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DNA replication in the 1-cell mouse embryo: stimulatory effect of histone acetylation

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Summary

Temporal and spatial distribution of the sites of DNA replication were examined in 1-cell mouse embryos. Embryos were labelled with bromodeoxyuridine (BrdU) at hourly intervals after fertilisation, and the incorporation of BrdU was examined by laser-scanning confocal microscopy following immunostaining with an anti-BrdU antibody. DNA replication first started uniformly in both the male and female pronuclei in the intranuclear region and then was observed in the peripheral regions of nucleus and nucleolus. These changes, however, occurred asynchronously in that the female pronucleus required a longer time to complete replication in the intranuclear region but not in the peripheral regions. Inhibiting transcription with α-amanitin had no effect on the temporal and spatial patterns of DNA replication. Treatment of the embryos with trapoxin, a specific inhibitor of histone deacetylase, accelerated the completion of replication in the peripheral regions but not in the intranuclear region. These results suggest that DNA replication is temporally and spatially regulated in the 1-cell embryos and that acetylation of histones, but not transcription, is involved in the regulation of DNA replication.

Keywords: DNA replication, Histone acetylation, Pronuclei, Trapoxin

Introduction

Although the male and female pronuclei of the 1-cell mouse embryo reside in a common cytoplasm and therefore would be anticipated to respond in a similar fashion to cytoplasmic changes, such is not the case. For example, fusion of zygotic halves containing either a female or a male pronucleus with a metaphase II-arrested egg results in more rapid premature chromosome condensation of the maternal chromatin, which also achieves a greater degree of condensation than its paternal counterpart (Ciemerych & Czolowska, 1993). The capacity of the two pronuclei to support transcription also differs. The male pronucleus supports higher levels of total endogenous transcription than does the female pronucleus (Aoki *et al.*, 1997), as well as a more robust expression of microinjected plasmid-borne reporter genes (Ram & Schultz, 1993; Wiekowski *et al.*, 1993; Henery *et al.*, 1995).

These differences probably reflect underlying differences in nuclear structure/composition of the two pronuclei. In particular, protamine–histone exchange is pivotal to the remodelling of sperm DNA and constitutes a core process in the transformation of the sperm head into the male pronucleus (Nonchev & Tsanev, 1990). This exchange does not occur during the formation of the female pronucleus, since the maternal chromosomes are already complexed with histones and present as chromatin. Protamine–histone exchange could provide a window of opportunity for maternal transcription to gain access to their *cis*-cognate DNA binding sequences that is not present for the female pronucleus. This could account for the observation that the male pronucleus has higher concentrations of transcription factors (Worrad *et al.*, 1994) and supports higher levels of transcription than the female pronucleus (Ram & Schultz, 1993; Wiekowski *et al.*, 1993; Henery *et al.*, 1995).

Protamine–histone exchange could also provide a window of opportunity for maternal histones to

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participate in chromatin assembly, and in fact the male pronucleus initially has higher concentrations of hyperacetylated histone H4 (Adenot *et al.*, 1997). The linkage between histone acetylation and transcriptionally permissive chromatin is rapidly garnering wide support (Wolffe & Pruss, 1996 and references therein). The observation that nucleosomes assembled from hyperacetylated histones bind transcription factors better than nucleosomes assembled from hypoacetylated histones (Lee *et al.*, 1993; Vettese-Dadey *et al.*, 1996) suggests that the increased concentration of hyperacetylated histones in the male pronucleus may also contribute to the increased concentration of transcription factors.

There is a previous report that in the mouse the timing of DNA replication differs between the two pronuclei: e.g. the male pronucleus initiates DNA replication prior to the female pronucleus (Luthardt & Donahue, 1973). In a similar fashion, the aforementioned differences in the formation of the male and female pronuclei could markedly contribute to the difference in the timing of DNA replication. For example, as is the case with the assembly of a transcription complex at its promoter, the assembly of the DNA replication machinery at origins of replication is also hindered by chromatin. Thus, protamine–histone exchange would provide a similar window of opportunity for the assembly of these complexes. Likewise, transcription factors have been implicated in stimulating simple origins of replication by either facilitating the binding of origin-recognising proteins, enhancing the activity of the initiation complex subsequent to its formation, or maintaining chromatin in a structure that is capable of directly binding the initiation factors (DePamphilis, 1993*a*, *b*). It should be noted that in these instances the transcription factors act independently of their transcription function and in fact the domain required for activation of DNA replication is not always the same domain that is required for activation of transcription (e.g. Mul & van der Vliet, 1992). Nevertheless, transcription may also stimulate DNA replication, perhaps by locally disrupting chromatin structure and thereby providing access for the replication machinery to origins of replication. In support of this hypothesis is the observation that most transcriptionally active genes are replicated earlier than inactive ones (Goldman *et al.*, 1984; Dhar *et al.*, 1989); that transcriptionally inactive heterochromatin is late-replicating (O'Keefe *et al.*, 1992; Spector, 1993 and references therein); and that the sites of replication and transcription are co-localised in human cells (Hassan & Cook, 1994).

In the present study we examined the temporal and spatial patterns of DNA replication during the first cell cycle, and the role of transcription and histone hyperacetylation in these changes. We confirm the findings that the spatial changes in the pattern of DNA replication are similar for both the male and female pronuclei, that DNA replication initiates essentially synchronously in the two pronuclei, and that DNA replication is first completed in the male pronucleus. We also found that while inhibiting transcription has no effect on the temporal and spatial changes in the pattern of DNA replication in either pronucleus, histone hyperacetylation accelerates the rate of DNA replication in both pronuclei.

Materials and methods

In vitro fertilisation and culture of embryos

Female ddY mice, 21–23 days of age, and male matured ICI mice were purchased from SLC Japan (Shizuoka, Japan). Female mice were superovulated with 5 IU of pregnant male gonadotrophin and 5 IU of human chorionic gonadotrophin (hCG) 48 h later. Unfertilised metaphase II-arrested eggs were collected in Whitten's medium (WM; Whitten, 1971) from the ampullae of oviducts 14–15 h after hCG injection. Sperm were obtained in WM medium from the cauda epididymis. The eggs were inseminated with capacitated sperm that had been incubated for 2 h at 37 °C. One and a half hours after insemination, the eggs were washed with glucose-free CZB medium (Chatot *et al.*, 1989) and cultured in a humidified atmosphere of 5% CO_2/95% air at 37 °C. The embryos were synchronised by removing those that had already formed pronuclei 4.5 h after insemination and then harvesting those that formed a pronucleus during the next hour of culture. In some experiments the embryos were transferred in CZB medium containing 10 ng/ml trapoxin or 24 μg/ml α-amanitin 2 h after insemination. The treatment with trapoxin did not affect the formation of pronuclei. Similar results for each set of experiments were also obtained with eggs derived from CF-1 mice (data not shown).

Detection of DNA synthesis

Embryos were labelled with 10 μM bromodeoxyuridine (BrdU) for 30 min at several time points following pronucleus formation. The embryos were incubated in BrdU from 15 min before and to 15 min after each time point shown in the figures. Following incubation in BrdU, the embryos were washed with phosphate-buffered saline (PBS) containing 0.3% bovine serum albumin (BSA) (PBS/BSA) and then fixed with 3.7% paraformaldehyde. The DNA was denatured by incubating the embryos with 2 N HCl at 37 °C for 1 h and the sample was then neutralised by the addition of 0.1 M borate buffer, pH 8.5, for 15 min. The incorporated BrdU was detected as previously described (Aoki *et al.*,

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1997). Briefly, after washing with PBS/BSA, the embryos were incubated with an anti-BrdU monoclonal antibody (Boehringer-Mannheim) for 45 min, washed, and then incubated with an anti-mouse IgG antibody conjugated with Texas Red (Jackson ImmunoResearch Laboratories, West Grobe, PA) for 45 min. The embryos were mounted on a glass slide with VectaShield (Vector Laboratories, Burlingame, CA) and observed on a Leicca TCS 4D laser-scanning confocal microscope.

Results

Temporal and spatial patterns of DNA replication in male and female pronuclei

The activation of the numerous origins of DNA replication, which are distributed throughout the chromosome (Drouin *et al.*, 1991; DePamphilis, 1993*a*, *b*), occurs in a defined temporal and spatial pattern. For example, in many types of mammalian cells DNA replication starts uniformly in the intranuclear euchromatin-rich region and then becomes localised in the heterochromatin-rich peripheral region (Nakayasu & Berezney, 1989; Fox *et al.*, 1991; O'Keefe *et al.*, 1992); the latest-replicating DNA is associated with perinucleolar and internal heterochromatin. Initial experiments were conducted to ascertain whether a similar pattern of DNA replication occurred in male and female pronuclei.

Pulse-labelling with BrdU revealed temporal changes in the sites of DNA replication during the first cell cycle. Although no incorporation of BrdU was detected in either male or female pronuclei 6 h after insemination, a uniform nuclear distribution of BrdU incorporation was observed in both male and female pronuclei in most embryos 8 h after insemination (Fig. 1*A*). The intensity of labelling appeared higher in the female pronucleus. At 9 h after insemination, approximately 50% of the male pronuclei incorporated BrdU predominantly in perinuclear and perinucleolar regions, although most of the female pronuclei still incorporated BrdU uniformly in nucleoplasm (Fig. 1*B*). In some of these pronuclei, BrdU was also incorporated uniformly in the intranuclear region, but the intensity of signal was lower than that in peripheral regions. At 10 h post-insemination both male and female pronuclei displayed the peripheral staining pattern in approximately 50% of embryos (Fig. 1*C*); in the remaining embryos only the male pronucleus showed the peripheral pattern and the female pronucleus still showed a uniform pattern. In approximately 50% of the male pronuclei showing a peripheral staining pattern, fluorescence was detected in the perinuclear but not the perinucleolar region at this time (Fig. 1*C*). At 12 h, no incorporation of BrdU was detected in a large percentage of the male pronuclei. Incorporation was detected, however, in approximately 50% of the female pronuclei. In some of these pronuclei fluorescence was observed in the perinucleolar but not the perinuclear region (Fig. 1*D*). Thus, the spatial pattern of DNA replication changed in a similar fashion for both the male and female pronuclei. Replication initially occurred uniformly in the intranuclear region and then only in the peripheral regions of nucleus and nucleolus in both the male and female pronuclei, although the order of completion of DNA synthesis in the peripheral regions was different: it was completed in the perinuclear region and perinucleolar regions first in the female and male pronuclei, respectively.

The temporal pattern of DNA replication for these spatial changes, however, differed for the male and female pronuclei. This difference was revealed by pulse-labelling with BrdU for 30 min and calculating the percentage of embryos showing a uniform and peripheral staining pattern at each time point (Fig. 2). A uniform staining pattern was first observed in the intranuclear regions in both the male and female pronuclei in about 60% of embryos at 7 h after insemination. In a small fraction of the embryos (9.2%) BrdU incorporation was detected only in the female pronucleus. These results indicated that DNA synthesis initiated slightly earlier in the male than the female pronucleus. Although a uniform staining pattern was still observed in 60% of the female pronuclei at 10 h after insemination, the uniform staining pattern was observed in only a few of the male pronuclei. This suggests that it took a longer time for the female pronuclei to complete DNA replication in the intranuclear region. Using the data presented in Fig. 2, one can estimate that this difference was about 1 h.

The peripheral staining pattern was observed earlier in the male than the female pronuclei. Although 53.7% of the male pronuclei showed the peripheral pattern at 9 h, only 4.8% of the female pronuclei displayed this pattern at that time; most female pronuclei still showed the uniform pattern. The time at which DNA replication was completed in the peripheral regions, however, was essentially the same for the male and female pronuclei.

Effect of inhibiting transcription on DNA replication

Although the 1-cell embryo is transcriptionally active (Bouniol *et al.*, 1995; Aoki *et al.*, 1997), transcription is clearly not required for DNA replication in the 1-cell mouse embryo, since 1-cell embryos incubated in medium containing α-amanitin under conditions that inhibit transcription, divide to the 2-cell stage (e.g.

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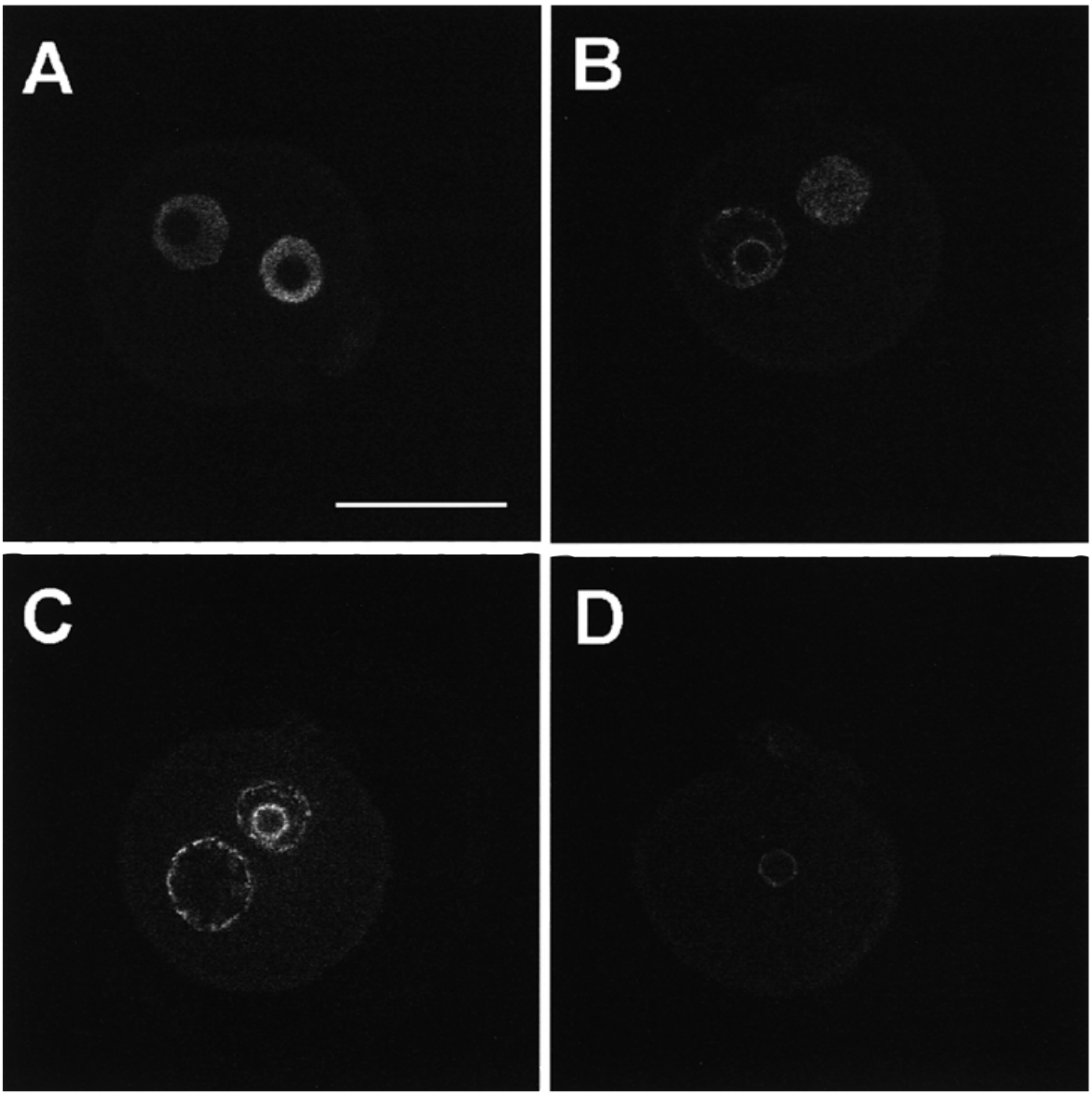

Figure 1 Spatial and temporal distribution of the sites of DNA replication in the 1-cell mouse embryos. Embryos were incubated with BrdU for 1 h at (*A*) 8 h, (*B*) 9 h, (*C*) 10 h or (*D*) 12 h after insemination. Distribution of incorporated BrdU in the pronuclei was observed by laser-scanning confocal microscopy following the immunostain with anti-BrdU antibody. Larger and smaller pronuclei were considered to be male and female pronuclei, respectively.

Stein *et al.*, 1997). Nevertheless, given the potential role of transcription in facilitating the formation of origins of replication, and that the male pronucleus is transcriptionally more active than the female pronucleus (see Introduction), it was possible that the temporal patterns of DNA replication in the male and female pronuclei were linked to transcription.

To test this hypothesis, the embryos were treated with α-amanitin, an inhibitor of RNA polymerase II, and BrdU incorporation was examined 11 h after insemination. α-Amanitin treatment had no effect on the percentage of embryos in which both the male and female pronuclei had completed replication (Fig. 3). Thus, transcription *per se* was not involved in the regulation of DNA replication in either the male or female pronuclei.

Effect of the histone deacetylase inhibitor trapoxin on DNA replication

As discussed in the Introduction, transcription factors may play a role in DNA replication that is independent of their ability to activate the transcription machinery. The higher nuclear concentration of transcription factors and hyperacetylated histones in the male pronucleus could contribute to its earlier initiation of DNA

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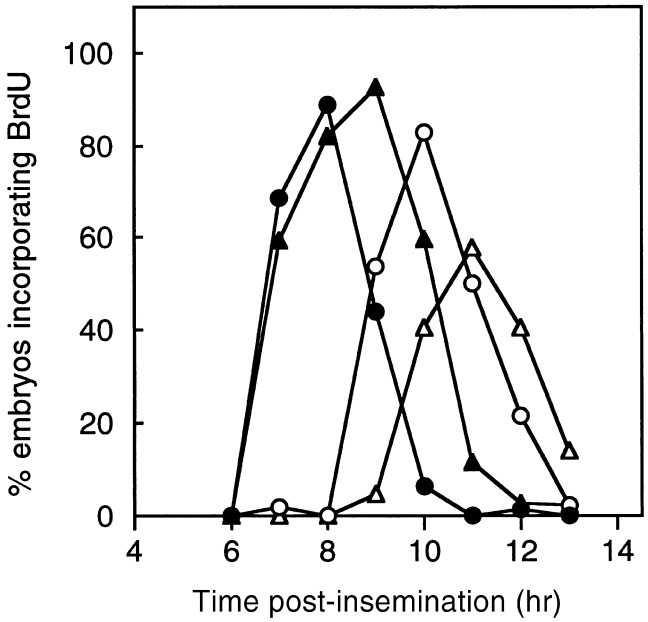

Figure 2 Changes in the sites of DNA replication during S-phase in 1-cell mouse embryos. Percentages of pronuclei showing a uniform or peripheral pattern were calculated at each time point. Filled circles, male pronuclei, uniform pattern; open circles, male pronuclei, peripheral pattern; filled triangles, female pronuclei, uniform pattern; open triangles, female pronuclei, peripheral pattern. The number of embryos examined was 26, 54, 45, 41, 47, 52, 74 and 43 at 6, 7, 8, 9, 10, 11, 12 and 13 h after insemination, respectively.

replication. Since histone hyperacetylation could enhance transcription factor binding to chromatin, we examined whether trapoxin, which is an irreversible inhibitor of histone deacetylase (Kijima *et al.*, 1993) and results in an increase in the level of acetylation of histones in the 1-cell embryo (Worrad *et al.*, 1995; Stein *et al.*, 1997), accelerated the rate of DNA replication.

Treatment of 1-cell embryos with trapoxin showed little effect on the rate of DNA synthesis in the intranuclear regions in either the male or female pronuclei. The percentages of PN showing the uniform staining pattern were almost the same in the embryos treated with trapoxin as in the untreated control embryos at all time points (Fig. 4*A, B*). The treatment did, however, significantly affect the sites and timing of completion of DNA synthesis in the peripheral regions of both male and female pronuclei. In the embryos treated with trapoxin, the incorporation of BrdU was not detected in the perinucleolar region in the male pronucleus, but was observed only in the perinuclear region. Although the time at which a significant number of pronuclei showing the peripheral pattern was first observed was the same between treated and control embryos (9 h, male; 10 h, female), the percentages of pronuclei showing the peripheral pattern were, thereafter, always lower in the treated embryos than in the control embryos. This result suggested that trapoxin treatment accelerated the rate of DNA synthesis in the peripheral regions. This was more apparent by plotting the percentages of pronuclei showing no fluorescence in which DNA replication had been completed (Fig. 5). At 11 h after insemination, the percentages of pronuclei that had completed DNA synthesis were significantly higher ($p < 0.005$) in both the male and female pronuclei in the trapoxin-treated embryos than in control embryos. At 12 and 13 h they were also significantly higher ($p < 0.05$) in the female pronuclei in the treated embryos. These results suggest that acetylation of histones accelerates the rate of DNA synthesis in the peripheral regions but not in the intranuclear region.

Discussion

While our studies were in progress two other reports appeared that described the spatial changes in the pattern of DNA replication in the 1-cell mouse embryo

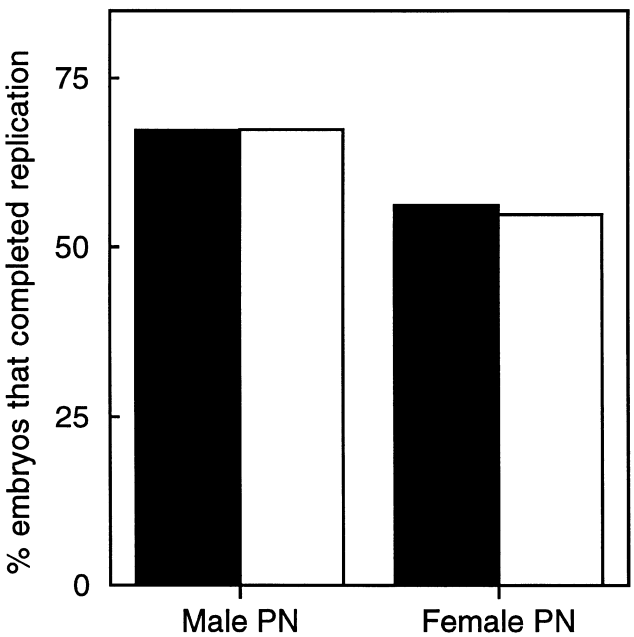

Figure 3 Effect of α-amanitin on the rate of DNA replication in 1-cell mouse embryos. Embryos were treated with α-amanitin from 2 h after insemination. Percentages of embryos that showed no incorporation of BrdU were calculated 11 h after insemination. Since almost all pronuclei showed incorporation of BrdU at 9 h after insemination, the embryos showing no incorporation thereafter could be considered as those that had completed DNA replication. The number of pronuclei examined was 95 and 98 for the α-amanitin group and the control group, respectively. Filled bars, control embryos; open bars, α-amanitin-treated embryos.

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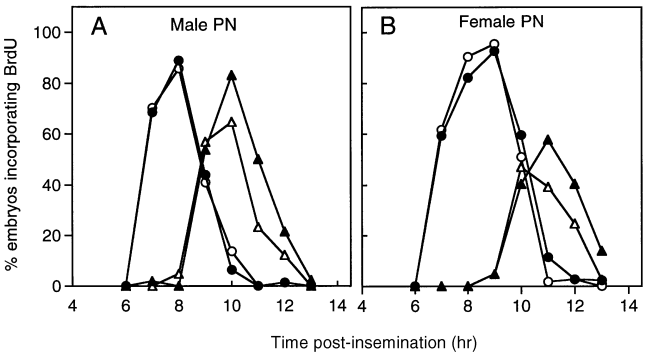

Figure 4 Effect of trapoxin on DNA replication in male and female pronuclei in 1-cell mouse embryos. Embryos were treated with trapoxin from 2 h after insemination. Percentages of (*A*) male and (*B*) female pronuclei showing a uniform or peripheral pattern were calculated. Open circles, trapoxin, uniform partner; filled circles, control, uniform pattern; open triangles, trapoxin, peripheral pattern; filled triangles, control, peripheral pattern. The number of pronuclei examined was 24, 47, 42, 44, 51, 56, 73 and 39 at 6, 7, 8, 9, 10, 11, 12 and 13 h after insemination, respectively, in the embryos treated trapoxin, and was 26, 54, 45, 41, 47, 52, 74 and 43 at 6, 7, 8, 9, 10, 11, 12 and 13 h after insemination, respectively, in the control embryos.

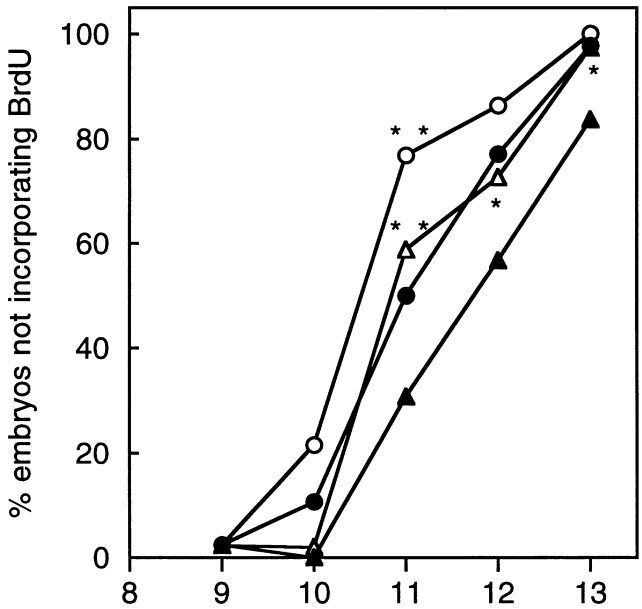

Figure 5 Effect of trapoxin on the rate of DNA replication in 1-cell mouse embryos. Embryos were treated with trapoxin from 2 h after insemination. Percentages of embryos that showed no incorporation of bromodeoxyuridine (BrdU) were calculated at each time point. Since almost all pronuclei showed incorporation of BrdU at 9 h after insemination, the embryos showing no incorporation thereafter could be considered as those that had completed DNA replication. Open circles, trapoxin, male pronuclei; filled circles, control, male pronuclei; open triangles, trapoxin, female pronuclei; filled triangles, control, female pronuclei. Asterisks indicate a significant difference from the control at the corresponding time (**$p < 0.005$; *$p < 0.05$, χ^2-test).

(Bouniol-Baly *et al.*, 1997; Ferreira & Carmo-Fonseca, 1997). Our study differed from these in that we used highly synchronised embryos obtained by the culling procedure in contrast to using naturally mated mice (Ferreira & Carmo-Fonseca, 1997) or mice that had mated within a 30 min window (Bouniol-Baly *et al.*, 1997), which nevertheless results in a substantial asynchrony of fertilisation. In addition, microinjected dig-11-dUTP (Bouniol-Baly *et al.*, 1997) or permeabilised embryos incubated with biotin-16-dUTP (Ferriera & Carmo-Fonseca, 1997) were used in the other studies, whereas we incubated embryos in medium containing BrdU. Despite these differences in experimental design, we report, as did the other two studies, that as in most somatic cells (Nakayasu & Berezney, 1989; Fox *et al.*, 1991; O'Keefe *et al.*, 1992), DNA replication occurs first in the intranuclear region and then in the perinuclear and perinucleolar regions in both the male and female pronuclei, even though the maternal and paternal genomes are compartmentalised. This spatial pattern of DNA replication corresponds with euchromatin, which is predominately localised in the nucleoplasm and nuclear periphery in the 1-cell embryo, replicating before heterochromatin, which is predominantly localised at the perinucleolar region; note that in contrast to somatic cells, little heterochromatin is associated with the nuclear envelope in the 1-cell embryo (Ferreira & Carmo-Fonseca, 1997).

Although the spatial patterns of DNA replication are similar in both male and female pronuclei, the temporal pattern of DNA replication differs. DNA replication appears to start fairly synchronously in both pronuclei. We found that a small fraction (9%) of the recently inseminated embryos incorporated BrdU in only the male, but not the female, pronucleus; a similar finding was also reported by Bouniol-Baly *et al.* (1997). In contrast, Ferreira & Carmo-Fonseca (1997) never observed staining in only the male pronucleus. The reason for this difference is not known. We found that although DNA replication appears to start relatively synchronously, it took the female pronucleus about 1 h longer to complete replication of the intranuclear region. In contrast, the female pronucleus may complete replication in the peripheral regions in about the same time or less than that required for the male pronucleus. The basis for this conclusion is that if the female pronucleus takes longer than the male pronucleus to complete replication in the peripheral regions, one would expect that the female pronucleus would complete replication in this region more than 1 h later than the male pronucleus. The difference, however, was less than 1 h. The net result is that the male pronu-

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cleus completes DNA replication prior to the female pronucleus. A similar conclusion was also reached in other recent studies (Adenot *et al.*, 1997; Bouniol-Baly *et al.*, 1997; Ferreira & Carmo-Fornseca, 1997).

The co-localisation of sites of transcription and DNA replication suggests that transcription may facilitate the formation of replication complexes on origins of replication (Hassan & Cook, 1994). In fact, the higher rate of transcription supported by the male compared with the female pronucleus could provide an explanation for the more rapid completion of DNA replication in the male pronucleus. Several lines of evidence, however, suggest that transcription has no apparent role in DNA replication in the 1-cell embryo. First, transcription starts in the 1-cell embryo in both pronuclei around the middle of S-phase, i.e. following the initiation of DNA replication (Aoki *et al.*, 1997; Bouniol-Baly *et al.*, 1997). Second, there is little co-localisation of transcription and DNA replication sites in the 1-cell embryo (Bouniol-Baly *et al.*, 1997). Third, results presented here demonstrate that α-amanitin has no discernible effect on the temporal and spatial patterns of DNA replication. The protamine–histone exchange that occurs during the transformation of the sperm head into the male pronucleus could still provide a window of opportunity for the assembly of replication complexes on origins of replication that could in turn contribute to the modest advantage of the male pronucleus regarding it ability to complete DNA replication prior to the female pronucleus.

Histone acetylation is now widely accepted to play a stimulatory role in transcription, perhaps by promoting the binding of transcription factors to their *cis*-cognate DNA binding sequences that are normally inaccessible in nucleosomes bearing hypoacetylated histones. In fact, treatment of 1- and 2-cell mouse embryos with histone deacetylase inhibitors relieves the requirement for an enhancer for efficient expression from microinjected plasmid-borne reporter genes (Henery *et al.*, 1995; Wiekowski *et al.*, 1993), as well as relieving the global repression of transcription that develops during the 2-cell stage (Worrad *et al.*, 1995; Aoki *et al.*, 1997). A paucity of information exists, however, regarding the role, if any, of histone acetylation in DNA replication. In a manner analogous to the recruitment of transcription factors to their promoters, the binding of specific transcription factors could directly or indirectly stimulate the assembly or activity of the replication machinery. Consistent with such a role is the observation that induction of histone hyperacetylation by trapoxin results in a reduction of the time to complete DNA replication in the peripheral regions but not the intranuclear regions. Laser-scanning confocal microscopy of 1-cell embryos treated with inhibitor revealed a marked enrichment of hyperacetylated histones at the nuclear periphery (Worrad *et al.*, 1995; Stein *et al.*, 1997). While the underlying molecular basis for this preferential localisation of hyperacetylated histones is not known – there is no evidence for enrichment of DNA at the nuclear periphery and fewer acetylated isoforms of histones are uniformly localised within the nucleoplasm (Worrad *et al.*, 1995; Stein *et al.*, 1997) – this preferential localisation may be directly linked to the more rapid replication of DNA in the peripheral regions. In the only other report (to our knowledge) that found an effect of histone acetylation on DNA replication, both butyrate and trichostatin A advanced the relative timing of replication of two imprinted genes that tend to be late-replicating (Bickmore & Carothers, 1995).

It should be noted that while the two pronuclei respond to either trapoxin or trichostatin treatment in a similar fashion regarding DNA replication, they respond differently regarding their requirement for an enhancer for expression obtained from microinjected plasmid-borne reporter genes. Whereas an enhancer is not required following injection of the male pronucleus (similar levels of expression are obtained with or without the enhancer), an enhancer is required following injection of the female pronucleus (Wiekowski *et al.*, 1993). Induction of histone hyperacetylation has no stimulatory effect on the expression from the male pronucleus but relieves the enhancer requirement for expression following injection of the female pronucleus (Wiekowski *et al.*, 1993; Henery *et al.*, 1995). Thus, histone hyperacetylation has differential effects on DNA replication and transcription that depend on the origin of the pronucleus.

The stimulatory effect of histone acetylation on DNA replication could be due to the more efficient recruitment of the replication machinery to *bona fide* origins of replication via the direct or indirect action of transcription factors. Alternatively, histone hyperacetylation and the attendant binding of transcription factors could lead to the recruitment of the replication machinery at promiscuous origins of replication. DNA replication from these promiscuous sites could depend on active transcription.

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