Cyclin D3 in the mouse uterus is associated with the decidualization process during early pregnancy

S K Das^{1,2}, H Lim¹, B C Paria¹ and S K Dey¹

¹Department of Molecular and Integrative Physiology, Ralph L Smith Research Center, University of Kansas Medical Center, Kansas City, Kansas 66160-7338, USA

²Department of Obstetrics and Gynecology, Ralph L Smith Research Center, University of Kansas Medical Center, Kansas City, Kansas 66160-7338, USA

(Requests for offprints should be addressed to S K Das, Departments of Molecular and Integrative Physiology and Obstetrics and Gynecology, University of Kansas Medical Center, MRRC 37/3007, 39th and Rainbow Boulevard, Kansas City, Kansas 66160-7338, USA)

ABSTRACT

In the mouse, the attachment reaction between the blastocyst trophoctoderm and the receptive uterine luminal epithelium occurs at 2200–2300 h on day 4 of pregnancy and is rapidly followed by transformation of stromal cells into decidual cells (decidual cell reaction). This process can also be induced experimentally (deciduoma) by intraluminal oil infusion in the uterus on day 4 of pseudopregnancy. The decidual cell reaction is associated with up- and down-regulation of many genes in a cell-specific manner. Using mRNA differential display, we identified cyclin D3 as one of the genes that is upregulated in the uterus at the sites of blastocyst apposition during the attachment reaction. The levels of expression were low in the morning of days 1–4 as determined by Northern hybridization. *In situ* hybridization analysis showed that on days 1 and 2, signals were primarily localized in uterine epithelial cells, while signals were detected in both the stromal and epithelial cells on days 3 and 4. In contrast, with the initiation and progression of decidualization on days 5, 6 and 7, the levels of cyclin D3 mRNA were remarkably upregulated in stromal cells both at the mesometrial and the

antimesometrial poles. However, on day 8, signals were primarily localized in stromal cells at the mesometrial decidual bed. Implanting blastocysts on these days also expressed cyclin D3 mRNA. In the progesterone-treated delayed implanting mice, the uterine levels of cyclin D3 mRNA were modest at the sites of blastocyst apposition, but were upregulated with the onset of implantation by estradiol-17 β . However, the decidual expression of cyclin D3 mRNA was not dependent on the presence of blastocysts, since increased expression also occurred in experimentally induced deciduoma in the absence of blastocysts. The importance of cyclin D3 in decidualization was further examined in *Hoxa-10*-deficient mice which show defective decidualization. The expression of cyclin D3 mRNA in *Hoxa-10*(–/–) uteri on day 5 was severely compromised after application of a decidualogenic stimulus on day 4 of pseudopregnancy. Collectively, the results suggest that cyclin D3 could be important for the process of decidualization.

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INTRODUCTION

The differentiation of the uterus to support embryo development and implantation is primarily coordinated by progesterone (P₄) and estrogen (Psychoyos 1973, Paria *et al.* 1993a). In the rodent, the first conspicuous sign of implantation is an increased endometrial vascular permeability at the sites of blastocyst apposition (Psychoyos 1973, Paria *et al.* 1993a). This increased vascular permeability

coincides with the initial attachment reaction between the blastocyst trophoctoderm and uterine luminal epithelium (Enders & Schlafke 1967). In the mouse, this reaction occurs in the evening (2200–2300 h) of day 4 of pregnancy and is preceded by uterine luminal closure, which in turn results in an intimate apposition of the trophoctoderm with the luminal epithelium (Psychoyos 1973, Enders 1976, Enders & Schlafke 1967, Das *et al.* 1994). Ovariectomy in the morning of day 4 of pregnancy,

prior to preimplantation ovarian estrogen secretion, results in blastocyst dormancy and failure of implantation, a condition known as delayed implantation. Delayed implantation can be maintained by continued P_4 treatment, but is terminated by an injection of estrogen which induces blastocyst activation and initiation of the attachment reaction (Yoshinaga & Adams 1966, Paria *et al.* 1993a,b). The luminal closure and apposition occur during the P_4 -treated delayed implantation, but the attachment reaction (uterine blue reaction) and the subsequent events do not occur unless estrogen is provided (Psychoyos 1973, Nilsson 1974, Enders 1976).

The uterus is composed of heterogeneous cell types which respond uniquely to estrogen and P_4 . In the adult ovariectomized mouse uterus, estrogen stimulates proliferation of epithelial cells, while this process in the stroma requires both P_4 and estrogen (Huet-Hudson *et al.* 1989). A similar steroid hormonal regulation occurs during the peri-implantation period. On days 1 and 2, preovulatory ovarian estrogen directs epithelial cell proliferation. On day 3, P_4 from newly formed corpora lutea initiates stromal cell proliferation, which is further potentiated by preimplantation ovarian estrogen secretion on day 4. In contrast, epithelial cells cease to proliferate and become differentiated on this day. With the initiation of blastocyst attachment reaction, stromal cells at sites of blastocyst apposition undergo proliferation and differentiation into decidual cells (Huet-Hudson *et al.* 1989). In pregnant mice, the stimulus for decidualization is the implanting blastocyst. On day 5 at the beginning of decidual cell reaction, only stromal cells surrounding the implanting blastocyst proliferate. On day 6, the stromal cells in immediate proximity to blastocyst apposition cease to proliferate and form the primary decidual zone (PDZ). However, the cells outside the PDZ continue to proliferate and form the secondary decidual zone (SDZ) (Dey 1996). This process can also be induced artificially in the pseudopregnant or steroid hormonally prepared uterus by intraluminal infusion of oil (Lim *et al.* 1997).

The best known regulators of mammalian cell proliferation are the three D-type cyclins (D1, D2 and D3), commonly referred as G1 cyclins, which are considered to be the key regulators of cell proliferation and transition of G1/S phase in the cell cycle (Hunter & Pines 1994, Sherr 1994, Grana & Reddy 1995, Herzinger & Reed 1998). These proteins mediate their action after binding to catalytic subunits of specific cyclin-dependent kinases (cdk-2, -4, -5 and -6) (Sherr 1994, Grana & Reddy 1995). The association of D-type cyclins

to the holoenzymes of $p34^{cdk-4}$ and $p38^{cdk-6}$ is particularly important for these cyclin proteins to function as positive regulators of G1 progression, whereas the cyclin D- $p32^{cdk-2}$ holoenzyme complex is inactive during the G1 phase (Pines 1995). However, the latter is required later during the S-phase progression and S/G2 transition. The actions of D-type cyclins are negatively controlled by the retinoblastoma proteins (pRB) and the progression through G1 phase requires inactivation of the pRB protein by phosphorylation (Weinberg 1995).

During embryo implantation, the proliferation and differentiation events in the endometrial compartment are associated with up- or down-regulation of several genes in the uterus at the sites of blastocyst attachment. Using the technique of differential display of mRNAs, we sought to identify the genes which are primarily regulated at the time of implantation in the uterus. In the present investigation, we provide evidence that the expression of cyclin D3 mRNA is distinctly upregulated in the mouse uterus at the sites of implantation and the expression is correlated with the process of decidualization.

MATERIALS AND METHODS

Animals and tissue preparation

CD-1 (Charles River Laboratories, Raleigh, NC, USA) and *Hoxa-10*(-/-) were housed in the animal care facility at the University of Kansas Medical Center in accordance with NIH standards for the care and use of experimental animals. Adult females were mated with fertile or vasectomized males of the same strain to induce pregnancy or pseudopregnancy respectively (day 1=day of vaginal plug). For differential display studies, mice were killed on day 4 of pregnancy or pseudopregnancy at 1800 h and on day 4 of pregnancy at 2300 h (the time of the attachment reaction). Pregnant uteri were gently flushed with saline to recover blastocysts. Whole uteri at 1800 h or the implantation sites at 2300 h were collected and processed for RNA.

Pregnancy on days 1–4 was confirmed by recovering embryos from the reproductive tracts. On the evening of day 4 (2300 h) at the time of attachment reaction or on days 5–6, implantation sites were identified by monitoring the localized uterine vascular permeability at the sites of blastocysts after intravenous injection of Chicago Blue B solution in saline. Implantation sites were demarcated by discrete blue bands along the uterus (Paria *et al.* 1993a). On days 7 and 8, implantation sites are distinct and their identification does not

require any special manipulation. Mice on days 1–8 were killed at 0830–0900 h and their uteri were collected for RNA extraction and *in situ* hybridization. To induce and maintain delayed implantation, mice were ovariectomized in the morning (0800–0900 h) of day 4 of pregnancy and received daily injections of P_4 (2 mg/mouse) from days 5–7 (Yoshinaga & Adams 1966, Paria *et al.* 1993b). To terminate delayed implantation and to induce blastocyst activation, the P_4 -primed delayed implanting mice were given an injection of estradiol-17 β (E_2 , 25 ng/mouse) on the third day of the delay (day 7). Mice were killed 24 h after treatment with the respective steroid hormones and their uteri were collected for *in situ* hybridization. The first visually detectable implantation sites after blue dye injection normally become evident 18–24 h after an E_2 injection.

To induce artificial decidualization, sesame oil (25 μ l) was infused intraluminally in one uterine horn on day 4 of pseudopregnancy; the contralateral horn served as a control. Decidual cell reaction was confirmed by recording uterine weight and histological examination of uterine sections on days 7 and 8 of pseudopregnancy (Lim *et al.* 1997).

Differential display of mRNA

Differential display was performed as described previously with some modifications (Liang *et al.* 1993). Total RNA was extracted from tissues by a modified guanidine thiocyanate procedure (Han *et al.* 1987, Das *et al.* 1994). DNA-free RNA was obtained by treatment of total cellular RNA (50 μ g) with RNase-free DNase I (Sigma Chemical Co., St Louis, MO, USA) in the presence of placental RNase inhibitor (BRL, Gaithersburg, MD, USA) for 30 min at 37 °C. After phenol:chloroform (3:1) extraction, RNA was precipitated with 0.3 M sodium acetate and ethanol. The RNA pellet was dissolved in diethyl pyrocarbonate-treated water, and 1.0 μ g total RNA was used for reverse transcription (RT) reactions using three different one-base anchored primers as described by Liang *et al.* (1994), with a modification as follows: LHT₁₁C (5'-TGCCGAAGCTTTT'TTTT'TTTC-3'), LHT₁₁G (5'-TGCCGAAGCTTTT'TTTT'TTTTG-3') and LHT₁₁A (5'-TGCCGAAGCTTTT'TTTT'TTTT TTA-3'). The protocol for the RT reaction was the same as previously described (Paria *et al.* 1993b). The polymerase chain reaction (PCR) was performed in a reaction mixture containing 0.1 volume of the RT product, 1 $\times$ PCR buffer (10 mM Tris-HCl, pH 8.3; 2.5 mM MgCl₂ and 50 mM KCl), 600 μ M each of dATP, dTTP, dGTP and dCTP, 500 μ Ci/ml ³⁵S-dATP (1200 Ci/mmol, New

England Nuclear, Boston, MA, USA), 0.5 μ M of the respective primer: LHT₁₁C, LHT₁₁G or LHT₁₁A, 0.5 μ M of the arbitrary primer and 20 units/ml Ampli Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA). The arbitrary primers were designed as described by Liang *et al.* (1994), with some modifications as follows: LHAP1, 5'-TGCCGAAGCTTGATT GCC-3', LHAP2, 5'-TGCCGAAGCTTCGAC TGT-3' or LHAP3, 5'-TGCCGAAGCTTTTGGT CAG-3'. PCR reaction was performed in a Perkin-Elmer 480 thermocycler, using cycling parameters as follows: first cycle at 94 °C for 1 min, 40 °C for 4 min and 72 °C for 1 min followed by 35 cycles at 94 °C for 45 s, 60 °C for 2 min and 72 °C for 1 min. The amplified cDNAs were separated on a 6% DNA sequencing gel. After electrophoresis, gels were transferred to 3MM paper (Whatman, Maidstone, Kent, UK), dried and exposed to Kodak XAR-5 film for 36–72 h. Differentially displayed bands of interest were identified, cut out from the gel, and DNA was eluted as described (Liang *et al.* 1993). The individual eluted bands were reamplified by PCR using the appropriate primers and the reaction conditions as described above. Aliquots (5 μ l) of the reamplified bands were examined on 1% agarose gels stained with ethidium bromide to ensure that correct sizes of fragments were generated during the second round of amplification. The remaining amplified products were then used to clone into the pCR-Script SK(+) vector (Stratagene cloning systems, Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions.

Sequencing of cDNA subclones of PCR fragments, Northern and Southern blot analysis

Double-stranded DNA sequencing was carried out with either T7 or T3 primers using the SequiTherm long-read cycle sequencing kit LC (Epicenter Technologies, Madison, WI, USA) according to the manufacturer's specification. The nucleotide sequences were analyzed by the BLAST Sequence Similarity Searching Program (blastn) using the GenBank sequence databases available at the National Center for Biotechnology Information, National Institute of Health, USA.

Radiolabeled [³²P]cRNA antisense probes were generated from cDNA clones by T7 or T3 RNA polymerases and analyzed by Northern and Southern blot hybridization. Total RNA (6.0 μ g) was used for Northern blotting, and mouse tail DNA (5.0 μ g) digested with different restriction enzymes (BamHI, EcoRI, BamHI+EcoRI, HindIII, SmaI,

HindIII+SmaI) was subjected to Southern blotting. In general, both Northern and Southern blots were prehybridized and hybridized at high stringency using the same protocol as previously described (Das *et al.* 1994, 1995). Briefly, hybridization was carried out for 20 h at 68 °C in 3 × SET (1 × SET=150 mM NaCl, 5 mM EDTA and 10 mM Tris-HCl, pH 8.0), 20 mM phosphate buffer (pH 7.2), 250 µg/ml tRNA, 10% dextran sulfate and $\approx 2 \times 10^6$ counts/min 32 P-labeled antisense RNA probe/ml of the hybridization buffer. After hybridization, the blots were washed once in 1 × SSC, 0.1% SDS for 1 h at 68 °C, followed by a second wash in 0.3 × SSC, 0.1% SDS for 1 h at 68 °C; the hybrids were detected by autoradiography. Stripping of the hybridized probe before subsequent rehybridization for the Northern blots was achieved as previously described (Das *et al.* 1995). Each blot was first hybridized to a clone-specific probe and then to the ribosomal protein L-7 (rpL7) probe to confirm integrity, equal loading and blotting of RNA samples.

Hybridization probes

By differential display cDNA cloning, we obtained a clone (3CBo) of approximately 450 bp, exactly identical to the sequences of the 3'-end of the mouse cyclin D3 cDNA. In addition, by RT-PCR amplification, we obtained an additional cDNA fragment containing the sequences, 1682–1980 nts (GenBank accession # U43844) for mouse cyclin D3 using total RNA on day 5 of pregnancy and subcloned this fragment into the pCR-Script SK(+) vector. The authenticity of this clone was determined by sequence analysis. This clone (Cd3) was used to compare our results obtained with the original clone, 3CBo. For Northern hybridization, 32 P-labeled antisense cRNA probes were generated using either T7 or T3 RNA polymerases. For *in situ* hybridization, sense and antisense 35 S-labeled cRNA probes were generated. A part of the rpL7 cDNA (246 bp, nts 359–604; GenBank accession # M29016) was subcloned into pCR-Script SK(+) vector and used as a template for synthesis of 32 P-labeled antisense cRNA probe with T7 polymerase. The probes had specific activities of $\approx 2 \times 10^9$ d.p.m./µg.

In situ hybridization

In situ hybridization was performed as previously described (Das *et al.* 1994, Lim *et al.* 1997). Frozen uterine sections (10 µm) were mounted onto poly-L-lysine coated slides. When required, frozen sections were cut serially to detect the sites of blastocysts. Sections were fixed in 4% paraformaldehyde

in PBS for 15 min at 4 °C. Following prehybridization, sections were hybridized to 35 S-labeled antisense cRNA probes for 4 h at 45 °C. As negative controls, uterine sections were hybridized with the 35 S-labeled sense probes. After hybridization and washing, the sections were incubated with RNase-A (20 µg/ml) at 37 °C for 20 min. RNase-A resistant hybrids were detected within 5–7 days of autoradiography using Kodak NTB-2 liquid emulsion. The slides were post-stained with hematoxylin and eosin.

RESULTS

Detection of genes preferentially expressed in the mouse uterus at the time of attachment reaction by mRNA differential display

To identify genes that are preferentially expressed in the uterus during initial embryo–uterine interactions, the mRNA differential display method was employed to compare mRNA species expressed in the uterus prior to the attachment reaction at 1800 h with those expressed at the time of attachment reaction (2300 h) on day 4 of pregnancy. Several mRNA species were obtained which exhibited upregulation in the uterus with the initiation of embryo implantation. One of the eight genes found to be upregulated in the implantation sites at 2300 h was designated 3CBo (Fig. 1a). The induction of this gene was also confirmed by Northern blot hybridization using the same RNA samples used for differential display (Fig. 1b). The sequence analysis of 3CBo identified the clone as cyclin D3. To further verify the identity of this clone, we obtained an additional cDNA clone (Cd3) for mouse cyclin D3 by RT/PCR. Southern blot analysis of genomic tail DNA, using both clones (3CBo and Cd3) as probes showed the similar pattern of hybridization (Fig. 1c), providing evidence that both clones were of the same gene product. Subsequently, all Northern and *in situ* hybridization experiments were performed using the clone 3CBo.

Northern blot analysis of cyclin D3 mRNAs in the peri-implantation mouse uterus

As shown in Fig. 2a, Northern blot hybridization detected a transcript of cyclin D3 mRNA (2.3 kb) in the heart, kidney, lung, ovary, spleen, stomach, testis and uterus. During the peri-implantation period (days 1–8), the abundance of cyclin D3 mRNA was lower on days 1 and 2, modest on days 3–5, and highest on days 6–8 (Fig. 2b). The levels of a house-keeping gene, rpL7, did not show much variation during this period.

Cell-specific expression of cyclin D3 mRNA in the peri-implantation mouse uterus and in artificially induced deciduoma

If cyclin D3 is important for implantation/decidualization, it should exhibit temporal and cell-specific expression in the peri-implantation uterus. Thus, the distribution of cyclin D3 mRNA in the peri-implantation uterus was examined by *in situ* hybridization (Fig. 3). On days 1 and 2 of pregnancy, low levels of autoradiographic signals of cyclin D3 mRNA were noted in the luminal and glandular epithelia (Fig. 3a), whereas on days 3 and 4, low levels of signals were localized in both stromal and epithelial cells (Fig. 3b). On day 5, increased autoradiographic signals were noted primarily in the decidualizing stroma both at the mesometrial and the antimesometrial poles (Fig. 3c). The luminal and glandular epithelial cells also showed low levels of signals on this day. On days 6 and 7, strong positive signals were detected in the SDZ at both the mesometrial and the

antimesometrial poles (Fig. 3d). Moreover, low levels of signals were detected in the circular muscle layer. In contrast, with the progression of decidualization on day 8, signals in the SDZ at the antimesometrial pole decreased, but strong signals were retained in cells in the mesometrial decidual bed, including in a subpopulation of 'cords' of cells. These latter cells are perhaps the endothelial cells which participate in angiogenesis and placentation, since vascular endothelial growth factor receptors are also expressed in these cells (Chakraborty *et al.* 1995). The autoradiographic signals were also noted in the embryo (Fig. 3e).

In contrast to decidualization during normal pregnancy, the artificially induced deciduoma on day 6 of pseudopregnancy showed the accumulation of cyclin D3 mRNA in the decidual bed, primarily at the antimesometrial pole (Fig. 3f). The levels of accumulation were low compared with those in normal decidua (Fig. 3f vs 3d). In the case of artificially induced deciduoma on day 8, the accumulation of mRNA in decidual cells occurred in a similar fashion as that of normal pregnancy on day 8, although in the latter case the expression was more intense in decidualized stroma at the presumptive site of placentation (Fig. 3g).

FIGURE 1. (a) Differential display of uterine mRNAs on day 4 of pregnancy and pseudopregnancy. Three different RNA samples from day 4 uterus during pseudopregnancy (Pseudo preg) or pregnancy (Preg) at 1800 h, and the implantation sites (Imp. sites) at 2300 h on day 4 of pregnancy were compared by differential display using one base anchored modified primer, LHT11C (Liang *et al.* 1994), with the arbitrary primer, LHAP3 (Liang *et al.* 1994). The PCR amplified cDNA fragment (3CBo) was subsequently cloned for cyclin D3. (b) Northern blot hybridization of the uterine RNA samples used for differential display with a cRNA probe obtained from the 3CBo clone. Three different total RNA samples (6.0 µg) from day 4 uterus during pseudopregnancy or pregnancy as described above for differential display, were separated by agarose-formaldehyde gel electrophoresis, transferred, UV cross-linked to nylon membrane and hybridized sequentially to ³²P-labeled 3CBo and rpL7 antisense cRNA probes. (c) Southern blot hybridization of the 3CBo clone using the genomic tail DNA following digestion with different restriction enzymes. Genomic tail DNA (5.0 µg) was digested with BamHI, EcoRI, BamHI+EcoRI, HindIII, SmaI and HindIII+SmaI overnight at 37 °C, Southern blotted and hybridized to the 3CBo cRNA probe. The autoradiographic signals for the Southern hybridization were detected after 3 days of exposure.

FIGURE 2. (a) Northern hybridization of cyclin D3 mRNA in adult mouse tissues. Br, brain; Ht, heart; K, kidney; Lv, liver; Lu, lung; Ov, ovary; Sp, spleen; St, stomach; T, testis; and Ut, day 4, uterus on day 4 of pregnancy. (b) Northern blot hybridization of cyclin D3 mRNA in the peri-implantation mouse uterus. Total RNA (6 µg) was separated by agarose-formaldehyde gel electrophoresis, transferred, UV cross-linked to nylon membrane and hybridized sequentially to ^{32}P -labeled 3CBo and rpL7 antisense cRNA probes. Days of pregnancy (days 1–8) are indicated. Autoradiographic exposures were 6 h for cyclin D3 and 2 h for rpL7.

***In situ* hybridization of cyclin D3 mRNAs in the delayed implanting mouse uterus**

To examine whether the expression of cyclin D3 gene in the uterus requires the presence of an active blastocyst, *in situ* hybridization was carried out on uterine sections obtained from P_4 -treated delayed implanting mice or after the initiation of blastocyst activation for implantation by an E_2 injection. Low levels of hybridization signals for cyclin D3 were

detected in the epithelial and stromal cells surrounding dormant blastocysts during P_4 treatment (Fig. 4a,b). In contrast, following the termination of delayed implantation by an E_2 injection, the accumulation of cyclin D3 mRNA became distinctly evident in the decidualizing stromal cells surrounding the implanting blastocysts (Fig. 4c,d). It should be recalled that the attachment reaction (blue reaction) occurs at 18–24 h after E_2 injection in P_4 -treated delayed implanting mice. Thus, the cyclin D3 gene expression at 24 h following termination of delayed implantation was similar to that detected in normal pregnancy on the morning of day 5 (Fig. 4c,d vs 3c).

Cyclin D3 expression in *Hoxa-10*(–/–) uteri

Although the results obtained thus far suggested that the expression of cyclin D3 is induced in parallel with the onset of the decidualization during normal pregnancy or pseudopregnancy, whether this expression is essential for decidualization is not known. In furthering this question, we examined the expression of cyclin D3 in *Hoxa-10*(–/–) mice, which exhibited defective implantation and decidualization (Benson *et al.* 1996). Intraluminal oil infusions were performed in both horns on day 4 of pseudopregnancy in both the wild-type and *Hoxa-10*(–/–) mice. The expression of cyclin D3 mRNA was examined in one horn on day 5, and the extent of decidualization of the remaining contralateral horn was examined on day 8. As shown in Fig. 5, the expression of cyclin D3 mRNA was severely affected in *Hoxa-10*(–/–) uteri (Fig. 5a) as compared with that in wild-type (+/+) uteri (Fig. 5b). This reduced expression of cyclin D3 in the *Hoxa-10*(–/–) uteri was correlated with reduced decidualization of the remaining horn on day 8 (30% of wild-type) (data not shown). The results suggest that the decreased expression of cyclin D3 could be a cause for the failure of decidualization.

DISCUSSION

The technique of differential display of mRNA is being extensively used to identify differential expression of genes under specific experimental or physiological conditions (Liang & Pardee 1992). Applying this technique, we searched for genes which could be involved in implantation and decidualization processes. Cyclin D3, one of the eight genes showing upregulation in the uterus at the sites of implantation, was primarily expressed in the decidualizing stromal cells during implantation.

FIGURE 3. *In situ* hybridization of cyclin D3 mRNA in the peri-implantation mouse uterus and in the experimentally induced deciduoma on days 6 and 8. Darkfield photomicrographs of representative longitudinal sections of uteri at 0900 h on days 1 (a) and 4 (b) are shown at $\times 100$. Darkfield photomicrographs of cross-sections of uteri on days 5 (c), 6 (d) and 8 (e) of pregnancy and of deciduoma on days 6 (f) and 8 (g) of pseudopregnancy are shown. Pictures are shown at $\times 40$ for days 5 and 6, and at $\times 20$ for day 8. bl, blastocyst; le, luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium; pdz, primary decidual zone; sdz, secondary decidual zone; M, mesometrial pole; AM, antimesometrial pole; em, embryo.

This is the first report demonstrating cell-specific expression of cyclin D3 mRNA in the uterus during early pregnancy in the rodent and provides evidence that uterine cyclin D3 could be important for implantation/decidualization. The expression of this gene in decidualizing stromal cells is not dependent upon the presence of blastocysts, because the induction also occurs in experimentally induced deciduoma.

The uterus is the major target for estrogen and P_4 actions. Although these steroids primarily direct uterine cell proliferation and differentiation during the peri-implantation period in a spatiotemporal manner, the molecular and cellular mechanisms involved in these processes are poorly understood. In the mouse, an extremely low level of estrogen is required to elicit implantation/decidualization in a

P_4 -primed uterus, suggesting that estrogen effects in the uterus are amplified by other local mediators. This speculation is consistent with recent observations of uterine cell-specific expression of several growth factors and their receptors during implantation (reviewed in Das *et al.* 1995). The expression of cyclin D3 mRNA in the uterine epithelium on day 1 of pregnancy under the influence of preovulatory estrogen is correlated with epithelial cell proliferation, while stromal cell proliferation under the influence of rising P_4 and preimplantation ovarian estrogen secretion on day 4 could be associated with stromal cell proliferation. The expression of cyclin D3 mRNA in the decidualizing stromal cells suggests that this gene product is another factor involved in potentiating estrogen effects in the uterus during implantation/

FIGURE 4. *In situ* hybridization of cyclin D3 mRNAs in uteri from P_4 -treated delayed implanting mice before and after an E_2 injection. Bright- and dark-field photomicrographs of longitudinal uterine sections are shown at $\times 100$. (a and b) Representative sections from P_4 -treated delayed implanting mice hybridized with cyclin D3 cRNA probes. (c and d) Representative sections from P_4 -treated delayed implanting mice after an E_2 injection showing signals for cyclin D3 mRNA at the implantation sites. bl, blastocyst; le, luminal epithelium; ge, glandular epithelium; s, stroma.

decidualization. Indeed, there is evidence that the expression of D-type cyclins and the cyclin-dependent kinases are regulated by estrogen in the rodent uterus (Altucci *et al.* 1997, Geum *et al.* 1997, Prall *et al.* 1997). The heightened expression of cyclin D3 mRNA in decidualizing stroma could be due to combined effects of estrogen and P_4 in the presence of a deciduogenic stimulus. This finding suggests that the regulation of this gene in the uterus could be modulated by estrogen and/or P_4 in a cell-specific manner. This is consistent with cell-specific expression of cyclin D3 mRNA in the ovariectomized uterus after estrogen and/or P_4 treatments (data not shown).

Normally, estrogen induces progesterone receptors (PR) in the uterus. However, the nature of the interactions between these two steroids which trigger the implantation and decidualization processes remain undefined. The dramatic downregu-

lation of the nuclear estrogen receptor- α (ER- α) (Das *et al.* 1996) and very low levels of ER- β (our unpublished observation) in the deciduum suggest that the participation of nuclear ERs in the process of decidualization is limited (Das *et al.* 1996). This is consistent with the observation that P_4 , not estrogen, is an absolute requirement for sustained decidualization in the rodent. However, it is to be noted that estrogen is essential for the initiation of the implantation in the P_4 -primed uterus (reviewed in Dey 1996).

The expression of cyclin D3 mRNA in the rapidly proliferating stromal cells (decidual cell reaction) surrounding the blastocyst on day 5 and in the SDZ, but not in the PDZ, on days 6 and 7 suggest that this gene is involved in extensive cell proliferation during decidual cell reaction. In contrast, low levels of cyclin D3 mRNA in the SDZ at the antimesometrial pole on day 8 are associated

FIGURE 5. *In situ* hybridization of cyclin D3 mRNA in Hoxa-10(-/-) and in wild-type mouse uteri. Hoxa-10(+/+) or Hoxa-10(-/-) mice received intraluminal oil infusion in both horns on day 4 of pseudopregnancy. On day 5, one horn was removed and *in situ* hybridization was performed for cyclin D3, the remaining horn was left untouched for recording decidualization. Darkfield photomicrographs of representative longitudinal sections of uteri from Hoxa-10(-/-) (a) or Hoxa-10(+/+) (b) are shown at $\times 40$. le, luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium.

with diminishing cell proliferation. This suggestion is supported by our observation in Hoxa-10(-/-) mice. Hoxa-10 is expressed in the stroma on day 4 and in decidualizing stroma on day 5 of pregnancy, suggesting that this homeobox gene is important for stromal cell proliferation and subsequent decidualization. Indeed, the null mutation of this gene causes decidualization failure in the mouse (Benson *et al.* 1996, Ma *et al.* 1998). Reduced levels of cyclin D3 mRNA in Hoxa-10(-/-) uteri after appli-

cation of a deciduogenic stimulus suggests that the expression of this D-type cyclin could be associated with Hoxa-10 effects during decidualization. Alternatively, reduced uterine expression of cyclin D3 in these mice could be secondary to decidualization failure.

The expression of cyclin D3 mRNA at the mesometrial pole on day 8 suggests that this protein could be involved in angiogenesis and placentation. D-type cyclins are involved in cell growth and are

known to possess distinct signaling pathways (Mann *et al.* 1997, Sun *et al.* 1996). A recent report indicates that the expression of cyclin D2 is critical for the hormone-induced proliferation and differentiation of granulosa cells (Robker & Richards 1998). A distinct switch in the expression of cyclin D3 to D1 during trophoblast giant cell differentiation in the rodent has also been reported (MacAuley *et al.* 1998). Although D-type cyclins are expressed in an overlapping and apparently redundant fashion in the proliferating tissues (Peters 1994, Sherr 1994), spatiotemporal expression of cyclin D1 and cyclin D2 in the peri-implantation uterus has not yet been examined.

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