryos developed *in utero* were examined by *in situ* hybridization. In uterine sections obtained from Day 4 pregnant mice, perlecan mRNA was uniformly expressed throughout the stromal compartment and abundantly expressed in the myometrial smooth muscle; however, no signal was apparent in the unhatched embryo (data not shown). Elevated levels of perlecan mRNA were detected in the subepithelial stroma surrounding Day 4.5 hatched embryos (Fig. 1C). Expression in the peripheral portion of the stromal compartment resembled that of Day 4. In contrast, expression of the perlecan transcript was negligible in uterine epithelial cells. Sections of implantation sites probed with a cRNA sense probe detected only background hybridization signals, establishing the specificity of the antisense probe (Fig. 1A). Due to high levels of perlecan mRNA expression in the endometrial stroma surrounding the implantation site and thin cell bodies of trophectoderm in hatched blastocysts, uniform localization of the perlecan transcript was difficult to obtain in sections of implantation sites. Therefore, in the remainder of the studies, embryos were allowed to develop *in vivo* and then flushed from the uterus at different developmental stages to facilitate interpretation of *in situ* hybridization results for embryos.

There was no significant difference in grain densities between morulae stage embryos probed with a sense or antisense perlecan cRNA probe (Figs. 2A, 2B, 3A). Perlecan mRNA was initially detected at low levels in Day 4 unhatched blastocysts (Figs. 2C, and 2D). Hatching from the zona pellucida in mice occurs between Days 4 and 4.5, after which the blastocysts become attachment competent. As shown in Fig. 2F, hatched blastocysts expressed high levels of perlecan mRNA. The transcript was present in both the
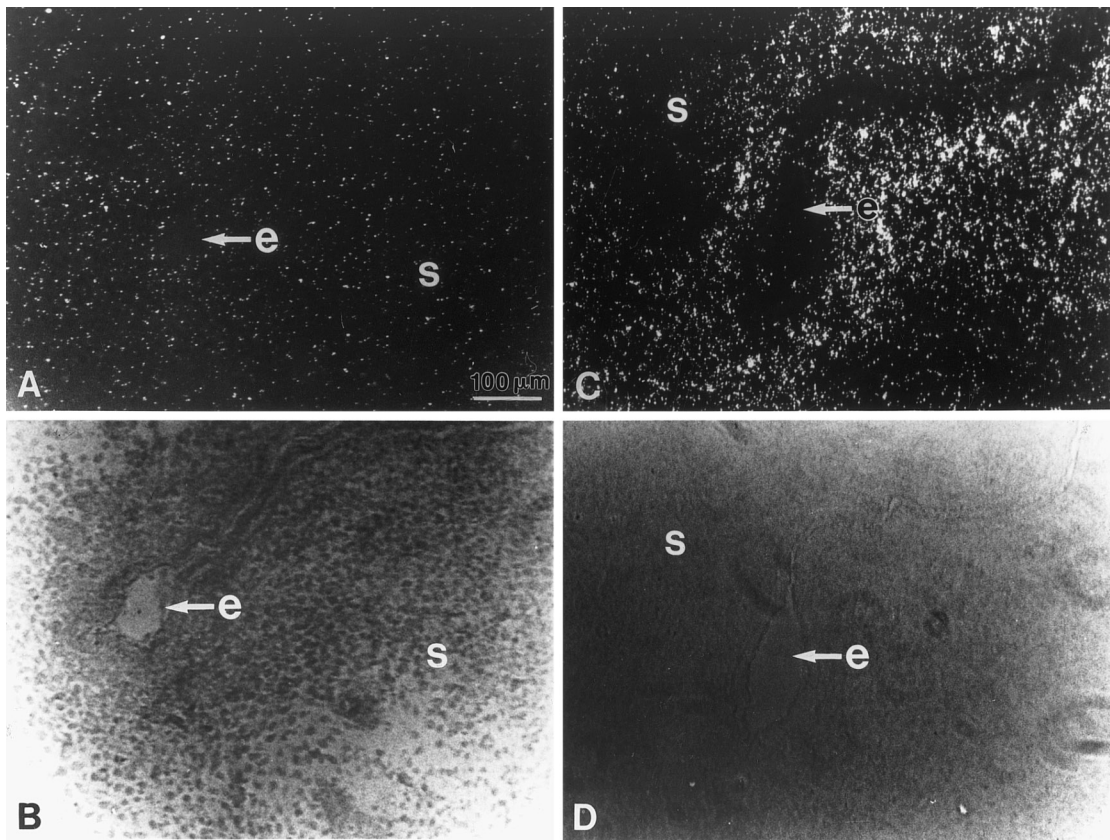
FIG. 1. Perlecan mRNA expression in Day 4.5 embryos detected by *in situ* hybridization. Dark-field (A, C) and corresponding bright-field (B, D) photomicrographs of an implantation site in frozen uterine sections obtained from a Day 4.5 pregnant mouse. Sense cRNA controls are shown in A and B and antisense cRNA was used in C and D. *In situ* hybridization using the sense probe does not show localization to a specific region. Perlecan mRNA localization detected by the antisense probe is abundant in the stromal cells (s) subjacent to the uterine epithelium surrounding the embryo (e).

mural and polar trophectoderm. These data indicate that perlecan mRNA expression increased closely following or coincident with the process of hatching. To quantitate the increase in perlecan mRNA expression between unhatched stage blastocysts and hatched blastocysts, the number of silver grains per embryo was counted for morulae, unhatched blastocysts, and hatched blastocysts, for both sense and antisense probes. The mean number of grains/embryo, shown in Fig. 3A, indicates a significant difference between the hatched blastocyst stage embryo and all groups, with $P < 0.0001$. The relative increase in antisense probe hybridization between unhatched (Day 4.0) and hatched (Day 4.5) blastocysts was approximately sixfold. Similar results were obtained using an RT–PCR assay. RNA encoding a constitutively expressed ribosomal protein, L19, was used as an internal control as described previously (Wegner *et al.,* 1996). As shown in Fig. 3B, a marked (sevenfold) increase was observed for perlecan mRNA relative to L19 transcript between Day 4.0 (unhatched) and Day 4.5 (hatched, attachment-competent) blastocysts. These results indicated that, like perlecan protein, increased perlecan mRNA expression

closely accompanied acquisition of attachment competence during normal embryonic development.

Perlecan protein and mRNA expression in blastocysts during delayed implantation. Given the results obtained during normal pregnancy, it was of interest to determine the consequences of implantation delay on perlecan mRNA and protein expression. In this model, embryos hatch from the zona pellucida, but do not become attachment competent until a nidatory dose of estrogen is administered. Expression of several other blastocyst genes is associated with this activation event (Paria *et al.,* 1992, 1993a,b). Immunohistochemical staining techniques were used to detect perlecan core protein and HS chain expression. Specificity controls included use of nonimmune ascites fluid versus ascites fluid containing rat monoclonal IgG directed at perlecan (compare Fig. 4, panels A and B) and predigestion of embryos with heparinases to remove HS chains (Fig. 4, panels C and D) or competition with excess heparin (Fig. 6, panels B and D) for the HS detection technique. While it is possible that bFGF might bind to components other than HS, the efficient competition with heparin and inhibition of bFGF binding
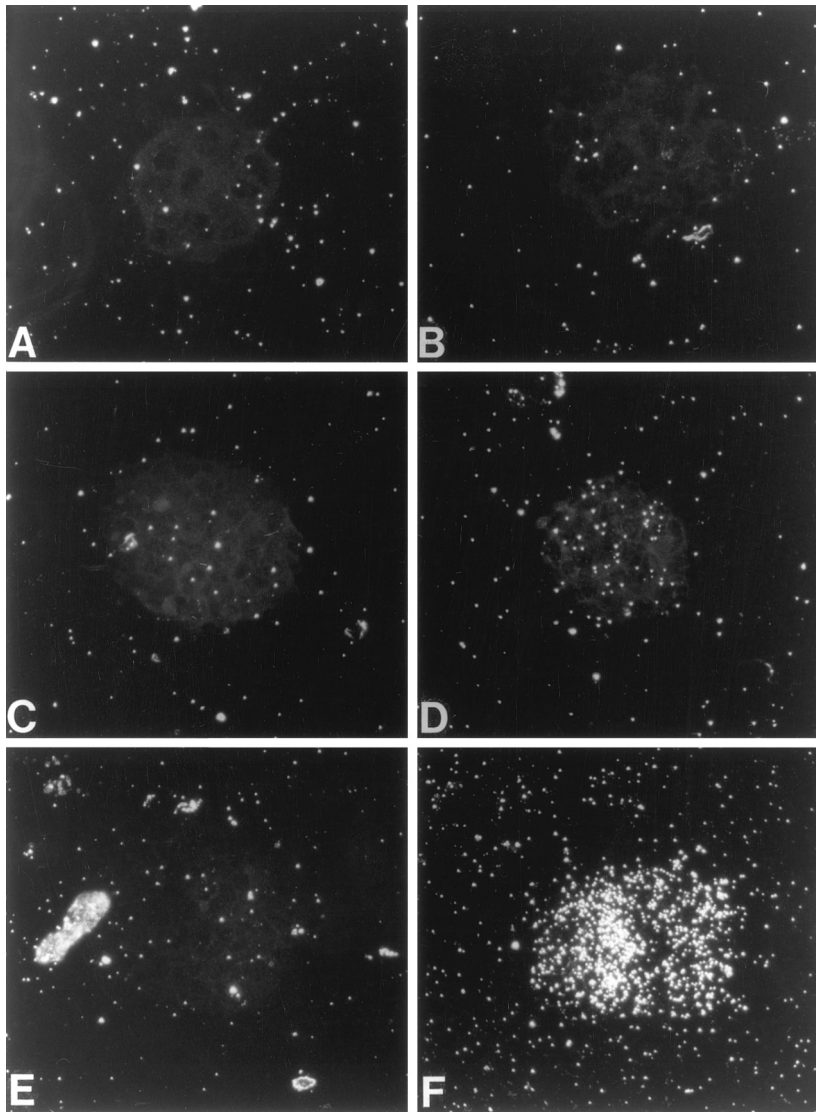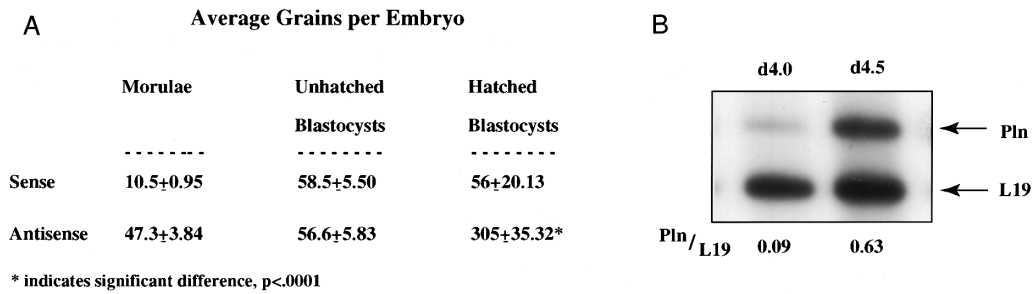
FIG. 2. *In situ* hybridization of perlecan mRNA in mouse embryos. *In situ* hybridizations of cytospun embryos were performed as described under Materials and Methods. Localized hybridization was not observed in the embryos in A, C, and E probed with sense cRNA. Perlecan mRNA, as detected by hybridization with antisense perlecan cRNA probe, was below detectable levels in Day 3 morulae (B), began to appear in unhatched Day 4 blastocysts (D), and was maximally expressed in hatched Day 4.5 blastocysts (F), at a time when initial adhesion to the uterus normally occurs *in vivo.* These experiments were repeated twice with 15–20 embryos each time from each developmental stage. Embryos chosen for illustration are representative. Magnification, 270×.

by heparinase predigestion strongly suggest HS is the predominant bFGF ligand detected in these experiments.

Increased levels of perlecan protein were detected in blastocysts at 12 hr (Fig. 5B) and 24 hr (Fig. 5C) after estradiol activation compared to dormant embryos maintained on progesterone alone (Fig. 5A). In addition to the perlecan core protein, HS expression, as detected by bFGF staining (Siedlak *et al.,* 1991), was elevated following estradiol activation, with a similar temporal pattern (Fig. 6). Thus, both perlecan core protein and HS polysaccharide expression were elevated in dormant blastocysts in response to estrogen activa-

tion. In contrast to protein expression, *in situ* hybridization analysis demonstrated that the transcript for perlecan was present in both dormant (Fig. 7B) and estrogen-activated (Fig. 7D) blastocysts. As observed with normal blastocysts, the transcript appeared to be present in the inner cell mass as well as the mural trophectoderm. Again, an absence of localized signal observed with a sense probe demonstrated the specificity of the antisense perlecan probe (Fig. 7F). These data, together with those obtained with normally implanting embryos, indicate that elevated expression of perlecan mRNA does not require estrogen activation. In

A

Average Grains per Embryo

	Morulae	Unhatched Blastocysts	Hatched Blastocysts
	--------	---------	--------
Sense	10.5+0.95	58.5+5.50	56+20.13
Antisense	47.3+3.84	56.6+5.83	305+35.32*

* indicates significant difference, p<.0001

B

FIG. 3. Quantitation of increase in perlecan mRNA expression. Cytospun embryos from *in situ* hybridization experiments were examined by light microscopy and total silver grains on the embryos were counted for 10–30 embryos in each case (A). Morula, unhatched blastocyst (Day 4.0), and hatched blastocyst (Day 4.5) stage embryos were examined with both sense and antisense probes. There is a significant difference, $P < 0.0001$, between the number of grains on hatched blastocysts using the antisense probe and all other groups of embryos. (B) RT–PCR analysis using unhatched blastocyst and hatched blastocyst RNA demonstrates a substantial increase in perlecan mRNA in comparison to mRNA for the constitutively expressed ribosomal protein, L19. The ratio of intensity of Pln bands to L19 bands, as determined by densitometry, was calculated as 0.09 for Day 4.0 embryos and 0.63 for Day 4.5 embryos. Approximately 60 embryos were used for each analysis.
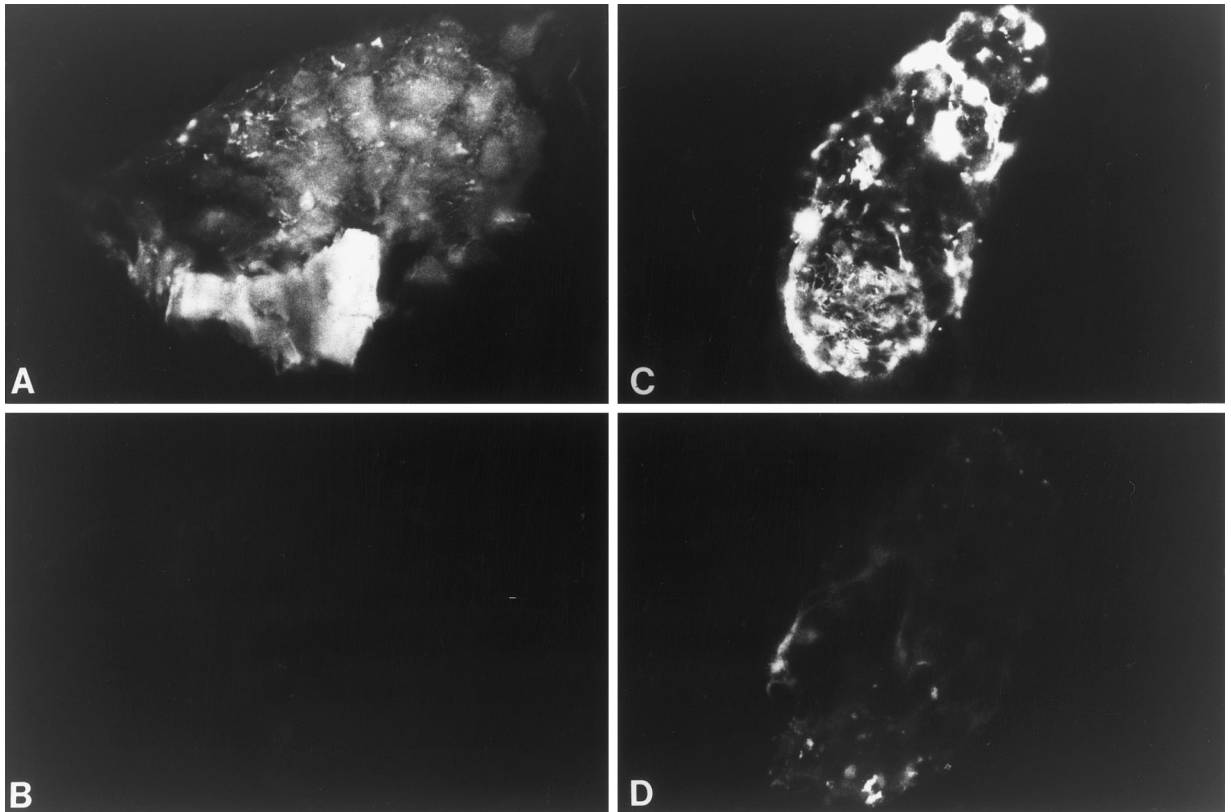
FIG. 4. Specificity controls for detection of perlecan and heparan sulfate in blastocysts. Normal hatched blastocysts were cytospun and probed with the rat antibody from ascites fluid to perlecan core protein (A), a rat ascites fluid control for the Pln antibody (B), recombinant bFGF to detect HS chains as described under Materials and Methods (C), or with bFGF after predigestion of the embryos with heparinase to remove HS chains (D). The signal present in the controls (B, D) is greatly decreased or absent versus that seen in the experimental embryos (A, C), demonstrating the probes specificity in both cases. Magnification, 430×.
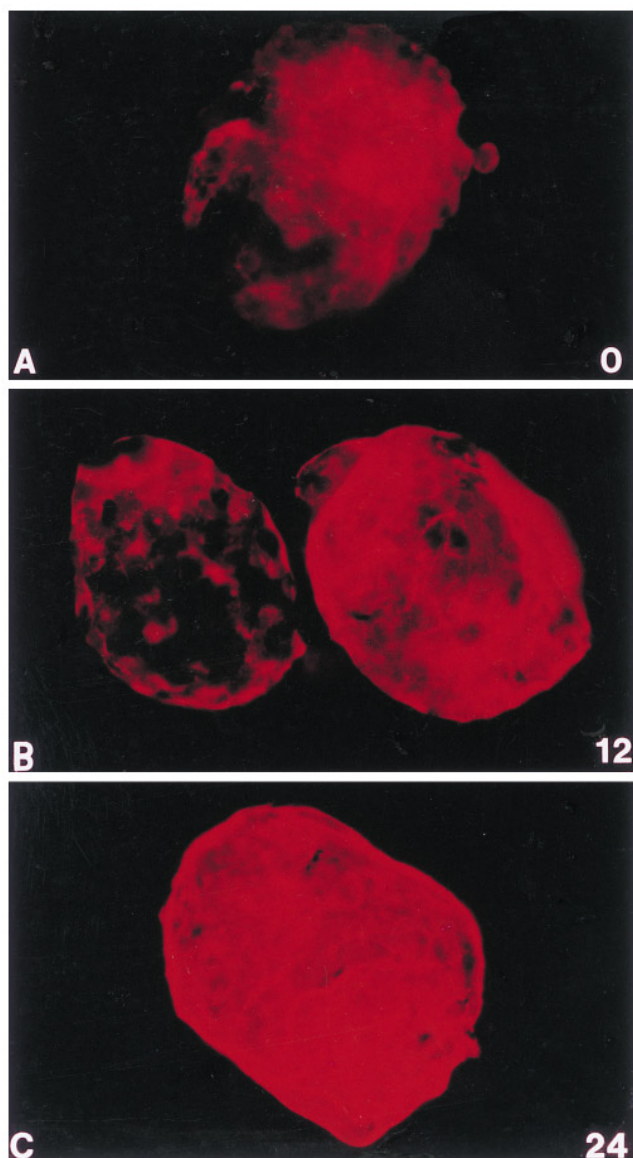
FIG. 5. Detection of perlecan core protein in delayed and activated mouse blastocysts by indirect immunofluorescence. Blastocysts collected during delay (A) or 12 (B) and 24 (C) hr after injection of nidatory estradiol were cytospun and probed with an antibody to perlecan as described under Materials and Methods. Low levels of perlecan protein was detected in the progesterone-maintained, dormant embryos, as shown in A; however, little definition of cell peripheries was evident in these embryos and this pattern of staining was similar to that observed with nonimmune rat IgG. In contrast, cell peripheries were evident in B and C, and the overall intensity of staining was markedly increased. Perlecan protein expression increased within 12 hr of estrogen activation (B) and persisted through 24 hr (C). Original magnification, 430×.

contrast, enhanced expression of perlecan core protein, as well as HS chains, requires additional developmental events that can be triggered by estrogen stimulation.

DISCUSSION

In the present study, we have established that perlecan mRNA is initially detected in unhatched Day 4 blastocysts. After hatching from the zona pellucida, the blastocysts become attachment competent and perlecan mRNA is abundantly expressed. This correlates with perlecan protein expression displayed on the trophectodermal surface of Day 4.5 blastocysts, but absent in Day 4 unhatched blastocysts (Carson *et al.,* 1993). Previous studies have detected extracellular matrix components in early preimplantation embryos prior to the formation of a basement membrane (see review by Leivo, 1983). These components include collagen IV, laminin, HSPG molecules, and nidogen (Leivo *et al.,* 1980; Sherman *et al.,* 1980; Wu *et al.,* 1983; Dziadek *et al.,* 1985; Dziadek and Timpl, 1985). After blastocyst formation, discontinuous patches of collagen type IV and fibronectin are exhibited on the inner aspects of the mural trophectoderm (Leivo *et al.,* 1980; Wartiovaara *et al.,* 1979). It is possible that these proteins expressed on the inner trophectodermal lining aid in the migration of parietal endoderm cells which deposit Reichert's membrane. Consistent with this view, we noted accumulation of perlecan mRNA in the inner cell mass region. In addition to deposition in the blastocyst interior, perlecan is present on the outer surface of trophectoderm cells at the time of attachment competence (Carson *et al.,* 1993). The presence of this HSPG on the outer surface of trophectoderm cells suggests a possible role for perlecan in embryo–uterine attachment.

In normal developing blastocysts, both perlecan protein (Carson *et al.,* 1993) and mRNA increase markedly during the transition from unhatched to hatched, attachment competent blastocysts. During delayed implantation *in vivo,* blastocysts hatch, but do not attach providing an additional test of potential HSPG function in the implantation process. It was of interest to determine if perlecan mRNA and protein were present in dormant blastocysts or induced in dormant blastocysts activated by estrogen (Huet-Hudson and Dey 1990; Paria *et al.,* 1992). Under these conditions, we detected perlecan protein in blastocysts 12 hr post-estrogen injection, with maximal expression at 24 hr. Since HS chains on the surface of blastocysts may be involved in interacting with uterine cell surface components to aid embryo adhesion with the luminal epithelium (see review Carson *et al.,* 1994), we also determined whether HS chains were correlated with the expression of perlecan protein. Results from the present study indicate that both perlecan core protein and HS expression are elevated in delayed blastocysts in response to estrogen. While it is possible that bFGF might bind to other ligands, e.g., FGF receptors, the heparinase digestion control (Fig. 4D) suggests that the majority of bFGF is binding to HS chains, located on the amino terminus of the Pln core protein, or one of the HS chains must interact with cell surface proteins. This would allow the remaining HS chains to interact with bFGF. There is also a possibility that the Pln core protein could be decorated with chondrotin sulfate (CS) chains or with no glycosaminoglycan chains at all (Couchman *et al.,* 1996). Com-
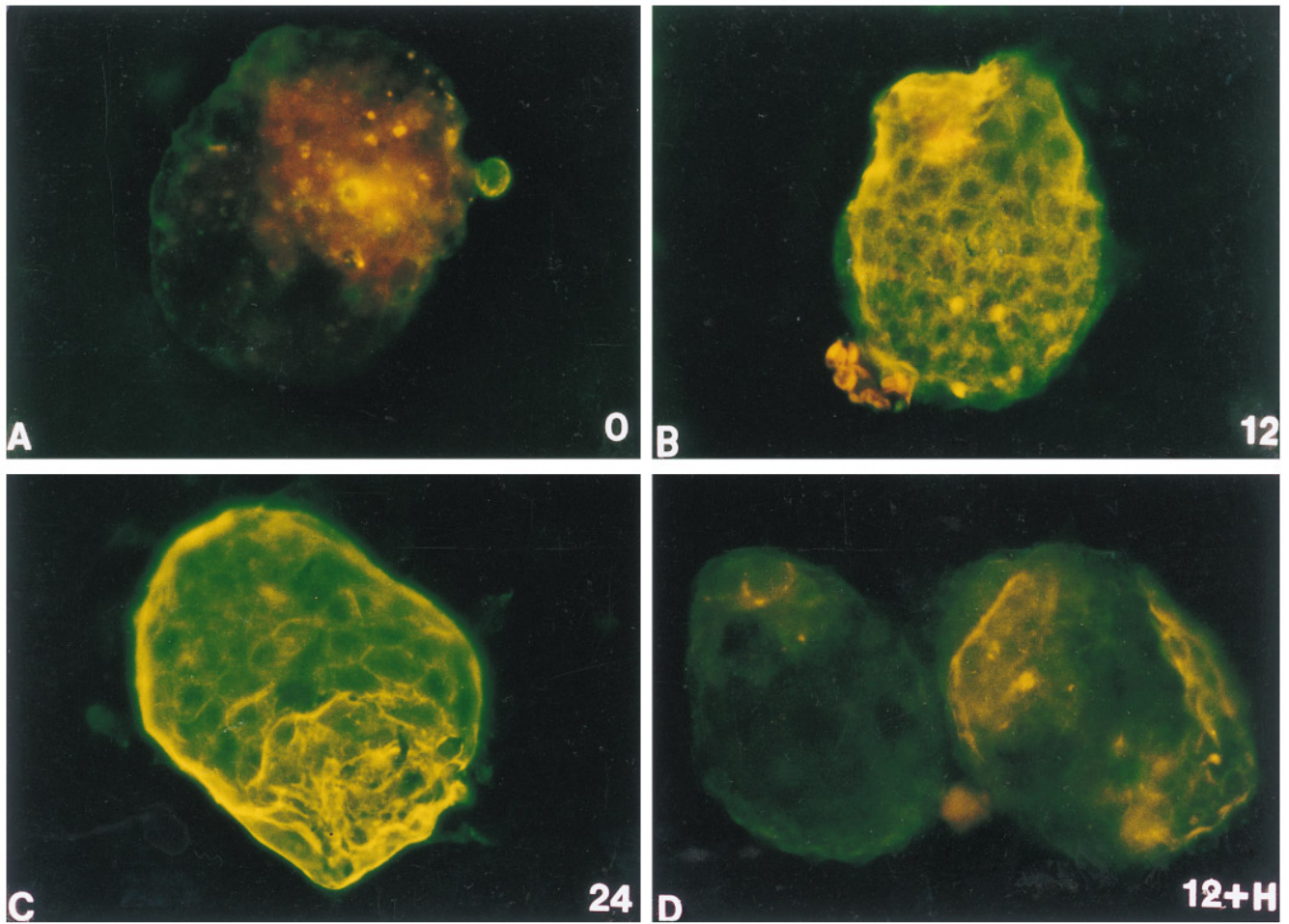
FIG. 6. Heparan sulfate (HS) chain expression increases in response to activation of dormant blastocysts. bFGF staining was used to detect HS chain expression in cytospun, dormant, or estrogen-activated blastocysts, as described under Materials and Methods. The figure shows staining of delayed embryos (A) and estradiol-activated (B and D, 12 hr; C, 24 hr) embryos incubated with bFGF in the absence (A, B, C) or the presence (D) of 1 mg/ml heparin. Note the increased staining of the activated embryos at 12 (B) and 24 (C) hr post-estrogen injection. This staining is markedly reduced by inclusion of heparin during the bFGF staining step (D). The staining observed in A and D is comparable to that observed if bFGF was omitted from the staining procedures. Original magnification, 430×.

pared to HS, relatively little CS is synthesized at this stage of development (Farach *et al.,* 1987). Furthermore, given the coordinate increase in expression of perlecan mRNA and protein with the HS chains on the blastocyst surface, it seems likely that, in this instance, Pln is decorated with HS chains.

In contrast, perlecan mRNA was expressed in blastocysts maintained in delay and was, therefore, present prior to estrogen activation and protein expression. Following activation, perlecan mRNA was still present in the inner cell mass and trophectoderm of activated blastocysts. Since perlecan mRNA is present prior to nidatory estrogen and perlecan protein expression increases after nidatory estrogen, it appears that perlecan may be subject to translational regulation. However, it is not clear whether the accumulation of perlecan mRNA was due to continued transcription or a

slower rate of perlecan mRNA degradation in the dormant blastocyst. This is in contrast to the finding of a rapid disappearance of EGF receptor mRNA in the blastocyst coinciding with the initiation of delayed implantation (Paria *et al.,* 1993a).

The expression of an HSPG molecule on the surface of attachment-competent blastocysts at the time of implantation suggests a role for this molecule in implantation. At the time embryos become attachment competent, the uterus must also be in a state of receptivity for implantation to occur (Psychoyos, 1986; Paria *et al.,* 1993b). Interactions between the trophectoderm and uterine luminal epithelial cells are critical for attachment in all species (Enders and Schlafke, 1974). Recently, the heparin binding EGF-like growth factor (HB-EGF) gene was found to be locally induced in mouse uterine luminal epithelial cells at the sites
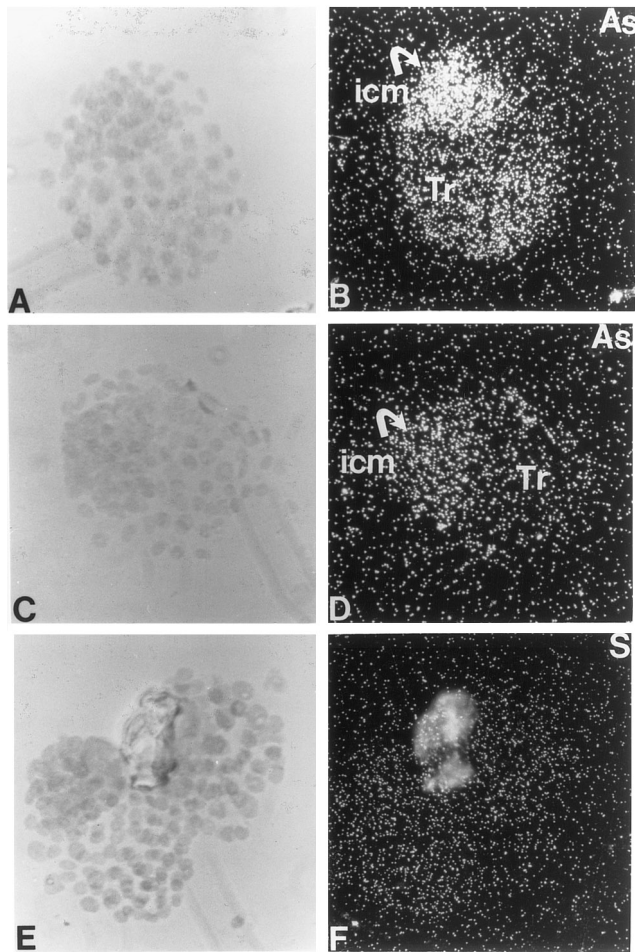
FIG. 7. Expression of perlecan mRNA in delayed and activated embryos. Bright-field (A, C, E) and dark-field photomicrographs of delayed (B) and estrogen-activated (D, F) embryos. Delayed embryos were obtained from mice injected with progesterone and assessed by *in situ* hybridization as described under Materials and Methods. Activated embryos were obtained from mice 24 hr after coinjection with progesterone and estrogen. Perlecan mRNA expression in delayed (B) and estrogen-activated embryos (D) is present in the mural trophectoderm (Tr) and inner cell mass (icm). (B and D) Demonstrate hybridization pattern of the antisense probe (AS) and F with the sense probe (S). These experiments were repeated twice with 10–15 blastocysts per experiment. Blastocysts chosen for illustration are representative of total blastocysts examined. Original magnification, 270×.

of blastocyst apposition at the time of attachment reaction (Das *et al.,* 1994). Since perlecan is present in attachment-competent blastocysts and HB-EGF is present on the apical surface of luminal epithelial cells, an interaction between HSPG and HB-EGF is possible for cell–cell interaction during implantation. This interaction may result in blastocyst attachment, enhanced HB-EGF actions on the blastocyst, or both (juxtacrine growth factor signaling). Other heparin binding proteins and growth factors may bind embryonic

HS chains of perlecan, promoting attachment and stimulate embryonic development. Description of HSPG expression in mouse embryos may provide a model to determine the precise role of these molecules in the implantation process.

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