INTRODUCTION

Eukaryotic cells duplicate their DNA during a well defined period of the cell cycle, the S-phase, and the extent, timing and accuracy of DNA replication are carefully controlled to assure fidelity of genome propagation (reviewed by Kornberg and Baker, 1992). In vertebrates, DNA replication occurs at 100-300 discrete intranuclear foci, each of which contains numerous replication forks (Hand, 1978; Nakamura et al., 1986; Nakayasu and Berezney, 1989; Mills et al., 1989). In yeast, origins of replication are specified by narrowly defined DNA regions, but in higher eukaryotic cells it appears that certain DNA sequences may direct initiation anywhere within a broad initiation zone (for a recent review see Hamlin and Dijkwel, 1995).

It has been recently proposed that each initiation zone in the mammalian chromosome contains many sites where replication can originate, but chromatin structure and/or the nuclear matrix may repress some sites while activating others (DePamphilis, 1993). The concept that nuclear organisation plays a major role in replication is further supported by the finding that early replicating DNA sequences are spatially segregated from late replicating DNA sequences in the nucleus, suggesting that replication of the mammalian genome follows a dynamic four-dimensional order: specific regions of DNA occupying defined locations in the nucleus replicate at precise times during S-phase (reviewed by Spector, 1993). Contrasting with these observations, in cell-free extracts from Xenopus eggs the foci of replication of either Xenopus sperm nuclei, chicken erythrocyte nuclei, Drosophila polytene nuclei, or bacteriophage λ are monotonously maintained during S-phase (Mills et al., 1989; Cox and Laskey 1991; Leno and Laskey 1991; Sleeman et al., 1992), suggesting that initiation sites fire more or less simultaneously. In addition, it has been shown that initiation of replication occurs at random sites on SV40 DNA injected into Xenopus eggs (Harland and Laskey, 1980; McTiernan and Stambrook, 1984) and apparently there are no specific sequence requirements for replication of DNA in these cells (Méchali and Kearsey, 1984; Hyrien and Méchali, 1993). Thus, in contrast with the data obtained in mammalian somatic cells, it appears that chromatin assembled in frog eggs does not organise into early and late replicating domains.

A striking characteristic of fertilised eggs from Xenopus laevis is their ability to undergo many divisions producing approximately 20,000 cells in the same time as a mammalian egg divides only once (see Newport and Kirschner, 1982; Leno and Laskey, 1991). In the fast dividing cells from amphibian embryos, DNA replication is completed within 15 minutes, whereas in the mouse egg the duration of S-phase is 6-7 hours (Callan, 1973; Howlett and Bolton, 1985). Since S-phase lasts 6-10 hours in adult somatic cells from both amphibia and mammals, it is likely that at least some aspects of replication may be distinct in amphibian embryos. We therefore decided to compare the replication pattern previously described in Xenopus eggs with that of mammalian embryos.

Following fertilisation, the mouse developmental program proceeds in a series of easily identifiable and well characterised steps. At approximately 4-9 hours after fertilisation the egg
contains two haploid pronuclei, the male pronucleus originating from the sperm and the female pronucleus originating from the oocyte germinal vesicle. About 12 hours after fertilisation each pronucleus replicates, and at approximately 20 hours after fertilisation the first mitosis occurs producing a 2-cell embryo with two diploid nuclei (reviewed by Telford et al., 1990). Although overt transcriptional activity is first detected in 2-cell embryos, it has been recently demonstrated that 1-cell embryos acquire a transcriptionally permissive state and that these cells may have a minor transcriptional activity during late S and G2 (Schultz, 1993; Bouiniol et al., 1995).

Here we have analysed the distribution of replication sites in 1-, 2- and 16/32-cell mouse embryos using high resolution confocal and video microscopy. The results show that distinct replication patterns occur in both maternal and paternal pronuclei of 1-cell embryos as well as in nuclei from 2- and 16/32-cell embryos. Each of these patterns represents DNA replication temporally restricted to a defined nuclear region, indicating that replication origins are activated at different times during S-phase.

MATERIALS AND METHODS

Animals and embryo recovery
Random-bred Swiss albino mice (6- to 10-week-old; Charles River Breeding Laboratories) were used. Superovulation was achieved by intraperitoneal injection of 5 i.u. of human chorionic gonadotrophin (PMSG) followed 46-48 hours later by an additional intraperitoneal injection of 5 i.u. of human chorionic gonadotrophin (HCG) as previously described (Hogan et al., 1986).

Mated females were used to obtain 1-cell embryos (23-27 hours post-HCG), 2-cell embryos (31-34 hours post-HCG) and morulae-stage (16/32-cell) embryos (72-79 hours post-HCG).

Cell culture
NIH 3T3 fibroblasts, Chinese hamster fibroblasts (CCL-39 cells from ATCC) and PtK2 cells were cultured in RPMI-1640 medium supplemented with 2 mM glutamine, antibiotics (penicillin 100 μg/ml and streptomycin 100 μg/ml) plus 10% FCS and maintained at 37°C with an atmosphere of 5% CO2. For immunofluorescence studies cells were plated and grown onto glass coverslips placed in the culture dishes.

Visualisation of replication sites
The in situ visualisation of DNA replication sites in mouse embryos was adapted from a previously described method (Hozák et al., 1993). In brief, embryos were washed in PB buffer (see Jackson et al., 1993) at 4°C and the plasma membrane permeabilised with 0.05% Triton X-100 in PB buffer at 4°C, until a slight swelling of the cells was observed (1-2 minutes). After three washes in cold PB buffer (2-3 minutes each) the embryos were incubated with replication mix containing 0.1 mM biotin-16-dUTP (Boehringer Mannheim) for 20-30 minutes at 33°C (Hozák et al., 1993). In control experiments replication mix was supplemented with aphidicolin to a final concentration of 5 or 10 μg/ml. At the end of the incubation period the embryos were washed twice in PB at 4°C and the nucleus was permeabilised with 0.2% Triton X-100 at 4°C for 3-4 minutes. The cells were then washed in cold PB and fixed in 3.7% paraformaldehyde in PB at room temperature for 15 minutes with gentle agitation. Detection of the incorporated biotin-dUTP was achieved using extravidin conjugated to either fluorescein or rhodamine (Sigma). Alternatively, embryos were pulse-labelled in vivo with bromo-deoxyuridine (BrdU). In this case, the embryos were cultured in M16 medium (Hogan et al., 1986) supplemented with BrdU to a final concentration of 50 μM for 30 minutes. At the end of the incubation period the embryos were rinsed twice in PB buffer at 4°C, permeabilised in 0.2% Triton X-100 at 4°C for 5 minutes and fixed in 3.7% paraformaldehyde in PB at room temperature for 15 minutes. After 3 washes (~5 minutes each) in PBS containing 0.05% Tween (PBS-Tween) the cells were placed in 4 M HCl at room temperature for 25-30 minutes to denature the DNA (O’Keefe et al., 1992). The cells were then extensively washed in PBS-Tween and either used immediately for immunolabelling or stored at 4°C for up to 3 days. Immunodetection of BrdU-DNA was performed by indirect immunofluorescence using a monoclonal antibody anti-BrdU (Boehringer Mannheim, clone BMC 9318) and a secondary antibody coupled to either fluorescein or rhodamine (Dianova, Germany). To detect DNA replication in cultured mammalian cells (3T3, Chinese hamster fibroblasts and PtK2) BrdU was added to the culture medium to a final concentration of 10 μM for 30 minutes. The cells were then rinsed in PBS and fixed in 2.4% paraformaldehyde in HPEM buffer (30 mM Hepes, 65 mM Pipes, 10 mM EGTA, 2 mM MgCl2, pH 6.9) plus 0.5% Triton X-100 for 15 minutes at room temperature. Denaturation of DNA and immunodetection of BrdU substituted DNA were performed as described above for mouse embryos. For fluorescence microscopy analysis the samples were mounted in 50% glycerol in PBS containing 100 mg/ml DABCO (as an anti-fading agent) and 0.5 μg/ml DAPI (as a DNA stain).

Fluorescence microscopy
Digital fluorescence microscopy was performed using a Zeiss LSM 310 microscope equipped with a Hamamatsu SIT-camera and an ARGUS 10 image processor (Hamamatsu Photonics, Japan). For confocal microscopy, the specimens were scanned with an Argon Ion laser (488 nm), and the images were either digitally recorded or photographed on Kodak TMAX 100 film, using a Polaroid freeze frame recorder.

Electron microscopy
For electron microscope analysis, embryos were recovered by flushing the oviducts with M2 medium (Hogan et al., 1986). The embryos were then incubated with 0.5% pronase (Sigma) in M2 medium for 6-9 minutes at 37°C to promote dissolution of the zona pellucida, rinsed in PBS, attached to poly-L-lysine coated Petri dishes and fixed in freshly prepared 1.6% glutaraldehyde in PBS for 60 minutes. After fixation the embryos were washed in PBS (3×15 minutes), dehydrated in ethanol and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEOL 100CXII electron microscope operated at 80 kV.

RESULTS

Origins of DNA replication in the one-cell mouse embryo are activated at different times during S-phase
Initially we were interested in determining whether the foci of DNA replication in mouse fertilised eggs are maintained during S-phase, as observed in nuclei assembled in cell-free extracts from Xenopus eggs, or change in distribution as observed in mammalian adult somatic cells (see Spector, 1993). To address this question, we have made use of fluorescence microscopy to map DNA replication sites in either permeabilised one-cell embryos that were incubated with biotinylated deoxyuridine (bio-dUTP) or nonpermeabilised embryos grown in the presence of bromodeoxyuridine (BrdU). The results show that DNA replication in both the maternal and paternal pronuclei occurs at several hundred discrete foci and the distribution of these foci is detected in distinct spatial con-
figurations. Four clearly distinct labelling patterns were identified: (1) diffuse, finely granular fluorescence distributed throughout the nucleoplasm (Fig. 1A); (2) perinuclear staining (Fig. 1B and B'); (3) perinucleolar staining (Fig. 1C); and (4) intranuclear fluorescent patches (Fig. 1D, arrowhead).

At the electron microscope level, heterochromatin in the pronuclei of 1-cell embryos is scant and predominantly distributed at the perinucleolar region and as intranuclear patches (Fig. 2). When cells are labelled with DAPI and observed with the fluorescence microscope, these heterochromatic regions are readily identified as brightly stained areas (Fig. 1A',C',D'). The simultaneous visualisation of replication sites and total DNA indicates that incorporation of deoxynucleotides at the perinucleolar region and intranuclear patches represents preferentially the replication of heterochromatin (Fig. 1C and C', D and D'). On the other hand, the nucleoplasmic diffuse labelling pattern is more likely to reflect replication of euchromatin. In contrast with most somatic cells, the pronuclei contain little heterochromatin associated with the nuclear envelope (Figs 1A', 2A). Therefore, the peripheral pattern of deoxynucleotide

**Fig. 1.** Chromatin replication patterns in one-cell embryos. Biotin 16-dUTP was incorporated into the replicating DNA of permeabilised 1-cell mouse embryos. Biotinylated DNA was visualised after detection with fluorochrome-coupled avidin (A-D) and total DNA was stained with DAPI (A',C',D'). The spatial organisation of DNA replication sites changes throughout S-phase giving rise to four distinct 'replication patterns': (1) diffuse labelling of nucleoplasmic euchromatin (A), (2) labelling of the nuclear periphery (B,B'), (3) labelling of perinucleolar heterochromatin (C), (4) labelling of intranuclear heterochromatin patches (D,D', arrowhead; note that in this cell the peri-nucleolar heterochromatin is also replicating). Bar, 10 μm.

**Fig. 2.** Ultrastructural aspects of pronuclear chromatin in 1-cell embryos. Electron micrographs were obtained from embryos collected between 27 and 29 hours post-hCG. (A) The chromatin associated with the nuclear envelope (delineated by arrowheads) appears predominantly loose. In contrast, blocks of heterochromatin (arrowheads) are clearly visible around the precursor nucleoli (B, No) or scattered in the nucleoplasm (C). Bars, 0.5 μm.
incorporation may also correspond, at least in part, to replication of euchromatin.

Since the embryos were obtained by natural fertilisation and thus are asynchronous, we cannot conclude on the temporal order of appearance of the different replication patterns. However, in a total of 93 embryos collected at 23-27 hours pCG we frequently observed pronuclei exhibiting a mixed labelling which simultaneously combined features of distinct patterns. For example, in some pronuclei the labelling was either diffuse and perinuclear, diffuse and perinucleolar, perinuclear and perinucleolar or perinucleolar and patchy. However, the combination of either diffuse or peripheral labelling with staining of intranuclear heterochromatin patches was never detected. This suggests that during S-phase replication of the nucleoplasmic and peripheral euchromatin is temporally far apart from replication of intranuclear heterochromatin regions, while replication of perinucleolar heterochromatin occurs at an intermediate stage. This is consistent with the finding that in somatic cultured cells the nucleoplasmic euchromatin replicates predominantly during early S-phase whereas heterochromatin replicates preferentially during mid and late S-phase (reviewed by Spector, 1993).

Assuming that S-phase of mouse 1-cell embryos lasts 6-7 hours (Howlett and Bolton, 1985), we have calculated the approximate time required to replicate each chromatin region in these cells based on the frequency of pronuclei in S-phase that exhibit each replication pattern (Table 1).

In summary, the data show that origins of DNA replication are activated at different times during S-phase of 1-cell mouse embryos, as observed in adult somatic cells.

### Maternal and paternal pronuclei replicate their genomes asynchronously

Having established that specific chromatin regions are replicated at different times in one-cell mouse embryos, we next compared the timing of replication of each chromatin region in the maternal and paternal pronuclei. The results show that similar DNA replication patterns are present in male and female pronuclei (Fig. 3). However, the simultaneous appearance of the same pattern in the two pronuclei of the

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**Table 1. Chromatin replication patterns in the pronuclei of one-cell mouse embryos**

<table>
<thead>
<tr>
<th>Nucleoplasmic</th>
<th>Peripheral</th>
<th>Perinucleolar</th>
<th>Intranuclear patches</th>
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<tbody>
<tr>
<td>50%</td>
<td>5%</td>
<td>57%</td>
<td>13%</td>
</tr>
<tr>
<td>3-3.5 hours</td>
<td>18-21 minutes</td>
<td>3.8-4 hours</td>
<td>47-55 minutes</td>
</tr>
</tbody>
</table>

A total of 134 pronuclei from 93 embryos was analysed. The sum of percentages is higher than 100 because replication of two distinct chromatin regions may partially overlap in the same pronucleus (for example, see Fig. 1D). The time required to replicate each chromatin region was calculated based on a total duration of S-phase of 6-7 hours (Howlett and Bolton, 1985). The table depicts the relative number of pronuclei replicating each chromatin domain.

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**Table 2. Chromatin replication in maternal and paternal pronuclei from one-cell mouse embryos**

<table>
<thead>
<tr>
<th>Both pronuclei are replicating</th>
<th>With similar patterns</th>
</tr>
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<tbody>
<tr>
<td>n=41*</td>
<td>20%</td>
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</table>

<table>
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<tr>
<th>Only female pronucleus is replicating</th>
<th>With different patterns</th>
</tr>
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<tbody>
<tr>
<td>n=52*</td>
<td>24%</td>
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*A total of 93 embryos was analysed.

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**Fig. 3.** Asynchronous versus synchronous DNA replication in the pronuclei of 1-cell embryos. (A and B) The male and female pronuclei replicate their DNA synchronously (note that the female pronucleus can be readily identified because it is smaller than the male pronucleus). (C and D) A replicating female pronucleus and a non-replicating male pronucleus (delineated by arrowheads). Bar, 10 μm.
same embryo was only observed in 20% of the cells (Fig. 3 and Table 2). In most embryos (56%) replication was only detected in the female pronucleus, and in 24% of cells the maternal and paternal pronuclei displayed distinct replication patterns. Embryos with a replicating male pronucleus and a non-replicating female pronucleus were never observed, even when embryos were collected at either earlier (<23 hours phCG) or later (>27 hours phCG) time points after fertilisation. This indicates that replication of the maternal and paternal genomes in one-cell mouse embryos is asynchronous.

Analysis of DNA replication in two-cell embryos revealed the occurrence of similar replication patterns. However, in contrast with the data observed in one-cell embryos, the two diploid nuclei from each blastomere always exhibited the same replication pattern (Fig. 4). Thus, we conclude that the blastomeres of two-cell embryos replicate their DNA co-ordinately.
The time required to replicate the peripheral chromatin increases during development

To study whether the replication patterns observed in early embryos are maintained during development, we analysed the nuclei of 100 blastomeres from 28 embryos at the morulae stage (16/32 cells). In these cells, the replication foci were either diffusely distributed in the nucleoplasm (Fig. 5A), localised at the periphery of the nucleus and nucleoli (Fig. 5B), localised at the nuclear periphery and in nucleoplasmic patches (Fig. 5C), or restricted to nucleoplasmic patches (Fig. 5D). Thus, contrasting with the results observed in early embryos, staining of the nuclear periphery in morulae was always associated with labelling of either the perinucleolar chromatin or the nucleoplasmic patches of heterochromatin.

We next compared the replication patterns observed in mouse embryos with those detected in rodent adult somatic cells. Asynchronous cultures of mouse fibroblasts (3T3), Chinese hamster fibroblasts (CCL-39) and rat kangaroo kidney epithelial cells (PtK2) were incubated in vivo with bromodeoxyuridine for 30 minutes and analysed as described in Materials and Methods. In each cell line, the tempo-spatial patterns of chromatin replication were essentially similar to those observed in mouse embryos at the morulae stage, i.e. nucleoplasmic diffuse (Fig. 6A), peripheral and perinucleolar (Fig. 6C), peripheral and patchy (Fig. 6E), or patchy (Fig. 6G).

Interestingly, a comparative analysis of the frequency of each replication pattern in early embryos, morulae and cultured adult cells reveals a striking increase in the relative number of nuclei replicating the peripheral chromatin (5% in 1-cell embryos, 43% in morulae and 50-64% in adult somatic cells; Fig. 7), indicating that a major change affecting the time required to replicate this specific chromatin region occurs during early development. As the timing of replication of a particular chromatin region appears to reflect its structural organisation, this observation suggests that a major structural remodelling may occur at the peripheral chromatin. Consistent with this idea, the nuclei from morulae contain significantly more peripheral heterochromatin than the pronuclei from 1-cell embryos (cf. Figs 8A,B and 2A).

In summary, we conclude that the replication dynamics of specific chromatin regions change during development and this is likely to reflect a structural remodelling process associated with differentiation.

DISCUSSION

In this study we show that, in mouse one-cell embryos, specific chromatin regions are replicated at different times during S-phase. This provides the first evidence that mechanisms controlling temporally and spatially the replication of DNA are already present in the haploid pronuclei of the mammalian zygote.

Numerous studies have previously demonstrated the presence of different patterns of DNA replication throughout S-phase in mammalian somatic cells (Nakayasu and Berezney, 1989; van Dierendonck et al., 1989; Mazzotti et al., 1990; Fox et al., 1991; O’Keefe et al., 1992). These studies have shown that replication of transcriptionally active chromatin (euchromatin) occurs early in S-phase and is visualised as a multitude of fine foci widespread in the nucleoplasm, whereas replication at the periphery of the nucleus has been detected during either mid S-phase (O’Keefe et al., 1992) or end of S-phase (Fox et al., 1991), and replication of perinucleolar and internal heterochromatic regions was shown to occur in mid to late S-phase. Since similar DNA replication patterns (i.e. nucleoplasmic diffuse, perinuclear, perinucleolar and internal heterochromatic patches) were identified in the pronuclei of one-cell embryos, we conclude that the dynamic temporal and spatial organisation of DNA replication is already defined in the primordial and totipotent fertilised mammalian egg.

Although a fixed temporal order of DNA replication has been demonstrated in yeast (Burke and Fangman, 1975) and
insect polytene chromosomes (Rudkin, 1972), there is no
evidence for a temporal structure of S-phase in insect or
amphibian eggs. In fact, *Drosophila* and *Xenopus* early
embryos replicate their DNA in approximately 3 or 15 minutes,
respectively, and such fast replication is achieved by an
apparent random increase in the number of initiation points
(Callan, 1973; Blumenthal et al., 1973). However, in these
organisms the length of S-phase increases about 200-fold

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![Image](image-url)

**Fig. 7.** The time required to replicate peripheral chromatin increases during development. The figure depicts the relative number of nuclei replicating each chromatin domain in 1-cell embryos (*n=134*), 16/32-cell embryos (*n=100*), 3T3 cells (*n=126*), PtK cells (*n=115*) and CCL cells (*n=506*). *n*, total number of nuclei analysed.

**Fig. 8.** Ultrastructural aspects of peripheral chromatin in morulae. Electron micrograph from an embryo collected 72 hours post-hCG. Distinct blocks of heterochromatin are associated with the nuclear envelope (delineated by arrowheads). Bar, 0.5 μm.
during development, and the chromosomal pattern of replication becomes more complex (Remington and Flickinger, 1971). In contrast, in the mouse zygote the length of S-phase is already similar to that of adult somatic cells (Howlett and Bolton, 1985). Possibly, this long period of DNA synthesis reflects the complex temporal and spatial order of chromatin replication, which is maintained during mammalian development but does not occur in the fast-dividing insect and amphibian embryos.

In one-cell mouse embryos the maternal and paternal genomes are physically compartmentalised in male and female pronuclei, and our data clearly demonstrate that they do not replicate synchronously. The finding that replication is often detected in the female pronucleus alone but is never restricted to the male pronucleus suggests that S-phase is longer in the female pronucleus. This is consistent with the idea that functional differences exist between the male and female pronuclei. It is well known that the paternal chromosomes are complexed with protamines, which replace histones during spermatogenesis. In the male pronucleus, histones replace the protamines 2 to 4 hours before the onset of DNA synthesis (Nonchev and Tsanev, 1990), and it has been recently proposed that the exchange of protamines for histones could facilitate the access of maternally-derived transcription factors to the male chromatin and therefore enhance its ability to support transcription (Schultz and Worrad, 1995). Likewise, replication factors would have more opportunity to bind to the male DNA, while the mature nucleosomes present in the female pronucleus may restrict the access of the replication machinery to the DNA and therefore slow down S-phase.

It has been proposed that transcriptional activity plays a critical role in setting the time of replication of a particular DNA sequence in the nucleus, since most actively transcribing genes replicate earlier than inactive genes (reviewed by Hatton et al., 1988a,b). During early Xenopus and Drosophila embryogenesis replication appears to occur in the absence of transcriptional activity (Newport and Kirschner, 1982; Edgar and Schuiberger, 1986), and in the mouse transcription is first detected following S-phase of 1-cell embryos (Schultz, 1993; Bouniol et al., 1995). Thus, our data indicate that some chromatin regions are replicated earlier than others in the transcriptionally inactive pronuclei of mouse embryos, arguing that ongoing transcription is not required to impose the temporal order of replication. Rather, this observation supports the view that the replication time of a gene or chromatin region is related to its potential for transcription rather than to actual transcriptional activity (Heintz, 1992; Friedman et al., 1995).

Following fertilisation, the transcriptionally silent early embryo must activate expression of zygotic genes in order to continue development, and there is increasing evidence that chromatin structure transitions are implicated in this process (see DePamphilis, 1993; Schultz and Worrad, 1995). As chromatin structure is known to affect the timing of replication of a particular DNA sequence (Calza et al., 1984; Hatton et al., 1988a,b), the dynamics of embryonic genome replication is likely to change during development. In fact, we show here that replication of peripheral chromatin occupies a significantly larger fraction of the S-phase in adult somatic cells and in morulae than in early embryos (Fig. 7). Furthermore, the nuclei from morulae contain significantly more peripheral heterochromatin than the pronuclei from 1-cell embryos (Fig. 8), suggesting that a structural remodelling of peripheral chromatin occurs during early mouse development. Consistent with this view, it has been recently reported that antibodies specific for hyperacetylated histone H4 and RNA polymerase II produce a transient staining of the nuclear periphery during the G2-phase of mouse two-cell embryos (Worrad et al., 1995).

In summary, a major conclusion from this work is that DNA synthesis in the mammalian zygote follows a fixed temporal and spatial order. Probably, this reflects a defined intranuclear organisation of the embryonic genome, from which differentiated complex structural patterns may evolve. Clearly, further studies are necessary to unravel the mechanisms responsible for nuclear organisation in ontogenically primordial cells and to understand their role during development and differentiation.

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Replication patterns in early mouse embryos


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