Progesterone blocks estrogen-induced DNA synthesis through the inhibition of replication licensing

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In the uterus, progesterone (P_4) acts early in G_1 as a physiological inhibitor of estradiol-17β (E_2)-induced epithelial cell proliferation. Gene expression profiling of uterine epithelial cell RNA isolated 3 h after hormonal treatment of ovariectomized mice revealed the co-coordinate down-regulation by P_4 of >20 genes whose functions are associated with DNA replication. This group included all of the minichromosome maintenance (MCM) proteins that are required for DNA replication licensing. E_2 regulated loading of these MCM proteins onto chromatin in parallel with its induction of DNA synthesis. E_2 caused this chromatin loading by retention of MCM proteins in the nucleus and through the induction of the loading factor Cdt1, which is necessary for the MCM heterohexamer to bind to the origin of DNA replication. P_4 dramatically reduced the binding of the MCMs to chromatin by a number of mechanisms. First, MCM mRNA and protein abundance was down-regulated. Second, P_4 inhibited the E_2 induction of Cdt1. Third, P_4 treatment sequestered the normally nuclear MCM proteins into the cytoplasm. This reduced MCM binding resulted in the complete inhibition of E_2-induced DNA synthesis by P_4. These data reveal mechanisms not only for female sex steroid hormone action but also in the regulation of DNA replication licensing.

DNA replication | minichromosome maintenance | uterus | cell cycle | microarray

In uteri of mice and women, the female sex steroid hormones estradiol-17β (E_2) and progesterone (P_4) interact to regulate cell proliferation (1). In mice, E_2 synthesized at proestrus stimulates uterine luminal and glandular epithelial cell proliferation (2). In contrast, P_4 synthesized by corporea lutea formed after mating blocks the E_2-induced epithelial cell proliferation but permits E_2 to induce a single wave of stromal cell division (3, 4). P_4 also induces differentiation of these epithelial cells so that they are receptive to the hatched blastocysts in order that implantation can proceed. These uterine cellular dynamics can be mimicked by administration of exogenous E_2 and P_4 to ovariectomized mice in regimens that parallel their physiological secretion (2, 5, 6). This hormone treatment provides a controllable model in which to study the mechanism of E_2 induction of cell proliferation in target epithelial tissues and also the action of a physiological inhibitor of cell division, in this case P_4. These studies are highly relevant because exposure to the mitogenic effects of E_2 is thought to be the major risk factor for the development of endometrial and breast cancer (7).

DNA replication needs to be a highly regulated process, and most cells seek to ensure one (and only one) round of replication per cell cycle. DNA synthesis is initiated by the formation of the prereplicative complex (pre-RC) at origins of replication during early G_1, a process known as replication licensing (8, 9). Pre-RC formation involves the sequential assembly of >20 replication factors in a process that is largely conserved from yeast to human. The origin of DNA replication is first marked by the origin recognition complex, a heterohexameric complex, which serves as a scaffold for the loading of additional proteins. The binding of Cdc6 and Cdt1 to the origin recognition complex facilitates the loading of the minichromosome maintenance (MCM) proteins (MCM2–7) onto the replication origins in stoichiometric amounts to form the pre-RC. Once loaded, origin firing can be activated by two kinases, Cdc7-Dbf4 kinase and cyclin-dependent kinase (CDK), to commence DNA synthesis. This origin firing involves the orderly recruitment of additional replication factors including Cdc45, proliferating cell nuclear antigen (PCNA), and DNA polymerase α, the latter of whose primase activity initiates DNA replication. Pre-RC formation confers competence on the origins to replicate only once in S phase. Studies in different organisms suggest that this process of pre-RC formation is a key mechanism that coordinates DNA replication with cell-cycle division.

Our previous studies in the adult mouse uterus have identified the nuclear localization of the cell-cycle regulatory molecule cyclin D1 as a major event in steroid hormone regulation of epithelial cell proliferation (10). E_2 causes the nuclear accumulation of cyclin D1 together with its CDK partners, CDK4 and CDK6, to phosphorylate the retinoblastoma family of proteins. Thereafter, cyclin E/CDK2 is activated, cyclin A is induced, and the cells are propelled into DNA synthesis. P_4 pretreatment completely blocks this cyclin D1 nuclear localization, and, as a consequence, retinoblastoma phosphorylation and cell-cycle progression are inhibited. Our recent studies have identified the mechanism that links the antagonistic actions of these two hormones and the subcellular localization of cyclin D1 (11). In the uterine epithelial cells E_2 causes an inhibitory phosphorylation of GSK-3β at Ser^9 through phosphatidylinositol 3-kinase activation of AKT. The inhibition of phosphatidylinositol 3-kinase activity by P_4 results in active GSK-3β activity that phosphorylates cyclin D1 at Thr^{286} and its export from the nucleus (12). These data were confirmed by direct inhibition of GSK-3β that reversed the cyclin D1 accumulation into the nucleus in response to E_2 even in the presence of P_4. This cyclin D1 nuclear accumulation caused progression of the cells toward S phase as shown by the induction of the S phase markers PCNA and Ki67. However, despite the induction of these markers, no true DNA synthesis could be detected by the incorporation of BrdU. These data suggest that a second pathway (independent of the retinoblastoma one) needs to be activated by E_2 for DNA synthesis to be initiated and that this pathway is also inhibited by P_4.

In this study, we used analysis of gene expression patterns of the uterine luminal epithelial cells after E_2 and P_4E_2 treatment to attempt to identify pathways that might be regulated by E_2 and inhibited by P_4. Remarkably, >20 genes associated with DNA replication were rapidly down-regulated by P_4. Noticeably among this group were all six MCM proteins, suggesting that replication licensing is a key regulatory point in sex steroid hormone regulation of cell proliferation. This article shows that this is the case, with P_4 regulating the abundance, activity, and cellular localization of MCM proteins indicating mechanisms controlling replication licensing in the uterine epithelium by sex steroid hormones *in vivo*.

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Abbreviations: MCM, minichromosome maintenance; CDK, cyclin-dependent kinase; QRT-PCR, quantitative real-time PCR; pre-RC, prereplicative complex; E_2, estradiol-17β; P_4, progesterone; IHC, immunohistochemistry; PCNA, proliferating cell nuclear antigen.

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Table 1. List of genes associated with cell cycle and DNA replication whose transcripts are down-regulated by P_4

Name	Symbol	ID*	Average $M^{\dagger}$
DNA pre-RC licensing genes			
MCM-deficient 4 homolog	*Mcm4*	AA259788	−1.444577042
		AI325074	−1.371608965
MCM-deficient 5	*Mcm5*	AA031056	−1.286345349
		AW536273	−1.263153464
MCM-deficient 3	*Mcm3*	AW536712	−1.236508743
MCM-deficient 2 mitotin	*Mcm2*	AA011839	−1.02171739
		AW553939	−1.004775339
MCM-deficient 7	*Mcm7*	AA064230	−0.9295692
MCM-deficient 6	*Mcm6*	AA016759	−0.883079258
DNA replication genes			
Flap structure-specific endonuclease 1	*Fen1*	AW538437	−1.114619134
PCNA	*Pcna*	AW545318	−0.990405638
Ligase I, DNA, ATP-dependent	*Lig1*	C77364	−0.91547759
Chromatin assembly and modification genes			
Chromatin assembly factor 1, subunit B (p60)	*Chaf1b*	AW547084	−1.468701553
		AA387585	−1.070805707
		W64706	−0.91775875
Helicase, lymphoid specific	*Hells*	AW555541	−1.397495102
Chromatin assembly factor 1, subunit A (p150)	*Chaf1a*	AU016029	−1.196072702
Protein phosphatase 1, regulatory (inhibitor) subunit 1B	*Ppp1r1b*	AU040756	−1.194267115
Other cell-cycle-related genes			
Stratifin	*Sfn*	AA009229	−1.556398182
		AU043198	−0.915968886
		AW536416	−1.423732936
Antigen identified by monoclonal antibody K_i 67	*Mki67*	AW536416	−1.423732936
E2F transcription factor 1	*E2f1*	AA396123	−1.206181967
CDK-like 2 (CDC2-related kinase)	*Cdkl2*	AA414632	−1.072730785
Cyclin D1	*Ccnd1*	AU015041	−1.01337925
		AI894115	−0.940600249
MAD2 (mitotic arrest deficient, homolog)-like 1 (yeast)	*Mad2l1*	AA174630	−0.993491411
		C87726	−0.913837172
Thymidine kinase 1	*Tk1*	AW544533	−0.959189527
Myeloblastosis oncogene	*Myb*	AA267899	−0.977284174
Myeloblastosis oncogene-like 2	*Mybl2*	AW555561	−0.916437637
Cyclin E1	*Ccne1*	AA465987	−0.893640476

*NCBI nonredundant BLAST.
$^{\dagger}\log_2 P_4E_2/E_2$.

Results

Microarray Analysis Revealed That Genes Associated with DNA Replication Are Down-Regulated by P_4. To study the molecular mechanism of P_4 inhibition of the E_2-induced proliferation in the uterine epithelium, a cDNA microarray comparison was performed between mice treated with E_2 and P_4E_2. Ovariectomized adult mice were pretreated with P_4 for 3 days before a final combined injection of P_4 and E_2 on the fourth day in regimens that parallel the physiological concentrations (13). The RNA samples obtained were compared with mice given E_2 treatment that mimicked the preovulatory surge of the estrus cycle (14). Given these different responses in different cell types of uteri and because P_4 exerts its inhibitory action on the uterine epithelium within the first 3 h (15), we isolated luminal epithelial cells at >95% purity (16, 17) over a time window of 3–4 h after P_4E_2 or E_2 treatment and prepared RNA samples from groups of three mice. These samples were cross-compared by using cDNA microarrays as described in *Supporting Methods*, which is published as supporting information on the PNAS web site.

After LOWESS normalization, data analysis, and data cutoffs by the filter criteria described in *Materials and Methods*, our analysis revealed that of the 27,396 gene array transcripts, 222 (0.810%) and 208 (0.759%) gene sequences were up-regulated and down-regulated, respectively, in the uterine epithelia after the treatment of P_4E_2 compared with E_2. The complete list of transcripts whose abundance was decreased is shown in Table 2, which is published as supporting information on the PNAS web site, and those 222 gene transcripts that were up-regulated have been discussed in another publication (17). Our analysis in the down-regulated group focused on the genes with known function, and this resulted in removal of 82 unknown genes such as EST and RIKEN cDNA gene sequences. To gain insight into the biological significance of the down-regulation in gene expression, classification of the remaining down-regulated gene sequences by Gene Ontology annotations showed that 32 of 126 known sequences were involved in cell-cycle control, DNA replication, and modification processes (Table 1). This percentage is a significant enrichment of this function category (25.3%). Well defined cell-cycle-related genes, such as *Mki-67*, *E2f1*, *Ccnd1*, *Ccne1*, and *Tk1*, are present. Striking is the coordinate down-regulation of all MCM-deficient (*Mcm2–7*) genes. These data were further validated because *Mcm2*, *Mcm4*, and *Mcm5* are

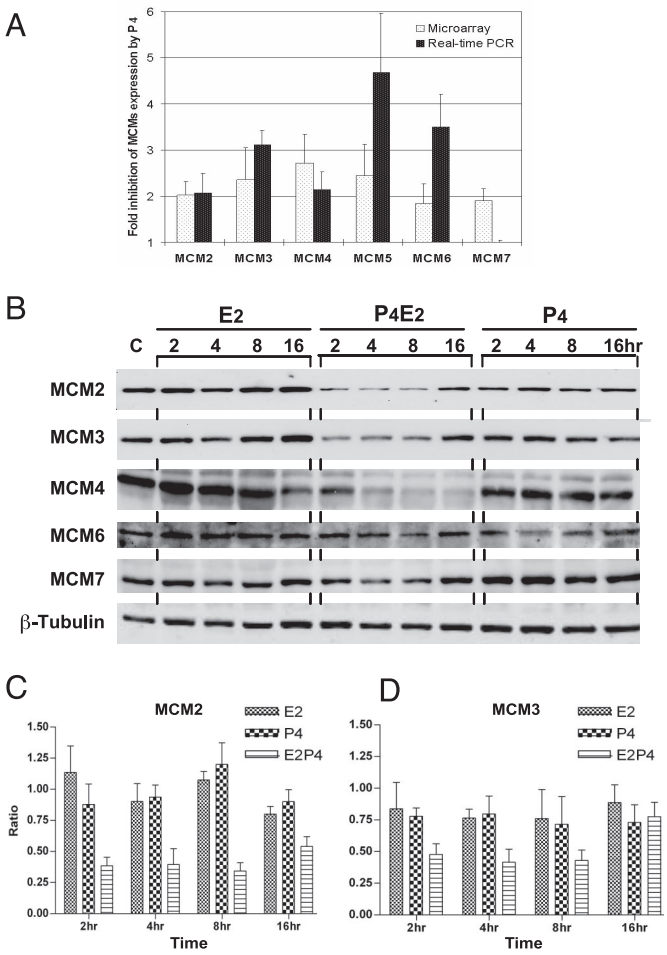

Fig. 1. Hormonal regulation of *Mcm* expression. (*A*) Validation of cDNA microarray data by QRT-PCR by using the gene-specific primers shown in Table 3, which is published as supporting information on the PNAS web site. The *y* axis shows the amplitude of down-regulation determined by Microarray or QRT-PCR of the genes shown on the *x* axis. Data shown are the mean ± SD of three experiments and show significant down-regulation by P_4 compared with E_2 treatment. (*B*) Synergistic down-regulation of MCM2, MCM3, and MCM4 protein concentration by E_2 and P_4. Ovariectomized mice were killed at the times shown after the different hormone treatments as indicated. Equivalent amounts (60 μg) of protein isolated from the total epithelial cell lysates were separated by SDS/PAGE, blotted onto Nylon membranes, and probed with the antibodies against the proteins listed on the side. Detection of β-tubulin with an anti-β-tubulin antibody was used as a protein loading control. The Western blots shown are representative of those obtained from three independent experiments. (*C* and *D*) Densitometric analysis of the expression of MCM2 (*C*) and MCM3 (*D*) after the various treatments shown. Data shown are the mean ± SD of three experiments.

repeated twice on the chips by different cDNA sequences. Additionally, there was a concomitant reduction of transcripts of DNA elongation genes including *Fen1*, *Pcna*, and *Lig1*. Finally, transcripts of a number of genes involved in chromatin assembly and modification were also down-regulated. Examples included *Chaf1a*, *Chaf1b*, *Hells*, and *Ppp1r1b* (Table 1). Collectively, our data indicate that the entire DNA replication process in the uterine luminal epithelium, including DNA prereplication assembly, replication elongation, and nucleosome assembly and modification, is a major target of P_4 in early G_1 phase.

Down-Regulation of MCM Protein Requires the Synergistic Action of P_4 and E_2. MCM family members are highly conserved proteins found in all eukaryotes and play an important role in DNA replication licensing (8, 9). We focused on this family of proteins and the assembly of the pre-RC in the uterine epithelium. First, we performed quantitative real-time PCR (QRT-PCR) analysis for all *Mcm* genes to confirm the validity of the microarray result. As shown in Fig. 1*A*, there was similar inhibition pattern by P_4 of 2- to 4-fold on *Mcm* transcript expression as determined by the microarrays and QRT-PCR, although the down-regulation of *Mcm5* and *Mcm6* was estimated to be greater by QRT-PCR than by the microarrays. In each case, this down-regulation was statistically significant. However, *Mcm7* was an exception because QRT-PCR did not confirm the microarray result. To determine which of the two was correct, two other pairs of primers to *Mcm7* were designed for QRT-PCR, and both of these indicated that there was no change in *Mcm7* expression.

To examine the hormonal regulation of the MCM proteins in the uterine luminal epithelium, we treated three independent cohorts of mice with the appropriate hormonal regimens followed by the preparation of epithelial cell lysates over a 16-h period after treatment that were subjected to Western blotting. Compared with the hormone-primed but otherwise untreated control mice (referred to as controls), the concentration of MCM2, MCM3, MCM4, MCM6, and MCM7 proteins barely showed any change after E_2 or P_4 treatment alone (Fig. 1*B*). In contrast, P_4 pretreatment in combination with E_2 caused an ≈40% down-regulation of MCM2 (Fig. 1 *B* and *C*) and MCM3 (Fig. 1 *B* and *D*) between 2 and 4 h after treatment. A similar down-regulation of MCM4 was also seen (Fig. 1*B*) However, the protein concentrations of MCM6 and MCM7 did not show a significant reduction after P_4E_2 treatment. There was a similar kinetic in the regulation of MCM2 and MCM3, with a drop 2 h after treatment to reach a nadir at ≈8–9 h, followed by a progressive return until 16 h. However, P_4E_2 induced a gradual decrease of MCM4 from 2 h until 16 h. These data show that E_2 and P_4 synergize to down-regulate MCM2, MCM3, and MCM4 protein abundance, although with different kinetics.

P_4 and E_2 Regulate MCM Cellular Distribution. In metazoans, MCM are nuclear proteins organized in heterohexameric complexes that are primarily retained in the nucleus in a cell-cycle-independent manner (9). Unexpectedly, when we analyzed the cellular distributions of the MCMs by immunohistochemistry (IHC) of transverse sections of uteri, we found that the different hormone treatments altered their cellular localization. MCM3 had a predominant nuclear localization in the uterine epithelium of control mice, although there was some weak cytoplasmic staining (Fig. 2*a*). E_2 treatment caused, as determined by the intensity of the IHC stain, a recruitment of this minor cytoplasmic fraction into the nucleus, and by 11 h after E_2 treatment there was prominent nuclear staining with very little detected in the cytoplasm (Fig. 2*b*). In contrast, P_4 treatment alone compared with the control reduced the nuclear MCM3 signal and concomitantly increased it in the cytoplasm (Fig. 2*c*). In the P_4E_2-treated uteri more MCM3 signal was detected in the cytoplasm within 1–2 h together with a concordant reduction in the nuclear signal (Fig. 2*d*). Consistent with the decrease in total protein caused by P_4E_2 treatment, the intensity of MCM3 staining in both the nucleus and cytoplasm was decreased by 4 h and reached its lowest level ≈8 h after hormone treatment (Fig. 2*e*), followed by a gradual increase in total MCM3 staining intensity at 11 h with some nuclei staining positive (data not shown). As a control for antibody specificity, we incubated the anti-MCM3 antibody with the cognate peptide before IHC, and this treatment completely inhibited the signal, confirming that MCM3 was being detected (Fig. 2*f*).

Examination of MCM2 by IHC demonstrated that the cellular distribution of MCM2 in the uterine epithelium was also reversely regulated by E_2 and P_4 treatments (Fig. 6, which is published as supporting information on the PNAS web site). The kinetics of redistribution of MCM2 in the P_4E_2-treated uteri was similar to that of MCM3. However, unlike MCM3, MCM2 was solely retained in

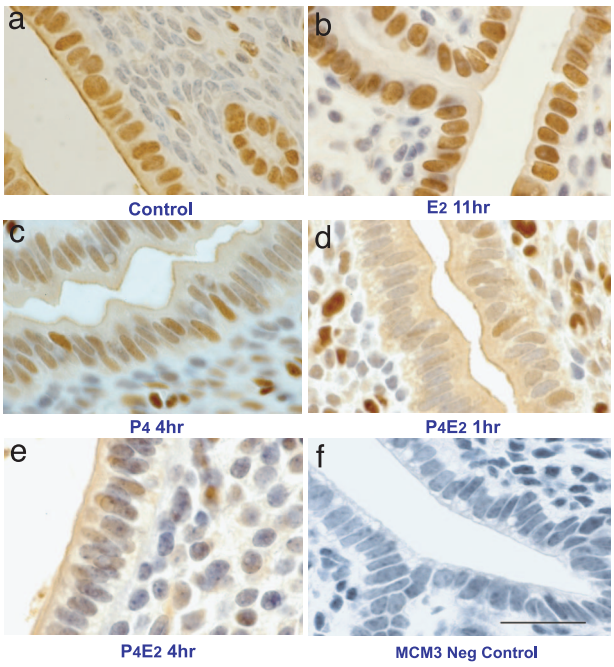

Fig. 2. MCM3 localization is controlled by E_2 and P_4 in the mouse uterine epithelium. Shown is immunostaining for MCM3 of uterine transverse sections isolated at the indicated times after the different hormonal treatments. (*a*) Control. (*b*) Fifty nanograms of E_2 at 11 h. (*c*) One milligram of P_4 for 4 days. (*d* and *e*) One milligram of P_4 for 4 days and killed at 1 h (*d*) and 4 h (*e*) after 50 ng of E_2 on the fourth day. (*f*) Inhibition of the IHC signal by a competitive MCM3 peptide. Brown indicates positive staining, and the columnar cells are the luminal epithelium. (Scale bar: 50 μm.)

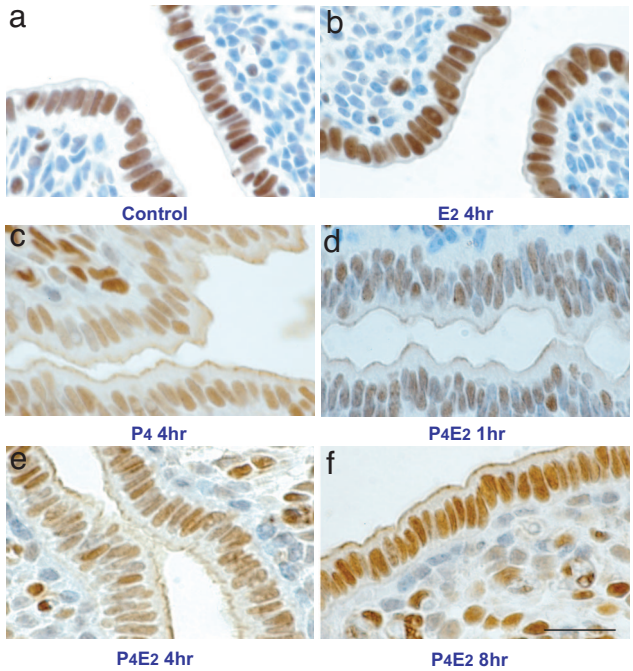

Fig. 3. P_4 transiently excludes MCM6 from the nucleus. Shown is immunostaining for MCM6 of uterine transverse sections isolated at the indicated times after the different hormonal treatments. (*a*) Control. (*b*) Fifty nanograms of E_2 at 4 h. (*c*) One milligram of P_4 for 4 days. (*d–f*) One milligram of P_4 for 4 days and killed 1 h (*d*), 4 h (e), and 8 h (*f*) after 50 ng of E_2 on the fourth day. (Scale bar: 50 μm.)

the uterine epithelial cell nuclei in both the untreated control and E_2-treated uteri. Similar to MCM3, preincubation with cognate peptide reduced the signal to background (Fig. 6*g*).

We further analyzed the distribution of MCM6 and MCM7 in the uterine epithelium after the different hormone treatments by IHC. Because MCM6 and MCM7 showed exactly the same pattern, only the distribution of MCM6 in the uterine epithelium is reported here (Fig. 3). MCM6 was found only in nuclei of control and E_2-treated uterine epithelial cells (Fig. 3 *a* and *b*). P_4 treatment caused a redistribution of MCM6 from the nucleus to the cytoplasm of the epithelial cells (Fig. 3*c*). In contrast to MCM2 and MCM3, after P_4E_2 treatment, although some of MCM6 stayed in the cytoplasm at 1 h (Fig. 3*d*), MCM6 shifted back to the nucleus from the cytoplasm from 2 h after hormone administration, and the majority of MCM6 had a nuclear localization by 8 h (Fig. 3 *e* and *f*). No competitive peptide was available for either MCM6 or MCM7, although omission of primary antibody reduced the IHC signal to background (Fig. 6*h*). The localization of MCM4 and MCM5 in the uterine epithelium could not be ascertained because of the lack of appropriate antibodies for IHC.

Thus, in contrast to the observations in almost all species that MCM are solely nuclear proteins, in the mouse uterine epithelium MCMs shift between the nucleus and cytoplasm under the influence of the different hormone regimens.

MCM Complex Loading onto the Chromatin Is Induced by E_2 and Inhibited by P_4. The stepwise recruitment of origin recognition complex, Cdc6 and Cdt1, followed by MCM complex onto the chromatin for pre-RC assembly appears to be conserved in eukaryotes and is required for the initiation of DNA replication (18, 19). Therefore, MCM are present in the nucleus in two different forms: one is soluble and extractable by nonionic detergents, and the other is tightly associated with nuclear structure and resistant to this extraction (20, 21). The redistribution of MCMs from nucleus to cytoplasm combined with the reduction of the total MCM protein led us to examine the chromatin association of MCMs regulated by female steroid hormones. Using lamin A/C as a loading control, some chromatin-associated MCM2 and MCM3 protein could be detected by Western blotting in control samples (Fig. 4). This finding is consistent with the low rate of epithelial cell proliferation in these control mice (≈5%). After E_2 treatment, additional MCM2 and MCM3 chromatin binding occurred cumulatively throughout G_1 phase, starting from 4 h and peaking between 8 and 11 h. After this time there was a gradual dissociation from the chromatin with a return to almost the basal level at 14 h (Fig. 4). This loading of MCMs onto the chromatin is restricted to the G_1 phase of the cell cycle, consistent with the role of MCM in

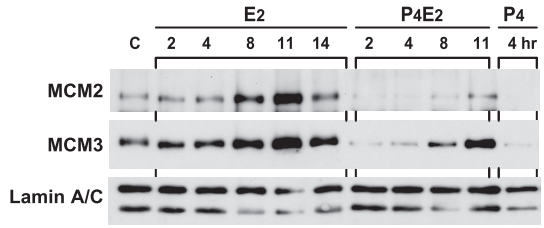

Fig. 4. P_4 inhibits the E_2-induced chromatin binding of MCM complexes in the mouse uterine epithelium. Uterine luminal epithelial cells were purified at indicated times after treatment with vehicle alone (C), E_2, P_4, or P_4E_2 as described. Chromatin-bound insoluble proteins were separated from total epithelial lysates as described in *Materials and Methods*. Equal amounts (30 μg) of protein were separated by SDS/PAGE and blotted onto nylon membranes. These blots were probed as indicated with antibodies to MCM2, MCM3, and lamin A/C. Lamin A/C served as the loading control for the chromatin-bound MCM2 and MCM3 proteins. Shown are representative Western blots from three independent experiments.

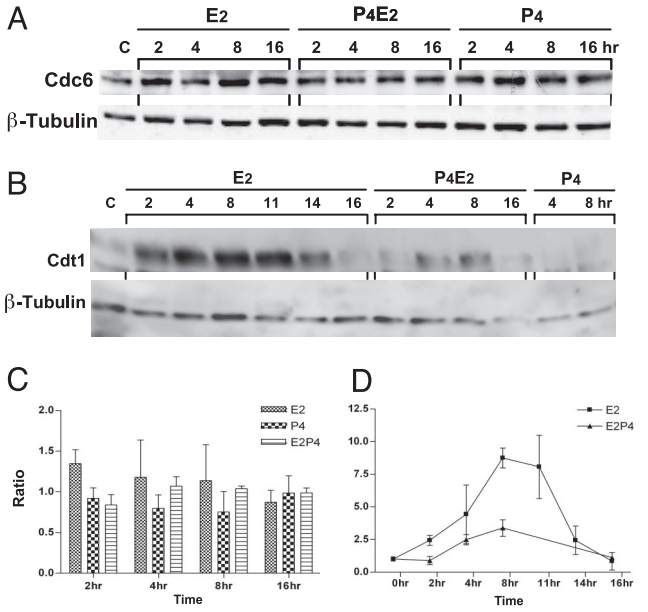

Fig. 5. P_4 and E_2 differentially regulate the two DNA replication initiation factors Cdc6 and Cdt1 in the uterine epithelium. Shown are representative Western blots from three independent experiments of total uterine luminal epithelial cell extracts prepared at various time points after hormonal treatments as indicated and probed with anti-Cdc6 antibody (*A*) and anti-Cdt1 antibody (*B*). Detection of β-tubulin was used as the loading control. (*C* and *D*) Densitometric analysis of the expression of Cdc6 (*C*) and Cdt1 (*D*) at different time points after the hormonal treatments as indicated. Data shown are the mean ± SD of three experiments.

replication licensing. P_4 treatment of the mice alone resulted in the dissociation of MCM2 and MCM3 from the epithelial chromatin to an almost undetectable level (Fig. 4). This pretreatment with P_4 almost completely abolished the E_2-induced MCMs loading onto chromatin through the first 8 h of E_2 treatment. However, after 8 h of P_4E_2 treatment some MCM2 and MCM3 was detected in the chromatin fraction, although the level of chromatin-bound MCMs remained significantly lower when compared with that bound in the samples treated with E_2 alone (Fig. 4). Thus, P_4 not only dissociated the existing MCMs from chromatin during the first 3 days of pretreatment but also significantly delayed and lowered the E_2-induced MCMs binding onto chromatin (Fig. 4).

E_2 and P_4 Exert Their Regulation Through Cdt1. At the replication origin, the binding of the members of the pre-RC to chromatin is strictly ordered. Origin recognition complex must first bind to chromatin to allow Cdc6 and Cdt1 binding, and this is a prerequisite for MCM binding (18, 19). To investigate the E_2 regulation of MCM recruitment onto the chromatin and its inhibition by P_4 pretreatment, we explored the hormonal regulation of Cdc6 and Cdt1 in the uterine epithelial cells. Cdc6 protein level in the total cell lysates of the uterine epithelial cells was found to remain approximately constant over the entire 16-h time course after the different hormone treatments (Fig. 5 *A* and *C*). No significant differences in the subcellular localization of Cdc6 in the uterine epithelium were observed after the different hormone treatments (data not shown). We further investigated Cdt1 protein levels in the total cell lysates of the epithelial cells after hormonal treatment. Interestingly, the Cdt1 level in the epithelial cells was very low in control mice, and similar levels were found after P_4 treatment (Fig. 5 *B* and *D*). However, E_2 treatment increased Cdt1 protein abundance within 2 h followed by an ≈10-fold accumulation that reached a plateau at the G_1/S phase transition between 8 h and 11 h, followed by a rapid loss to basal levels at 16 h (Fig. 5 *B* and *D*). The tight control of Cdt1 protein level by E_2 stimulation is precisely coincident with the loading of MCM onto chromatin during the cell-cycle progression of these epithelial cells (Fig. 4). P_4 pretreatment significantly attenuated the E_2-induced elevation of Cdt1, although there was still a rise that began from 4 h and reached a maximum by 8 h before declining to the basal level at 16 h (Fig. 5*B*). This induction parallels the kinetics of MCM loading onto chromatin in the P_4E_2-treated sample (Fig. 4). Taken together, P_4 and E_2 differentially regulate the two pre-RC loading factors, Cdc6 and Cdt1. Cdt1 rather than Cdc6 is the limiting factor for either the association of MCM with chromatin in the cell-cycle epithelial cells or the displacement of MCM complexes from chromatin in the P_4E_2-induced differentiation cells.

Discussion

P_4 blocks the mitogenic activity of E_2 in the uterine luminal and glandular epithelium of mice and women. In mice, if given 2 days before the E_2, mimicking the physiological condition, this suppression of cell division is complete (2). Studies altering the timing of P_4 administrations indicate that its inhibitory actions occur early in G_1 and acts within the first 3 h (4).

Both E_2 and P_4 require their transcription factor receptors for their actions on cell proliferation, suggesting that they work via transcriptional mechanisms (22, 23). Thus, there have been several studies that have profiled gene expression patterns in the uterus after sex steroid hormone treatment of ovariectomized mice or of endometrial samples isolated at different stages of the menstrual cycle in humans (24–27). In general these studies have taken RNA extracted from whole uterine tissue to profile. However, the uterus is a complex multicellular tissue, and it also shows dynamic changes in cell type under different hormonal conditions. Thus, the approach of taking whole-organ lysates for analysis obfuscates the cell-type-specific responses, particularly those in the uterine epithelium, which comprises only 5% of the uterine cell mass. In the current studies we isolated the uterine luminal epithelium before RNA purification and expression profiling on cDNA microarrays. We chose the 3-h time point after E_2 and P_4E_2 treatments because of the aforementioned fact that P_4 acts early in G_1.

This study revealed ≈200 expressed gene sequences whose transcripts were down-regulated 3 h after P_4E_2 treatment. Of these, Gene Ontology analysis showed that >20 genes (32 sequences) were involved in the DNA replicative process, including replication elongation and nucleosome assembly (Table 1). Remarkably, transcripts for five *Mcm* genes (*Mcm2–6*) involved in the prereplication licensing were down-regulated at this time, suggesting that this pathway is a major target of P_4 action.

Interestingly, unlike other cell types in which reduction of DNA synthesis is always accompanied by a concomitant down-regulation of RNA splicing and transport and protein synthesis during the cell differentiation process (28), these processes are unaffected by P_4. Indeed, the hypertrophy induced by E_2 is comparable between the epithelial cells regardless of whether the mice have been treated with P_4 (29). E_2 also stimulates the induction of immediate early gene expression, including the protooncogenes *c-fos*, *c-myc*, and a little later *c-ras*^{Ha}, and this expression is also unaffected by P_4 (1). This result was confirmed by analysis of the present cDNA microarray expression data (Table 2).

In this study we concentrated on the MCM proteins because of their importance in the regulation of DNA replication licensing. MCM proteins were originally identified in genetic screens for mutants defective in MCM in *Saccharomyces cerevisiae* and then characterized in a variety of eukaryotes, including mouse and human (8, 9). All six MCM members are closely related in sequence and structure with several highly conserved regions. Most of the MCM protein members cosediment on glycerol gradients and coelute after gel filtration as a complex with a molecular mass of ≈600 kDa, and which is composed of each MCM protein in an equimolar stoichiometry (8, 30, 31). MCM complex assembly

occurs in the cytoplasm followed by entry into the nucleus. Because only MCM2 and MCM3 possess identifiable nuclear localization sequences (32, 33), these two MCM members probably provide the nuclear targeting signal for the entire complex. Although MCM proteins function as a hexameric complex, each MCM member is uniquely required for DNA replication because mutation in any single MCM gene in budding, and fission yeast has an equal effect on inhibiting DNA replication and cell viability (34, 35).

Our study shows that preinitiation licensing is tightly controlled during the cell cycle in the E_2-induced uterine epithelium. Pre-RC assembly is regulated mostly at the level of loading of the MCM proteins onto the chromatin without change in overall MCM protein level and distribution in the luminal epithelium. This finding is consistent with the previous finding in most organisms that MCM proteins do not fluctuate during the cell cycle and are constitutively retained in the nucleus (8, 9). E_2 stimulates MCM complex chromatin binding early in G_1 phase, starting from 2 h and peaking at 8–11 h at the G_1-to-S phase transition.

The data presented in this study show that P_4 completely inhibits this E_2-induced uterine epithelial cell proliferation by targeting replication licensing in early G_1 phase through several mechanisms. First, measurements with both cDNA microarray and QRT-PCR showed that P_4 reduced the transcript abundance of *Mcm2–6*. Furthermore, Western blotting indicated that MCM2, MCM3, and MCM4 protein concentration was also reduced by P_4. This down-modulation required the E_2 to be in combination with P_4, because treatment with P_4 alone did not have a significant effect on the concentrations of these proteins compared with the hormone-untreated control mice. Because a modest reduction in any one of MCM levels in other systems results in a dramatic inhibition of DNA synthesis (34, 35), it seems probable that this P_4-induced down-regulation of MCM is part of the cause of the profound block of DNA synthesis by P_4.

P_4 pretreatment significantly lowered the E_2-induced chromatin binding of MCM proteins. This reduction in chromatin binding paralleled the reduction of Cdt1 but not Cdc6 protein. Several lines of evidence indicate that Cdc6 and Cdt1 physically interact with each other and functionally cooperate in the recruitment of MCM complex onto chromatin (36, 37). However, our data indicate that female steroid hormone differentially regulates these two licensing factors in the uterine epithelial cells during both proliferation and differentiation. This finding strongly suggests that P_4 regulates DNA replication licensing by inhibiting MCM complex loading through reducing the abundance of Cdt1 in exact opposition to E_2 action that enhances Cdt1 concentration and consequently MCM loading.

A third level of regulation by P_4 was achieved by alterations in cellular localization of MCM proteins. In all other cases documented, MCM are predominantly nuclear proteins (9). Such a nuclear localization for MCM2, MCM3, MCM6, and MCM7 was observed in uterine epithelial cells in control and E_2-treated mice. However, as assessed by IHC, P_4 pretreatment resulted in a redistribution of these proteins to the cytoplasm, with the effect being greatest on MCM2 and MCM3. The subsequent E_2 treatment in the face of P_4 resulted in a further depletion from the nucleus over the first 2 h of treatment, although there was some rebound at 8 h after E_2. The exact mechanism of this exclusion remains to be determined, but these results show a mechanism for the control of replication licensing through changes in MCM cellular localization.

Our previous studies indicated that P_4 inhibited the canonical cell-cycle regulatory machinery by blocking cyclin D1/CDK4 nuclear localization (10, 11). Here we demonstrate that P_4 inhibits the replication licensing machinery at many levels. Thus, P_4 exerts its complete block of cell proliferation by inhibiting two E_2-induced pathways in early G_1 phase that apparently run in parallel but both of which are required for the E_2 induction of DNA synthesis.

Materials and Methods

Animal Treatment and Purification of Uterine Epithelial Cells. Adult female CD1 mice (Charles River Laboratories, Wilmington, MA) were ovariectomized at 8–10 weeks of age under anesthesia and rested for 2–3 weeks before any hormonal treatment. E_2 and P_4 were purchased from Sigma Chemical (St. Louis, MO) and given s.c. in peanut oil as described before (10). All experiments and all time points were independently repeated at least three times with similar results.

After hormone treatment, uteri were removed, split longitudinally, and vortexed with Teflon beads (Small Parts, Miami, FL) in 1 ml of extraction buffer for 2.5 min as described to obtain an epithelial cell preparation that is >95% pure (16). This preparation was used for either protein or total RNA isolation as described (10, 38).

Supporting Information. For further details, see *Supporting Methods*.

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