The EMBO Journal Vol.16 No.20 pp.6250–6262, 1997

Nuclear translocation and carboxyl-terminal domain phosphorylation of RNA polymerase II delineate the two phases of zygotic gene activation in mammalian embryos

Sylvain Bellier, Sylvie Chastant[1,2], Pierre Adenot[2], Michel Vincent[3], Jean Paul Renard[2] and Olivier Bensaude[4]

Génétique Moléculaire, URA 1302 CNRS, Ecole Normale Supérieure, 46 rue d'Ulm, 75230 Cedex 05, [1]Service de Reproduction, Ecole Nationale Vétérinaire, 7 Avenue du Général De Gaulle, 94704 Maisons-Alfort Cedex, France, [2]Biologie du Développement, Institut National de la Recherche Agronomique, 78352 Jouy en Josas, France and [3]Recherche en Sciences de la Vie et de la Santé, Pavillon Marchand, Université Laval, Sainte-Foy, Québec G1V 4G2, Canada

[4]Corresponding author
e-mail: bensaude@biologie.ens.fr

S.Bellier and S.Chastant contributed equally to this work

In mammalian embryos, zygotic gene transcription initiates after a limited number of cell divisions through a two-step process termed the zygotic gene activation (ZGA). Here we report that RNA polymerase II undergoes major changes in mouse and rabbit preimplantation embryos during the ZGA. In transcriptionally inactive unfertilized oocytes, the RNA polymerase II largest subunit is predominantly hyperphosphorylated on its carboxy-terminal domain (CTD). The CTD is markedly dephosphorylated several hours after fertilization, before the onset of a period characterized by a weak transcriptional activity. The largest subunit of RNA polymerase II then lacks immunological and drug-sensitivity characteristics related to its phosphorylation by the TFIIH-associated kinase and gradually translocates into the nuclei independently of DNA replication and mitosis. A phosphorylation pattern of the largest subunit, close to that observed in somatic cells, is established in both mouse and rabbit embryos at the stage when transcription becomes a requirement for further development (respectively at the 2- and 8/16-cell stage). As these events occurred in the presence of actinomycin D, the nuclear translocation of RNA polymerase II and the phosphorylation of the CTD might be major determinants of ZGA.
Keywords: embryo/phosphorylation/RNA polymerase/transcription

Introduction

In many animals, shortly after fertilization, the embryo is transcriptionally inactive and the onset of development does not require RNA synthesis (reviewed in Davidson, 1986; Yasuda and Schubiger, 1992). Transcription resumes and becomes an absolute requirement for development after a number of cell cycles, this number being a characteristic of each species. In mammals, zygotic gene activation (ZGA) involves a two-step process. This process has been particularly well defined in mice (reviewed in Telford *et al.*, 1990; Schultz, 1993). In this species, a 'minor' ZGA phase initiates at the late 1-cell stage (G_2 phase) with a very weak transcriptional activity (Latham *et al.*, 1992; Ram and Schultz, 1993; Matsumoto *et al.*, 1994; Temeles *et al.*, 1994; Bouniol *et al.*, 1995; Christians *et al.*, 1995; Aoki *et al.*, 1997). As a consequence, a small set of proteins are synthesized at the early 2-cell stage (G_1/S phase) (Flach *et al.*, 1982; Latham *et al.*, 1991): the 70 kDa heat-shock proteins (Bensaude *et al.*, 1983), the TRCs (transcription-requiring complexes) (Conover *et al.*, 1991), the U2afbp-rs splicing factor (Latham *et al.*, 1995) and the translation initiation factor, eIF-4C (Davis *et al.*, 1996). Reporter genes microinjected into the pronuclei of a 1-cell mouse embryo can be transcribed during the minor ZGA phase (for reviews, see Schultz, 1993; Majumder and DePamphilis, 1995; Nothias *et al.*, 1995; see also Christians *et al.*, 1995; Henery *et al.*, 1995). However, this transcription is repressed after the first mitosis unless the microinjected genes possess appropriate enhancers. At the late 2-cell stage (G_2 phase), a sharp transcriptional activation and increase of translational activity characterize the 'major' ZGA and result in a complete change in the pattern of protein synthesis (Van Blerkom and Brockway, 1975; Flach *et al.*, 1982; Howlett and Bolton, 1985; Taylor and Piko, 1987; Latham *et al.*, 1991; Nothias *et al.*, 1996). In summary, three transcriptional transitions have been characterized in the early mouse embryo: (i) the onset of a minor ZGA phase at the late 1-cell stage; (ii) the repression of enhancer-less promoters after the first mitosis; and (iii) the occurrence of a major ZGA at the late 2-cell stage after the second round of DNA replication.

In other mammalian species, the ZGA spans a longer developmental period, postponing the requirement in transcription. Depending on the species, the major ZGA generally occurs after two or three cleavage divisions (Telford *et al.*, 1990) and up to four in the rabbit embryo (Manes, 1977; Delouis *et al.*, 1992). In this species, although zygotic transcripts have not yet been detected before the 2-cell stage (Cotton *et al.*, 1980; Kanka, 1993), the minor ZGA phase may begin at the end of the 1-cell stage, as in the mouse (Christians *et al.*, 1994). The same study indicated that microinjected enhancer-less genes are repressed after the first mitosis.

Conserved mechanisms appear to be involved in the control of ZGA in groups as distinct as amphibians and mammals. In *Xenopus laevis*, the major ZGA occurs at the mid-blastula transition (MBT) and might rely on titration of inhibitory factors by the replicating genomes (Kirschner *et al.*, 1985); the large excess of maternal histones stored in the oocyte of that species might compete for the recruitment of the basal transcription machinery (Prioleau *et al.*, 1995). A deficiency in the activity of transcriptional activators has also been suggested to con-

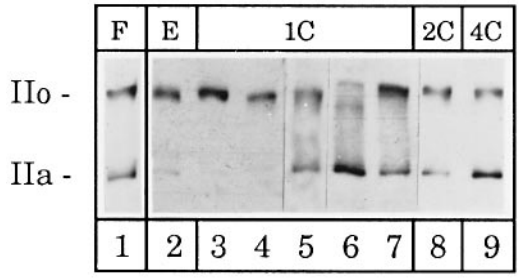

Fig. 1. Phosphorylation state of the RPB1 subunit in mouse embryos. Western blot analysis of the RPB1 subunit in whole lysates using the POL3/3 antibody. NIH 3T3 fibroblasts (F) (lane 1); metaphase II-arrested oocytes (E) (lane 2); 1-cell embryos (1C) lysed respectively before (18 h post-hCG) (lane 3) and <1 h after the appearance of the pronuclei (20 h post-hCG) (lane 4); 1-cell embryos (1C) respectively at 22, 24 and 30 h post-hCG (lanes 5–7); 2-cell embryos (2C) 42 h post-hCG (lane 8); 4-cell embryos (4C) 65 h post-hCG (lane 9). All the lanes correspond to the same exposure in the same experiment. The positions of the IIa and IIo forms are indicated.

tribute to the transcriptional inactivity before MBT (Almouzni and Wolffe, 1995). In the mouse, the first cell cycles are characterized by extensive chromatin rearrangements and changes in nuclear protein composition which are likely to control the ZGA (Debey *et al.*, 1989; Adenot *et al.*, 1991; Clarke *et al.*, 1992; Prather and Schatten, 1992; Vautier *et al.*, 1994; Worrad *et al.*, 1994, 1995; Thompson *et al.*, 1995). Taken together, these studies suggest that the accessibility of the transcription machinery to DNA is controlled by the extensive chromatin modifications taking place during ZGA (reviewed in Patterton and Wolffe, 1996). In the mouse, a 'zygotic clock' which might involve protein kinases, has also been proposed to determine the time-lapse separating fertilization from the beginning of the minor ZGA phase (Schultz, 1993).

Oocytes from *X.laevis* (Roeder, 1974; Toyoda and Wolffe, 1992; Bellier *et al.*, 1997) and from the mouse (Latham *et al.*, 1992) contain large amounts of functional RNA polymerase II. The onset of embryonic development must use this maternal store. The present study was initiated to examine the possibility that phosphorylation of RNA polymerase II largest subunit (RPB1 subunit) might be involved in ZGA. Indeed, both *in vitro* and in somatic cells, transcription involves a cycle of phosphorylation/dephosphorylation of the carboxy-terminal domain (CTD) of this subunit (reviewed in Emili and Ingles, 1995; Dahmus, 1996). Moreover, phosphorylation of the CTD is altered in response to stimuli which have a general influence on the transcriptional activity of the cells, such as viral infection (Rangel *et al.*, 1987; Rice *et al.*, 1994), growth-factor release from quiescence (Dubois *et al.*, 1994b), heat-shock (Venetianer *et al.*, 1995) and meiotic maturation (Bellier *et al.*, 1997).

Mouse and rabbit embryos were used as model systems because they differ markedly in timing and number of cell cycles delimiting the ZGA. In both species, we show that in addition to sharp and specific changes in the pattern of RPB1 phosphorylation, the RPB1 subunit translocates into the nucleus during the period of zygotic gene activation. These time-controlled events which provide a molecular support to define the minor ZGA phase, might control the onset of zygotic gene activation.

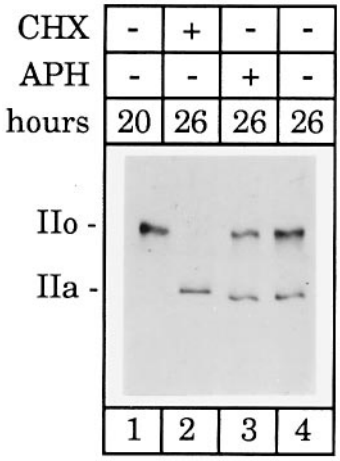

Fig. 2. Influence of cycloheximide or aphidicolin on the phosphorylation state of the RPB1 subunit. Western blot analysis using the POL3/3 monoclonal antibody. Mouse embryos were exposed or not during 6 h to 10 μg/ml cycloheximide or 2 μg/ml aphidicolin Lane 1, control 1-cell embryos at 20 h post-hCG; lane 2, 1-cell embryos from 20 h to 26 h post-hCG in cycloheximide (CHX); lane 3, 1-cell embryos from 20 h to 26 h post-hCG in aphidicolin (APH); lane 4, control 1-cell embryos at 26 h post-hCG. The positions of the IIa and the IIo form are indicated. All lanes correspond to the same exposure in the same experiment.

Results

Dephosphorylation of RPB1 after fertilization

The POL3/3 antibody which reacts with an internal epitope distinct from the CTD, recognized two forms of RPB1 on a Western blot from murine fibroblast lysates (Figure 1, lane 1). A hypophosphorylated form, IIa, migrated at a position similar to that of a 210×10^3 M_r protein and a hyperphosphorylated form, IIo, to that of a 240×10^3 M_r protein (Dubois *et al.*, 1994c; Dahmus, 1995). Both forms gave signals of similar intensity. This pattern remained constant when synchronized fibroblasts were investigated at different stages of the cell cycle (Kim *et al.*, 1997 and our data not shown). In contrast, a form co-migrating with the IIo form predominated in metaphase-arrested mouse oocytes (Figure 1, lane 2) and several hours after fertilization (Figure 1, lanes 3–4). A transition then occurred, at 2–3 h after the appearance of the pronuclei (22 h post-hCG), with the decrease in the IIo-like form and a concomitant sharp increase in the IIa form (Figure 1, lane 5). Forms with intermediate electrophoretic mobilities were observed during a short period at the 1-cell stage, at ~24 h post-hCG (Figure 1, lane 6). A two-band pattern similar to that of transcriptionally active somatic cells was observed at the end of the 1-cell stage (30 h post-hCG) (Figure 1, lane 7). This pattern did not change significantly at the 2-cell stage (lane 8) and throughout further development (not shown). The above-described dephosphorylation was not linked to RPB1 neosynthesis since cycloheximide added to mouse embryos just after the formation of the pronuclei did not prevent the appearance of the hypophosphorylated form of RPB1 (Figure 2, lane 2). However, in the presence of cycloheximide, the hyperphosphorylated forms of RPB1 were no longer detectable. Inhibition of DNA replication with aphidicolin did not affect the phosphorylation status of RPB1 (Figure 2, lane 3).

From these results, we conclude that in the mouse embryo, two sharp time-controlled transitions in the phosphorylation status of the RPB1 subunit take place during interphase of the first zygotic cycle and are completed at the onset of the major ZGA phase.

An embryonic form of RPB1 in preimplantation embryos

Experiments were next designed to establish whether the RPB1 phosphorylation transitions were related to ZGA or were dependent on events occurring during the first cycle. As a first investigation of embryonic events, a comparison between mouse and rabbit embryos was undertaken because in the latter species, the ZGA spans over five cell cycles instead of two. In rabbit fibroblasts, as in mouse fibroblasts, the POL3/3 antibody recognized two bands which corresponded to the IIa and IIo forms of the RPB1 subunit (Figure 3A, lane 1). In rabbit metaphase-arrested oocytes, a RPB1 form co-migrating with the IIo form predominated (Figure 3A, lane 2) as already seen in mouse oocytes. This phosphorylated form dominated for several hours following fertilization (Figure 3A, lanes 3 and 4). A first transition was observed at 18 h post-coitum (hpc) (2–3 h after the appearance of the pronuclei), with the increase in the hypophosphorylated IIa form (Figure 3A, lane 6). The mobility of the hyperphosphorylated form increased gradually and stabilized into a form with an intermediate electrophoretic mobility (Figure 3A, lanes 5–7). This form, which was designated as IIe (embryonic), predominated from the 2-cell to the 8-cell stage (Figure 3B, lanes 1–5). Meanwhile, a faint band co-migrating with the IIo form could be seen on overexposed autoradiograms. The RPB1 subunit dephosphorylated normally in rabbit embryos enucleated just after the formation of the pronuclei and analysed at the late 1-cell stage (data not shown). Thus, in both mouse and rabbit 1-cell stage embryos the increase in hypophosphorylated form, IIa, of RPB1 and the replacement of the oocyte form IIo by an embryonic RPB1 form, IIe, are linked to the onset of the minor ZGA.

Actinomycin D does not prevent a new RPB1 phosphorylation pattern to be established at the onset of the major ZGA

In rabbit embryos, a second transition in the RPB1 phosphorylation state initiated at the 6/8-cell stage (the beginning of the fourth cell cycle), when a form co-migrating with the IIo form gradually replaced the IIe form (Figure 3B, lanes 4–6). At the late 8/16-cell stage, the IIe form was no longer detectable and the RPB1 phosphorylation pattern was very similar to that observed at the morula stage (Figure 3B, lane 7) and in fibroblasts (lane 8). The mouse intermediate form observed during a short period at the 1-cell stage (Figure 1, lane 6) may be the counterpart of the rabbit IIe form.

Since transcription involves a cycle of CTD phosphorylation and dephosphorylation, we next examined the influence of transcription inhibitors on the evolution of the RPB1 phosphorylation pattern at the major ZGA. The non-specific transcription inhibitor, actinomycin D was used because the RNA polymerase II-specific inhibitor, α-amanitin promoted the degradation of RPB1 (Nguyen *et al.*, 1996). Actinomycin D was efficient in inhibiting

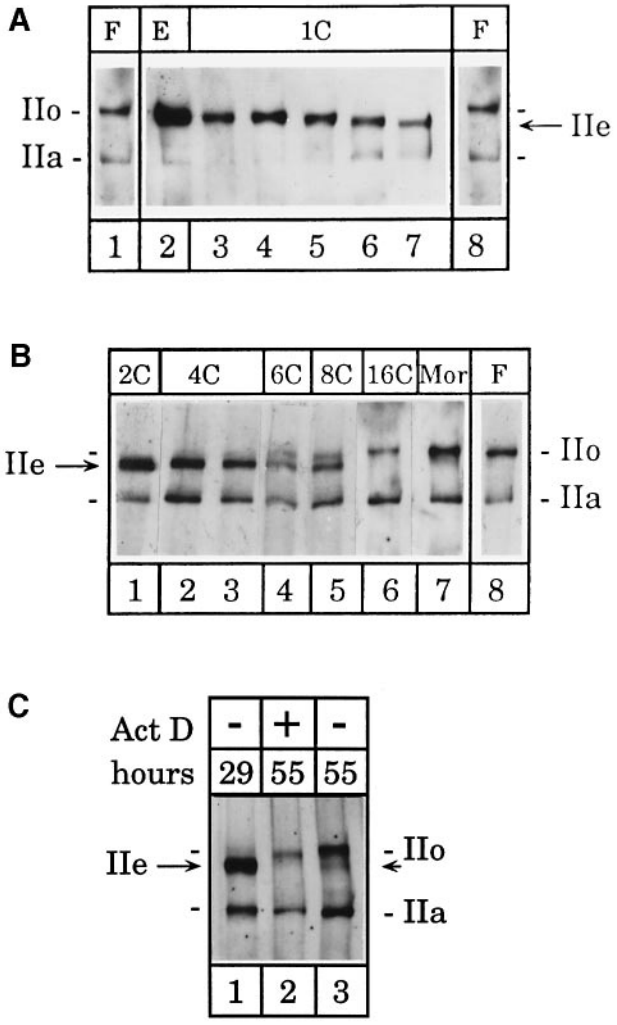

Fig. 3. Phosphorylation state of the RPB1 subunit in rabbit embryos. Western blot analysis of the RPB1 subunit in whole lysates using the POL3/3 antibody. (**A**) Rabbit primary fibroblasts (F) (lanes 1 and 8); unfertilized metaphase II-arrested oocytes (E) 19 hpc (lane 2); 1-cell embryos (1C) lysed 15, 16, 17, 18, 20 hpc (lanes 3–7, respectively). (**B**) 2-cell embryos (2C) 26 hpc (lane 1); 4-cell embryos (4C) 30 and 35 hpc (lanes 2 and 3); 6-cell embryos (6C) 39 hpc (lane 4); early 8/16-cell embryos (8C) 45 hpc (lane 5); late 8/16-cell embryos (16C) 52 hpc (lane 6); morulae (Mor) 72 hpc (lane 7); rabbit primary fibroblasts (F) (lane 8). All lanes correspond to the same exposure in the same experiment. (**C**) 2-cell rabbit embryos were exposed or not to actinomycin D (2 μg/ml) during 26 h. In the presence of actinomycin D (which also inhibits transcription and DNA replication; Kornberg and Baker, 1992), rabbit embryos were prevented from dividing but did not degenerate. Lane 1, 2-cell embryos at 29 hpc; lane 2, 2-cell embryos left from 29 hpc to 55 hpc in actinomycin D; lane 3, untreated 16-cell embryos at 55 hpc. Within each panel, all lanes correspond to the same exposure in the same experiment. The positions of the IIa, IIe and IIo forms are indicated.

luciferase expression driven by an HSP70 promoter after microinjection of the N3Luc plasmid (Christians *et al.*, 1994) into the rabbit pronuclei at the 1-cell stage (data not shown). In embryos left in actinomycin D from 29 hpc (2-cell stage) to 55 hpc, the content in RPB1 was significantly lower than that in controls (Figure 3C). However, in the actinomycin-treated embryos the embryonic form of RPB1, IIe, disappeared completely and the phosphorylation pattern was close to that observed in 16-cell embryos. Thus, a new RPB1 phosphorylation pattern

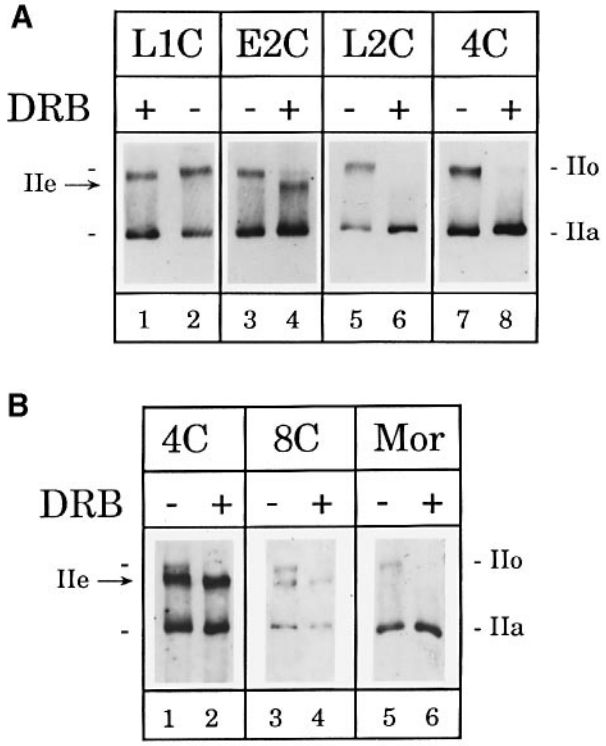

Fig. 4. Influence of DRB on the phosphorylation state of the RPB1 subunit. Western blot analysis using the POL3/3 monoclonal antibody. (**A**) Mouse embryos were exposed (+) or not (–) during 2 h to 100 μM DRB. Late (G_2) 1-cell stage embryos (L1C) from 28 to 30 h post-hCG (lanes 1 and 2); early (G_1/S) 2-cell embryos (E2C) from 31 to 33 h post-hCG (lanes 3 and 4); late (G_2) 2-cell embryos (L2C) from 46 to 48 h post-hCG (lanes 5 and 6); 4-cell embryos (4C) from 63 to 65 h post-hCG (lanes 7 and 8). (**B**) Rabbit embryos were exposed (+) or not (–) during 3 h to 100 μM DRB. Four-cell stage embryos (4C) from 28 to 31 hpc (lanes 1 and 2); early 8/16-cell stage embryos (8C) from 42 to 45 hpc (lanes 3 and 4); morula stage embryos (Mor) from 72 to 75 hpc (lanes 5 and 6). The positions of the IIa, IIe and IIo forms are indicated. All lanes correspond to the same exposure in the same experiment.

is established at the onset of the major ZGA and is unlikely to be a consequence of transcriptional activation.

Embryonic forms of RPB1 insensitive to DRB, a CTD-kinase inhibitor

Several serine/threonine kinases have been demonstrated to phosphorylate the CTD *in vitro* (for a review, see Dahmus, 1996). To characterize the phosphorylation status of the RPB1, embryos were exposed to 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). DRB is a CTD-kinase inhibitor (Yankulov *et al.*, 1995; Herrmann *et al.*, 1996; Marshall *et al.*, 1996) which promotes RPB1 dephosphorylation in fibroblasts (Dubois *et al.*, 1994c).

In mouse embryos, DRB also promoted the dephosphorylation of RPB1 at the 4-cell stage, after the onset of the major ZGA (Figure 4A, lanes 7 and 8). Although the phosphorylation profiles remained identical in untreated embryos from the late 1-cell to the late 2-cell embryos, the DRB effects were markedly different. DRB had no effect on RPB1 phosphorylation in late 1-cell embryos (G_2 phase) (Figure 4A, lanes 1 and 2), led to a partial dephosphorylation in early 2-cell embryos (G_1/S phases) (lanes 3 and 4) and to a complete dephosphorylation in the late 2-cell embryos (G_2 phase) (lanes 5 and 6) at the onset of the major ZGA.

In rabbit embryos, DRB also promoted the dephosphorylation of the RPB1 subunit at the morula stage, long after the onset of the major ZGA (Figure 4B, lanes 5 and 6). Furthermore, DRB provoked the disappearance of a band co-migrating with the IIo form and detectable at the 4-cell or at the 8/16-cell stages (Figure 4B, lanes 1–4). However, it did not affect the IIe form present in the embryos either at the 4-cell or at the 8/16-cell stage.

Our observations show that the predominant slow-migrating forms of RPB1 present during the minor ZGA phase are not DRB-sensitive. Strikingly, the changes in DRB sensitivity are linked to the onset of the major ZGA in both rabbit and mouse embryos.

The embryonic RPB1 form IIe lacks a phosphoepitope generated by TFIIH-associated kinase phosphorylation

DRB is an inhibitor of the TFIIH-associated kinase (Yankulov *et al.*, 1995) which phosphorylates the CTD within preinitiation complexes of transcription (reviewed in Hoeijmakers *et al.*, 1996; Svejstrup *et al.*, 1996). To characterize further the RPB1 form, IIe, present in the early rabbit embryo, we investigated its recognition by the monoclonal antibody, CC-3. Indeed, this antibody, which is directed against a phosphorylated epitope of the CTD, binds to RPB1 phosphorylated *in vitro* by the TFIIH-associated kinase (Dubois *et al.*, 1997). In rabbit fibroblasts, the CC-3 antibody reacted with a major band which co-migrated with the IIo form (Figure 5A) and a non-identified protein of 180 kDa (Vincent *et al.*, 1996). In 8/16-cell stage embryos, the two RPB1 forms, IIo and IIe, were detected in equivalent amounts with the POL 3/3 antibody, but only the slower-migrating one, IIo, was stained with the CC-3 antibody. In 16-cell embryos and in embryos treated with actinomycin D (from 29 to 55 hpc), the CC-3 antibody clearly reacted with both the non-identified 180 kDa protein and the slow-migrating form of RPB1 (Figure 5B). Thus, in contrast to the IIo form, the IIe form of RPB1 present from the 1-cell to the 8/16-cell rabbit embryo lacks a TFIIH-generated phosphoepitope on the CTD.

The embryonic form IIe of RPB1 is not bound to nuclear structures

Since transcribing RNA polymerase is firmly bound to chromatin (Jackson and Cook, 1985; Razin *et al.*, 1985), we next investigated the association of RPB1 with embryonic nuclear structures. Rabbit embryos at the 8/16-cell stage were used because they contained similar amounts of IIe and IIo forms. When embryonic lysates were fractionated, most of the IIe form remained in the cytosolic fraction (Figure 6, lane 6); the hypophosphorylated IIa form was clearly present in the nuclear fraction but recovered mainly in the cytosolic fractions obtained from embryos whereas the major part of the hyperphosphorylated IIo form was associated with the nuclear fraction (Figure 6, lanes 5 and 6, respectively). When lysates from rabbit fibroblasts were fractionated using the same procedure, the hypophosphorylated IIa form distributed equally between the cytosolic and nuclear fractions, whereas the hyperphosphorylated IIo form remained almost totally in the

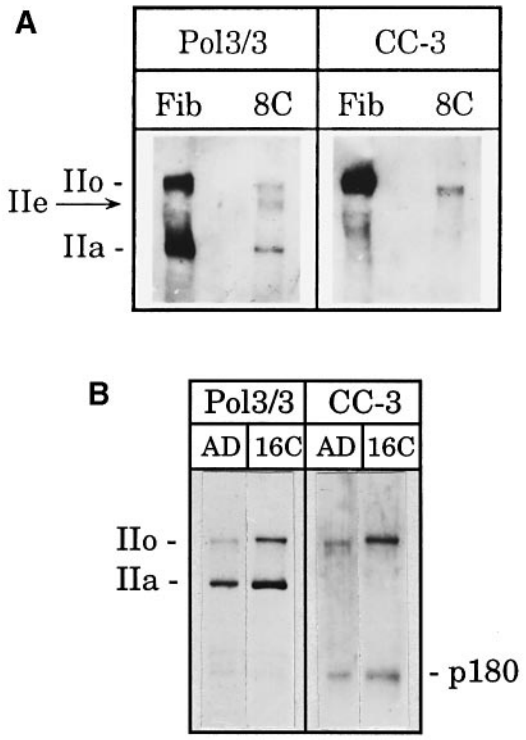

Fig. 5. (**A**) The embryonic form of RPB1 lacks the phosphoepitope CC-3. Rabbit embryos at the 8/16 cell stage (45 hpc) (8C) and rabbit fibroblasts (Fib) were processed for Western blot analysis with the POL3/3 antibody. After destaining in 1% SDS, the same nitrocellulose membrane was restained with the CC-3 antibody. (**B**) 2-cell stage rabbit embryos incubated with actinomycin D from 29 hpc to 55 hpc (AD) and 16-cell embryos 55 hpc (16C) were processed for Western blot analysis with either the POL3/3 or the CC-3 antibodies. The positions of the IIa, IIe and IIo forms are indicated as well as p180, a protein which reacts with the CC-3 antibody.

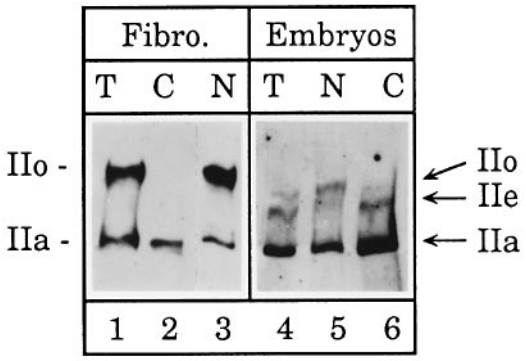

Fig. 6. Fractionation of RPB1 in rabbit fibroblast and embryo lysates. Crude lysates (T) in the non-denaturing salt buffer F were fractionated by centrifugation into nuclear (N) and cytosolic fractions (C). Crude lysates from five rabbit embryos, and fractions from 15 embryos at the 8/16-cell stage (45 hpc) were loaded on the gel. The positions of the IIa, IIe and IIo forms are indicated. All lanes correspond to the same exposure in the same experiment.

nuclear fraction (Figure 6, lanes 2 and 3). As a whole, most of the RPB1 is linked to the nuclear fraction in fibroblasts but remains in the cytosolic fraction in the 8/16-cell embryos.

Gradual nuclear accumulation of the RPB1 subunit before the major ZGA

The presence of RPB1 in the cytosolic fraction does not preclude its compartmentalization and might be due to its leaking from the nucleus. Indeed, several nuclear proteins have been found to be extracted in low-salt buffers (Krek *et al.*, 1992; Michels *et al.*, 1995). Therefore, to investigate further the localization of RPB1 in the rabbit embryos, an immunofluorescence investigation was undertaken using the POL3/3 antibody, visualizing the nuclei with propidium iodide. No staining was evident in metaphase II oocytes and 1-cell stage embryos in which the pronuclei had just formed (Figure 7A, panels 1 and 2). A very faint nuclear staining was visible at the late 1-cell stage (panel 3), increasing slightly at the 2-cell stage (panel 4), more pronounced at the 4-cell stage (panel 5) and still increasing up to the 8/16-cell stage (panel 6) which corresponds to the onset of the major ZGA. The POL3/3 antibody is directed against an RPB1 epitope located outside the CTD (Krämer *et al.*, 1980) and might have been masked in the early stages. Therefore, the immunofluorescence study was repeated using another antibody, 8WG16, which binds an epitope on the CTD (Thompson *et al.*, 1989). Again, the nuclear staining was faint at the 1-cell stage (Figure 7B, panel 1), weak but clearly visible at the 2-cell stage (panel 2), stronger at the 4-cell stage (panel 3) and more intense at the 8/16-cell stage (panel 4). From these concordant results we conclude that, in the rabbit embryo, the RPB1 subunit accumulates gradually in the nuclei throughout the first four cell cycles.

To question whether RPB1 localization would rely on cell cycling, the immunocytology was repeated with mouse embryos. In metaphase II-arrested oocytes (Figure 8, panel 1) and 1-cell embryos within 1 h after formation of the pronuclei (Figure 8, panel 2), the POL 3/3 anti-RPB1 antibody stained the cytoplasm and the pronuclei very weakly but uniformly; however, the nucleoli (arrowheads) did not stain. Weak staining of the pronuclei (excluding the nucleoli) became visible only 3–4 h following their formation (Figure 8, panel 3). It was more pronounced 6–7 h later (at ~10 h after formation of the pronuclei) corresponding to the end of the first cell cycle (panel 4). At the 2-cell stage, the nuclear staining continued to increase (Figure 8, panels 5 and 6) and became comparable with that found in fibroblasts (data not shown). In embryos and fibroblasts, the RPB1 staining was excluded from the nucleoli. Interestingly, the polar bodies which have been reported to be transcriptionally active (Bouniol *et al.*, 1995) were also heavily stained for RPB1 (arrows) (Figure 8, panels 3 and 5).

Hence in both species, RPB1 accumulates into the nuclei along the ZGA period. The accumulation occurs more abruptly in the mouse than in the rabbit, in agreement with the respective ZGA chronologies.

Nuclear translocation of RPB1 does not require either protein or nucleic acid synthesis

It has been reported that protein synthesis and DNA replication are required for the increase in nuclear concentration of the general transcription factors, SP1 and the TATA box binding protein (TBP), that occurs during the first cell cycle of the 1-cell embryo (Worrad *et al.*, 1994). To document this requirement for RPB1 accumulation, 1-cell mouse embryos that had recently formed a pronucleus were cultured in the presence of cycloheximide.

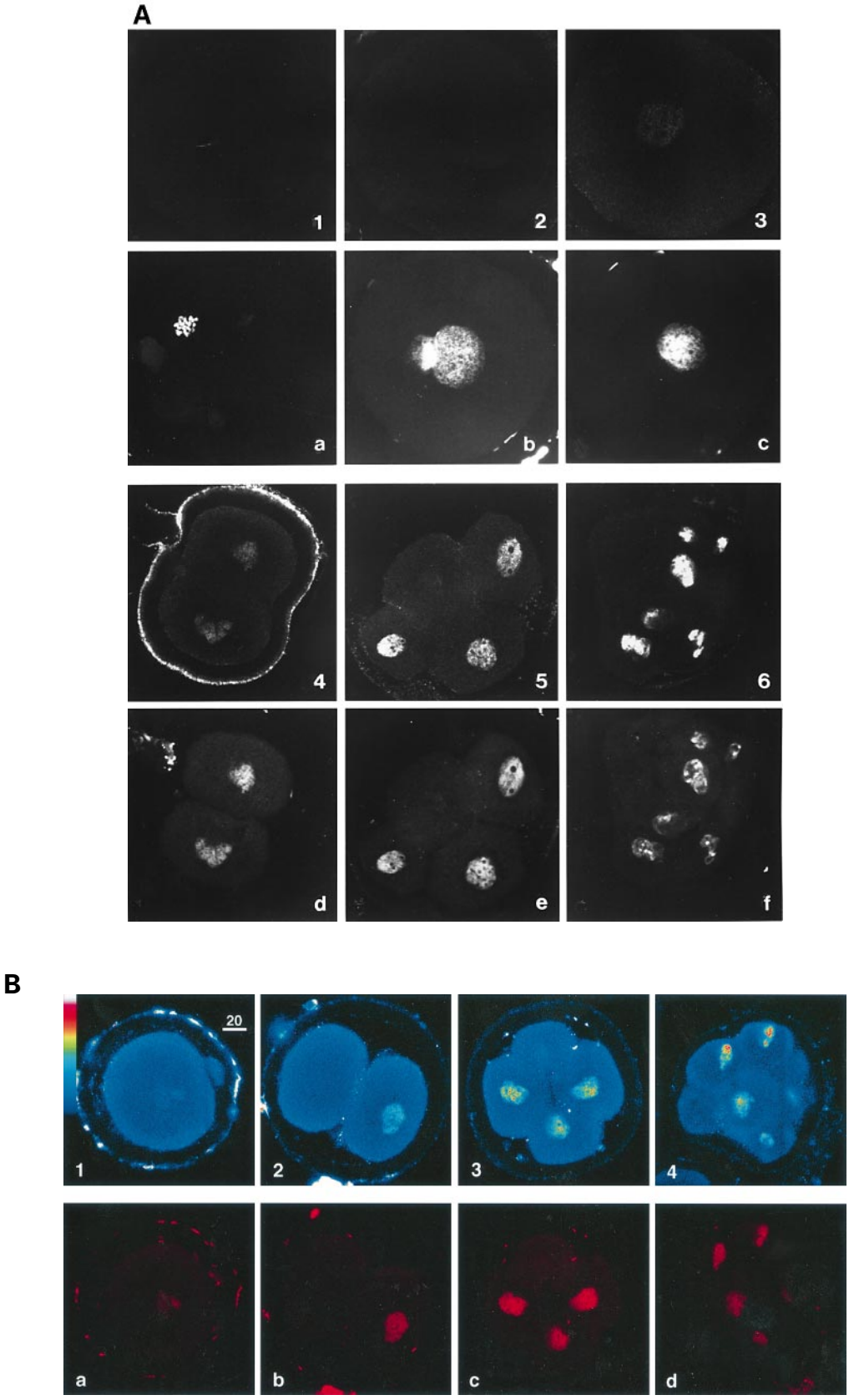

Fig. 7. Immunolocalization of RPB1 subunit during rabbit early development. (**A**) The RPB1 subunit was stained using the POL3/3 antibody (panels 1–6). Chromatin was stained by propidium iodide (panels a–f). Metaphase II-arrested oocytes (1, a); 1-cell embryos, 15 hpc (2, b) and 19 hpc (3, c); 2-cell embryos 24 hpc (4, d); 4-cell embryos 30 hpc (5, e); early 8/16-cell embryos 45 hpc (6, f). (**B**) The RPB1 subunit was stained using the 8WG16 antibody (panels 1–4). Chromatin was stained by propidium iodide (panels a–d). One-cell embryos, 15 hpc (1, a); 2-cell embryos, 24 hpc (2, b); 4-cell embryos, 30 hpc (3, c); early 8/16-cell embryos, 45 hpc (4, d). More than 12 embryos were analysed at each stage.

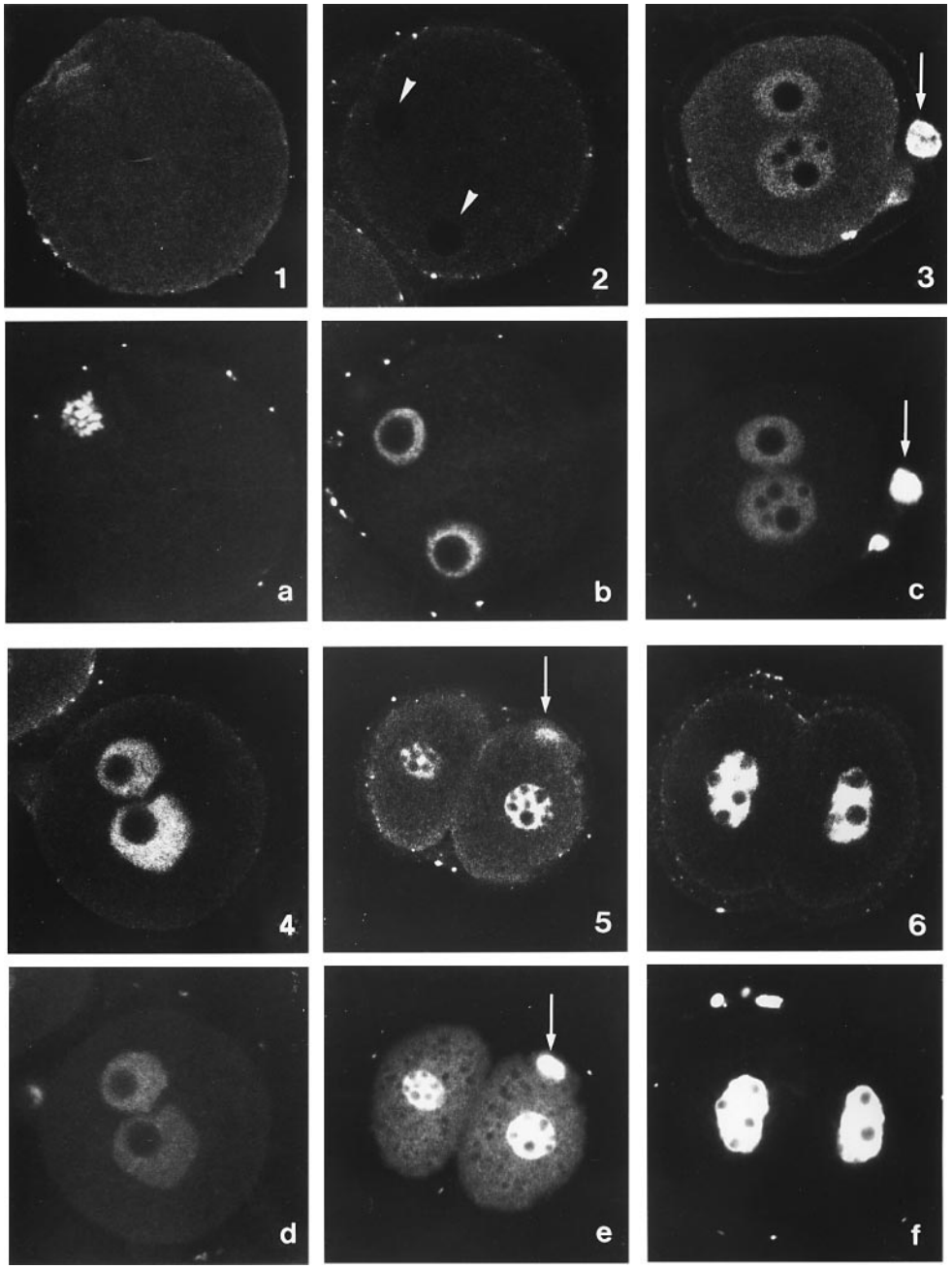

Fig. 8. Immunolocalization of RPB1 subunit during mouse early development. The RPB1 subunit was stained using the POL3/3 antibody (panels 1–6). Chromatin was stained by propidium iodide (panels a–f). Metaphase II-arrested oocytes (1, a); 1-cell embryos 0–1 h (2, b), 3–4 h (3, c), 9–10 h (4, d) following the appearance of the pronuclei (respectively 20 h, 24 h and 30 h post-hCG); 2-cell embryos 0–1 h (5, e), 10–12 h (6, f) following cleavage were stained with the POL3/3 antibody (1–6). Embryos were collected 16 h post-hCG and maintained in M16 medium (1–6). On panel 2, arrowheads point to the nucleoli and on panels 3, 5, c and e, arrows point to the polar bodies. More than 15 oocytes or embryos were analysed at each stage.

Inhibition of protein synthesis did not prevent the nuclear accumulation of RPB1 at the 1-cell stage (Figure 9). Inhibiting DNA replication with aphidicolin, or RNA synthesis with actinomycin D, did not prevent the nuclear translocation of RPB1 either (data not shown). These observations demonstrate that the nuclear accumulation of RPB1 does not result from neosynthesis but is due to a translocation process which does not require transcription, DNA replication or mitosis.

Discussion

Based on the results presented in this study, we propose that compartmentalization of the RPB1 subunit, together with phosphorylation of the CTD, might control ZGA in mammalian embryos. In two different species, mouse and rabbit, with different ZGA chronologies, four major changes in RNA polymerase II properties were closely linked to the minor and major ZGA transitions: (i) the RPB1 subunit translocated gradually into the nucleus

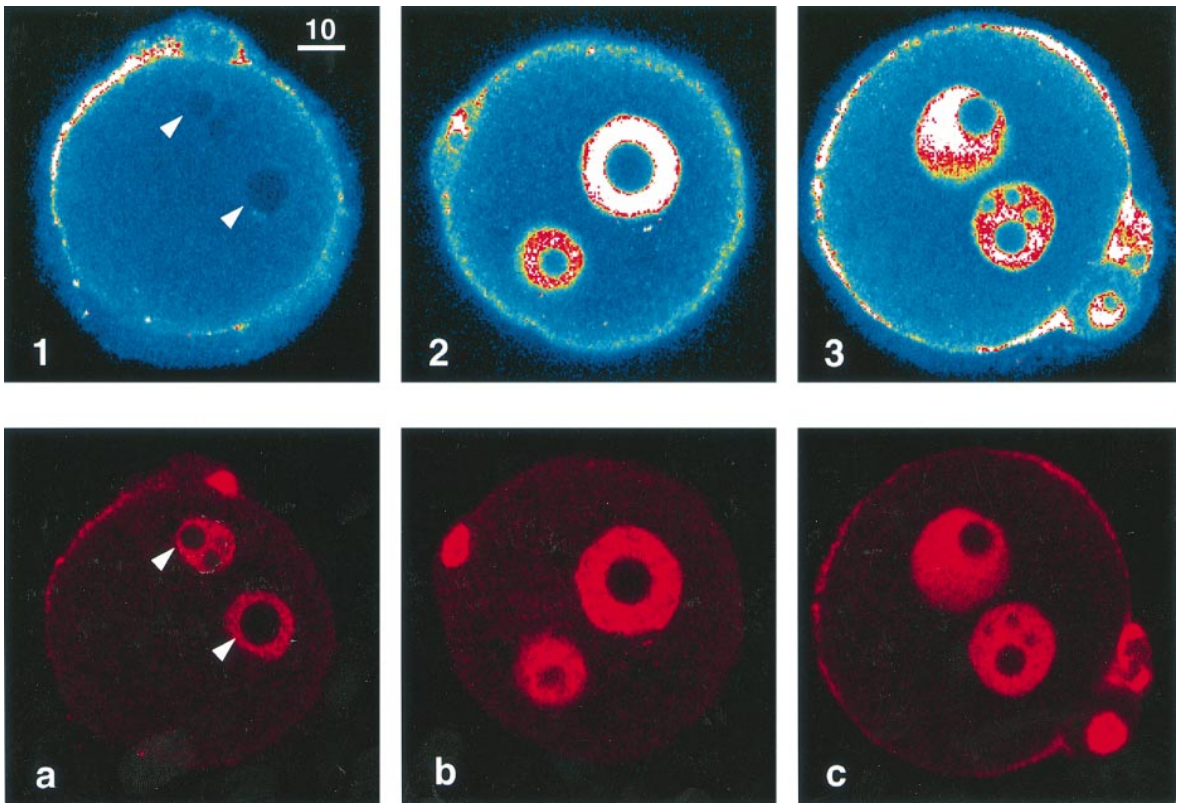

Fig. 9. Immunolocalization of RPB1 subunit in mouse 1-cell embryos treated or not with 10 μg/ml cycloheximide from 19 h post-hCG to 29 h post-hCG. The RPB1 subunit was stained using the 8WG16 antibody (panels 1–3). Chromatin was stained by propidium iodide (panels a–c). One-cell embryos, 19 h post-hCG (1, a); cycloheximide treated 1-cell embryos, 29 h post-hCG (2, b); 1-cell embryos, 29 h post-hCG (3, c). On panels 1 and a, arrowheads point to the nucleoli. More than 20 embryos were analysed in each group.

throughout the minor ZGA phase; (ii) the RPB1 subunit was dephosphorylated during the first cell cycle before the onset of the minor ZGA phase; (iii) throughout the minor ZGA phase, the RPB1 subunit did not show the characteristics of TFIIH phosphorylation; and (iv) at the onset of the major ZGA, the profile of RPB1 hyperphosphorylated forms evolved into that observed in somatic cells.

Nuclear translocation of the RPB1 subunit along the minor ZGA phase

Our results demonstrate that in mammals the nuclear accumulation in RPB1 can be assigned to a gradual translocation of RPB1 from the cytoplasm to the nuclei. This translocation spans over a 24 h period corresponding to one cell cycle in the mouse and throughout at least four cell cycles in the rabbit. In fibroblasts, RNA polymerase II is spread throughout the cytoplasm during mitosis from late prophase to anaphase and concentrates abruptly within minutes into the nucleus during telophase (Warren *et al.*, 1992; Bregman *et al.*, 1995; also our unpublished data). In contrast, RPB1 translocation clearly occurs along interphase in the 1-cell mouse embryo. We also show, using cycloheximide, that the translocation of RPB1 in mouse embryos does not require protein synthesis. It has been suggested that in somatic cells, specific CTD kinase(s) may influence the subnuclear localization of RPB1 (Bregman *et al.*, 1995). Since cycloheximide prevented RPB1 phosphorylation, it can be inferred that its translocation into the nucleus does not require CTD phosphorylation. Since evidence for translocation of RPB1 coincides temporally with the appearance of a transcriptionally permissive phase at the late 1-cell stage, and since in the rabbit it proceeds up to the major ZGA, translocation of RPB1 might be considered as delineating and controlling the minor ZGA phase. It has been proposed that the first round of DNA replication governs the onset of the minor ZGA phase by providing an opportunity for the transcription machinery to gain access to promoters after disruption of the assembled nucleosomes (Davis *et al.*, 1996). In support of this hypothesis, the TBP as well as transcription factors have been shown to translocate into the pronuclei along the first cell cycle in the mouse embryo (Worrad *et al.*, 1994). However, in contrast to TBP, the translocation of RPB1 was not under the control of DNA synthesis. Thus, the nuclear translocation of RPB1 and TBP may not rely on connected molecular events. The RNA polymerase II core enzyme is an assembly of several subunits (Sawadogo and Sentenac, 1990; Young, 1991; Shpakovski *et al.*, 1995), and further studies will examine whether it translocates as such, or whether the various subunits translocate individually and assemble within the nucleus.

RPB1 dephosphorylation at the 1-cell stage: a requirement to turn on the minor ZGA?

In unfertilized mouse and rabbit oocytes, when most of the RPB1 subunit is hyperphosphorylated, an increase in the hypophosphorylated form, IIa, can be detected only several hours after fertilization, when the pronuclei have

formed. The increase in the IIa form of RPB1 does not result from *de novo* synthesis as it also appeared in cycloheximide-treated embryos; it is very likely the consequence of a dephosphorylation process. In *X.laevis*, RPB1 phosphorylation has been attributed to MAP kinase during oocyte maturation; the RPB1 subunit is dephosphorylated after fertilization coincidentally with MAP kinase deactivation (Bellier *et al.*, 1997). In parthenogenetically activated and in fertilized mouse embryos, the decline in MAP kinase activity correlates with the formation of the pronuclei (Kalab *et al.*, 1996; Moos *et al.*, 1996). Thus, in the 1-cell mouse embryo, the dephosphorylation of RPB1 also follows MAP kinase inactivation.

In the mouse, the hypophosphorylated form, IIa, increases from 2–3 h after the appearance of the pronuclei, that is before class II gene transcription, which only initiates 5–6 h after formation of the pronuclei (Bouniol *et al.*, 1995; Aoki *et al.*, 1997). In the rabbit, where transcription from a microinjected plasmid cannot be detected until 24 hpc (Christians *et al.*, 1994), the IIa form also increases from 2 h after formation of the pronuclei (18 hpc). As the CTD was also dephosphorylated in rabbit embryos enucleated just after formation of the pronuclei (data not shown), RPB1 subunit dephosphorylation at this stage relies neither on its nuclear localization nor on transcriptional activation. In both species, dephosphorylation of RPB1 precedes the minor ZGA. This event might be a requirement for the onset of the minor ZGA phase since the hyperphosphorylated RPB1 form, IIo, is reported to be unable to initiate transcription (Dahmus, 1996).

Embryonic forms of RPB1 insensitive to DRB are present during the minor ZGA phase

A previously undescribed slow-migrating form of RPB1, IIe, is now reported to be present at the onset of embryonic development. The IIe form is mainly found in a cytosolic fraction which strongly suggests that it is not engaged in transcription. Indeed, the transcribing RNA polymerase molecules remain bound to the chromatin in low-salt buffers similar to that used for fractionation (Jackson and Cook, 1985; Linial *et al.*, 1985; Razin *et al.*, 1985). The period during which this IIe form is detected, closely coincides with the minor ZGA phase in the rabbit, up to the fifth cell cycle (8/16-cell stage). In the mouse, a similar IIe form is observed but very transiently and not as clearly as in the rabbit. In both species, the bulk of the slow-migrating forms of RPB1 is not affected by DRB during the minor ZGA phase. The differences in DRB susceptibilities between the early and late 2-cell stage mouse embryos are particularly striking. Indeed, in the mouse, numerous studies have pointed out that the major ZGA initiates precisely at the late 2-cell stage (Flach *et al.*, 1982; Howlett and Bolton, 1985; Taylor and Piko, 1987; Latham *et al.*, 1991). Hence in both species, the establishment of the major ZGA is linked to the complete dephosphorylation of RPB1 in the presence of DRB.

Inefficient phosphorylation of the RPB1 subunit by the TFIIH-associated kinase throughout the minor ZGA phase

In yeast, inactivation of the TFIIH-associated kinase results in the rapid disappearance *in vivo* of the hyperphosphorylated form, IIo, of RPB1 (Valay *et al.*, 1995, 1996). Other CTD-kinases have been detected under stressful or developmental conditions (Dubois *et al.*, 1994b; Venetianer *et al.*, 1995; Baskaran *et al.*, 1996; Bellier *et al.*, 1997). The rabbit IIe form of RPB1 is unlikely to be derived from phosphorylation by the mammalian TFIIH-kinase. Indeed, the IIe form lacks the CC-3 phosphoepitope which was shown to be generated *in vitro* by phosphorylation with the TFIIH-associated kinase (Dubois *et al.*, 1997). Furthermore, both the rabbit IIe form and the slow-migrating RPB1 forms found in the 1-cell mouse embryo do not respond to DRB, a TFIIH-kinase inhibitor (Yankulov *et al.*, 1995). The phosphorylation of the CTD by the TFIIH-associated kinase occurs within the preinitiation complex of transcription and the formation of such a complex indeed requires a DNA template plus a transactivator-assisted recognition of promoters by the general transcription factors (reviewed in Hoeijmakers *et al.*, 1996; Svejstrup *et al.*, 1996). Several CTD kinases have been described in eukaryotes (reviewed in Dahmus, 1996) and the involvement of kinase(s) specific to the early embryos might be considered. Alternatively, the gradual increase in the electrophoretic migration of the phosphorylated RPB1 subunit observed in rabbit embryos at the 1-cell stage, may suggest that the IIe form directly derives from the oocyte hyperphosphorylated form through a partial dephosphorylation.

The embryonic characteristics of RPB1 phosphorylation are compatible with transcription of the HSP70.1 gene, a landmark of early zygotic gene activity

The CTD mediates the interaction of RNA polymerase II with transcriptional activators (Gerber *et al.*, 1995; Koleske and Young, 1995; Björklund and Kim, 1996) and the pre-mRNA processing components (Greenleaf, 1993; Mortillaro *et al.*, 1996; Yuryev *et al.*, 1996; Du and Warren, 1997; McCracken *et al.*, 1997). Phosphorylation of the CTD releases the interaction of the 'core' RNA polymerase II with TBP (Usheva *et al.*, 1992), TFIIE (Maxon *et al.*, 1994), and is involved in pre-mRNA processing (Greenleaf, 1993; Mortillaro *et al.*, 1996; Kim *et al.*, 1997). In yeast, phosphorylation of the CTD by the TFIIH-associated kinase is necessary for the polymerase to carry out transcription elongation (Cismowski *et al.*, 1995; Valay *et al.*, 1995, 1996; Akhtar *et al.*, 1996). The hyperphosphorylated form of RPB1 recognized by the CC-3 antibody is generated *in vitro* by phosphorylation with the TFIIH-associated kinase (Dubois *et al.*, 1997) and associates with spliceosomes (Bisotto *et al.*, 1995; Chabot *et al.*, 1995; Vincent *et al.*, 1996). It is therefore intriguing to note that the heat-shock-inducible HSP70.1 gene (which has no introns) is transcribed during the minor ZGA phase in the mouse embryo, from the late 1-cell to the early 2-cell stage (Christians *et al.*, 1995). In heat-shocked cells, although DRB inhibits heat-shock gene expression (data not shown), the bulk of the phosphorylated RPB1 is insensitive to DRB and the CC-3 epitope is also lost (Dubois *et al.*, 1994a, 1997).

DRB-sensitive hyperphosphorylated form of RPB1: a requirement to turn on the major ZGA?

The comparative study of mouse and rabbit embryos shows that the onset of the major ZGA is linked to the

disappearance of the embryonic IIe form and to the marked increase in a IIo form associated with the predominance of a DRB-sensitive turnover of RPB1 phosphorylation. This new phosphorylation pattern is unlikely to be a consequence of transcriptional activation. First, a RPB1 hyperphosphorylated form very similar to the IIo form was generated in the rabbit embryo, even in the presence of actinomycin D which inhibited transcription. Although this form showed CC-3 immunoreactivity, it was unlikely to be generated by the TFIIH-associated kinase as its CTD-kinase activity is exerted within a preinitiation complex of transcription and actinomycin D prevents the formation of such complexes (J.-M.Egly, personal communication). However, transcription-independent kinases such as $p34^{cdc2}$ may also generate the CC-3 epitope on the CTD (Dubois *et al.*, 1997). Second, the number of polymerase molecules present in the rabbit embryos during the ZGA period extends far beyond the fraction likely to be involved in transcription as, on average, a single rabbit embryo contains as much RPB1 as 650 rabbit fibroblasts and a single mouse embryo contains as much RPB1 as 80 mouse fibroblasts (data not shown). Taking into account the number of cells at the ZGA (8/16 in the rabbit, two in the mouse), the number of polymerase molecules per genome is ~40- to 80-fold higher in mammalian embryonic cells than in fibroblasts. Therefore, a fast turnover of the bulk RPB1 phosphorylation due to transcriptional activity is improbable, as a very minor fraction of the RPB1 molecules is likely to be involved in transcription in these early stages.

Thus, a progressive translocation of RPB1 combined with low levels in DRB-sensitive hyperphosphorylated IIo form may account for the time-controlled restricted zygotic gene activity that follows fertilization. The onset of zygotic transcription is also delayed in non-mammalian metazoans such as *Caenorhabditis elegans* and *Drosophila melanogaster* (Yasuda and Schubiger, 1992). In these species, transcription is initiated in the presomatic cells first, and in the primordial germ cells afterwards. It has recently been shown that in the transcriptionally inactive primordial germ cells, the CTD lacks the H5 phosphoepitope which is present in the transcriptionally active presomatic cell nuclei (Seydoux and Dunn, 1997); the H5 phosphoepitope is associated with transcription foci (Zeng *et al.*, 1997). Therefore, we propose that deficient phosphorylation of the CTD is linked to the transcriptional repression which characterizes early embryos from different phyla.

Materials and methods

Oocyte and embryo collection

F_1 hybrid (C57BL/6×CBA) mice females, superovulated by injection of 10 IU pregnant mare serum gonadotrophin (PMSG, Intervet) followed 46–48 h later by injection of 5 IU of human chorionic gonadotrophin (hCG), were mated or not with F_1 hybrid males. Fertilization occurs at ~12 h post-hCG injection. Unfertilized oocytes or 1-cell zygotes were collected 16 h post-hCG injection and cultured in an incubator in 8% CO_2 at 37°C, in M16 medium under oil (light paraffin, BDH) (Christians *et al.*, 1995). The appearance of the pronuclei was followed by visual inspection every 30 min and allowed batches of synchronized embryos to be collected.

New Zealand White rabbits were mated after superovulation and sacrificed 14 hpc for the experiment reported in Figure 3A or 19 hpc for all other experiments. Embryos were recovered as described previously (Christians *et al.*, 1994) and cultured in 199 medium supplemented with 10% fetal calf serum in a 5% CO_2 atmosphere at 39°C. In this species, fertilization occurs at ~12 hpc. Fertilization was assessed by the presence of two polar bodies. Asynchronous cell division is a general feature of rabbit embryonic development after the second cleavage division (4-cell stage) leading to an 8/16-cell stage and a 16/32-cell stage occurring at a relatively precise timing from mating (Ménézo and Renard, 1993).

Antibodies

Monoclonal antibody POL3/3 (a generous gift of Prof. E.K.Bautz) was directed against an epitope located in the core of the RPB1 subunit (Krämer *et al.*, 1980). The 8WG16 monoclonal antibody (a generous gift of Dr N.Thompson) was targeted against the CTD (Thompson *et al.*, 1989). The CC-3 monoclonal antibody was directed against a hyperphosphorylated form of RPB1 (Vincent *et al.*, 1996; Dubois *et al.*, 1997).

Lysis, fractionation and Western blot analysis

Whole lysates prepared from batches of five rabbit embryos or 50 mouse embryos in denaturing buffer L [20 µl of 50 mM Tris–HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol] were electrophoresed in denaturing 5% polyacrylamide–SDS gels and blotted onto nitrocellulose. For fractionation, batches of 15 rabbit embryos were depellucidated in pronase, lysed in 20 µl of buffer F (20 mM sodium β-glycerophosphate, pH 7.4, 1 mM orthovanadate, 0.2 mM EGTA, 2 mM $MgCl_2$, 0.5% Nonidet P40, 0.1% 2-mercaptoethanol) and the lysate was loaded on a 20 µl cushion consisting of buffer F supplemented with sucrose (20%) and stained with bromophenol blue. After 5 min of centrifugation at 1000 *g*, the supernatant which remained above the cushion was designated as the 'cytosolic' fraction and was supplemented with buffer L. The nuclear pellet was dissolved in buffer L. Immunodetection of the RPB1 subunit involved the use of either the POL3/3 or the CC-3 antibody followed by a peroxidase-labelled anti-mouse immunoglobulin antiserum and enhanced chemiluminescence (New England Nuclear). All Western blot experiments were repeated at least three times and showed essentially the same results on each occasion.

Immunofluorescence studies

For immunofluorescence studies with the POL3/3 monoclonal antibody, oocytes and embryos were fixed in phosphate-buffered saline (PBS), 4% paraformaldehyde for 15 min, permeabilized in PBS, 0.2% Triton X-100 for 15 min, blocked in PBS, 3% bovine serum albumin (BSA) for 30 min and incubated with the POL3/3 antibody in PBS, 0.1% Triton X-100, 0.3% BSA for 1 h, then washed three times in PBS, 0.1% Triton X-100 followed by incubation with fluorescein (FITC)-labelled anti-mouse antibody. Nucleic acids were counterstained with 10 µg/ml propidium iodide in PBS for 15 min. The fluorescence was detected with a Zeiss laser-scanning confocal microscope LSM-310 using a Zeiss plan neofluor 100× (NA 1.3) oil immersion objective and 1 µm sections were made through the embryos.

For immunofluorescence studies with the 8WG16 antibody, embryos were treated as above except that: (i) the paraformaldehyde fixation step lasted 18 h; (ii) an RNase step was included as described (Worrad *et al.*, 1995); and (iii) the fluorescence was detected by a Molecular Dynamics laser-scanning confocal microscope with a Zeiss Plan-Apochromat 63×/1.40 oil immersion objective.

Series of embryos corresponding to different stages of development were collected, processed and analysed together under the same staining conditions and using the same settings (filter, gain, background levels). Image processing did not involve either background or contrast adjustments. All the embryos presented in a given figure were selected from a single experiment, which was repeated at least three times and showed essentially the same results on each occasion.

Acknowledgements

This work was supported by grants from the Association pour la Recherche sur le Cancer (ARC 6250), and ACC SV4 (contract No. 504152). We are much indebted to Evelyne Campion and Patrick Chesné for their help in experiments, to Geneviève Almouzni, Michael Dahmus and Marie-Françoise Dubois for their interest in this work and for critical reading of the manuscript, and to Susan Joyce for English proof-reading.

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Received on February 10, 1997; revised on August 4, 1997