Hormonal regulation of transcription of rDNA: glucocorticoid effects upon initiation and elongation *in vitro*

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ABSTRACT

Various parameters of transcription of cloned mouse rDNA have been examined using extracts from control P1798 cells and from cells treated 24h with 0.1 μ M dexamethasone. Highly purified RNA polymerase I from either source catalyzes nucleotidyl transfer (elongation) at a rate of approximately 30 nucleotides/sec. Extracts from hormone-treated cells are capable of forming stable, preinitiation complexes. The rates of stable complex formation are the same in extracts from control and hormone-treated cells. Nevertheless, initiation of transcription does not occur in extracts from hormone-treated cells. Initiation in such extracts may be restored by the addition of a partially purified RNA polymerase I initiation factor, designated TFIC. The data indicate that initiation by RNA polymerase I is a multi-step process. The first step involves the formation of stable, preinitiation complexes, as demonstrated by a number of groups. Initiation, *per se*, requires an additional protein, TFIC. Glucocorticoids and perhaps other mitogenic agents regulate transcription of rDNA by influencing the amount or activity of TFIC.

INTRODUCTION

In rapidly growing cells, approximately half of the transcriptional activity is attributable to RNA polymerase I (EC 2.7.7.6 DNA-directed nucleoside triphosphate:RNA nucleotidyl transferase, eucaryotic type I or A). This enzyme catalyzes the synthesis of 45S pre-rRNA and although the process is subject to physiological regulation, little is known about the details of any regulatory mechanism. The activity of this enzyme is increased in mitotically stimulated cells (for review see 1) and decreases as a result of serum or amino acid starvation in culture (2, 3, 4). Serum starvation apparently involves regulation of a transcription factor (5). The synthesis of pre-rRNA is also decreased during glucocorticoid-mediated inhibition of proliferation of lymphosarcoma P1798 cells in culture (6) and extracts from hormone-treated cells cannot transcribe cloned mouse rDNA *in vitro* (7). The experiments described in this paper were undertaken to study the biochemistry of initiation by RNA polymerase I *in vitro* and to elucidate the defect in transcription exhibited by extracts from

hormone-treated cells. A number of groups have reported that transcription of rDNA requires association of the template with one or more proteins to form stable, preinitiation complexes (8-11). One possible effect of glucocorticoids could be loss or inactivation of stable complex proteins. With respect to rDNA, the relationship between preinitiation complexes and functional initiation complexes is not well defined and it is possible that the hormone might cause inhibition of some reaction that is required for conversion of the preinitiation complex to the initiation complex. Alternatively, glucocorticoids might cause catalytic inactivation of RNA polymerase I leading to a decreased rate of chain elongation. To discriminate among these possibilities, various aspects of transcription have been examined including the rate of elongation by RNA polymerase I and the formation of preinitiation complexes in extracts from control and hormone-treated cells. The data indicate that glucocorticoids regulate transcription of rDNA by a decrease in the amount or activity of a diffusible protein that is required for conversion of preinitiation complexes to initiation complexes.

EXPERIMENTAL PROCEDURES

Cell culture and preparation of extracts: P1798 cell culture lines were established from an ascites tumor population (12) and the clonal isolate P1798-D2 was used in these experiments (13). Mid log phase cultures (1L, 10^6 cells/ml) were grown in suspension culture and S100 extracts were prepared as previously described (7). S100 extracts were prepared from control cells and from cells treated 24h with $0.1 \mu\text{M}$ dexamethasone. A single control extract containing 12 mg/ml protein was used throughout these experiments. Extracts from control and hormone-treated cells were not significantly different with respect to protein concentration or RNA polymerase activity (7).

Estimation of the rate of elongation by purified RNA polymerase I: RNA polymerase I was extensively purified from extracts of control and hormone-treated P1798 cells using a procedure described elsewhere (14). The specific activity of the purified enzyme was 4.3 and 2.3 nanomoles uridine monophosphate/min/mg protein for control and hormone-treated enzyme, respectively. These differences are within the range of expected variation from one polymerase preparation to another and are not thought to be significant. The specific activity of highly purified P1798 RNA polymerase I was not significantly different from that estimated for RNA

polymerase I purified to apparent homogeneity from mouse hepatoma tissue (4.1 nmol/min/mg, ref. 14) or rat hepatoma nuclei (3.3 nmol/min/mg, ref. 22) but was less than that reported for plasmacytoma RNA polymerase I (19 nmol/min/mg, ref. 23). The apparent difference in specific activity may be due to the low overall recovery of the enzyme from P1798 cells (<5%). Additional properties of the highly purified polymerase are described elsewhere (14).

The rate of elongation was estimated by a pulse-chase experiment using a homopolymerically tailed template (15). The template was pRL102 (16), made linear by digestion with KpnI, and homopolymerically tailed by the addition of 20-30 dCMP residues using terminal deoxynucleotidyl transferase as described elsewhere (14). The elongation reaction was initiated by addition of 25 μ l of enzyme (3-4 μ g protein) to a tube containing 75 μ l of 50 mM Tris-HCl (pH 7.9), 5 mM β -mercaptoethanol, 10% glycerol, 0.5 mM each of ATP, CTP, GTP, 8 μ M UTP, 6 mM $MgCl_2$, 2 μ g of poly(dC)-pRL102, and 50 μ Ci of [α - 32 P]UTP (New England Nuclear, >600 Ci/mmol). Elongation was terminated by the addition of 5 μ l of 2% SDS containing 25 μ g carrier tRNA and 5 μ g of proteinase K. The samples were processed and resolved by electrophoresis on 5% polyacrylamide:urea gels as previously described (7) and autoradiography was performed at -70°C using preflashed Kodak XAR film and intensifying screens. Standards were 5' end-labeled fragments from bacteriophages ϕ X174 and λ digested with HaeIII and HindIII, respectively.

Transcription in vitro: was carried out in a 50 μ l reaction containing 0.4 pmol of template and 5 μ l of S100 extract as previously described (7). Electrophoresis and autoradiography were carried out as described above. Autoradiograms were scanned with a Beckman DU8 spectrophotometer with programmable electronic peak selection and integration mode. A standard curve was constructed and the relationship between 32 P disintegrations and area of the autoradiographic peak was determined to conform to the following relationship: 32 P dpm = $[5.21 \times 10^4 (\text{peak area}) + 247] \times (\text{exposure time in min})^{-1}$. The curve was linear through a peak area of 3 arbitrary units ($r = 0.998$).

Partial purification of transcription factors: Approximately 5 ml of S100 extract was used for each preparation. All manipulations were performed at 4°C. The extract was dialyzed overnight against 500 ml of 20 mM Hepes (pH 7.9), 20% glycerol, 0.2 mM EDTA, and 1 mM dithiothreitol (HGED). The dialyzate was then centrifuged for 10 min at 8500 g and the

supernatant removed. This supernatant was loaded onto a 5 ml DE52 column (Whatman) equilibrated in HGED and the column was washed with 10 ml of HGED. Protein was eluted in step-wise fashion with 10 ml aliquots of HGED buffer containing 50 mM, 100 mM and 350 mM KCl. Protein concentration was estimated by A_{280} and the protein peak that eluted at 350 mM KCl was pooled. The salt concentration of this solution was measured with a Thomas Model 275 conductivity meter, and the solution was diluted with 20 mM Hepes (pH 7.9) 0.2 mM EDTA to achieve a final salt concentration of 100 mM KCl. This fraction was concentrated to its original volume using a Centriflo CF-25 (Amicon) apparatus. The protein pool was loaded onto a 1 ml phosphocelulose column (Whatman, P-11) equilibrated with HGED buffer containing 100 mM KCl. The column was washed with 5 ml of HGED buffer containing 100 mM KCl and protein eluted in a step-wise fashion with 5 ml aliquots of HGED buffer containing 400 mM, 600 mM and 1 M KCl. According to the convention of Mishima *et al.* (17) the phosphocellulose fractions eluting under these conditions are designated A-D, respectively. The protein peaks were pooled and precipitated by the addition of crystalline $(\text{NH}_4)_2\text{SO}_4$ (Schwarz-Mann) at a ratio of 0.335 gm/ml followed by centrifugation at 8500g for 10 min. The pellet was dissolved in a minimum volume of HGED buffer containing 100 mM KCl and then dialyzed overnight against 100 volumes of the same buffer. Factor preparations were stored in liquid N_2 and were completely stable to repeated freezing and thawing.

RESULTS

Estimation of the rate of elongation of RNA chains: The rates of elongation were estimated by transcription of a homopolymerically tailed template as described by Kadesch and Chamberlain (15). Purified RNA polymerase I from control or hormone-treated cells was incubated for 15 sec at 30°C with poly(dC)-pRL102 in the presence of ribonucleotide triphosphates plus 8 μM [$\alpha\text{-}^{32}\text{P}$]UTP; thereafter the tracer was diluted by the addition of unlabeled UTP to a final concentration of 250 μM and 40 $\mu\text{g/ml}$ heparin was added to prevent further initiation. Aliquots were withdrawn at intervals, labeled transcripts were extracted and resolved by electrophoresis, and the size of the labeled RNA was estimated from autoradiograms by comparison with 5' end-labeled standards. Representative data are summarized in Figure 1 in which chain length is plotted as a function of time after the addition of heparin. RNA polymerase I from control cells catalyzed elongation at a rate of ~30nt/sec (closed circles) and

Figure 1: Elongation rates of purified RNA polymerase I. RNA polymerase I was purified to near homogeneity from control cells (closed circles) and hormone-treated cells (open circles). A pulse chase experiment was carried out to assess the rates of elongation upon poly(dC)-pRL102, as described in Experimental Procedures.

there was no significant difference in the rate of elongation exhibited by the polymerase from hormone-treated cells (open circles).

Stable complex formation: Several groups have demonstrated that transcription of rDNA requires formation of stable, preinitiation complexes (8-11). Such complexes may be demonstrated by template competition experiments such as that shown in Figure 2. In this experiment, 5 μ l of an S100 extract from control cells was incubated with 0.4 pmol prMAB/Pvu for 15 min at 4°C in a reaction mixture without nucleoside triphosphates. Thereafter, the reaction was fortified by the addition of 0.4 pmol of prMAB that had been truncated at +155bp by digestion with SmaI (prMAB/Sma) and transcription was initiated by the addition of nucleoside triphosphates. As shown in lane a, only the preincubated template was transcribed to yield the expected 292nt RNA. In the reaction shown in lane b, an extract from control cells was preincubated with prMAB/Sma and then challenged by the subsequent addition of prMAB/Pvu. In lane c, the extract was preincubated with supercoiled prMAB and challenged with

Figure 2: Formation of stable preinitiation complexes. Lane a: An S100 from control cells was preincubated with prMAB/Pvu and challenged with prMAB/Sma. Lane b: A control extract was preincubated with prMAB/Sma and challenged with prMAB/Pvu. Lane c: A control extract was preincubated with prMAB supercoiled template and challenged with prMAB/Pvu. The calculated positions of the transcripts from prMAB/Pvu (292nt) and prMAB/Sma (155nt) are indicated by closed and open arrows, respectively. Lane d: A control extract was preincubated with prMAB supercoils and mixed with a hormone-treated extract that had been preincubated with prMAB/Pvu and challenged with prMAB/Sma.

prMAB/Pvu. Only high molecular weight transcripts were formed. These data are typical of template competition experiments and indicate that preincubation of a control extract with a given template results in formation of stable preinitiation complexes and precludes transcription of a second template added after complex formation is complete. Competition experiments were carried out to determine the amount of template required to saturate those factors involved in stable complex formation. The data indicate that saturation is achieved by preincubation with plasmid concentrations ≥ 0.08 pmol/5 μ l of S100 (not shown). All subsequent experiments were carried out with 0.4 pmol of both preincubated and competitor template.

In the experiment shown in Figure 2 lane d, an S100 extract from control cells was preincubated with supercoiled prMAB and a corresponding extract from hormone-treated cells was preincubated with prMAB/Pvu. After preincubation with prMAB/Pvu, the extract from hormone-treated cells was challenged by the addition of prMAB/Sma. Five μ l of control and hormone-treated extracts were mixed and the reaction was initiated by the addition

Figure 3: Fractionation of control cell extracts to yield TFIC. Five ml of control cell extracts were fractionated as described in the text. The protocol is shown schematically in the insert. The protein elution profile from the phosphocellulose column is also shown. The protein peak labeled A was obtained by washing the column with 100 mM KCl. Peak B was obtained by the addition of 400 mM KCl; peak C by the addition of 600 mM KCl and peak D by addition of 1 M KCl. The arrows indicate the point at which the salt step was added. Protein eluting in peak C was designated TFIC.

of nucleoside triphosphates. Under these circumstances the supercoiled plasmid was transcribed to yield high molecular weight RNA, prMAB/Pvu was transcribed to yield the 292 nt run off RNA, and prMAB/Sma was not transcribed. Since the extract from control cells was preincubated with excess supercoiled prMAB transcription of prMAB/Pvu cannot result from stable complexes formed between this template and factors from control cell extracts. Transcription of prMAB/Pvu must result from stable complexes formed by association of the template with factors present in the extract from hormone-treated cells. The observation that prMAB/Sma was not transcribed indicates that stable complexes with prMAB/Pvu did not form subsequent to mixing of the extracts.

Although extracts from hormone-treated cells are capable of forming stable, preinitiation complexes, such complexes are not sufficient for transcription. To determine what other factors are required for transcription, a control extract was fractionated and fractions tested for the ability to restore transcription to hormone-treated extracts. The fractionation protocol is outlined in Figure 3, which also illustrates a

Figure 4: Reconstitution of transcription with TFIC. Transcription of prMAB/Pvu was carried out with a control extract (lane a) and a hormone-treated extract (lane b). Five μ l of each fraction from the phosphocellulose column were then mixed with 5 μ l of the hormone-treated extract and incubated with prMAB/Pvu in a standard transcription reaction; fraction A + extract (lane c), fraction B + extract (lane d), fraction C + extract (lane e), fraction D + extract (lane f). Lane g contains the products of a template competition experiment in which an extract from the hormone-treated cells was preincubated with prMAB/Pvu and then mixed with TFIC that had been preincubated with prMAB/Sma. Open and closed arrows indicate the calculated positions of the 155 nt and 292 nt transcripts, respectively.

typical protein elution pattern from phosphocellulose. None of the phosphocellulose fractions were capable of supporting faithful initiation on rDNA (not shown). The fractions were tested for ability to reconstitute transcription and these data are shown in Figure 4. Lane a contains the products of a reaction in which prMAB/Pvu was transcribed by a control extract to yield the expected 292 nt run off transcript. This transcript was not formed by extracts from hormone-treated cells as shown

in lane b. Lanes c through f contain the products of transcription reactions in which phosphocellulose fractions A through D (respectively) were mixed with an extract from hormone-treated cells. Only fraction C (lane e) was able to reconstitute transcription to a significant degree. Fraction C contains less than 0.1% of the protein and less than 1% of the RNA polymerase I activity that was present in the S100 preparation from which this fraction was prepared. According to the convention of Mishima *et al.* (17), we designate the transcription factor, present in this fraction, as TFIC.

The ability to reconstitute transcription with TFIC was used to confirm formation of preinitiation complexes in extracts from hormone-treated cells. Such an extract was preincubated with prMAB/Pvu and TFIC was preincubated with prMAB/Sma. These were mixed and transcription was initiated by the addition of NTPs. As shown in Figure 4, lane g, only prMAB/Pvu was transcribed. Functional stable complexes did not form with prMAB/Sma during preincubation with TFIC or after mixing with the hormone-treated extract. Therefore transcription of prMAB/Pvu must be due to stable association of this template with factors that are present in extracts from hormone-treated cells.

Template competition experiments were carried out to examine the kinetics of formation of stable complexes in extracts from control and hormone-treated cells. A reaction mixture from control cells was prewarmed to 30°C and stable complex formation was initiated by the addition of prMAB/Pvu. prMAB/Sma was added at intervals thereafter and transcription was initiated with nucleoside triphosphates and allowed to proceed for 15 min. RNA was extracted and resolved on polyacrylamide:urea gels and the relative amounts of the 292nt and 155nt transcripts were determined by densitometry. As shown in Figure 5A, approximately equal amounts of 292nt and 155nt transcript were formed when both templates were added simultaneously (at 0 sec). Thereafter the relative amount of 155nt transcript decreased as a function of the length of preincubation with prMAB/Pvu. Stable complex formation in control extracts was complete within 1 min as evidenced by the observation that transcription of prMAB/Sma was reduced to less than 5% of that observed from the preincubated template.

A similar approach was used to estimate the rate of formation of stable complexes in extracts from hormone-treated cells. Extracts were mixed with prMAB/Pvu and challenged with prMAB/Sma at intervals. In this

Figure 5: Rate of formation of stable complexes. **A.** S100 extract from control cells was preincubated with prMAB/Pvu (292nt) and challenged by the addition of prMAB/Sma (155nt), as described in the text. The ratio of 292nt/155nt and the total amount of RNA synthesized (292nt + 155nt) were estimated by quantitative densitometry. **B.** S100 extract from hormone-treated cells was preincubated with prMAB/Pvu and challenged with prMAB/Sma. Transcription was initiated by the addition of NTPs plus TFIC.

experiment, however, transcription was initiated by the addition of nucleoside triphosphates plus TFIC. As shown in Figure 5B, stable complex formation in extracts from hormone-treated cells was complete within 60 sec. Within the limits of this assay, there was no significant difference in the rates of formation of stable complexes in extracts from control and hormone-treated cells.

DISCUSSION

Initiation of transcription of rDNA in vitro occurs via an ordered, multi-step mechanism. As reported by a number of groups, the first step involves the interaction of the template with one or more proteins to form a stable, preinitiation complex (8-11). By definition, these complexes do not dissociate under reaction conditions so that the rate of association does not contribute to the steady state rate of product formation. Stable complexes form at 4°C and do not require the addition of nucleoside triphosphates (11). Unpublished data from our laboratory indicate that heat inactivation of RNA polymerase I in S100 extracts does not preclude the formation of stable complexes and Meisfeld and Arnheim (10) have shown that stable complex formation can be observed using a partially purified fraction (TFID) that is devoid of polymerase activity.. Such data suggest that functional polymerase I is not required for formation of stable, preinitiation complexes with rDNA.

Formation of stable complexes is required but not sufficient for initiation to occur. Initiation, per se, requires at least two additional diffusible components: RNA polymerase I and TFIC. A fourth component that elutes from phosphocellulose at 100 mM KCl (TFIA) has also been implicated in transcription of rDNA (10, 17). We have previously demonstrated that RNA polymerase I from hormone-treated P1798 cells is capable of initiation if supplemented with factors from control cells (7). Therefore, failure of initiation in glucocorticoid-treated P1798 cells is not attributable to the polymerase. Hormone-treated cells are depleted of active TFIC. Lack of TFIC does not affect the ability to form stable complexes or the rate at which this process occurs. Moreover, the chromatographic properties of TFIC differ from those of the stable complex factors (10, 17). We conclude that TFIC is distinct from the stable complex factors and does not participate in formation of preinitiation complexes.

Our working hypothesis proposes that the preinitiation complexes serves as a binding site for RNA polymerase I and/or TFIC. Assembly of

the quaternary complex constitutes the initiation complex from which transcription proceeds. According to this model the steady state rate of synthesis of pre-rRNA should be determined by the steady state concentration of the initiation complex and the rate of elongation by RNA polymerase I. Analysis of transcription in vitro indicates that glucocorticoids regulate rRNA synthesis at the level of the initiation complex rather than by influencing the rate of elongation. We suppose that a similar situation prevails in vivo and such data as are available indicate that initiation of synthesis of pre-rRNA in isolated nuclei conforms to this expectation (18).

This mechanism provides for three different levels of regulation: (i) a long term regulatory event involving more or less permanent gene activation via stable complex formation; (ii) rapid regulation of the rate of transcription at the level of the factor (TFIC) that is required for initiation; and (iii) regulation of the rate of elongation by RNA polymerase I. We are unaware of any well characterized physiological regulatory event that involves stable complex factors although this possibility has not been carefully researched. Species specific transcription of rDNA is apparently attributable to stable complex factors (10) and nucleolar dominance in somatic hybrids may involve loss or suppression of genes encoding stable complex factors (19). Regulation at the level of initiation is well documented by the data presented in this paper as well as other data from our laboratory (7). Such a mechanism accounts for the effects of glucocorticoids upon rRNA synthesis in P1798 cells and recent data from our laboratory indicate that initiation factor TFIC is involved in inhibition of rRNA synthesis in cells treated with inhibitors of protein synthesis (20). We suspect that this may be a common mechanism for regulation of the rate of synthesis of rRNA by substances that influence that rate of cell proliferation. Regulation of the rate of elongation by RNA polymerase I is controversial and has been invoked to account for increased activity of RNA polymerase I in transformed cells (21). However, we have not observed differences in the rate of elongation by purified or partially purified polymerase from liver and hepatoma (14). We concur that this is a potentially significant regulatory site but submit that physiological regulation at this level remains to be demonstrated.

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