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Presence of an inhibitor of RNA polymerase I mediated transcription in extracts from growth arrested mouse cells

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ABSTRACT

Extracts obtained from mouse cells growth arrested at stationary phase or under serum starvation exhibit no specific rDNA transcription activity. Experiments with mixed transcriptionally active and inactive whole cell extracts (WCE) obtained from rapidly dividing or growth arrested cells, respectively, demonstrate that rRNA synthesis in vitro can be suppressed by a polymerase I transcription inhibitory activity (PIN), present in inactive extracts. This inhibition effect is not related to increased nuclease activity and affects neither the non-specific Pol I transcription, nor a polymerase II promoter. A comparison of WCE isolated under different growth conditions indicates that PIN changes according to the physiological state of the cell. It reaches a maximal level soon after serum depletion and disappears rapidly when cells are allowed to recover in serum-rich medium. PIN can be clearly demonstrated in WCE but not in nuclear or cytoplasmic extracts and can be also obtained by an additional high salt extraction of nuclei. Furthermore, gel retardation and transcription-in-pellet assays demonstrate that rDNA promoter binding and preinitiation complex stability are similar in active and inactive WCE. This indicates that some later stage(s) of rDNA transcription, rather than the preinitiation complex formation, are attenuated by inactive extracts. Analysis of partially fractionated extracts suggests that PIN is not associated with but can be separated from polymerase I.

INTRODUCTION

Ribosome production changes according to the rate of cell proliferation. A key step in the control of ribosome formation in eukaryotes is the regulation of pre-ribosomal RNA synthesis that produces 18 S, 5.8 S and 28 S ribosomal RNA (rRNA). Its rate is rapidly modulated in response to changes in cell growth. In dividing cells, the rRNA genes are highly expressed while under conditions of arrested growth due to attainment of stationary phase, serum or aminoacid starvation, or after cyclochexamide treatment, rapid down regulation of rRNA synthesis occurs (for review see 1). Decreased rate of rRNA accumulation is also observed during cell differentiation (2, 3), heat shock (4, 5) or in some cases after virus infection (6). Hormone treatment could induce both up- or down-regulation of rRNA gene expression. For example, the activity of rRNA genes in myoblasts or rat liver is increased in response to insulin or glucocorticoid treatment (7, 8). In contrast, glucocorticoid treated lymphosarcoma cells exhibit strongly reduced rRNA synthesis (9 and ref. therein). The development of cell-free studies on polymerase I transcription along with the key finding that the transcriptional potential of cell extracts can mirror the activity of the rRNA genes in vivo have allowed delineation of rDNA promoter and enhancer sequences and characterization of several Pol I transcription factors (reviewed in 10). The mechanisms of signal transduction bringing about the modulation of rDNA transcription, however, are still unclear. An important question concerning the modulation of rRNA synthesis according to the physiological state of the cell is whether it involves modification of the polymerase itself, associated growth regulated factor(s), or both. Data on Acantamoeba and mouse cells have shown that two forms of polymerase I, template-active and non-active, can be functionally distinguished. In rapidly dividing cells the fraction of the active Pol I molecules engaged in transcription of rRNA genes is increased while the total Pol I activity remains unchanged (11, 12). According to these findings, the rRNA gene transcription might be subject to positive regulation by subtle modification of some polymerase subunit(s). In contrast, some recent studies have demonstrated that factors responsible for growth regulation of rDNA transcription are separable from the polymerase I complex. Thus, it has been shown that in mouse ascites cells the amount or activity of at least two Pol I related factors, TIF-IA and UBF, is increased during cell proliferation and these factors play a critical role in restoring the transcriptional capacity of inactive extracts (13, 14). Furthermore, it was demonstrated that the

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reversible inhibition of rRNA synthesis observed in mouse lymphosarcoma cells after glucocorticoid treatment is due to reduction of the activity of a Pol I related factor, TFIC, which in purified form is devoid of polymerase I activity (9). Despite the contradictive points in these results, they in common suggest a positive growth dependent manner of regulation of rRNA gene transcription. The possibility, however, that Pol I inhibitory factor(s) could also contribute to the down-regulation of rRNA synthesis can not be ruled out. For example, intriguing are the results on nucleolin, a growth regulated nucleolar protein, which according to its processing, could both activate or inhibit rDNA transcription in vitro (15) and probably in vivo (16). However, there are still no physiologically relevant data on negative mode of regulation of rRNA synthesis via repressor factors. On the other hand, in growing number of cases, negative regulators of Pol II transcribed genes are found (reviewed in 17). In this line, the present work was directed to test for existence of specific inhibitor(s) of Pol I transcription system. To this end we have performed a series of in vitro assays for polymerase I activity using extracts from rapidly dividing and growth arrested mouse cells. The results indicate that the Pol I mediated transcription can be specifically suppressed by an efficient inhibitory activity that changes according to the phisyological state of the cell and is present only in non-dividing cells. This activity (refered to as PIN) can be obtained by a high salt extraction of nuclei from growth arrested cells and could be demonstrated in whole cell extracts but not in cytoplasmic or nuclear extracts, most probably due to less efficient protein extaction in these two preparations. DNA binding and transcription-in-pellet assays have indicated that the action of PIN does not concern the preinitiation complex formation, but rather attenuates later stage(s) of transcription. Also, experiments with partially fractionated extracts suggest that PIN is not associated with the Pol I complex.

METHODS

Cell culturing and extracts preparation

FM3A mouse cells were cultivated in ES medium complemented with 5% fetal calf serum. Transcriptionally active extracts were prepared after growing the cells to a density of about 0.6×10^6 cells/ml. Transcriptionally inactive extracts were obtained after cell growth to maximal density (about 2.5×10^6 cells/ml) and additional cultivation for 24 hours or, alternatively, by transferring the cells in 1/8 volume of serum deficient medium and additional incubation for 1 or 3 hours. In some cases, after serum starvation cells were allowed to recover by 10 fold dilution in serum containing medium and incubated further for 1 or 3 hours. Whole cell extracts were prepared according to the procedure described by Manley et al. (18). Briefly, cells were collected and suspended in four packed-cell volumes of buffer M1 (0.01 M Tris.HCl, pH 7.9/0.001 M EDTA/0.005 M dithiothreitol (DTT). After 20 min cells were lysed by 8−10 strokes with Dounce homogenizer, using a 'B' pestle. Four packed-cell volumes of buffer M2 (50 mM Tris.HCl, pH 7.9/10 mM magnesium chloride/2 mM DTT/0.25 mM phenylmethylsulfonyl fluoride (PMSF)/25% sucrose/50% (v/v) glycerol) were then added, followed by precipitation with one packed-cell volume of saturated ammonium sulfate. The extract was centrifuged at 60,000 rpm for 1.5 hrs in Sorvall RP 65-Ti rotor and the supernatant was subjected to a second protein precipitation by addition of ammonium sulfate to 0.33 g/ml. The precipitate, collected by centrifugation at 15,000×g for 20 min, was resuspended in one packed-cell volume of buffer A (20 mM Tris.HCl, pH 7.9/8 mM magnesium chloride/0.2 mM EDTA/1mM DDT/0.25 mM PMSF) containing 100 mM KCl and dialyzed against the same buffer. Nuclear (NE) and cytoplasmic (S-100) extracts were obtained as previously described (19). A nuclear fraction, NM, was obtained by additional high salt extraction of the nuclei imediately after NE preparation. In this case the nuclear remnants, obtained after a standard 0.42 M NaCl extraction (19) were resuspended in 20 mM Tris.HCl, pH 7.9/0.8 M NaCl/1.5 mM magnesium chloride/0.2 mM EDTA/0.25 mM PMSF/0.5 mM DTT/25%(v/v) glycerol and extracted for 30 min at 4° by slow stirring. This fraction was further proceeded according to the protocol for WCE preparation with addition of buffers M1 and M2 (4 volumes of each) and double ammonium sulfate precipitation as described above.

In some experiments the whole cell extracts were partially fractionated on Heparin-Sepharose, S-Sepharose and phosphocellulose columns by stepwise salt elution with buffer A, containing the respective KCl concentrations given in figure legends. All extract and fraction preparations were dialyzed against buffer A/100 mM KCl. The protein content was quantitated by Bradford's method (20).

DNA templates and oligonucleotides

Plasmid pMrPPH having mouse rDNA promoter sequences from position −330 to +291 according to the transcripton start site (21) was used as template in in vitro transcription experiments after linearizing with Bam HI. In some control experiments pSV2NEO DNA (22) linearized with Bam HI was used as a polymerase II template. Two double stranded oligonucleotides, pMD and mUCE, were synthesized and used in gel retardation experiments, as binding probes and as specific competitors. pMD contains the core promoter sequences of mouse rDNA, spanning the region −43/+13 relative to the transcription initiation site (21, 23−26, 30) (GGGTTGTGATCTTT TCTATC TGTT-CCTATTGGACCTGGAGATAGGTACTGACACG CTGT); mUCE includes the region of mouse rDNA, supposed to bind UBF (27, 28) (GGGCCACCTCCACAGGTATGACTTCCAGG TATTCTCTGTGGCCTGT), placed in position −112/−70.

Transcription *in vitro*

Transcription reactions were carried out in 25 μl volume for 60 min. at 30° as previously described (21), using 250 ng of pMrPPH DNA (21) as template, (alpha-^{32}P)-GTP as labeled nucleotide and different amounts of cell extracts or fractions as indicated in figure legends. The synthesized RNA products were purified and electrophoresed on 4−6% polyacrylamide gel. In some experiments, preincubation with competitor DNAs was done for 15 min at 30° and after that, transcription was started with addition of template and nucleotides. All reactions except those using pSV2NEO as template DNA contained alpha-amanitin in concentration 100μg/ml. Transcription-in-pellet reactions (30, 31) were carried out after incubation of template DNA in transcription mix without nucleotides for 10 min at 30°, followed by centrifugation at 4° at 12000×g for 8 min. The pellets containing rDNA:protein complexes were washed with buffer A100 and gently resuspended in transcription mix including nucleotides, in the presence or absence of extract proteins. The transcription reactions and RNA analysis were proceeded as mentioned above.

Non-specific polymerase I activity assay

The non-specific Pol I activity was assayed as described earlier (13). 100 or 200 μg/ml poly(dA:dT) was used as random template in standard transcription reactions, including 10 μl of A-WCE, N-WCE, or a mix of them. All reactions contained 100 μg/ml alpha-amanitin. ^{3}H-UTP incorporation in RNA was measured after TCA precipitation and filtration through glass-fiber filters.

Gel retardation assay

pMD and mUCE oligonucleotides were used as protein binding probes after 3'-labeling with (alpha ^{32}P)-dCTP and Klenow fragment of DNA polymerase I following conventional protocol (29). Binding reactions (25 μl) contained 12 mM Tris, pH 7.9, 80 mM KCl, 8 mM $MgCl_2$, 1 mM dithiothreitol, 12% glycerol, 0.2−0.4 μg poly(dI:dC) or poly(dA:dT) as non-specific competitor DNA, 10−15 fmoles labeled probe and 1 or 2 μl of whole cell extract. After preincubation of competitor DNAs with the extract proteins for 15 min. at 30°, the labeled probe was added and the incubation was continued for additional 45 min at 30°. The rDNA:protein complexes were electrophoresed on 6% polyacrylamide gel with Tris-glycine running buffer at 11 V/cm, at room temperature.

RESULTS AND DISCUSSION

Inhibitory effect of inactive WCE on Pol I mediated transcription

In order to test the possibility of negative regulation of rDNA transcription in cells with strongly decreased rate of rRNA synthesis, we carried out a series of in vitro transcription experiments with mixed extracts. Transcriptionally active whole cell extracts (A-WCE) were prepared from exponentially growing FM3A cells. For preparation of transcriptionally inactive extracts (I-WCE), the same cells were grown to stationary density and incubated for 3 hours in 1/8 volume of serum deficient medium. In control experiments (some of which are shown in figure 2), we found that cells can survive such treatment and recover after dilution in serum rich medium. A plasmid carrying mouse rDNA promoter region, pMrPPH (21), linearized with Bam HI to produce a 295 nt transcript, was used as template in the transcription reactions. The results shown in figure 1 have clearly demonstrated that the amount of the Pol I transcripts can be strongly reduced in the presence of a relatively small quantity of I-WCE added to A-WCE (figure 1, lanes 2−4). No or very weak Pol I transcriptional activity was detected when reaction was carried out with inactive extract alone (shown in figure 2). The simplest explanation of this finding might be that the so-called 'inactive' extract is not only unable to transcribe rDNA, but suppresses the rRNA accumulation by affecting the transcription rate or stability of RNA. To discriminate between these possibilities, we performed chase experiments which demonstrated that the suppression effect was not simply due to increased ribonuclease activity in the inactive extract. As shown in lanes 5 and 6 of figure 1, addition of I-WCE to the transcripton reaction after chase of the labeled nucleotide does not affect the level of the pre-synthesized transcripts. Independent controls including ribonucleases and proteases inhibitors, such as RNAsin and PMSF, as well as ethidium bromide staining of the DNA

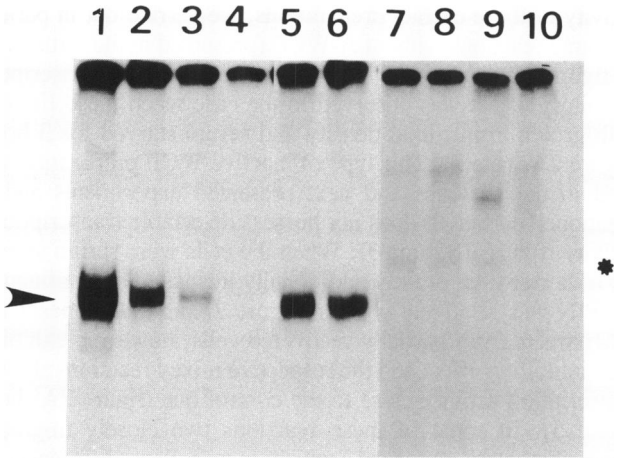

Figure 1. Inhibitory effect of inactive WCE on the specific Pol I transcription. Lanes 1−6: 250 ng of pMrPPH template DNA linearized with Bam HI were transcribed in 25 μl reaction in the presence of10 μl A-WCE (lane 1) with addition of 1, 2.5 or 5 μl of I-WCE, lanes 2, 3 and 4, respectively. In chase experiments, after 45 min transcription reaction as in lane 1, GTP to 1 mM was added and after next 5 min. 2.5 μl of transcription buffer (lane 5) or I-WCE (lane 6) were added followed by 15 min additional incubation. Lanes 7−10: 250 ng of Bam HI linearized pSV2NEO template DNA were transcribed in presence of 10 μl A-WCE (lane 7) with addition of 1 μl (lane 8) or 2.5 μl (lane 9) of N-WCE. A control reaction for alpha-amanitin sensitivity of transcription is shown in lane 10. All reactions except those in lanes 7−9 were performed in the presence of 100 μg/ml alpha-amanitin. The position of the specific Pol I and Pol II transcripts, run separately in 6% and 4% PAGE, are shown by arrow and asterisk, respectively.

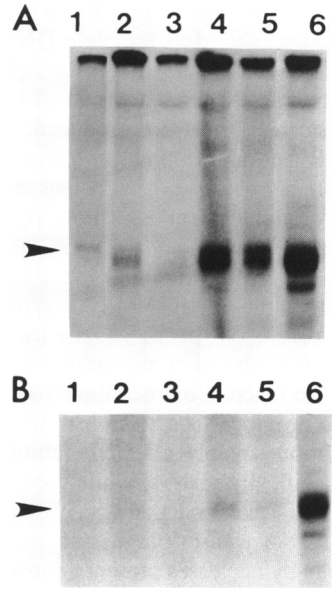

Figure 2. Changes in Pol I inhibitory activity in WCE obtained from cells in various physiological states. (A) Transcription inhibitory effect of WCE preparations. All reactions contained 7.5 μl (45 μg protein) of A-WCE as basic transcriptional activity (lane 6) with addition of 5 μg protein of WCE variants obtained as follows: Lane 1, cells grown for 24 hrs after attaining stationary density; Lanes 2 and 3, after reaching stationary density cells were concentrated 8 fold in serum deficient medium and incubated additionally for 1 or 3 hours, respectively; Lanes 4 and 5, as in lane 3, but after 3 hours of serum starvation cells were allowed to partially recover by 10 fold dilution in serum rich medium and further incubation for 3 or 1 hours, respectively. (B) Intrinsic Pol I transcription activity of the WCE variants. Lane 6, control transcription with A-WCE as above; Lanes 1−5, the same transcripton reactions as in A were carried out in the absence of A-WCE. The position of the pMrPPH/Bam HI transcripts is shown by an arrow.

Table 1. Non-specific Pol I activity of WCE prepared from exponentially growing and growth arrested FM3A cells.

SAMPLE /T. reaction/	Pol I ACTIVITY /c.p.m. in RNA/
No template	260
No extract**	60
A-WCE*	4720
A-WCE**	8450
I-WCE*	4380
I-WCE**	8130
A+I WCE*	4260
A+I WCE**	8060

Pol I activity of the whole cell extracts used in figure 1 was estimated by filter trapping assay using poly(dA:dT) as non-specific template DNA in concentration 80 μg/ml (*) or 160 μg/ml (**). All reactions include 100 μg/ml alpha-amanitin. Average values of three parallel samples are presented.

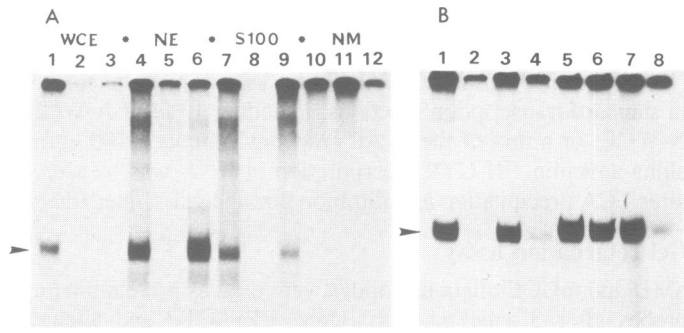

Figure 3. Distribution of PIN by different protein extraction procedures. (A) Effect of mixing active and inactive parallel preparations of WCE, S-100, NE and 0.8 M NaCl nuclear fraction (NM) on Pol I transcription. Transcription reactions were carried out with 15 μl (about 100 μg protein) of A-WCE, I-WCE or their 1:1 mixture, lanes 1–3, respectively. Next, the same order of active/inactive extract combinations was followed using NE (lanes 4–6), S-100 (lanes 7–9) and NM (lanes 10–12). (B) Inhibition of WCE and NE by NM fraction obtained from growth arrested cells. Transcription reactions were performed with 10 μl (70 μg protein) of A-WCE (lane 1) and addition of 35 μg protein of I-WCE (lane 2), A-NM (lane 3) or I-NM (lane 4). The next reactions include 70 μg protein of A-NE as basic activity (lane 5) with addition of half amount of I-NE (lane 6), A-NM (lane 7) or I-NM (lane 8). The arrow shows the position of the 295 nt pMrPPH transcript.

template in gel after transcription have also indicated that the inhibition effect of I-WCE is not related to incresed nuclease or protease activities (data not shown). This was further confirmed by a test for inhibitory effect of I-WCE on a polymerase II (SV 40) promoter. As can be seen in lanes 8 and 9 of figure 1, when pSV2NEO plasmid was used as template DNA, no significant inhibition of alpha-amanitin sensitive transcripts was observed. Interestingly, in this case in the presence of I-WCE some side transcrips with incorrect size were produced. The efficiency of polymerase II mediated transcription was lower than this of rDNA transcription, probably because the constant reaction conditions were optimized for Pol I dependent transcription.

Next, we estimated the non-specific polymerase I activity of A-WCE and I-WCE in filter trapping assay, using poly(dA:dT) as random template in the presence of 100 μg/ml alpha-amanitin. In contrast to the results on specific rDNA transcription, in this assay we have not observed transcription inhibition either in mixed extracts or in inactive extract alone (table 1). This result is consistent with previous data demonstrating that changes in cell growth conditions mainly affect the ability of polymerase I to transcribe specifically rDNA, while the total Pol I activity remains similar (12). Taken together, the presented data strongly suggest that the rDNA transcription is suppressed by a potent inhibitor rather than by nucleases present in growth arrested cells. This inhibition affects the specific rDNA transcripts but does not change Pol II mediated transcription.

Changes of Pol I transcription inhibitory activity in different physiological states of the cell

A major question is whether the inhibition of rDNA transcription we observe in vitro reflects any physiologically relevant function. In order to test the possibility that the Pol I inhibitory activity (PIN) found in I-WCE may reflect an aspect of negative regulation of rRNA gene activity in growth arrested cells, we analyzed the variation of PIN during different cell states (figure 2A). To this end, we standardized and compared the Pol I inhibitory effect of WCE obtained from FM3A cells either grown to maximal density and incubated additionally for 24 hours (figure 2A, lane 1) or 8-fold concentrated and serum depleted cells (lanes 2 and 3), as well as from cells allowed partially to recover after dilution in serum rich medium (lanes 4 and 5). Transcription reactions were carried out with a constant amount (45 μg protein) of A-WCE and the addition of 5 μg protein of the WCE variants described above. To compare also the intrinsic transcriptonal activity of these extract preparations, we carried out in parallel the same reactions without A-WCE (presented in the same order in figure 2B). As could be expect, the strongest transcription inhibitory effect was observed in the case when the cells were both grown to maximal density and serum starved for 3 hours (figure 2A, lane 3). This type of inactive WCE preparation was used in the previous and next presented experiments and as mentioned earlier, it does not possess detectable transcriptional activity (figure 2B, lane 3). When the cells were serum starved for a shorter time or only additionally incubated by confluency, the PIN was relatively weaker (figure 2A and 2B, lanes 1 and 2). Extracts from partially recovered cells, however, exhibited little inhibition effect and the respective mixed reactions showed transcription activity close to the control one (figure 2A, lanes 4 and 5). In some of these reactions two closely migrating transcripts were observed, which may reflect differences in discrete rRNA cleavage or processing activity in the WCE variants used. The results presented here indicate that the Pol I inhibition activity demonstrated in vitro fluctuates according to the physiological state of the cell, showing maximal level during growth arrest and rapidly disapearing in recovery phase. Comparing lanes 4 and 5 in figure 2A and 2B, it can also be seen that the drop of PIN under recovery conditions corelates with appearance of weak intrinsic Pol I activity. It is noteworthy that in the same period of time when PIN disappears, the Pol I transcriptional activity is not yet fully restored. This is compatible with the idea that the Pol I mediated transcription could be subject both to positive and negative regulation. Such hypothesis could well explain the rapid up- and down-amplitudes

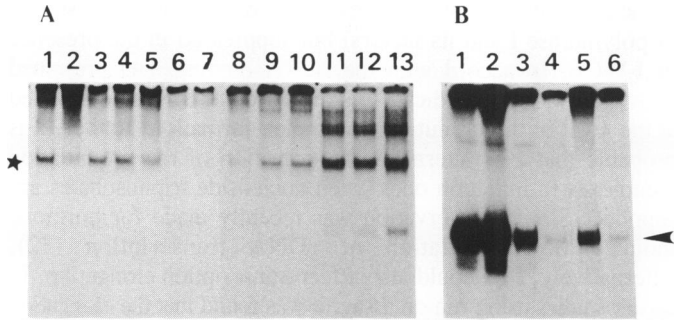

Figure 4. rDNA binding activity of transcriptionally active and inactive WCE. (A) Gel retardation assay for core promoter and UCE binding protein(s). Lanes 1–7, effect of DNA competitors on binding: 10–15 fmoles of core promoter labeled probe, pMD, were incubated in a 25μl binding reaction with 4 μl (lane 2) or 2 μl (all of the rest of the lanes) A-WCE after pre-incubation for 15 min at 30° without competitor (lane 1) or with 300 ng of poly(dA:dT) (lane 3), poly (dI:dC) (lane 4), linear pUC DNA (lane 5), pMD oligonucleotide (lane 6) or mUCE oligonucleotide (lane 7). Lanes 8–13, comparison of core promoter and UCE complexes formed in A-WCE and I-WCE. The binding reactions were carried out with labeled pMD probe (lanes 8–10) or mUCE probe (lanes 11–13) in the presence of 2 μl (15 μg) of A-WCE (lanes 8 and 11), the same amount of I-WCE (lanes 10 and 13) or mixed (1:1) A-WCE and I-WCE (lanes 9 and 12). In all reaction pre-incubation with poly(dA:dT) and poly(dI:dC), 150 ng of each, was done as above.The position of the rDNA:protein complexes, resolved in 6% PAGE, is shown by asterisk. For better resolution of these complexes the free oligonucleotide probes were run off the gel. (B) Transcripton competition by pMD and UCE oliginucleotides. 250 ng Bam HI linearized pMrPPH template DNA was transcribed with 10 μl A-WCE. The reactions were started with addition of the template and nucleotides after pre-incubation for 15 min. at 30° without competitor (lane 1) or with 300 ng of poly(dA:dT) (lane 2), 150 or 300 ng of pMD oligonucleotide (lanes 3 and 4) and 150 or 300 ng of mUCE oligonucleotide (lanes 5 and 6). The position of the 295 nt transcript is shown by arrow.

in the rDNA transcription rate in response to physiological changes.

Comparative analysis of WCE, NE and S100 extracts obtained from growth arrested cells for their ability to suppress Pol I mediated transcription

Experiments with mixed active and inactive extracts have been used in several previous reports on rDNA transcription regulation. In these cases, NE or S100 extract preparations are used and the inactive extracts show rather a neutral than an inhibitory effect on Pol I mediated transcription (9, 12, 13). The present results, however, demonstrating such inhibitory effect are based on using whole cell extracts. To analyze this discrepancy, we have followed in parallel the three most used protocols for obtaining WCE, NE and S100 extracts from growth arrested FM3A cells and tested them for Pol I inhibitory activity. The results, shown in figure 3, have confirmed that, at least by the cell type used, PIN can be demonstrated mainly in whole cell extracts (comp. lanes 3, 6 and 9 of fig.3A, representing transcription reactions with mixed active (A) and inactive (I) WCE, NE and S100, respectively). One reason for that could be that PIN related protein(s) is absent in NE and S100 preparations but not in WCE where, presumably, the overall protein extraction efficiency is higher. To test this possibility, after the standard nuclear extraction with 0.42 M NaCl for obtaining NE, we further extracted the nuclear remnants with 0.8 M NaCl and proceeded this high salt fraction (referred to as NM) according to the protocol for WCE preparation. When tested in transcription reaction, NM prepared from either dividing or growth arrested cells did not exhibit transcriptional activity by itself (figure 3A, lanes 10–12). Only the NM fraction obtained from growth arrested cells, however, was able to bring about the same inhibitory effect on rDNA transcription in active extracts as I-WCE (figure 3B, lanes 4 and 8, respectively). From these results we could infer that the absence or the low level of PIN in NE and S100 extracts prepared from gowth arresed cells is due to insufficient protein representation in them. In fact, PIN existing in such cells could be additionally obtained by high salt extraction of nuclei.

PIN is not related to changes in promoter rDNA binding activity and preinitiation complex formation in inactive whole cell extracts

To address the question of how PIN affects the rDNA transcription we have used two types of experiments. First, the promoter rDNA binding activity in A-WCE and I-WCE was compared. In gel retardation assay we used two mouse rDNA oligonucleotide probes: pMD, containing the core promoter region (nucleotide sequences from position −43 to +13 relative to the transcription initiation site) and mUCE (position −112 to −70), analogous to the UCE region in human rDNA. These rDNA regions are supposed to include the binding sites of the mouse specific transcription factor, TFID (TIF-IB), and the upstream binding factor, UBF (23–28). As shown in figure 4A, lanes 8–13, the two rDNA probes form a complex of the same mobility, which after pre-incubation with different DNA competitors proved to be specific (lanes 3–7). This complex is probably due to the interaction of the two rDNA promoter regions with TFID (TIF-B), as was suggested (24–27). Its formation was sensitive to the DNA:protein ratio in the binding reaction, and with increased amount of extract protein, the non-specific binding was stronger (lane 2, the top of the gel). The lower mobility bands observed with the UCE probe (lanes 11–13) probably represent double protein (TFID+UBF) complexes which is in agreement with the proposed interaction of these two factors in initiation complex formation (27). The binding reactions presented in lanes 8–13 of figure 4A demonstrate that the pattern of complex formation with both core promoter and UCE probes is the same in A-WCE, I-WCE or a mixture of them. This indicates that the transcription activity/inactivity of these extracts is not related to their rDNA promoter binding ability. Identical binding patterns of A-WCE and I-WCE was also observed by Southwestern technique using the same rDNA probes (unpublished results).

Control transcription reactions have shown that the two oligonucleotide probes used in the protein binding experiments are functionally active in competing for essential Pol I factor(s). As shown in figure 4B, the rDNA transcription was specifically inhibited after preincubation with pMD (lanes 3 and 4) or mUCE (lanes 5 and 6) oligonucleotides.

To analyze further the action of PIN, we next used a transcription-in-pellet assay which enables one to dissect the preinitiation complex formation from the next transcriptional stages. As was demonstrated earlier, transcriptionally active complexes formed on the rDNA template can be easily separated by a low speed centrifugation. Template commitment experiments have shown that the pelleted complexes are relatively stable and can maintain several rounds of re-initiation (30, 31).The results of transcription-in-pellet experiments using combinations of active and inactive WCE are presented in figure 5. The template rDNA, pMrPPH, was preincubated with A-WCE (lanes 1, 3 and 5) or

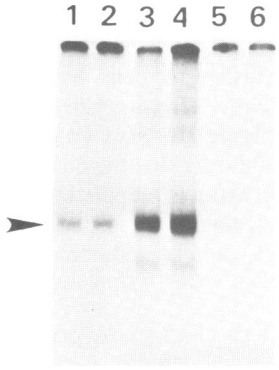

Figure 5. Pellet transcription of rDNA:protein complexes formed in A-WCE and I-WCE. Transcription-in-pellet reactions were performed as described in Methods. To allow preinitiation complexes to form, 250 ng of Bam HI linearized pMrPPH DNA was preincubated with 12 μl of A-WCE (lanes 1, 3 and 5) or a mix of 12 μl A-WCE + 3μl I-WCE (lanes 2, 4 and 6). Following centrifugation, the pelleted complexes were transcribed after addition of nucleotides in A-100 buffer (lanes 1 and 2), in the presence of 12 μl A-WCE (lanes 3 and 4), or mixed 12 μl A-WCE + 3 μl I-WCE (lanes 5 and 6). The arrow shows the position of the 295 nt pMrPPH transcript.

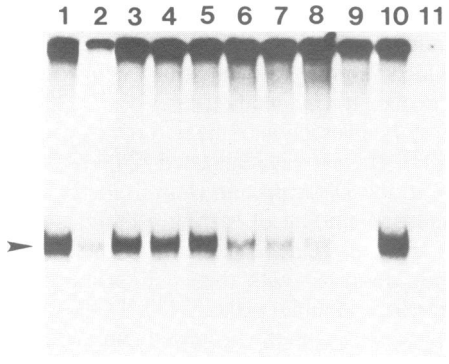

Figure 6. Pol I transcription inhibitory effect of I-WCE fractions. In standard transcription reactions, each containing 60 μug protein of A-WCE (lane 1), the inhibitory effect after addition of 6 μg protein of I-WCE or its fractions was compared as follows: lane 2, I-WCE; lanes 3−8, Heparin-Sepharose fractions eluted in 100 mM (flow through), 200, 300, 400, 600 and 800 mM KCl, respectively; lane 9, the 600 mM and 800 mM KCl fractions of Heparin-Sepharose were combined, two-fold diluted in buffer A, further retained on S-Sepharose and eluted in 600 mM KCl; lanes 10 and 11, phosphocellulose 'C'and 'D' fractions, eluted in 600 and 1000 mM KCl, respectively. The position of the 295 nt pMrPPH transcript is shown by arrow.

a mixture of A-WCE + I-WCE (lanes 2, 4 and 6). (As was shown in figure 1, such an extract mixture in standard transcription reaction exhibits a strong inhibition effect on specific rDNA tanscription). After centrifugation the pelleted complexes were transcribed in transcription buffer (lanes 1 and 2) in the presence of A-WCE (lanes 3 and 4) or a mixture A-WCE + I-WCE (lanes 5 and 6). The results demonstrate that PIN is not associated with the precipitated complexes formed on rDNA and Pol I transcription in the pellets does not change whether these complexes are formed in the presence or absence of I-WCE. As shown in lanes 1 and 2, in both cases they have the same stability and ability to maintain transcription. The overall RNA synthesis obtained with these complexes, however, was strongly increased in the presence of A-WCE (presumably due to additional supply of polymerase I and its factors) but suppressed in the presence of I-WCE. In accord with the rDNA binding data presented above, these results indicate that the action of PIN is not exerted at the level of the preinitiation complex formation. Rather, it is probable that PIN alternates the transition of the preinitiation complexes to initiation ones when nucleoside triphosphates are supplied. Similar observation was recently made for hormone induced down-regulation of rDNA transcription (32). Alternatively, PIN could also affect transcription elongation. In earlier studies using run-on assay, it was found that the elongation rate of rRNA synthesis in lymphocytes is regulated in response to physiological changes (34).

Distribution of PIN in fractionated inactive whole cell extract

In order to analyze the chromatographic properties of the Pol I inhibitor(s) present in growth arrested cells, we have initiated experiments with partially fractionated I-WCE. The PIN activity found in these cells was not dialyzable, implying that it probably has protein nature. To assess the inhibitory effect of different fractions obtained from several columns we used standard transcription reactions as those described in figure 2, keeping the protein amount constant. As was shown earlier, unfractionated WCE prepared from serum deprived mouse cells exhibits strong inhibitory effect on the specific Pol I transcription (figure 6, lane 2). After fractionation of that extract on Heparin-Sepharose by step-wise salt elution we have found that PIN elutes in the range of 400 to 800 mM KCl (figure 6, lanes 3−8). Next, the 600 mM and 800 mM KCl fractions were combined, two-fold diluted in buffer A and further applied on S-Sepharose. In the fraction eluted by 600 mM KCl, we obtained stronger PIN activity which completely inhibits rDNA transcription (lane 9). On the other hand, data of our laboratory have shown that this high salt S-Sepharose fraction is free of Pol I activity (C. Song et al., submitted). We have also fractionated I-WCE on phosphocellulose and tested for PIN activity in two fractions necessary to reconstitute transcription, C and D (33), eluted with 600 mM and 1000 mM KCI, respectively. Addition of fraction C, known to contain the bulk of polymerase I (33), had no inhibitory effect, while in presence of D fraction we observed again full inhibition of rDNA transcription (figure 6, lanes 10 and 11). Taken together, these results suggest that PIN is not associated with, but can be separated from, polymerase I complex. Interestingly, the strongest PIN activity we observed appears in fraction D, which is enriched in the mouse specific Pol I factor, TFID (25, 33). An intriguing question arises of whether PIN is related to some of the Pol I transcriptional factors. As was recently shown, at least two factors, TIF-IA and UBF, are involved in growth regulation of mouse rDNA transcription. In growth arrested cells, TIF-IA activity is not present and UBF is inactivated, probably by dephosphorylation (13, 14). One could speculate that PIN indirectly inhibits the rDNA transcription via rapid inactivation of these factors. According to the data presented here, it is the specific rDNA transcription, and not the activity of Pol I on nonspecific template, that is affected by PIN. Interestingly, in the cases of poliovirus infection or glucocorticoid induced inhibition of rRNA synthesis it was demonstrated that specific inactivation of certain transcription factor(s) occurs while the nonspecific Pol I activity remains unchanged (6, 9). Alternatively, the growh dependent suppression of rDNA transcription might represent direct action of a new type of Pol

I regulatory factor(s). Considering the fact that Pol I transcription machinery responses rapidly to physiological changes, it is conceivable that it could be subject to dual positive and negative regulation. The data presented in figure 2 are compatible with such hypothesis. They suggest that under growth arrest of the cell a transient, reversible inhibition of rRNA synthesis takes place. The question here is how extractable or stable is that inhibitory activity in cell-free systems? As was shown in this study, PIN can be obtained only in whole cell extracts or it needs an additional high salt extraction step to be released from nuclei. That could be the reason why in some other studies using different extraction protocols, Pol I inhibitory activity was not demonstrated. If so, in such cases only the final result of PIN function is found, i.e., transcriptionally inactive, yet neutral, non-inhibtory extracts. Regarding PIN stability, in our hands some aged inactive extracts or fractions used to show tendency to lose gradually their inhibitory effect and become neutral. It would be interesting to test if it is possible to reverse the inhibiton and restore the specific Pol I transcriptional activity in vitro (what presumably takes place in vivo). When this manuscript was completed a recent finding that rDNA transcription in mouse cells could be repressed in vitro was reported (35). The authors suggest that the Pol I transcription factor UBF relieves transcription inhibition by preventing binding of putative repressor(s) to promoter rDNA. It is still unclear if the inhibitory activity postulated in that study is growth regulated and whether it is related to the repression of rDNA transcription reported here. Fractionation and reconstitution transcription experiments designed to characterize the identity of PIN and its mechanism of action are now in progress.

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