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Glucocorticoid inhibition of initiation of transcription of the DNA encoding rRNA (rDNA) in lymphosarcoma P1798 cells

(transcription *in vitro*/rRNA synthesis/RNA polymerase I/initiation factors)

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ABSTRACT **Cell-free extracts of lymphosarcoma P1798 cell culture lines support faithful initiation upon the cloned mouse DNA encoding rRNA (rDNA) promoter, whereas extracts from cells treated for 16 hr with 0.1 μM dexamethasone cannot. Extracts from both sources transcribe the cloned 5S RNA gene *in vitro* and mixing experiments further demonstrate that inhibition of transcription of rDNA *in vitro* is not due to nucleases or inhibitors of transcription present in extracts from glucocorticoid-treated cells. Incubation of extracts from control cells at 45°C for 15 min inactivates RNA polymerase I and abolishes transcription. Activity can be restored by the addition of partially purified RNA polymerase I from control cells and hormone-treated cells. Moreover, extracts from hormone treated cells can be reconstituted by the addition of a partially purified, heat-stable transcription factor from control cells.**

In the presence of 0.1 μM dexamethasone, murine lymphosarcoma P1798 undergoes reversible inhibition of synthesis of mature rRNA and a concomitant decrease of >95% in the activity of RNA polymerase I bound to the chromatin and involved in synthesis of pre-rRNA (1). A corresponding increase is observed in the amount of RNA polymerase I that is not bound to the template; hence, total polymerase I activity remains constant. The observation that template-bound activity decreases as "disengaged" activity increases suggests that initiation of transcription is inhibited by treatment with glucocorticoids in culture.

Inhibition of initiation could occur at several levels. One possible effect could be modification of core RNA polymerase I to the extent that the enzyme can no longer form specific initiation complexes. Alternatively, the hormone might induce an inhibitor of initiation or inhibit the expression of some factor that is required for initiation of transcription at rRNA promoters. Finally, inhibition might be due to modification of the DNA encoding rRNA (rDNA)-associated chromatin so as to preclude initiation. To discriminate among these possibilities, studies have been carried out *in vitro* using cloned mouse rDNA restriction fragments and cell-free extracts prepared from P1798 cells. Faithful initiation by RNA polymerase I has been demonstrated in such extracts (2–5). The presumptive rRNA promoter has been located within a *Sal* I restriction fragment of mouse rDNA and the authentic origin of transcription has been established for 45S RNA synthesized *in vivo* and run-off transcripts synthesized *in vitro*. We therefore undertook to determine if faithful initiation could be obtained with cell-free extracts prepared from hormone-treated cells. Experiments were also carried out to determine if hormonal effects occurred at the level of RNA polymerase I or, alternatively, if hormonal inhibition was associated with changes in the amount or activity of ancillary transcription factors. Data indicate that extracts from control cells are capable of supporting faithful initiation of transcription, whereas extracts from hormone-treated cells are not. Mixing experiments indicate that RNA polymerase I from hormone-treated cells is capable of catalyzing faithful initiation *in vitro* if supplemented with heat-stable factors from control cells.

MATERIALS AND METHODS

Materials. Restriction endonucleases were purchased from New England BioLabs. Unless otherwise indicated, all other enzymes were purchased from Bethesda Research Laboratories. Labeled nucleoside triphosphates were obtained from Amersham Radiochemicals, ICN Pharmaceuticals, or New England Nuclear. All other reagents were obtained from Sigma or Fisher.

Plasmid pI23 (Fig. 1), originally cloned by Marshall Edgell, was obtained from Norman Arnheim. Detailed restriction maps of this plasmid have been published (6, 7). The plasmid contains a *Sal* I insert of 3,200 base pairs (bp) of mouse rDNA including the promoter region, and transcription of pI23 *in vitro* has been described in detail by others (2, 3). Plasmid prMAB (Fig. 1) was constructed by digestion of pI23 with *Pvu* II, which removes the majority of the mouse rDNA insert plus that portion of pBR322 extending from the *Sal* I to the *Pvu* II site of the vector. Ligation and transfection yielded a foreshortened plasmid of 3,425 bp containing the mouse rRNA promoter, 168 bp of 5′ nontranscribed spacer DNA, a unique *Sma* I restriction site at +155, and a unique *Pvu* II restriction site at +292 (relative to the origin of transcription of 45S RNA). Plasmid pTH1, which contains the Syrian hamster 5S RNA gene (8), was obtained from William Folk.

The cell line used in these experiments was P1798.S20, a glucocorticoid-sensitive murine (BALB/c) lymphosarcoma line established from an ascites tumor (9). Cells were maintained in RPMI 1640 medium supplemented with 5% fetal bovine serum, 25 mM Hepes, 5 mM glutamine, 10 mM glucose, and 20 μM 2-mercaptoethanol. Cells were grown in suspension at 37°C in an atmosphere of 95% air/5% CO_2.

Methods. Restriction endonuclease digestion of plasmid templates was carried out by using conditions recommended by the manufacturer. Truncated plasmids were extracted once with phenol and three times with $CHCl_3$, precipitated with ethanol, and dissolved in H_2O to a concentration of 1 μg/μl. Plasmid prMAB cut with *Pvu* II is designated prMAB/Pvu II. S100 extracts were prepared as described by Weil *et al.* (10). Starting material was ≈1 g of P1798 cells from control cultures or from cultures that had been treated with 0.1 μM dexamethasone for 16 hr. Protein concentration (mean ± SD) in S100 extracts was 8.2 ± 1.7 mg/ml and there was no statistically significant difference in this respect be-

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Abbreviations: nt, length in nucleotides of single-stranded nucleic acids; bp, length in base pairs of double-stranded nucleic acids; rDNA, DNA encoding rRNA.

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tween extracts from control and hormone-treated cells. Activity of RNA polymerase I in S100 extracts was estimated by using published procedures (11) and was not significantly different in extracts from control and hormone-treated cells (≈4 pmol of UMP incorporated per min/mg of protein). Transcription of cloned rDNA was carried out as described by Miller and Sollner-Webb (2). The reaction volume was 50 μl and all reactions contained 0.4 pmol of plasmid, 100 mM KCl, 6 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 30 mM Hepes (pH 7.9), 12% glycerol, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 10 mM creatine phosphate, 25 μM UTP, 50 μg of α-amanitin per ml, and 5–10 μCi of [α-^{32}P]UTP (≈ 400 Ci/mmol; 1 Ci = 37 GBq). Transcription of pTH1 was carried out in a similar reaction mixture in the absence of α-amanitin. Routinely, transcription was initiated by the addition of 10 μl of S100. In cases in which less S100 was used, an equivalent volume of S100 diluent was added to maintain the reaction components at the concentrations stated above. Reaction mixtures were incubated 30 min at 30°C and transcription was terminated by the addition of 5 μl of 2% $NaDodSO_4$ containing 25 μg of *Escherichia coli* tRNA with 5 μg of proteinase K (EM Laboratories). After incubation at 65°C for 15 min, the proteinase K digest was diluted 1:10 with H_2O and RNA was precipitated in the presence of 1 mM spermine at 4°C for 15 min (12). Spermine-precipitated RNA was washed with 75% ethanol, dried, and dissolved in 50 μl of 4 M urea in 90 mM Tris/borate, pH 8.3, with 0.025% xylene cyanol and bromphenol blue. Samples were heated at 95°C for 5 min and resolved on 5% denaturing polyacrylamide gels containing 8 M urea in Tris/borate/EDTA, pH 8.3 (13). Standards were 5′-end-labeled fragments from *Hae* III digestion of bacteriophage φX174RF (14). Autoradiography was performed at −70°C by using Kodak XAR film and DuPont Lightning Plus intensifier screens. Gels were routinely exposed 12–16 hr.

RNA polymerase I and the transcription factor were partially purified by step-elution chromatography on DEAE-Sephadex A-25 by standard procedures (11). One milliliter of S100 extract was adsorbed to a 3.0-ml Sephadex A-25 column previously equilibrated in TGED buffer (50 mM Tris, pH 7.9/25% glycerol/1 mM EDTA/0.5 mM dithiothreitol) containing 50 mM $(NH_4)_2SO_4$. The column was washed with 3 ml of TGED buffer containing 75 mM $(NH_4)_2SO_4$ and polymerase I was step-eluted with TGED buffer containing 150 mM $(NH_4)_2SO_4$. This procedure resolved polymerase I from polymerases II and III and resulted in an enzyme preparation that was ≈10% pure, as judged by specific activity (≈80 pmol of UMP per min/mg of protein).

RESULTS

Transcription of Truncated Plasmids by S100 Extracts from P1798.S20 Cells. Plasmid prMAB (Fig. 1) was digested with *Pvu* II (prMAB/Pvu II), which cleaves the DNA 292 bp downstream from the point at which transcription originates *in vivo* (2). The truncated plasmid was incubated with an S100 extract prepared from P1798.S20, and ^{32}P-labeled transcripts were extracted and resolved on polyacrylamide urea gels as shown in Fig. 2. Transcription of prMAB/Pvu II yielded a major transcript of ≈292 nucleotides (nt) (Fig. 2,

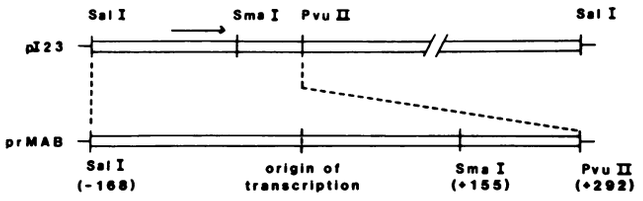

FIG. 1. Partial restriction map of the region surrounding the mouse rRNA promoter.

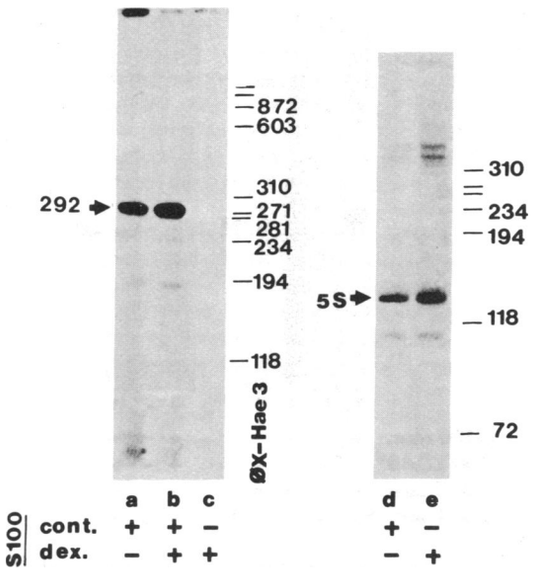

FIG. 2. Transcription of mouse rDNA and the Syrian hamster 5S RNA gene *in vitro*. Transcription was carried out as described in the text. The positions of *Hae* III-cut φX174RF standards are indicated to the right of each autoradiogram. The arrows indicate the calculated positions of the 292-nt run-off transcript from prMAB/Pvu II and 5S RNA from pTH1. Lanes a–c were exposed 16 hr and lanes d and e were exposed 6 hr. cont., Control; dex., dexamethasone treated.

lane a), which is consistent with other reports (2, 3) and indicates that S100 extracts of P1798 are capable of supporting faithful initiation upon mouse rDNA. The identity of transcripts synthesized *in vitro* has been confirmed by S1 nuclease mapping, run-off transcription of *Sma* I-truncated prMAB and *Xho* I-truncated pI23, and identification of the 5′-nucleoside triphosphate of the 292-nt RNA (data not shown).

Transcription of prMAB/Pvu II with S100 extract from hormone-treated cells did not result in formation of the expected 292-nt RNA (Fig. 2, lane c). However, a mixture of extracts from both sources was able to support faithful initiation of transcription *in vitro* (Fig. 2, lane b), indicating that dexamethasone-mediated inhibition of transcription *in vitro* was not due to the presence of an inhibitor or increased levels of nuclease activity. S100 extracts from control and hormone-treated cells were also incubated with a plasmid containing the cloned Syrian hamster 5S RNA gene (pTH1). When transcripts were resolved on denaturing polyacrylamide gels, a ^{32}P-labeled nucleic acid of ≈120 nt was observed (Fig. 2, lanes d and e). This nucleic acid, which resembles 5S RNA in electrophoretic mobility, was synthesized in the presence of 1 μg of α-amanitin per ml but not in the presence of 100 μg of α-amanitin per ml or the absence of pTH1. On this basis, it was concluded that this 120-nt species is 5S RNA transcribed from pTH1 *in vitro*. As shown in Fig. 2, the synthesis of 5S RNA was not reduced in extracts from hormone-treated cells.

Reconstitution of Initiation in S100 Extracts from Hormone-Treated Cells. RNA polymerase I is extremely thermolabile (11, 15); at 45°C, the half-time of denaturation of the enzyme in S100 extracts was ≈1 min (data not shown). S100 extracts from control cells were heated at 45°C and incubated with [α-^{32}P]UTP and prMAB/Pvu II, and transcripts were resolved on polyacrylamide urea gels as shown in Fig. 3. The ability to synthesize the 292-nt RNA was lost after 15 min of incubation at 45°C (Fig. 3, lanes b and e). To ascertain that loss of transcriptional activity was due to inactivation of RNA polymerase I, the enzymes from S100 extracts from control and

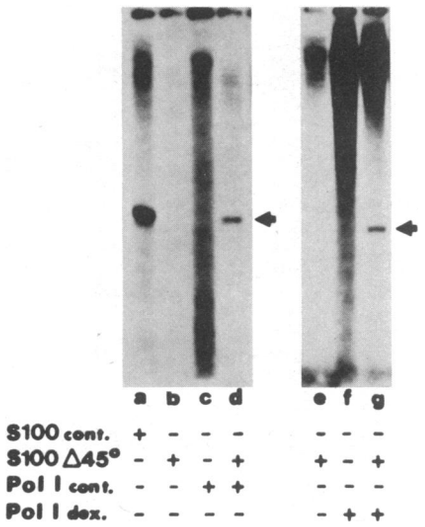

FIG. 3. Transcription by heat-treated extracts and partially purified polymerase I (Pol I). Lane a, transcripts synthesized by 10 μl of control (cont.) S100; lanes b and e, transcripts synthesized by 10 μl of control extract heated at 45°C for 15 min (S100Δ45°); lanes c and f, transcripts synthesized by 10 μl of partially purified RNA polymerase I from control (145 pmol of UMP per min/ml) and hormone-treated cells (195 pmol of UMP per min/ml), respectively; lanes d and g, products formed by a mixture of 5 μl of heat-treated extract with 5 μl of RNA polymerase I. The arrows indicate the position of the 292-nt transcript calculated from standards. dex., Dexamethasone treated.

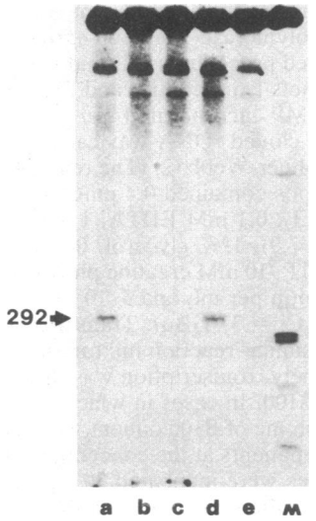

FIG. 4. Reconstitution of transcription in hormone-treated extracts. The lanes contain products formed from the following reactions: lane a, 5 μl of control S100; lane b, 5 μl of S100 from hormone-treated cells; lane c, 5 μl of S100 from control cells heated 45°C for 15 min; lane d, 5 μl of S100 from hormone-treated cells with 5 μl of heat-treated control S100 (45°C, 15 min); lane e, 5 μl of S100 from hormone-treated cells with 5 μl of control extract that was heated at 90°C for 5 min; lane M, standards. The arrow indicates the calculated position of a 292-nt transcript. High molecular weight bands observed in this experiment came from the batch of tracer employed and are not transcripts.

hormone-treated cells were partially purified by chromatography on DEAE-Sephadex A-25 and mixed with S100 extracts that had been inactivated by heating at 45°C for 15 min. As shown in Fig. 3, partially purified RNA polymerase I from control or hormone-treated cells was not able to carry out specific initiation (Fig. 3, lanes c and f). However, polymerase I from either source was able to reconstitute transcriptional activity in heat-treated extracts (Fig. 3, lanes d and g). The data shown in Fig. 3 may also be interpreted to indicate that partially purified RNA polymerase I from either source can support faithful initiation, which is not evident in lanes c and f because of the high levels of nonspecific transcription. According to this hypothesis, heat-treated extracts contain factors that suppress nonspecific transcription and decrease the background to the extent that the specific transcript may be seen. In any event, both interpretations demand that extracts from control and hormone-treated cells contain functional RNA polymerase I, which is the point addressed by this experiment.

Experiments were carried out to determine if the heat-treated extract from control cells contained factors that were capable of reconstituting transcriptional activity in S100 extracts from dexamethasone-treated cells. These data are shown in Fig. 4. In lane a, one observes the results obtained when 5 μl of control S100 was incubated with [α-^{32}P]UTP and prMAB/Pvu II. As expected, a 292-nt RNA was synthesized. This RNA was not synthesized in the presence of S100 extracts from hormone-treated cells (Fig. 4, lane b) or in the presence of S100 extract that had been heated for 15 min at 45°C (Fig. 4, lane c). Transcription was carried out in the presence of 5 μl of S100 from hormone-treated cells with 5 μl of heat-treated (45°C, 15 min) S100 from control cells. As shown in Fig. 4, lane d, this mixture was capable of synthesizing the authentic 292-nt transcript. A mixing experiment was carried out in which S100 extract from hormone-treated cells was mixed with S100 from control cells that had been heated 5 min at 90°C. As shown in Fig. 4, lane e, this mixture did not synthesize the 292-nt RNA. These data suggest that extracts from control cells contain a protein that is capable of reconstituting transcriptional activity in extracts from hormone-treated cells.

S100 extracts from control cells were fractionated by chromatography on DEAE-Sephadex. Proteins that were not adsorbed to DEAE-Sephadex were pooled and designated fraction I. The column was washed with TGED buffer containing 75 mM $(NH_4)_2SO_4$ and these fractions were pooled and designated fraction II. Fraction III was step-eluted with 150 mM $(NH_4)_2SO_4$ and pooled. This fraction contained >98% of the RNA polymerase I activity. The column was washed with TGED buffer containing 500 mM $(NH_4)_2SO_4$ to yield fraction IV. These fractions were dialyzed overnight against a buffer containing 100 mM KCl, 20 mM Hepes (pH 7.9), 20% glycerol, 1 mM dithiothreitol, and 0.2 mM EDTA. Individual fractions were tested for the ability to reconstitute initiation in S100 extracts from hormone-treated cells. As shown in Fig. 5, fraction II (lane c) contained a factor that restored the ability of such extracts to initiate at the rRNA promoter *in vitro*. Fraction II from a hormone-treated extract did not reconstitute transcription and none of the individual fractions synthesized the 292-nt specific transcript (data not shown).

DISCUSSION

Lymphosarcoma P1798 cells provide a useful system for studying hormonal regulation of rRNA synthesis. The rate of synthesis of pre-rRNA in intact cells may be altered 10- to 20-fold by the addition or withdrawal of physiological concentrations of a glucocorticoid (1). In contrast to the situation that usually obtains when studying transcription from RNA polymerase II or III promoters, regulation of transcription of rDNA is maintained in extracts that are free of cells or nuclei.

S100 extracts from control cells can support faithful initiation at rRNA promoters, whereas extracts from hormone-treated cells do not (within the limits of detection of the assay). Transcription of the 5S RNA gene is unaffected and the inhibitory effects of dexamethasone do not result from

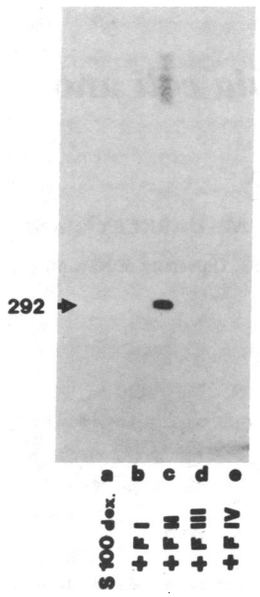

FIG. 5. Fractionation of S100 extracts. An S100 extract from control cells was fractionated and 5 μl of each pool was mixed with 5 μl of an extract from hormone-treated cells as indicated. The arrow indicates the position of the 292-nt transcript calculated from standards. dex., Dexamethasone treated; FI–FIV, fractions I–IV.

expression of an inhibitor or increased nuclease activity in extracts from hormone-treated cells. The observation that initiation is inhibited in cell-free extracts confirms an earlier conclusion that glucocorticoids inhibit initiation of transcription of rDNA *in vivo* (1). Furthermore, the observation that inhibition persists *in vitro* suggests that modification of nucleolar chromatin does not play a major role in glucocorticoid-mediated inhibition of rRNA synthesis in P1798 cells.

Hormonal inhibition could involve alteration of RNA polymerase I or ancillary transcription factors. Partially purified RNA polymerase I from control or hormone-treated cells can reconstitute heat-treated extracts from control cells. This suggests that the hormone is not acting upon the polymerase. The data indicate that untreated cells contain RNA polymerase I and relatively heat-stable factors that are required for initiation. One or more of these heat-stable factors is missing in hormone-treated cells. This is consistent with the hypothesis proposed by Feigelson and co-workers to account for glucocorticoid regulation of rRNA synthesis in rat liver (16). The factor is denatured at 90°C, is nondialyzable, may be precipitated with ammonium sulfate, and exhibits chromatographic properties that suggest that it may be a protein. Available data do not indicate the nature of the interaction between the factor(s), RNA polymerase I, and the template. Our working hypothesis is that the factor(s) is involved in formation of a ternary initiation complex. Alternative mechanisms include modification of template structure or activation of RNA polymerase I. Further elucidation of the mechanism requires additional purification and characterization of the components of the transcriptional complex.

The role of dexamethasone in regulation of transcription of rDNA is also unclear. The observation that transcription of the 5S RNA gene is unaffected suggests a rather specific effect. For example, the hormone may directly repress transcription of a gene(s) that encodes a rRNA initiation factor. Alternatively, loss of the initiation factor may be a secondary consequence of mitotic inhibition. Additional questions arise regarding the relationship between the glucocorticoid-regulated factor and those involved in hormonal regulation of rRNA synthesis in other tissues. Complete elucidation of the role of hormones in this process must await purification of the factor and ultimately characterization of the factor gene.

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