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Cell cycle-dependent regulation of RNA polymerase II basal transcription activity

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ABSTRACT

Regulation of transcription by RNA polymerase II (pol II) in eukaryotic cells requires both basal and regulatory transcription factors. In this report we have investigated *in vitro* pol II basal transcription activity during the cell cycle by using nuclear extracts from synchronized HeLa cells. It is shown that pol II basal transcription activity is low in the S and G2 phases and high in early G1 phase and TFIID is the rate limiting component of pol II basal transcription activity during the cell cycle. Further analyses reveal that TFIID exists as a less active form in the S and G2 phases and nuclear extracts from S and G2 phase cells contain a heat-sensitive repressor(s) of TATA box binding protein (TBP). These results suggest that pol II basal transcription activity is regulated by a qualitative change in the TFIID complex, which could involve repression of TBP, during the cell cycle.

INTRODUCTION

The eukaryotic cell cycle is a highly regulated process that is dependent on the temporal expression of specific genes. The cloning of $TAF_{II}250$ (1,2), a subunit of the RNA polymerase II (pol II) transcription factor TFIID, has provided a link between cell cycle regulation and the basal transcriptional machinery. This largest subunit of human TFIID is encoded by *CCG1*, a gene that overcomes a G1 arrest in the temperature-sensitive hamster cell line ts13 (3,4). Therefore $TAF_{II}250$ may be required for progression through G1 phase and may be involved in cell cycle control.

Heintz and Roeder (5) have previously analyzed the transcription activity of the human H4 histone gene promoter and adenovirus type 2 major late promoter (Ad2MLP) using nuclear extracts prepared from synchronized HeLa cells and have reported that while the transcription activity with H4 histone was higher in S phase than in non-S phase, the activity with Ad2MLP was higher in non-S phase than in S phase. Their observations suggested that pol II basal transcription could be regulated in a cell cycle-dependent manner, although they used transcription templates containing an upstream regulatory region in addition to a core promoter region (5,6). In this report we have further investigated the relationship between regulation of pol II basal transcription and the cell cycle. To analyze basal transcription activity we used template fragments containing only a core element (TATA box and initiation region) derived from Ad2MLP and other class II promoters. By using these templates and nuclear extracts of synchronized HeLa cells we found that the basal transcription activity of these promoters was low in the S and G2 phases and high in early G1 phase. We also observed that TFIID activity was rate limiting for this difference. A possible mechanism of cell cycle-dependent regulation of pol II basal transcription will be discussed.

MATERIALS AND METHODS

Cells and synchronization

HeLa S3 cells were grown at 37°C in RPMI medium with 10% fetal bovine serum (Upstate Biotechnology Inc.) in dishes (245 × 245 × 25 mm) at a density of $1–8 \times 10^5$ cells/ml. These cells were synchronized at the G1–S phase boundary by the double thymidine block protocol (7) with minor modifications. Log phase cells were incubated in complete medium with addition of 2 mM thymidine for 16 h. After release in thymidine-free medium for 8 h the cells were incubated again with 2 mM thymidine for 16 h. After release in thymidine-free medium the cells were harvested at the indicated time points. To monitor cell synchrony 1×10^6 cells from each sample were fixed in 70% ethanol, treated with RNase A (0.3 mg/ml) for 30 min at 37°C, stained with 0.5 ml propidium iodide (25 mg/ml) and then analyzed by flow cytometry on a FACScan (Becton Dickinson).

Extract preparation

Nuclear extracts were prepared as described by Dignam *et al.* (8) with minor modifications. Harvested cells (1×10^8) were washed with cold phosphate-buffered saline (PBS), suspended in 600 µl buffer A [10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dithiothreitol (DDT)] and homogenized by 20 strokes of a glass Dounce homogenizer (B pestle). The homogenate was spun at 2000 *g* for 10 min and the nuclear pellet suspended in 5 ml buffer A. After centrifugation at 25 000 *g* for 20 min the nuclear pellet was

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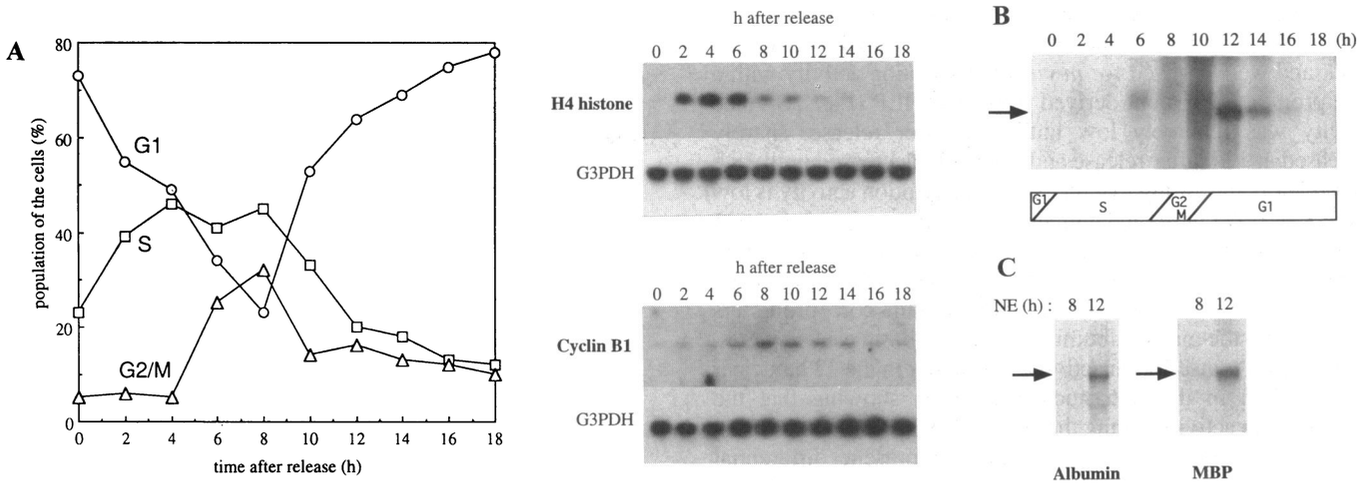

Figure 1. Cell cycle-dependent regulation of pol II basal transcription acitivity. (**A**) (Left) Synchrony of the cycling population of HeLa cells after release from double thymidine block monitored by flow cytometry. The population of G1 (circles), S (squares) and G2/M (triangles) phase cells are indicated as percentages. (Right) Northern blot analyses of the expression of H4 histone and cyclin B1 genes during the cell cycle. Bottom panels were glyceraldehyde 3-phosphate dehydrogenase (G3PDH) controls. The analyses were carried out with 15 µg each total RNAs. (**B**) Nuclear extracts prepared at various time points after release from double thymidine block were assayed for *in vitro* transcription activity with a core promoter derived from Ad2MLP. Nuclear extracts of 12 µl (32 µg total protein) were used in each reaction. (**C**) The 8 h and 12 h nuclear extracts were assayed for *in vitro* transcription activity with core promoters derived from mouse albumin and mouse myelin basic protein (MBP). Nuclear extracts of 12 µl (36 µg total protein) were used in each reaction.

suspended in 200 µl buffer C (20 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 600 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM PMSF, 0.5 mM DDT). The suspension was gently agitated for 30 min, spun at 25 000 *g* for 30 min and the supernatant dialyzed for 6 h against 100 ml buffer D (20 mM Hepes, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM PMSF, 0.5 mM DDT). The nuclear extracts after dialysis were clarified by centrifugation at 25 000 *g* for 30 min, quickly frozen in liquid nitrogen and stored at –80°C.

Transcription templates

pMLC2AT-Δ50 (9) contains the region–50 to +10 of Ad2MLP. A 450 bp *Xba*I–*Sma*I fragment of the plasmid containing the core promoter and a downstream G-less cassette was prepared by PCR and restriction enzyme digestion. Plasmids carrying the mouse albumin promoter from –35 to +10 (AL35/10) and mouse myelin basic protein promoter from –36 to +12 (McBP) between the *Eco*RI and *Hin*dIII sites of pBR322 (10) were gifts from T. Tamura (Chiba University, Chiba, Japan). Fragments containing the core promoters of the albumin and the myelin basic protein with 33 bp upstream and 422 bp downstream regions derived from pBR322 were prepared by PCR and Klenow fragment treatment.

In vitro transcription assay and preparation of heat-treated nuclear extracts

In vitro transcription activity was assayed as described previously (11) with minor modifications. The assays were carried out in a final volume of 20 µl. First, 12 µl nuclear extract was mixed with the core promoter fragments and incubated for 30 min at 30°C. Then a final concentration of 0.5 U/µl ribonuclease inhibitor, 1.5 mM creatine phosphate, 0.1 µg/µl creatine phosphate kinase, 625 µM ATP, 625 µM CTP, 15 µM UTP and 6 µCi [α-^{32}P]UTP were added to the mixture. In cases with the core promoters of albumin and myelin basic protein 625 µM GTP was also added. The reaction was stopped after incubation for 1 h at 30°C and radioactive RNA transcripts were analyzed on 6% polyacrylamide–7 M urea gel. Reconstituted transcription assays with purified general transcription factors and pol II were performed as previously described (12). Heat-treated nuclear extracts were prepared by incubating 10 µl aliquots of extracts in 1.5 ml Eppendorf tubes at 47°C for 6 min.

Other procedures

Total RNAs were isolated from synchronized cells with Isogen (Wako). The method for Northern blotting was as described by Sambrook *et al.* (13). DNA probes were prepared from pF0108X (14) containing the human H4 histone gene and pCycB1 (15) containing the cyclin B1 gene. They were radiolabeled using the BcaBest Labeling Kit (Takara). Western blotting was performed as in the Western Light protocol provided by Boehringer Mannheim.

RESULTS AND DISCUSSION

HeLa S3 cells were synchronized at the G1–S phase boundary by double thymidine block and synchrony of the cycling population was monitored by flow cytometry, as shown in Figure 1A, left panel. After release from the thymidine block the cells rapidly entered S phase, were predominantly in G2 phase at 8 h, divided at 8–10 h and were predominantly in G1 phase at 12–18 h. Figure 1A, right panel, shows Northern blot analyses of total RNA from the cells at various time points during the cell cycle. The amount of H4 histone mRNA rapidly increased at 2 h after release when the cells entered S phase, reached a peak at ~4–6 h and then gradually decreased until 10 h. The amount of cyclin B1 mRNA was, in contrast, most abundant at 8 h after release. The data agree with previous reports that H4 histone and cyclin B1 mRNA were abundant in S phase (16) and in G2/M phase (14) respectively.

Nuclear extracts were prepared from cells synchronized as in Figure 1A at various time points after release from the thymidine block and were assayed for *in vitro* transcription activity with a core promoter fragment derived from Ad2MLP (Fig. 1B). The activity was relatively low until 8 h after release, sharply increased at 12 h after release and gradually decreased until 18 h after release, indicating that the basal transcription activity is low in the S and G2 phases and high in early G1 phase. Quantification of the transcript showed that transcription activity was ~8-fold higher in early G1 phase than in the S and G2 phases. Figure 1C shows the *in vitro* transcription activity with core promoters derived from the mouse albumin and myelin basic protein genes. In both cases transcription activity was higher in the 12 h nuclear extracts than in the 8 h nuclear extracts, showing that the fluctuation in activity during the cell cycle was not an Ad2MLP-specific phenomenon. Thus it should represent differential activity of the basal transcription machinery consisting of general transcription factors and pol II.

It was possible that the change in basal transcription activity during the cell cycle reflected a quantitive change in the amount of general transcription factors or pol II. However, analyses of mRNA levels for TBP, TFIIB and TFIIF (RAP30/RAP74) and protein amounts of TBP, TFIIB, TFIIF and pol II by Northern and Western blotting respectively demonstrated that the mRNA levels and the protein amounts were constant during the cell cycle (data not shown; for Western blot analysis of TBP see Fig. 2B).

To clarify the biochemical mechanism underlying the difference in the basal transcription activity during the cell cycle we measured *in vitro* transcription activities of the 8 h and 12 h nuclear extracts after adding specific purified general transcription factors and pol II. Figure 2A, left panel, shows that increasing amounts of purified TFIID (fraction FD; 17) stimulated basal transcription activity of the 8 h nuclear extracts, while that of the 12 h extracts was unaffected. It should be noted here that the native TFIID was purified from log phase HeLa cells which contain a large population of G1 phase cells. In contrast, the addition of recombinant TFIIB, recombinant TFIIF and pol II purified from log phase HeLa cells had no effect on transcription activities in both the 8 h and 12 h extracts (data not shown). These data strongly suggest that TFIID activity is a limiting determinant of basal transcription activity in the 8 h extracts. Next, to confirm that TFIID is the only rate limiting initiation factor, endogenous TFIID activity in the nuclear extracts was eliminated by heat treatment. Nakajima *et al.* (18) have reported that TFIID in HeLa cell nuclear extracts was selectively inactivated by mild heat treatment. In our experiments endogenous TFIID was inactivated by incubation at 47°C for 6 min. Figure 2A, right panel, shows that the transcription activities of both the 8 h and 12 h nuclear extracts were totally abolished by the heat treatment, but were restored to the same extent in both extracts by addition of purified TFIID. This result demonstrated that TFIID activity was primarily responsible for the different basal transcription activity of the 8 h and 12 h extracts. Furthermore, it is also shown that the activities of other general initiation factors were apparently not different between the extracts. Western blot analyses of TATA box binding protein (TBP), a core subunit of the TFIID complex, revealed that the amounts of TBP in the 8 h and 12 h extracts were equivalent (Fig. 2B), indicating that the lower TFIID activity in the 8 h extracts did not derive from a lower content of TBP. These data strongly suggest that a qualitative change in the TFIID complex accounts for the cell cycle-dependent basal transcription activity, i.e. TFIID exists in a less active form in the S and G2 phases and an active form in early G1 phase.

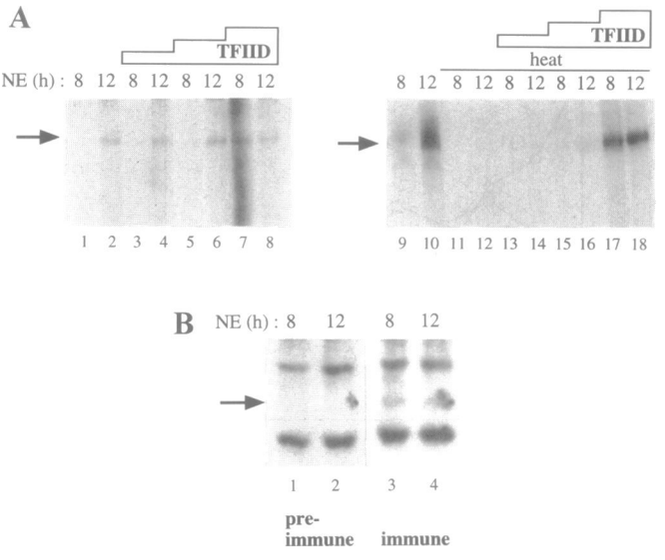

Figure 2. Effects of the addition of native TFIID fraction to S and G2 phases and early G1 phase nuclear extracts on pol II basal transcription activity. (**A**) (Left) HeLa TFIID fraction was added at 0 ng (lanes 1 and 2), 40 ng (lanes 3 and 4) and 400 ng (lanes 5 and 6) and 4 µg (lanes 7 and 8) to 10 µl (30 µg total protein) each of the 8 h (lanes 1, 3, 5 and 7) and 12 h (lanes 2, 4, 6 and 8) nuclear extracts and assayed for *in vitro* transcription activity with the core promoter derived from Ad2MLP. (Right) Aliquots of 10 µl (30 µg total protein) (lanes 11–18) nuclear extract were heated at 47°C for 6 min. Lanes 11–18 are the same as the left panel except that nuclear extracts were heat treated. Lanes 9 and 10 show the control experiments with the 8 h and 12 h extracts respectively before the heat treatment and addition of TFIID. (**B**) Protein amounts of TBP contained in the 8 h and 12 h nuclear extracts were equivalent. Aliquots of 10 µl (30 µg total protein) each of the 8 h (lanes 1 and 3) and 12 h (lanes 2 and 4) nuclear extracts were analyzed by Western blotting with pre-immune serum (lanes 1 and 2) and anti-TBP serum (lanes 3 and 4). The position of TBP is indicated by an arrow. Other bands which were detected with both sera were non-specific bands. It was confirmed that the amounts of the extracts used for Western blotting were within the linear range of response (data not shown).

In contrast to addition of native TFIID, addition of increasing amounts of recombinant TBP (rTBP) had no stimulative effect on the basal transcription activity of the 8 h extracts (Fig. 3A, left panel). However, as shown in Figure 3A, right panel, rTBP could stimulate basal transcription activity in the heat-treated 8 h extracts to a level comparable with that in the 12 h extracts (compare Fig. 3A, lanes 7 and 8 with lanes 15 and 16). These findings indicate that the 8 h nuclear extracts contain a heat-sensitive repressor(s) which can interact with exogeneously added free rTBP and inhibit rTBP-driven basal transcription. It was also shown that this putative repressor(s) interacted very weakly with TBP in the TFIID complex, since purified TFIID increased transcription in the 8 h extracts under the same conditions, whereas rTBP did not (compare Fig. 2A, lane 7 with Fig. 3A, lane 7). Furthermore, we compared the specific activity of TBP in the TFIID complex with that of rTBP by reconstituted transcription assay. As shown in Figure 3B, the TFIID fraction exhibited almost 10 times less activity than rTBP, while it contained ~100 times as much TBP protein as rTBP. TFIID from the HeLa cells exhibits far less activity than the free form of TBP (rTBP), possibly due to complex formation of TBP with inhibitory factors, including TAFs (19,20). Thus it is possible that

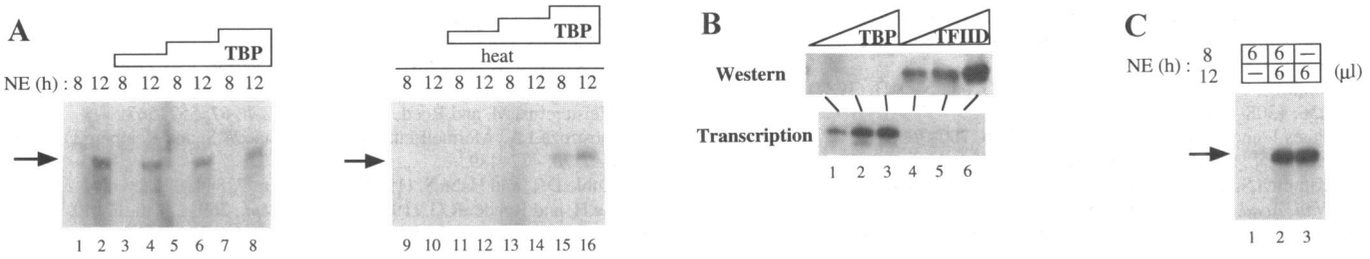

Figure 3. Effects of the addition of recombinant TBP to S and G2 phases and early G1 phase nuclear extracts on pol II basal transcription activity. (**A**) (Left) Recombinant TBP (rTBP) at 0 pg (lanes 1 and 2), 10 pg (lanes 3 and 4) and 100 pg (lanes 5 and 6) and 1 ng (lanes 7 and 8) was added to 10 μl (30 μg total protein) each of the 8 h (lanes 1, 3, 5 and 7) and 12 h (lanes 2, 4, 6 and 8) nuclear extracts and assayed for basal transcription activity. (Right) As the left panel except that the nuclear extracts were heat treated. (**B**) The specific activity of rTBP was far higher than that of the native TFIID. Recombinant TBP at 250 pg (lane 1) and 500 pg (lane 2) and 1 ng (lane 3) and the TFIID fraction at 1 μg (lane 4), 2 μg (lane 5) and 4 μg (lane 6) were analyzed by Western blotting and reconstituted *in vitro* transcription assay. (**C**) Effect of mixing S and G2 phases and early G1 phase nuclear extracts on pol II basal transcription activity. Aliquots of 6 μl (43 μg total protein) each of the 8 h and 12 h nuclear extracts either alone (lanes 1 and 3) or mixed (lane 2) were assayed for *in vitro* transcription activity.

interaction between the TBP repressor(s) and TBP was responsible for the observed difference in basal transcription activity during the cell cycle. It should be noted here that rTBP exhibited less activity than purified TFIID in the nuclear extracts, even after the extracts were heat treated (compare Fig. 2A, right panel and Fig. 3A, right panel). It is possible that the nuclear extracts also contain a heat-resistant repressor(s) which inhibits the free form of TBP; repressor(s) activity can be separated from TFIID during purification. At this moment, however, it is not clear whether the heat-resistant repressor(s) is involved in regulation of TFIID activity in the cell cycle.

Since the activity of log phase TFIID, which should contain the active form of TFIID existing in early G1 phase, was not reduced when added to the 8 h extracts (Fig. 2A, left panel), early G1 phase TFIID should be resistant to the putative TBP repressor(s). Consistent with this, a mixing experiment with the 8 h and 12 h extracts (Fig. 3C) demonstrated that the addition of 8 h extracts to 12 h extracts did not repress basal transcription. This result implies that the S and G2 phase extracts do not contain dominant repressor(s) and that the higher basal transcription activity of early G1 phase extracts does not derive from a decrease in the amount of such repressor(s).

Taken together our results lead us to propose a mechanism for modulating TFIID activity during the cell cycle. TFIID exists as a less active form in the S and G2 phases and as a more active form in early G1 phase, and TFIID determines the basal transcription activity in synchronized cells. The less active state of TFIID could be due to the presence of a heat-sensitive repressor(s) of TBP. In early G1 phase TFIID is converted to the active form, which decreases the interaction between the putative TBP repressor(s) and TBP and thereby increases basal transcription activity. However, there is no direct evidence that the low activity of TFIID and repression of free TBP in the S and G2 phase extracts are caused by a single mechanism. Also, we cannot exclude the possibility that an unknown activator which exists in early G1 phase affects TFIID activity. Further analyses will be required to understand the detailed mechanism that controls the activity of TFIID during the cell cycle.

Recently several factors that negatively control TBP have been reported. The *Drosophila* largest TFIID subunit (p230), a human $TAF_{II}250$/CCG1 homolog, has been shown to be a negative regulator of the TATA box binding activity of TBP (20). Furthermore, there are some factors which are not TAFs and interact directly with TBP to negatively regulate transcription. For example, NC1 (21), NC2 (22), which appears to be identical to Dr1 (23), yeast ADI (24) and HMG1 (25) can negatively regulate basal transcription by associating with TBP. It is possible that one of these factors represses TBP activity in the nuclear extracts of the S and G2 phases. However, it is not known whether these factors are heat sensitive at 47°C.

In conclusion, we suggest that the major target of regulation within TFIID activity is TBP. Colgan and Manley (26) have reported that TBP could be rate limiting for the basal transcription activity of TATA-containing promoters *in vivo*. Therefore, it is possible that cell cycle-dependent regulation of pol II basal transcription activity exists not only *in vitro*, but also *in vivo*.

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