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Regulation of Transcriptional Activity during the First and Second Cell Cycles in the Preimplantation Mouse Embryo

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Transcription of endogenous genes in preimplantation 1- and 2-cell mouse embryos was determined by monitoring the incorporation of BrUTP by plasma membrane-permeabilized embryos. Incorporation is observed starting by mid-S phase in the 1-cell embryo and increases progressively; the amount of incorporation by the 1-cell embryo in G2 is about 20% that of the 2-cell embryo in G2. Incorporation by the male pronucleus is always about four to five times greater than that of the female pronucleus. Nevertheless, the amount of incorporation by the female pronucleus present in parthogenetically activated eggs is similar to the total amount of incorporation in inseminated eggs, i.e., the transcriptional capacity of the female pronucleus is not inherently less than that of the male pronucleus. Inhibiting the first round of DNA replication does not prevent the initiation of transcription in the 1-cell embryo, but does inhibit the extent of BrUTP incorporation by 35%. The transcriptional machinery of the 1-cell embryo appears to be rate-limiting, since the total amount of BrUTP incorporation by parthenogenetically activated and dispermic eggs is similar to that in monospermic eggs; trispermic eggs incorporate BrUTP to only about 60% the level of monospermic eggs. A transcriptionally repressive state may start to develop in the 2-cell embryo, since inhibiting the second round of DNA replication results in an 50% increase in BrUTP incorporation. Trapoxin treatment, which induces histone hyperacetylation, enhances incorporation by 2-cell embryos 1.8-fold and suggests that histone hyperacetylation can relieve this repression.

INTRODUCTION

The fully grown meiotically competent mouse oocyte is a nonproliferating, differentiated cell; oocytes are the only cells in the female that undergo meiosis and express oocyte-specific genes, e.g., the *zona pellucida* genes. Following fertilization and the first round of DNA replication, the 2-cell stage blastomeres, which contain the first zygotic nuclei, are totipotent (Pedersen, 1986). This remarkable transformation presumably entails, in addition to the requirement for both the maternal and paternal genomes (McGrath and Solter, 1984), reprogramming the pattern of gene expression, and in fact a dramatic change in gene expression occurs during the 2-cell stage (Latham *et al.*, 1991). The molecular basis for how this reprogramming of gene expression, which must use maternally derived transcription factors and/or factors synthesized from maternally recruited mRNAs during oocyte maturation and following fertilization, is poorly understood.

Although a large body of experimental evidence indicates that zygotic gene activation has occurred by the 2-cell stage (Schultz, 1993, and references therein), results of more recent experiments indicate that the 1-cell embryo is transcriptionally active (Vernet *et al.*, 1992; Ram and Schultz, 1993; Matsumoto *et al.*, 1994; Christians *et al.*, 1995; Bounil *et al.*, 1995). Moreover, the male pronucleus (PN) supports a significantly higher level of transcription than the female PN (Ram and Schultz, 1993; Bounil *et al.*, 1995), and consistent with this is that higher concentrations of transcription factors are present in the male PN (Worrad *et al.*, 1994). Nevertheless, the magnitude of the changes in endogenous transcriptional activity during the 1- and 2-cell stages has not been quantified.

The first round of DNA replication, which disrupts nucleosomes that inherently repress transcription, could be a major locus of control for the reprogramming of gene expression, since it would provide a window of opportunity for transcription factors to gain access to their cognate *cis*-acting DNA-binding sequences (Wolffe, 1991, 1994). Consistent with this hypothesis is that the first round of DNA replication is critical for the maximal expression of two endogenous genes whose expression transiently increases during the 2-cell stage (Davis *et al.*, 1996). Although results of experiments using reporter genes suggest that zygotic

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gene activation is independent of DNA replication—their expression is not inhibited by blocking the first round of DNA replication (Majumder and DePamphilis, 1995, and references therein)—it should be noted that the reporter genes used in those studies do not undergo DNA replication (e.g., Henery *et al.*, 1995), and as carefully pointed out by Henery *et al.* (1995), their expression pattern is indicative of the promoter and enhancer requirements for their expression, as opposed to the role of DNA replication in their expression.

Changes in chromatin structure coupled to the second round of DNA replication appear critical for the reduced expression for genes whose expression transiently increases during the 2-cell stage (Davis *et al.*, 1996). Moreover, changes in chromatin structure have been proposed to mediate the repression of reporter gene expression in the 2-cell embryo (Henery *et al.*, 1995; Wiekowski *et al.*, 1991). Consistent with this hypothesis is that treatment of mouse embryos with inhibitors of histone deacetylases, e.g., butyrate (Kruh, 1982) or trapoxin (Kijima *et al.*, 1993), sustains the expression of these transiently expressed endogenous genes (Davis *et al.*, 1996), as well as stimulates the expression of plasmid-borne reporter genes that do not harbor an enhancer to levels similar to reporter genes that do possess an enhancer (Wiekowski *et al.*, 1993; Henery *et al.*, 1995).

In order to monitor developmental changes transcription of the total pool of endogenous genes by 1-cell and 2-cell mouse embryos, we quantified by laser-scanning confocal microscopy the incorporation of BrUTP, which is utilized by RNA polymerase II (Jackson *et al.*, 1993; Wansink *et al.*, 1993; Dundr and Raska, 1993). We then used this approach to assess the role of DNA replication and changes in chromatin structure in the expression of these genes. We report that transcription of the total pool of endogenous genes initiates during S phase of the 1-cell embryo such that the total amount of transcription in the 1-cell embryo in G2 is about 20% that of the 2-cell embryo in G2. Moreover, the amount of transcription supported by the male PN is about four to five times greater than that fostered by the female PN. Following cleavage to the 2-cell stage the overall amount of transcription increases continuously during the 2-cell stage. Inhibiting the first round of DNA replication does not prevent initiation of transcription but it does inhibit by ~35% the extent of transcription by G2 in the 1-cell embryo. In contrast, inhibiting the second round of DNA replication results in an increase of ~50% in the total amount of transcription in the 2-cell embryo. Treatment of 1-cell embryos with trapoxin results in a 50% decrease in transcription in both the male and female PN at G2 of the 1-cell stage, whereas transcription is increased by 80% at the 2-cell stage relative to control untreated embryos. Last, the female PN in parthenogenetically activated 1-cell embryos supports a level of transcription similar to that of fertilized eggs, and while the total amount of transcription supported by dispermic fertilized eggs is similar to that of monospermic eggs, that of trispermic eggs is substantially reduced.

MATERIALS AND METHODS

Collection and Culture of Mouse Embryos

One-cell mouse embryos were collected from superovulated female CF-1 mice (Harlan) that had been mated with B6D2F1 males (Jackson Laboratory) as previously described (Moore *et al.*, 1993). The embryos were cultured in KSOM plus amino acids in an atmosphere of 5% O_2/5% CO_2/90% N_2 at 37°C (Erbach *et al.*, 1994; Ho *et al.*, 1995).

Embryos were synchronized by culling embryos that had recently formed a PN as previously described (Ram and Schultz, 1993). Briefly, following collection of the fertilized eggs, those containing a visible PN were discarded. The remaining cells were examined 60 min later and those that formed a PN were culled and used for the experiments described below.

Parthenogenetic Activation of Mouse Eggs

Ovulated eggs were collected 17 hr post-hCG administration (5 IU); the collection medium was bicarbonate-free Whitten's medium (Whitten, 1971) in which the bicarbonate was replaced with 25 m*M* Hepes, pH 7.4, and the BSA was replaced with 3 mg/ml polyvinylpyrrolidone. The eggs were washed with calcium- and magnesium-free Whitten's medium and then incubated for 4 min in the same medium containing 5 μM A23187. The eggs were then washed in calcium- and magnesium-free Whitten's medium and incubated for 10 min prior to transferring them to KSOM plus amino acids.

Fertilization in Vitro

Sperm were collected from 12- to 24-week-old (C57BL/6J × SJL/J) F_1 males (Jackson Laboratories) (Moore *et al.*, 1993), and metaphase II-arrested eggs were collected from superovulated CF-1 females (Harlan) 14–15 hr post-hCG as previously described (Moore *et al.*, 1993). *Zona pellucida*-free eggs were obtained by removing the *zona pellucida* with acidic MEM-compatible buffer (MEMCO) as previously described (Evans *et al.*, 1995). Fertilization *in vitro* was performed as previously described (Moore *et al.*, 1993).

Incorporation of BrUTP and Quantification by Laser-Scanning Confocal Microscopy

All treatments were performed at room temperature unless otherwise specified. Embryos were washed in phosphate-buffered saline, and the plasma membrane was permeabilized by treating the embryos for 1–2 min with 0.05% Triton X-100 in "physiological buffer" (PB) that consisted of 100 m*M* potassium acetate, 30 m*M* KCl, 1 m*M* $MgCl_2$, 10 m*M* Na_2HPO_4, 1 m*M* ATP supplemented with 1 m*M* dithiothreitol, 0.2 m*M* phenylmethylsulfonyl fluoride, and 50 units/ml of RNasin (Promega, Madison, WI) (Jackson *et al.*, 1993; Ferreria and Carmo-Fonseca, 1995). Following this treatment the embryos were briefly washed three times with PB and then transferred to 100 m*M* potassium acetate, 30 m*M* KCl, 1 m*M* $MgCl_2$, 10 m*M* Na_2HPO_4 containing 2 m*M* ATP, 0.4 m*M* each of GTP, CTP, and BrUTP, and 2 m*M* $MgCl_2$. After a 10-min incubation at 33°C the embryos were washed briefly three times with PB and the nuclear membrane was permeabilized by a 3-min treatment in PB containing 0.2% Triton X-100. The embryos were then washed in PB three times and fixed overnight with 3.7% paraform-

aldehyde in PB. For polyspermic embryos, permeabilization of the nuclear membrane was performed after fixation, since these *zona pellucida*-free embryos were destroyed during the permeabilization procedure if they were not fixed.

The incorporated BrUTP was detected by immunostaining with anti-BrdU antibodies. The embryos were washed five times in 15 μl drops of PBS containing 3 mg/ml of BSA (PBS/BSA) over a period of 15 min and then incubated for 45 min with 2 μg/ml of the anti-BrdU monoclonal antibody (Boehringer-Mannheim). The embryos were then washed three times with PBS/BSA over the course of 15 min and subsequently incubated in PBS/BSA containing 0.5 μg/ml of an anti-mouse IgG antibody conjugated with Texas Red for 45 min. The samples were then washed with PBS/BSA and mounted with VectaShield (Vector Laboratories, Burlingum, CA). For the experiments in which polyspermic embryos were generated, the cells were stained for 20 min with 3 μg/ml of 4′,6′-diamidino-3-phenylindole (DAPI) before mounting for laser-scanning confocal microscopy.

Fluorescence was detected using a Leica TCS 4D laser-scanning confocal microscope and the signal was quantified as previously described (Worrad *et al.*, 1994). Briefly, the pixel value/unit area was measured from five different regions of the nucleus and five different regions of the cytoplasm, and the average cytoplasmic value was subtracted from the average nucleoplasm value. This value was then multiplied by the pronuclear/nuclear volume to yield the total amount of fluorescence, i.e., BrUTP incorporation, for the pronucleus/nucleus. Pronuclear/nuclear volume at each of the indicated times in the figures was calculated by measuring the diameter of the pronucleus/nucleus on a different set of embryos using an ocular micrometer attached to a microscope and equipped with Hoffmann optics.

In each experiment, the average value calculated for either the male PN or nuclei in 2-cell embryos at late G2 for either the first or second cell cycle, respectively, was set as 100% and the values obtained at other time points were expressed relative to this value. For polyspermic embryos, the procedure to calculate the total amount of fluorescence per PN was slightly modified, since the pronuclear volumes were highly variable. In these cases, the nuclear volume was determined by using images taken through the section containing the largest pronuclear cross-section.

Immunofluorescent Localization of TBP by Confocal Laser-Scanning Microscopy

The samples were prepared for laser-scanning confocal microscopy and the TATA box binding protein, TBP, was detected as previously described (Worrad *et al.*, 1994).

RESULTS

Characterization of BrUTP Incorporation by Permeabilized Preimplantation Mouse Embryos

The poor uptake, metabolism, and incorporation of [^{3}H]-uridine into acid-insoluble material by 1- and 2-cell mouse embryos have hampered quantification of developmental changes in the transcriptional activity at these early developmental stages (Clegg and Pikó, 1977). In addition, this approach does not discriminate nuclear transcription from mitochondrial transcription. Although an autoradiographic approach circumvents this latter problem, a meaningful interpretation of differences in signal intensity can only be made when the specific activity of the endogenous UTP pool is known. To address in a more direct fashion the transcriptional activity of the early preimplantation mouse embryo, we took advantage of the ability of RNA polymerase II to incorporate BrUTP into nascent transcripts (Jackson *et al.*, 1993; Wansink *et al.*, 1993; Dundr and Raska, 1993). Although incorporation of microinjected BrUTP by the 1- and 2-cell preimplantation mouse embryo has recently been reported (Bounil *et al.*, 1995), we elected to use permeabilized embryos, since, in addition to its relative technical ease when compared to microinjection, BrUTP incorporation by permeabilized embryos is independent of the endogenous UTP pool size, because the endogenous UTP pool is replaced by the BrUTP present in the transcription buffer. Thus, the extent of BrUTP incorporation likely measures the rate of transcription at the time of embryo permeabilization. In addition, the coupling of laser-scanning confocal microscopy and image analysis permits quantification of the data, which is not readily achievable using epifluorescence, which was previously used (Bounil *et al.*, 1995).

The 2-cell embryo is transcriptionally active, and we therefore defined conditions for BrUTP incorporation by permeabilized 2-cell embryos in G2. BrUTP incorporation was readily detectable in both nuclei (Fig. 1A). Incorporation was evenly distributed but exhibited a punctate pattern, and no apparent differences between the two blastomere nuclei were observed (Fig. 1A). It was unlikely that the uniform staining observed was an artifact of the permeabilization procedure, since a similar staining pattern was observed when embryos were microinjected with BrUTP (final intracellular concentration, 2.5 m*M*) and then fixed and processed for laser-scanning confocal microscopy 2–8 min following microinjection (data not shown). It should also be noted that although rRNA synthesis occurs in the 2-cell embryo (Knowland and Graham, 1972), no signal was observed in the nucleolus. This may reflect the inaccessibility of the antibodies to the nucleolar matrix, since TBP, which is required for transcription of rDNA (Hernandez, 1993), is also not detected in the nucleolus (Worrad *et al.*, 1994).

Several lines of evidence indicated that BrUTP incorporation was due to RNA synthesis. First, inclusion of 1 μg/ml of α-amanitin in the permeabilization and transcription buffer resulted in essentially no detectable signal (Fig. 1B). Second, treatment of the samples with RNase (Fig. 1C), but not DNase (Fig. 1D), following fixation resulted in a marked reduction in the signal. Third, omission of GTP from the transcription assay resulted in no detectable BrUTP incorporation (data not shown). In addition, we have previously demonstrated that the protein kinase A inhibitor, H8, inhibits transcription in 2-cell embryos (Manejwala *et al.*, 1991; Poueymirou and Schultz, 1989; Schwartz and Schultz, 1992). As anticipated, incubation of 1-cell embryos in medium containing H-8 (50 μM) also inhibited BrUTP incorporation by 1-cell embryos in G2 (Fig. 2).

The amount of BrUTP incorporated was a function of the

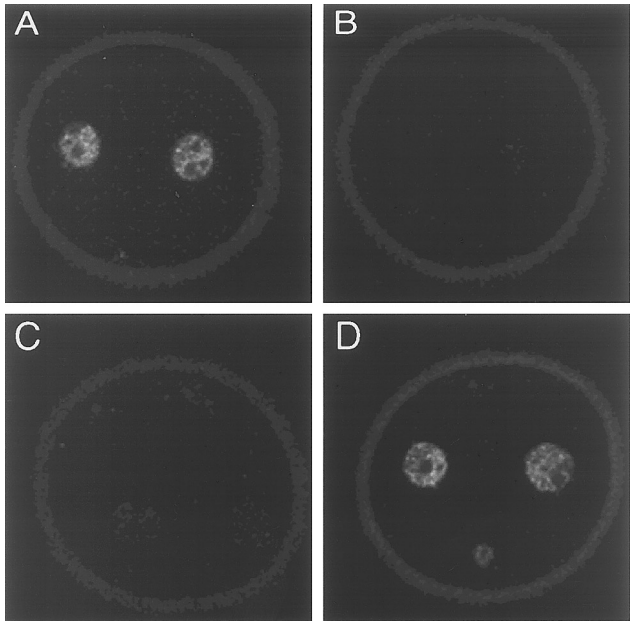

FIG. 1. Laser-scanning confocal micrographs of BrUTP incorporation by permeabilized 2-cell mouse embryos. Two-cell embryos in G2 were permeabilized and incubated in transcription buffer containing 0.4 m*M* BrUTP. (A) Control 2-cell embryo, (B) two-cell embryo in which 1 μg/ml of α-amanitin was included in the transcription buffer, (C) two-cell embryo treated with RNase (50 μg/ml) for 60 min after fixation, (D) two-cell embryo treated with DNase RQ (1 unit/ml) for 60 min after fixation. The staining observed at the periphery is due to nonspecific binding of the secondary antibody to the *zona pellucida* (Worrad *et al.*, 1995).

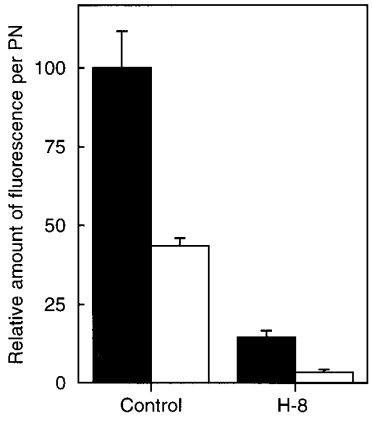

FIG. 2. Effect of H-8 on BrUTP incorporation by 1-cell embryos in G2. One-cell embryos were incubated in medium containing 50 μ*M* H-8; H-8 was added 1 hr following PN formation. The embryos were then cultured for 9 hr and assayed for BrUTP incorporation in which manganese chloride and ammonium sulfate were included in the transcription buffer (see legend to Fig. 6). Solid bars, male PN; open bars, female PN. The data are expressed as means ± SEM in which 10 control and 11 H-8-treated embryos were analyzed.

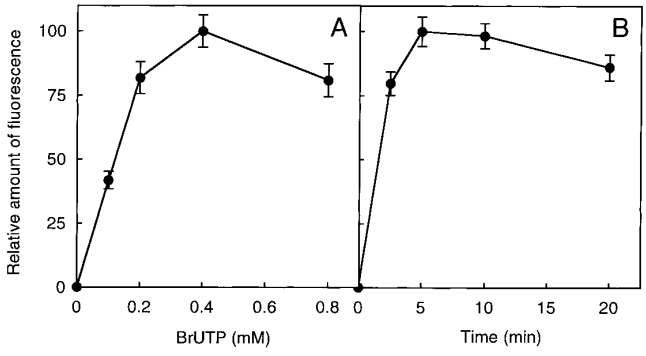

FIG. 3. Effect of BrUTP concentration and time of incubation on BrUTP incorporation. (A) Two-cell embryos in G2 were permeabilized and incubated for 10 min in transcription buffer containing the indicated concentrations of BrUTP. The number of nuclei analyzed at 0, 0.1, 0.2, 0.4, and 0.8 m*M* BrUTP was 20, 24, 24, 22, and 24, respectively. The amount of incorporation observed at 0.4 m*M* was set as 100%. The data in this and subsequent figures are expressed as the mean ± SEM. (B) Two-cell embryos in G2 were permeabilized and incubated for the indicated times in transcription buffer (2 m*M* ATP, 0.4 m*M* CTP, GTP, and BrUTP). The number of nuclei analyzed at 0, 2.5, 5, 10, and 20 min was 20, 16, 42, 46, and 44, respectively. The amount of BrUTP incorporation observed at 5 min was set as 100%.

concentration of BrUTP. In the presence of 2 m*M* ATP and 0.4 m*M* CTP and GTP, maximal incorporation was observed at 0.4 m*M* BrUTP (Fig. 3A); compared to these optimal conditions (2 m*M* ATP, 0.4 m*M* CTP and GTP, and 0.4 m*M* BrUTP), the extent of incorporation in the presence of 2 m*M* ATP, 0.1 m*M* CTP and GTP, and 0.4 m*M* BrUTP was 50% (data not shown).

The kinetics of BrUTP incorporation reached a plateau value within 5–10 min following incubation in BrUTP-containing transcription assay buffer (Fig. 3B). The time requirements mandated by washing the permeabilized embryos that were subsequently incubated in buffer containing BrUTP and then again washed prohibited using incubation times shorter than 5 min to obtain reproducible data. Relative to the amount of BrUTP incorporated by permeabilized embryos that were immediately incubated in transcription buffer, permeabilized embryos that were incubated in buffer 5 or 10 min prior to addition of BrUTP incorporated BrUTP to levels of 99 and 87%, respectively (data not shown). Thus, the transcription machinery appeared to be retained in these permeabilized embryos for at least the duration of the times used in these experiments.

Changes in Transcriptional Activity during the 1-Cell and 2-Cell Stages

Although the expression of either a paternally derived reporter transgene (Matsumoto *et al.*, 1994; Christians *et al.*, 1995) or a microinjected reporter gene (Ram and Schultz,

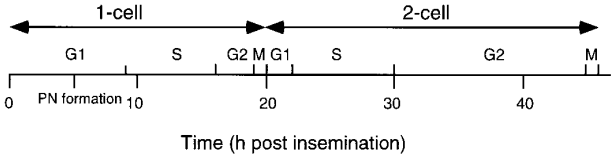

FIG. 4. Time course for cell cycle progression in the 1-cell and 2-cell stages. The data are taken from Moore *et al.* (1996).

1993; Wiekowski *et al.*, 1993) can be detected in G2 of the 1-cell embryo, these approaches do not permit quantification of the total transcriptional capacity of the 1-cell embryo, i.e., the transcriptional activity of both the male and female PN. Hence, these approaches exclude quantifying changes in the total transcriptional capacity that occur between the 1- and 2-cell stages. Moreover, these approaches do not permit these measurements to be made in the same embryo, e.g., transcriptional activity of the male and female PN. Accordingly, we quantified differences in the transcriptional activity of the male and female PN, as well as changes in transcriptional activity during the 1- and 2-cell stages by measuring the extent of BrUTP incorporation into permeabilized 1- and 2-cell embryos. A time course for cell cycle events is depicted in Figure 4 to facilitate relating the time following PN formation with cell cycle progression (Moore *et al.*, 1996).

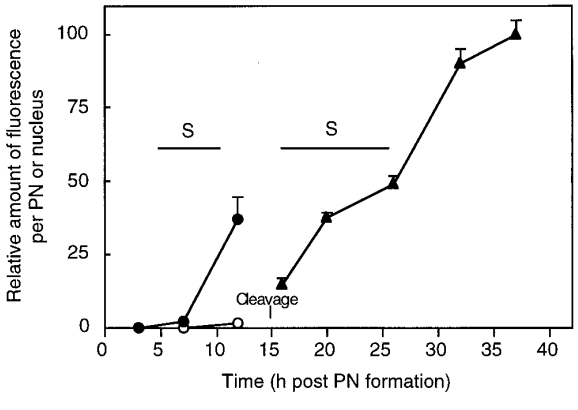

FIG. 5. Time course of BrUTP incorporation by 1-cell and 2-cell mouse embryos. At the indicated times following PN formation the embryos were permeabilized and assayed for BrUTP; the values shown reflect the total amount of incorporation by either male or female PN or the 2-cell nuclei, i.e., the intensity of the fluorescent signal times the nuclear volume. (●) male PN, (○) female PN, (▲) 2-cell nucleus. The bar with an S above it indicates the duration of S phase, and the time of cleavage to the 2-cell stage is also indicated. The number of male and female PN analyzed at 3, 7, and 12 hr post-PN formation was 56, 46, and 58, respectively, and the number of 2-cell nuclei analyzed at 16, 20, 26, 32, and 37 hr post-PN formation was 86, 76, 114, 114, and 126, respectively. The amount of BrUTP incorporation observed at 37 hr post-PN formation was set as 100%.

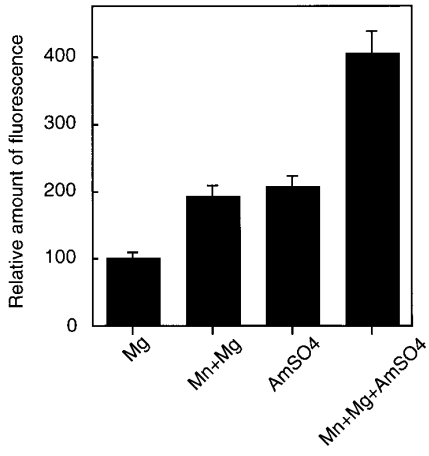

FIG. 6. Effect of $MnCl_2$ and ammonium sulfate on BrUTP incorporation by permeabilized 2-cell embryos. Mg, transcription buffer containing 2 m*M* $MgCl_2$; Mn + Mg, transcription buffer containing 1 m*M* $MnCl_2$ and 1 m*M* $MgCl_2$; $AmSO_4$, transcription buffer containing 50 m*M* ammonium sulfate; Mn + Mg + $AmSO_4$; transcription buffer containing 1 m*M* $MnCl_2$, 1 m*M* $MgCl_2$, and 50 m*M* ammonium sulfate. Eighteen pronuclei were analyzed for each treatment group, except for the Mn + Mg + $AmSO_4$ group in which 20 pronuclei were examined. The extent of BrUTP incorporation in transcription buffer containing 2 m*M* $MgCl_2$ was set as 100%.

While no BrUTP incorporation was observed 3 hr post-PN formation, a low level of incorporation was detected in the male PN by 7 hr post-PN formation. This value increased by 12 hr post-PN formation, i.e., G2 of the first cell cycle (Fig. 5). Consistent with previous observations (Ram and Schultz, 1993; Bounil *et al.*, 1995; Wiekowski *et al.*, 1993), the level of transcription fostered by the male PN was significantly greater than that supported by the female PN (Fig. 5). Although these results indicated that transcription initiated during S phase of the 1-cell embryo, the signal obtained was low, especially that observed for the female PN.

In order to refine the timing for the onset of ZGA and determine more accurately the transcriptional differences between the male and female PN during the first cell cycle, we took advantage of the ability of manganese and ammonium sulfate to increase the activity of RNA polymerase II *in situ* by stimulating the rate of elongation (Jackson and Cook, 1985). Accordingly, we first verified the stimulatory effect of these agents on BrUTP incorporation by permeabilized 2-cell embryos (Fig. 6). We then used this approach to quantify changes in transcriptional activity during the first cell cycle. Transcription was detected as early as 5 hr post-PN formation, i.e., at the initiation of DNA replication (Fig. 7). Moreover, the extent of transcription increased progressively during S phase and was always greater in the male PN than in the female PN; the ratio between the male and female PN (~4 or 5 to 1), however, remained essentially constant. The incorporation that occurred during S phase

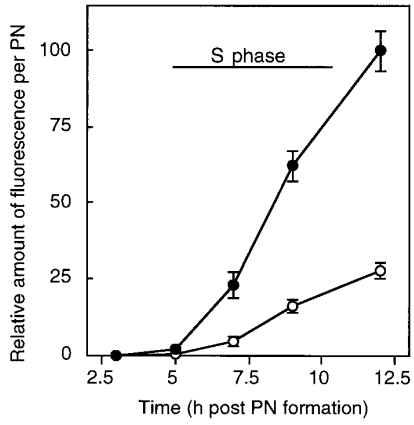

FIG. 7. Time course of BrUTP incorporation during the first cell cycle. BrUTP incorporation in permeabilized 1-cell embryos at the indicated times following PN formation was determined in which $MnCl_2$, $MgCl_2$, and ammonium sulfate were included in the transcription buffer. (●) male PN, (○) female PN. Twenty-seven embryos were examined at each time point, except 28 were analyzed at 3 hr post-PN formation. The extent of incorporation by the male PN at 12 hr post-PN formation was set as 100%. The reason for the difference in the ratio between the male and female PN shown in this figure and that in Fig. 5 is due to the low level of BrUTP incorporation observed in the PN, especially that for the female PN, in the absence of manganese and ammonium sulfate.

was unlikely due to DNA synthesis, since cellular dNTPs, which are not included in the transcription buffer, would be lost following permeabilization. In addition, α-amanitin- or RNase-treated 1-cell embryos in S phase did not reveal any detectable signal, whereas DNase-treated embryos revealed a signal of similar intensity as control, untreated embryos (data not shown). It should be noted that a previous study failed to detect BrUTP incorporation by permeabilized 1-cell embryos in S/G2 (Ferreria and Carmo-Fonseca, 1995). A likely explanation for this difference is that the stimulation of RNA polymerase II activity by manganese and ammonium sulfate was not used to enhance the detection of BrUTP incorporation in that study.

Cleavage to the 2-cell stage was associated with a drop in the extent of BrUTP incorporation, which again increased during S phase (16–24 hr post-PN formation) and continued to increase during G2 (Fig. 5). The total transcriptional activity of the 1-cell embryo in G2 was about 20% that of the entire 2-cell embryo. When the data are expressed as the amount of BrUTP incorporation per cellular DNA content (i.e., the 2-cell embryo in G2 has twice as much DNA as the 1-cell embryo in G2), the rate of transcription of the 1-cell embryo in G2 is about 40% that of the 2-cell embryo in late G2. Thus, transcription of endogenous genes in the 1-cell embryo, relative to a 2-cell blastomere, was quite robust.

It should be emphasized that the results obtained using BrUTP incorporation as a marker for total endogenous transcription were in excellent agreement with results obtained from the expression of plasmid-borne reporter genes (Wiekowski *et al.*, 1991, 1993; Ram and Schultz, 1993) and transgenes (Matsumoto *et al.*, 1994; Christians *et al.*, 1995) regarding the timing for the onset for detection of transcription, the differences in transcriptional capacity of the male and female pronuclei, and the differences in the extent of transcription between the 1- and 2-cell embryos. These similarities strongly indicated that BrUTP incorporation provides a satisfactory marker to quantify total transcription in the preimplantation mouse embryo and that the differences in BrUTP incorporation between male and female PN and between 1- and 2-cell embryos were not an artifact of the permeabilization procedure.

Effect of Inhibiting DNA Replication on BrUTP Incorporation by 1-Cell and 2-Cell Embryos

The observation that transcription was detected upon entry into S phase, coupled with the link between DNA replication and transcription in providing a window of opportunity for transcription factors to gain access to their cognate DNA-binding sequences (Wolffe, 1991, 1994) led us to examine the effect of aphidicolin, which is an inhibitor of replicative DNA polymerases (Ikegami *et al.*, 1978), on transcription. Addition of aphidicolin immediately after PN formation and examination of transcription in G2 of the 1-cell embryo revealed essentially no effect on incorporation supported by the female PN, whereas transcription supported by the male PN was inhibited relative to control embryos (Fig. 8); addition of aphidicolin just after PN formation inhibits BrdU incorporation (data not

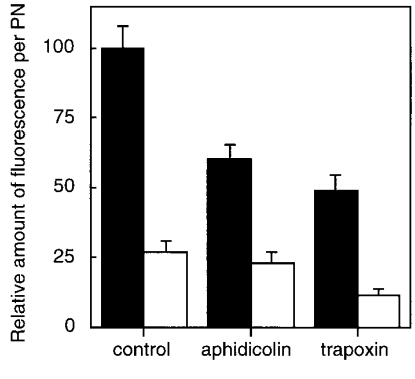

FIG. 8. Effect of aphidicolin or trapoxin on BrUTP incorporation by 1-cell embryos in G2. Either aphidicolin (3 μg/ml) or trapoxin (100 m*M*) was added in G1 and the embryos were cultured until G2, i.e., 12 hr post-PN formation. BrUTP incorporation in the presence of manganese and ammonium sulfate was then determined and the amount of incorporation observed in the male PN in control embryos was set as 100%. Solid bars, male PN; open bars, female PN. Twenty-six control and aphidicolin-treated embryos were analyzed, and 24 trapoxin-treated embryos were analyzed.

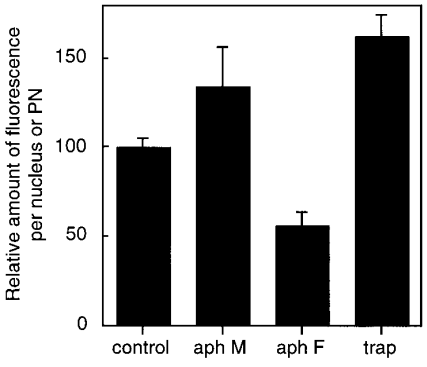

FIG. 9. Effect of aphidicolin or trapoxin added in G1 of the first cell cycle on BrUTP incorporation at G2 of the 2-cell stage. Aphidicolin (3 μg/ml) or trapoxin (100 n*M*) was added in G1, and the embryos were cultured until G2 of the 2-cell stage, i.e., 37 hr post-PN formation. BrUTP incorporation was then determined, and the amount of incorporation observed in the 2-cell control nuclei was set as 100%. Control, 2-cell embryos (96 nuclei examined); aph M and aph F, male and female PN, respectively, in aphidcolin-treated embryos (40 of each type of PN were analyzed); trap, trapoxin-treated embryos (84 nuclei were analyzed). The amount of BrUTP incorporated by nuclei in control 2-cell embryos was set as 100%.

shown). Nevertheless, it should be noted that the extent of transcription in these male PN from aphidicolin-treated embryos was ~60% that of the control embryos. Thus, transcription clearly initiated in the aphidicolin-treated embryos, albeit the total amount of BrUTP incorporation was reduced by 35%.

We next analyzed the extent of BrUTP incorporation by cleavage-arrested aphidicolin-treated embryos that corresponded to G2 of the 2-cell stage, since this is a frequently used protocol for analyzing reporter gene expression (e.g., Wiekowski *et al.*, 1991, 1993). Results of these experiments revealed that the amount of BrUTP incorporation supported by the female PN and male PN was about 55 and 135%, respectively, of that present in a control 2-cell nucleus (Fig. 9). Thus, in the absence of DNA replication, the extent of transcription continued to increase such that the total amount of BrUTP incorporation by these cleavage-arrested embryos was similar to that in control 2-cell embryos, i.e., the sum of BrUTP incorporation by the male and female PN equaled that of the two nuclei present in the 2-cell embryo. Expressing the data as the amount of BrUTP incorporation per cellular DNA content indicated that the total amount of BrUTP incorporation in the aphidicolin-treated 1-cell embryo (sum of incorporation by the male and female PN) was about four times that of a control 2-cell blastomere, since the sum of the two nuclei present in the 2-cell embryo in G2 contain four times the amount of DNA present in the cleavage-arrested aphidicolin-treated embryos and the total amount of BrUTP incorporation in the aphidicolin-treated embryo equaled that of the 2-cell embryo. This result suggested that a transcriptionally repressive environment developed between the 1- to 2-cell stages (see below).

Results of recent experiments implicate the second round of DNA replication in reducing the expression of genes that are transiently expressed during the 2-cell stage (Davis *et al.*, 1996). To ascertain if the second round of DNA replication resulted in a global reduction in transcription, aphidicolin was added to 1-cell embryos in G2. These embryos cleaved to the 2-cell stage, and when BrUTP incorporation was assayed at G2, a significantly higher level (50% increase) of incorporation was observed when compared to the control untreated 2-cell embryos that were analyzed in G2 (Fig. 10). Thus, the second round of DNA replication appeared associated with repression of transcription.

Effect of Inhibiting Histone Deacetylases on BrUTP Incorporation by 1-Cell and 2-Cell Embryos

Treatment of preimplantation mouse embryos with butyrate stimulates the expression of plasmid-borne reporter genes not bearing an enhancer to a level similar to that in untreated embryos injected with an enhancer-bearing reporter gene (Wiekowski *et al.*, 1993). Moreover, trapoxin prevents the decrease in expression of endogenous genes whose expression transiently increases during the 2-cell stage (Davis *et al.*, 1996). These results suggest that chromatin remodeling contributes to the regulation of gene expression in the preimplantation embryo (Patterton and Wolffe, 1996) and that histone acetylation may overcome a transcriptionally repressive state that appears to develop during the 2-cell stage (Wiekowski *et al.*, 1993; Henery *et al.*, 1995; and see above).

To explore the effect of these agents on the expression of the total pool of endogenous genes, trapoxin was added to 1-cell embryos immediately after PN formation and BrUTP

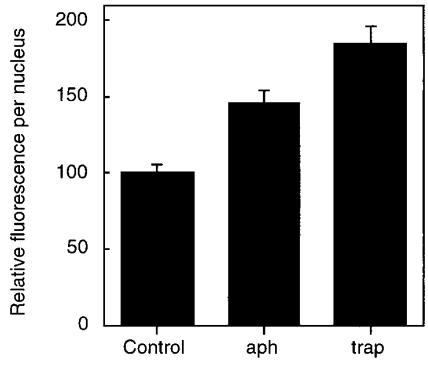

FIG. 10. Effect of aphidicolin or trapoxin added in G2/M of the first cell cycle on BrUTP incorporation in G2 of the second cell cycle. Either aphidicolin (3 μg/ml) or trapoxin (100 n*M*) was added 14 hr post-PN formation, and BrUTP incorporation was determined at 37 hr post-PN formation. The number of nuclei analyzed in the control, aphidicolin, and trapoxin-treated groups was 70, 70, and 72, respectively. The amount of BrUTP incorporated by nuclei in control 2-cell embryos was set as 100%.

incorporation was assayed at either G2 of the 1-cell stage or 2-cell stage; this treatment results in an increase in histone hyeracetylation (Worrad *et al.*, 1995). Whereas trapoxin resulted in a 50% decrease in the total amount of BrUTP incorporation supported by both the male and female PN relative to control 1-cell embryos in G2 (Fig. 8), trapoxin stimulated BrUTP incorporation by 60% when the embryos were in G2 at the 2-cell stage (Fig. 9). It should be noted that trapoxin treatment did not inhibit DNA replication, since the trapoxin-treated 1-cell embryos cleaved to the 2-cell stage. Last, addition of trapoxin to 1-cell embryos in G2 that were then cultured to G2 of the 2-cell stage also resulted in a 1.8-fold increase in BrUTP incorporation relative to control untreated 2-cell embryos (Fig. 10); trapoxin treatment did not inhibit DNA replication in these 2-cell embryos, since there was no effect on BrdU incorporation by these embryos (data not shown).

Transcriptional Activity in Parthogenetically Activated and Polyspermic Eggs

Results of experiments using plasmid-borne reporter genes indicate that the level of transcription is higher in the male PN than in the female PN (Ram and Schultz, 1993; Wiekowski *et al.*, 1993), and consistent with this observation is that the concentration of two transcription factors, Sp1 and TBP, is greater in the male PN (Worrad *et al.*, 1994). We have ascribed these differences to protamine–histone exchange that occurs in the male PN (Nonchev and Tsanev, 1990), since this exchange would provide a window of opportunity for maternally derived transcription factors go gain access to DNA binding sites in the male PN. This opportunity would not present itself for the female PN, since the DNA is already packaged into chromatin.

We examined the transcriptional capabilities of the female PN in the absence of the male PN, i.e., under conditions in which the female PN would not have to compete for transcription factors with the remodeling male PN, by determining the extent of BrUTP incorporation by parthenogenetically activated eggs. The total amount of transcription fostered by the female PN in parthenogenetically activated eggs was similar to the sum of transcriptional activities observed in the male and female PN in fertilized eggs (Fig. 11). In addition, the amount of TBP in these activated eggs was significantly greater than that in the female PN present in fertilized eggs; relative to the male PN, the amount of TBP present in the female PN or PN present in the activated eggs was 36 and 99%, respectively (data not shown). Thus, the transcriptional capacity of the female PN was not intrinsically less than that of the male PN, and in the absence of a male PN the female PN effectively recruited maternally derived transcription factors. These results also suggested that the increase in the DNA content after the first round of replication was not a rate-limiting factor that determines the initiation of transcription, since the DNA content of the parthenogenetically activated embryos was one half that of the fertilized eggs.

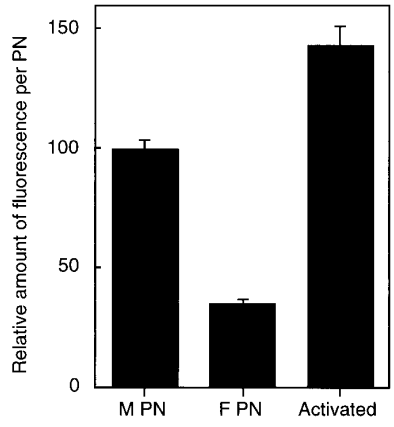

FIG. 11. BrUTP incorporation by parthenogenetically activated eggs. Following parthogenetic activation, BrUTP incorporation in the presence of manganese and ammonium sulfate was determined 12 hr post-PN formation. To synchronize the cell cycle, only embryos that had formed PN before 6.5 hr after insemination were used. Male (M PN) and female PN (F PN) in 33 fertilized eggs and the PN present in 30 parthogenetically activated eggs were analyzed. The amount of BrUTP incorporated by the male PN was set as 100%.

To determine if the capacity of the transcription machinery of the 1-cell embryo exceeded the observed transcriptional activity of the 1-cell embryo, i.e., the transcription machinery was not rate-limiting, we generated polyspermic eggs and determined the total amount of BrUTP incorporation. Results of these experiments indicated that the total amount of transcription supported by dispermic eggs was similar to that of monospermic eggs, and that of trispermic eggs was reduced by 40% (Fig. 12). These results suggested that the transcription machinery was in fact rate-limiting. It should also be noted that although it was difficult to differentiate the male and female PN on the basis of size in the trispermic eggs, the frequency in which one PN had no detectable signal was 80%, whereas it was only about 16% in dispermic eggs. If the PN that did not have a detectable signal was in fact the female PN, this result suggested that in the presence of an increasing number of male PN, the female PN apparently competed less effectively for maternally derived transcription factors.

DISCUSSION

Previous results indicate that the expression of plasmid-borne reporter genes following microinjection of the male PN in S phase is observed in G2 of the 1-cell embryo (Ram and Schultz, 1993; Vernet *et al.*, 1992) and that reporter gene expression is about four to five times greater following injection of the male PN (Wiekowski *et al.*, 1993; Henery *et al.*, 1995). Our results, based on the incorporation of BrUTP, extend these observations to the expression of

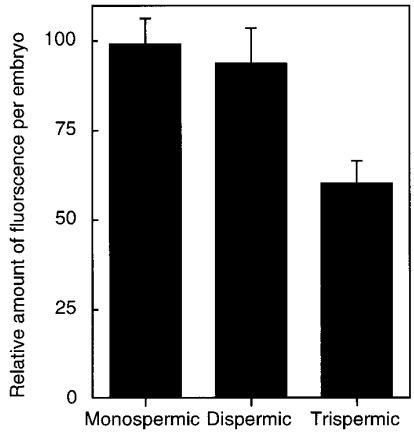

FIG. 12. BrUTP incorporation by polyspermic eggs. *Zona pellucida*-free eggs were fertilized *in vitro* under conditions that promote polyspermy, i.e., 2×10^5–4×10^6 sperm/ml. BrUTP incorporation in the presence of manganese and ammonium sulfate was determined 12 hr post-PN formation. The eggs were observed by epifluorescent microscopy to count the number of nuclei that stained with DAPI and then optically sectioned using the laser-scanning confocal microscope to measure the amount of BrUTP incorporated. The number of monospermic, dispermic, and trispermic eggs analyzed was 30, 31, and 10, respectively. The amount of BrUTP incorporation shown represents the total amount of incorporation by the embryo, i.e., is the sum of incorporation by all of the PN. The amount of incorporation by the monospermic eggs was set as 100%.

endogenous genes and demonstrate that the 1-cell mouse embryo efficiently transcribes endogenous genes such that by late G2 of the first cell cycle the extent of transcription is 20% that of the 2-cell embryo in G2. Transcription is first detected 1–2 hr following initiation of S phase in the 1-cell embryo and steadily increases thereafter. In addition, the male PN supports transcription at levels four to five times greater than the female PN at all times during the first cell cycle. The higher level of transcription in the male PN of endogenous genes is consistent with the male PN containing higher concentrations of transcription factors (Worrad *et al.*, 1994).

This early onset of ZGA of endogenous genes is in marked contrast to *Xenopus laevis* embryos, which do not exhibit readily detectable zygotic transcription until after 12 rounds of DNA replication (Newport and Kirschner, 1982). In *X. laevis*, DNA replication appears to be a critical factor regulating the initiation of zygotic gene activation. Although these embryos possess a functional transcription apparatus (Newport and Kirschner, 1982; Prioleau *et al.*, 1994, 1995), the large maternal stockpile of histones, which is sufficient to support the formation of 15,000–20,000 nuclei (Woodland and Adamson, 1977), appears to prevent the formation of stable basal transcription complexes (Prioleau *et al.*, 1995). Once the maternal histone pool is titrated out by the exponential increase in DNA following 12 rounds of DNA replication, stable basal transcription complexes can form and transcription can initiate.

In contrast to the situation in *X. laevis* embryos, the results presented here clearly demonstrate that DNA replication, and the resulting increase in the amount of DNA, is not critical to initiate ZGA in mouse embryos. Inhibiting the first round of DNA replication with aphidicolin does not prevent the initiation of BrUTP incorporation in 1-cell embryos, although it does inhibit the extent of BrUTP incorporation by 35% (see below for further discussion). Furthermore, parthogenetically activated 1-cell embryos, which have the same DNA content as aphidicolin-treated embryos (i.e., 2C), display a similar level of BrUTP incorporation as control 1-cell embryos. In contrast to *X. laevis*, the maternal histone pool in the mouse 1-cell embryo is probably sufficient for only 1–2 rounds of DNA replication (Wassarman and Mrozak, 1981) and hence insufficient to prevent effectively the assembly of stable basal transcription complexes. Thus, titration of the maternal histone pool by an increase in the mass of DNA does not appear to be a critical factor in regulating the onset of transcription in the mouse embryo. Moreover, the lack of a rapid S phase in the mouse embryo would permit sufficient time for productively assembled transcription complexes to transcribe genes.

Based on the observation that the extent of BrUTP incorporated in aphidicolin-treated 1-cell embryos is 65% that of control embryos, our results suggest that the transcription machinery in the 1-cell mouse embryo apparently can readily gain access to promoters in the absence of DNA replication and the associated chromatin remodeling. The recent finding that the SWI/SNF complex, which is part of the RNA polymerase II holoenzyme, can remodel chromatin by disrupting nucleosomes and facilitating TBP binding to nucleosomes (Wilson *et al.*, 1996) could explain, at least in part, the increase in BrUTP incorporation that is observed in these aphidicolin-treated 1-cell embryos. Nevertheless, the extent of BrUTP incorporation by aphidicolin-treated 1-cell embryos is 35% less than that of control embryos. This difference could reflect a requirement for DNA replication by providing a window of opportunity for maternally derived transcription factors to bind to their cognate *cis*-acting DNA sequences; DNA replication disrupts nucleosomes that intrinsically inhibit transcription (Wolffe, 1994). Consistent with this proposal is that, in contrast to the aphidicolin-treated embryos that display a reduced extent of BrUTP incorporation, the total amount of BrUTP incorporation in parthenogenetically activated eggs, which undergo DNA replication, is similar to that of control 1-cell embryos. It should also be noted that the increase in the number of genes as a consequence of DNA replication cannot totally account for the increase in the rate of transcription that occurs during the first cell cycle, since parthenogenetically activated 1-cell embryos support BrUTP incorporation to the same extent as control embryos, which contain twice the amount of DNA.

The accessibility of transcription factors to their DNA binding sequences created during DNA replication may also

be critical to reprogram the pattern of gene expression in the embryo. Thus, the genes that are expressed in the aphidicolin-treated embryos may reflect those for which the transcription machinery can gain access in the absence of replication and differ from the pattern of gene expression observed in normally replicating embryos. In this regard it is interesting to note that the expression of two genes whose expression transiently increases during the 2-cell stage appears to require the first round of DNA replication for efficient expression (Davis *et al.*, 1996). To address the role of DNA replication in reprogramming the pattern of gene expression, we are currently examining by high-resolution two-dimensional gel electrophoresis and mRNA differential display the gene expression profiles in control and aphidicolin-treated embryos.

The transcriptional capacity of the 1-cell embryo may be rate-limiting, since the total amount of BrUTP incorporation in parthenogenetically activated, mono- and dispermic eggs is similar; if the transcriptional capacity were not rate-limiting, an increase in the total amount of BrUTP as a function of an increase in the DNA content would have been observed. Efficient transcription results from the synergistic interaction of a battery of transcription factors assembled on the array of promoter/enhancer elements for a gene. The decrease in BrUTP incorporation observed in trispermic eggs may reflect titration and partition of transcription machinery components that reduces the synergistic interactions among the transcription factors assembled on their promoters/enhancers. Alternatively, the decrease in the extent of BrUTP incorporation that is observed in the trispermic eggs may reflect the limited capacity of the 1-cell embryo to form mature nuclei in which the chromatin is completely remodeled due to the limited pool of maternally derived histone (Clarke and Masui, 1987).

Throughout the first cell cycle, the female PN is about $\frac{1}{4}$–$\frac{1}{5}$ as transcriptionally active as the male PN. This reduction does not reflect an inherent deficiency in the transcriptional capacity of the female PN, since the total amount of BrUTP incorporated into parthogenetically activated eggs is essentially the same as that for fertilized eggs. This dramatic increase in the transcriptional capacity of the female PN in the absence of a male PN likely reflects the ability of the female PN to sequester transcription factors that are normally efficiently sequestered by the male PN during its formation, and, in fact, the PN in parthenogenetically activated eggs contains greater amounts of TBP than the female PN of fertilized eggs. As previously discussed, the exchange of sperm-derived protamines for maternally derived histones during formation of the male PN provides a window of opportunity for transcription factors to associate preferentially with the male PN, an opportunity that is not experienced by the female PN.

Based on the observation that the inhibiting the second round of DNA replication inhibits the decrease in expression of two genes whose expression transiently increases during the 2-cell stage, we suggested that the second round of DNA replication is transcriptionally repressive (Davis *et al.*, 1996). The second round of DNA replication could displace previously assembled transcription complexes and/or provide an opportunity for repressors to gain access to their DNA binding sites. This repression apparently extends to the pool of endogenous genes, since addition of aphidicolin subsequent to the first round of DNA replication but prior to the second round augments by 1.5-fold the extent of BrUTP incorporated into these aphidicolin-treated embryos, when compared to control 2-cell embryos in G2. When the data are expressed on a per cellular DNA basis, this increase is 3-fold. Thus, even though the rate of transcription increases during the 2-cell stage, the magnitude of this increase may be reduced by the development of a transcriptionally repressive state. In this regard, it is interesting to note that results of experiments in which expression of plasmid-borne reporter genes is assayed suggest that a transcriptionally repressive state develops in the 2-cell embryo (Wiekowski *et al.*, 1993; Majumder and DePamphilis, 1993; Henery *et al.*, 1995); the molecular basis of repression, however, is unknown.

The development of a transcriptionally repressive state in the 2-cell embryo may involve some aspect of chromatin remodeling that is coupled to the second round of DNA replication. The ability of trapoxin, which results in histone hyperacetylation, to increase by twofold the extent of BrUTP incorporation by 2-cell embryos in G2 is consistent with this hypothesis. Thus, histone hyperacetylation may counteract the transcriptionally repressive state that develops following the second round of DNA replication by promoting derepression.

In contrast to the stimulatory effect that trapoxin treatment has on BrUTP incorporation in the 2-cell embryo, trapoxin inhibits the extent of BrUTP incorporation by both the male and female PN of 1-cell embryos in G2 by ~50%. Sodium butyrate, which is not as specific as trapoxin, also inhibits by ~50% the expression of a plasmid-borne reporter gene microinjected into the male PN (Wiekowski *et al.*, 1993; Henery *et al.*, 1995). In contrast, butyrate treatment increases the reporter gene expression observed from the female PN to levels comparable to that observed for the male PN in the absence of butyrate (Wiekowski *et al.*, 1993; Henery *et al.*, 1995). The basis for this difference is not understood, although it should be noted that the analysis of reporter gene expression was conducted with cleavage-arrested aphidicolin-treated 1-cell embryos that were chronologically at the 2-cell stage (Wiekowski *et al.*, 1993; Henery *et al.*, 1995).

We previously demonstrated that during the 2-cell stage hyperacetylated, but not hypoacetylated, isoforms of histone H4 become transiently enriched at the nuclear periphery (Worrad *et al.*, 1995); hyperacetylated isoforms of histone H4 are highly correlated with transcriptionally permissive chromatin (Turner, 1991; Jeppesen and Turner, 1993; Hebbes *et al.*, 1994; O'Neill and Turner, 1995). Nevertheless, we do not observe enhanced BrUTP incorporation at the nuclear periphery in 2-cell embryos. This raises the in-

triguing possibility that a specific subset of genes is located in this chromatin domain and requires histone hyperacetylation for their transcription. In addition, the recent observation that the histone acetylase responsible for acetylating chromatin-associated histones may associate with transcription activators and/or RNA polymerase II (Brownell *et al.*, 1996) suggests that this enzyme may be transiently localized at the nuclear periphery during the 2-cell stage.

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