Volume 12 Number 21 1984 Nucleic Acids Research

# Hormonal regulation of transcription of rDNA: use of nucleoside thiotriphosphates to measure initiation in isolated nuclei

Zhen-yong Zhang, E.Aubrey Thompson and Michael R.Stallcup

Department of Biology, University of South Carolina, Columbia, SC 29208, USA

Received 10 July 1984; Revised 29 August 1984; Accepted 27 September 1984

ABSTRACT

Nuclei isolated from cultured mouse and rat cell lines initiated pre-rRNA chains at the correct site and with the correct nucleotide specificity (A for mouse, G for rat). Nucleic acid filter hybridization and S1 nuclease mapping were used to analyze the RNA products initiated with nucleoside (β-S)triphosphates. Initiation of pre-rRNA was completely resistant to α-amanitin but was inhibited by either actinomycin D or heparin. Experiments with P1798 mouse lymphoma cells indicated that the antiproliferative effects of glucocorticoids on lymphoid cells includes a reduction in the ability of nuclei to initiate pre-rRNA chains.

## INTRODUCTION

Proliferation of lymphosarcoma P1798 cells in culture may be reversibly regulated by the addition or withdrawal of the synthetic glucocorticoid dexamethasone (1). Inhibition of proliferation is accompanied by a rapid decrease in the rate of synthesis of 45S ribosomal precursor RNA (pre-rRNA) in cells. Transcription elongation measurements in isolated nuclei suggest that inhibition is due to a decrease in the number of RNA polymerase I molecules actively engaged in transcription of the rDNA genes that encode rRNA (2). In addition, extracts from hormone-treated cells are depleted of an initiation factor activity that is required for transcription of cloned mouse rDNA *in vitro* (3). These data indicate that glucocorticoids regulate the amount or activity of one or more initiation factors and suggest that inhibition of transcription in culture is due to a decrease in the rate of initiation of transcription. To confirm this hypothesis, experiments were undertaken to estimate the extent to which initiation of transcription was inhibited in nuclei isolated from hormone-treated cells.

Thiophosphoryl nucleotides have been used to study initiation of transcription in isolated nuclei (4, 5). The basis of this procedure is that thiophosphate residues from nucleoside (β-S)triphosphates or nucleoside (γ-S)triphosphates are incorporated only into the 5' termini of RNA chains;

© IRL Press Limited, Oxford, England.

thus when transcription is carried out in the presence of these analogues, the thiophosphate residue will be uniquely incorporated into transcripts that are initiated in vitro.

In the experiments described below, nuclei were isolated from rat or mouse cell lines and transcription was carried out in vitro in the presence of adenosine 5'-O-(2-thiotriphosphate) (ATPβS) or guanosine 5'-O-(2-thiotriphosphate) (GTPβS). Thiol-containing RNA was resolved by chromatography on mercury-Sepharose and analyzed by quantitative filter hybridization or S1 nuclease mapping, using cloned mouse and rat rDNA probes. The data reveal that correct initiation of pre-rRNA occurs in isolated nuclei and that nuclei from hormone-treated mouse P1798 lymphosarcoma cells are specifically inhibited in this respect.

## EXPERIMENTAL PROCEDURES

### Cell lines and tissue culture methods

GR-A3 is a cloned cell line derived from a mammary tumor in the GR strain of mouse (6). M1.54 is a cloned isolate from mouse mammary tumor virus-infected HTC (rat hepatoma) cells (7). P1798 is a mouse T-lymphosarcoma cell line (1). The specific P1798 lines used for these experiments had been infected with mouse mammary tumor virus (MMTV) to introduce a glucocorticoid-inducible marker. GR and M1.54 cells were grown in monolayer cultures in Dulbecco's modified essential medium supplemented with 5% horse serum; cells were harvested before the monolayer became confluent. P1798 cells were grown in spinner culture in RPMI 1640 medium supplement with 10 mM glucose, 20 μM 2-mercaptoethanol, and 5% fetal bovine serum; mid log phase cells were harvested at a density of approximately $10^6$ cells per ml. Whole cell extracts from P1798 cells were prepared as described (3) and used where indicated to supplement nuclei in the transcription reactions.

### Recombinant plasmids containing the 5'-ends of mouse and rat 45S rRNA genes

Plasmid pI23 (8) is pBR322 containing a 3.2-kb SalI insert that contains the promoter region and the first 3.0-kb of the mouse 45S rRNA gene (Fig. 1A). Plasmid pB4-5.1ΔH (9) is pBR322 with a 0.4-kb BamHI-HindIII insert that contains the promoter region and the first 125 nucleotides of the rat 45S rRNA gene (Fig. 1B). M13mp7.95 and M13mp82.55 DNAs are single-stranded M13 DNAs containing PstI inserts representing the minus strand of the MMTV long terminal repeat (LTR) and nonrepetitive (unique) regions, respectively (10).

Transcription in vitro and fractionation of RNA products by mercury-Sepharose chromatography

Previously described procedures (5) were modified as described below. Whole nuclei were prepared from GR cells, M1.54 cells, or P1798 cells by Dounce homogenization in the presence of NP40 detergent. The transcription reaction mixture for the nuclei contained either ATPβS or GTPβS (0.2 mM) substituted for the analogous natural substrate; the labeled substrate was either [$^{3}$H]CTP (66 μM, 2.5 Ci/mmole), or [α-$^{32}$P]UTP (20 μM, 20 Ci/mmole). RNA was purified from the reaction mixture by phenol extraction at pH 5. RNA chains initiated with ATPβS or GTPβS were separated from the remaining RNA by mercury-Sepharose (Hg-Sepharose) column chromatography. Samples were loaded onto 1-ml columns in urea buffer containing 7 M urea, 10 mM Tris-Cl (pH8.0), and 3 mM EDTA. The column was washed with 2 volumes of urea buffer and then washed with TNES buffer as previously described (5). Bound RNA was eluted with TNES buffer containing 10 mM dithiothreitol. The urea buffer helped to reduce nonspecific binding to the column. After ethanol precipitation the thiol-containing and thiol-free RNA fractions were analyzed by filter hybridization or S1 nuclease mapping.

Nucleic acid filter hybridization

Filters containing pI23, M13mp7.95 or M13mp82.55 DNA were prepared and used for filter hybridization as described (5). [$^{32}$P]RNA complementary to the SalI-PvuII fragment (5'-end of the 45S rRNA) of pI23 (Fig. 1A), or to the cloned double stranded MMTV DNA fragments, was synthesized by *E. coli* RNA polymerase as described (2, 10) and included in each hybridization reaction to monitor efficiency of hybridization.

S1 nuclease mapping

The DNA probes were prepared as described (11). Briefly, pI23 DNA (mouse ribosomal gene) was cut with PvuII, and pB4-5.1ΔH DNA (rat ribosomal gene) was cut with HindIII. The DNA was treated with alkaline phosphatase and digested with exonuclease III, which digests from the 3'-ends of double-stranded DNA only, to create partially single-stranded probe. T4 polynucleotide kinase and [γ-$^{32}$P]ATP were used to label the 5' ends.

Hybridization, S1 digestion, and polyacrylamide gel electrophoresis were performed as described (11). Thiol-containing or thiol-free RNA from the Hg-Sepharose columns were incubated in a total volume of 40 μl with 0.1 pmole of the appropriate labeled or unlabeled rat or mouse DNA probe: that is, [$^{3}$H]RNA was incubated with [$^{32}$P]DNA, and [$^{32}$P]RNA was incubated with unlabeled DNA. The hybridization products were digested in a volume of 250

µl with 30-100 units of S1 nuclease at 37°C for 30 min. Electrophoresis was carried out on an 8% denaturing (urea) polyacrylamide gel (12). The gels were dried and exposed to Kodak XAR-5 film with a Cronex Lightning Plus intensifying screen at -70°C.

## RESULTS AND DISCUSSION

### Filter hybridization analysis of pre-rRNA initiated in nuclei from mouse cells

Nuclei from GR cells, a mouse mammary tumor cell line, were incubated in cell free transcription reactions containing [$^3$H]CTP and either ATPβS or GTPβS substituted for the analogous natural substrate. The RNA products were purified from the reaction and then fractionated by Hg-Sepharose column chromatography. Thus two labeled RNA fractions were generated from each transcription reaction: 1) the Hg-Sepharose-bound (thiol-containing) RNA chains which were initiated in the cell free reaction with the thiol-nucleotide substrate; and 2) the non-binding (thiol-free) RNA chains which were elongated but not initiated in the cell free reaction. For each of these two fractions filter hybridization was used to measure the amount of [$^3$H]RNA representing the 5'-end of 45S rRNA. In this analysis the ATPβS-initiated RNA contained 5 times as much 45S rRNA (5' end) as the GTPβS-initiated RNA (Table 1, top half, line g). In contrast, the thiol-free RNA from the ATPβS and GTPβS reactions contained approximately equal amounts of the 5'-terminal sequences of 45S rRNA (Table 1, bottom half, line g).

Since 45S rRNA is known to initiate with ATP in mouse cells (13), nuclear preparations exhibited the proper specificity for the initiating nucleotide. The apparent initiation with GTPβS observed in this assay system could be due to nonspecific or non-physiological initiation in the cell free system or to nonspecific binding of RNA to the Hg-Sepharose column. Our analyses with S1 mapping, described below, indicate that a small amount of nonspecific binding to Hg-Sepharose was occurring.

### S1 nuclease mapping analysis of pre-rRNA initiated in nuclei from mouse cells

The ATPβS and GTPβS transcription reactions described above were repeated, again with GR cell nuclei, and the RNA products were fractionated on Hg-Sepharose columns. This time the thiol-containing and thiol-free fractions were analyzed by S1 nuclease mapping to determine the location(s) of the 5'-ends of the pre-rRNA sequences in each fraction. The DNA hybridization reagent used for the S1 mapping was plasmid pI23 (representing

TABLE 1
Initiation of pre-rRNA synthesis in mouse nuclei: Analysis by filter hybridization

| | Experiment I | | Experiment II | |
|---|---|---|---|---|
| | ATPβS | GTPβS | ATPβS | GTPβS |
| a. Total [$^3$H]RNA synthesis (cpm) | 5.5 x $10^6$ | 6.5 x $10^6$ | 5.3 x $10^6$ | 4.2 x $10^6$ |
| b. % [$^3$H]RNA bound to Hg-Sepharose | 2.3 | 2.4 | 2.0 | 3.3 |
| THIOL-CONTAINING RNA (INITIATED IN VITRO) | | | | |
| c. [$^3$H]RNA bound to pI23 filter (cpm) | 4291 | 754 | 3575 | 1005 |
| d. % [$^3$H]RNA bound to pI23 filter | 4.62 | 0.79 | 4.94 | 1.03 |
| e. hybridization efficiency of [$^{32}$P]cRNA | .263 | .263 | .207 | .349 |
| f. corrected % [$^3$H]RNA bound to pI23 filter | 17.6 | 3.0 | 23.9 | 3.0 |
| g. A/G ratio for pre-rRNA initiation | 5.6 | | 4.8 | |
| THIOL-FREE RNA (ELONGATED IN VITRO) | | | | |
| c. [$^3$H]RNA bound to pI23 filter (cpm) | 1439 | 3121 | 1641 | 2670 |
| d. % [$^3$H]RNA bound to pI23 filter | 0.244 | 0.498 | 0.269 | 0.492 |
| e. hybridization efficiency of [$^{32}$P]cRNA | .120 | .161 | .126 | .152 |
| f. corrected % [$^3$H]RNA bound to pI23 filter | 2.0 | 3.1 | 2.1 | 3.3 |
| g. A/G ratio for pre-rRNA elongation | .65 | | .64 | |

Transcription reactions, Hg-Sepharose chromatography, and filter hybridization were performed as described in the text.
Line c: scintillation counter background (6 cpm) and nonspecific binding to control DNA filters (20-100 cpm) have been subtracted.
Line d: d = 100 x c/a (Letters refer to lines in the table.)
Line f: f = d/e. (Corrected for efficiency of hybridization.)
Line g: $g = \frac{(f \times b) \text{ for ATP}\beta\text{S}}{(f \times b) \text{ for GTP}\beta\text{S}}$ for thiol-containing RNA
and $g = \frac{f \text{ for ATP}\beta\text{S}}{f \text{ for GTP}\beta\text{S}}$ for thiol-free RNA
Total RNA synthesis (a) was not included in calculating g, because differences in total RNA synthesis are random and/or due to differences of inhibitory activity in the ATPβS and GTPβS preparations; these factors are not relevant to the relative pre-rRNA synthesis in the presence of the two analogues.

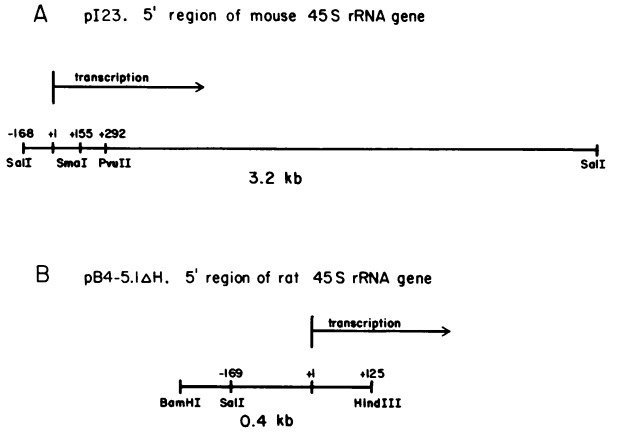


Figure 1. Partial restriction maps of the 5' regions of mouse and rat 45S rRNA genes.

the promoter region and 5'-end of mouse 45S rRNA), cut with PvuII (+292 relative to the transcription start site), digested with exonuclease III, and 5'-end-labeled with T4 polynucleotide kinase (Fig. 1A). Authentic 45S rRNA protects a 292-nucleotide fragment of this DNA probe. Because the DNA probe is $^{32}$P-labeled, both endogenous pre-rRNA and pre-rRNA synthesized in vitro are detected by this procedure.

The thiol-free RNA fractions from both the ATPβS and the GTPβS reactions protected a 292-nucleotide (±5 nucleotides) DNA fragment (Fig. 2, lanes 1 and 2). The nuclear preparations contained large amounts of endogenous pre-rRNA that elutes with the thiol-free RNA. Thus, this fraction served as a control to indicate the length of DNA probe protected by authentic pre-rRNA. The thiol-containing RNA (initiated in vitro) from the ATPβS reaction (lane 3) yielded a protected fragment of the predicted length (292-nucleotides). The amount of this protected fragment was much less when the probe was hybridized with thiol-containing RNA synthesized in the GTPβS reaction (lane 4), demonstrating a clear preference for initiation of pre-rRNA with ATP.

As stated above for the filter hybridization results, we believe that the GTPβS band (lane 4) is due to a small amount of non-specific binding of thiol-free RNA to the Hg-Sepharose column. In order to eliminate the signal from all endogenous rRNA sequences, the ATPβS and GTPβS reactions were repeated with [α-$^{32}$P]UTP as the radioactively labeled substrate. The fractions from the Hg-Sepharose columns were then analyzed by S1 mapping

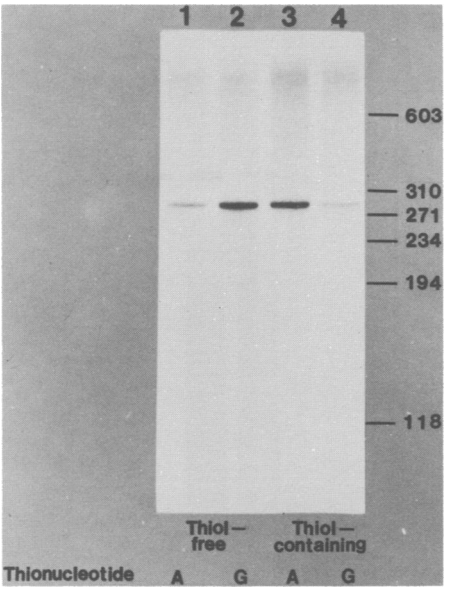


Figure 2. S1 nuclease mapping anlaysis of unlabeled mouse pre-rRNA initiated in vitro. Transcription reactions contained [$^{3}$H]CTP and approximately $10^{8}$ GR cell nuclei in a total volume of 0.5 ml. 40% of the thiol-containing RNA and 0.4% of the thiol-free RNA fractions were analyzed by S1 nuclease mapping, using 5'-$^{32}$P-end-labeled PvuII-cut pI23 DNA as the hybridization probe. Lane 1, thiol-free RNA, ATPβS reaction; lane 2, thiol-free RNA, GTPβS reaction; lane 3, thiol-containing RNA, ATPβS reaction; lane 4, thiol-containing RNA, GTPβS reaction. Numbers at the right indicate positions and sizes (in nucleotides) of HaeIII-cut ΘX174 DNA fragments.

with unlabeled PvuII-cut, exonuclease III-treated pI23 DNA. With this procedure, only RNA synthesized in vitro was detected on the autoradiogram. The thiol-free fractions from the ATPβS and GTPβS reactions (Fig. 3, lanes 1 and 2) yielded a protected RNA fragment that was somewhat larger than the predicted size. The apparent increase in size is an artifact of this particular gel, since all other experiments (including Fig. 4, Fig. 6, and many other experiments not shown here) produced [$^{32}$P]RNA fragments of the expected size (292 ±5 nucleotides). The smaller fragments are degradation products from the 5'-end of pre-rRNA that were detected in this assay but not when unlabeled RNA and 5'-end-labeled DNA probe were used (Fig. 2).

The thiol-containing RNA fraction from the ATPβS reaction yielded a band of the appropriate size after S1 mapping (Fig. 3, lane 3), but the GTPβS-initiated fraction produced very little or no band of this size (lane

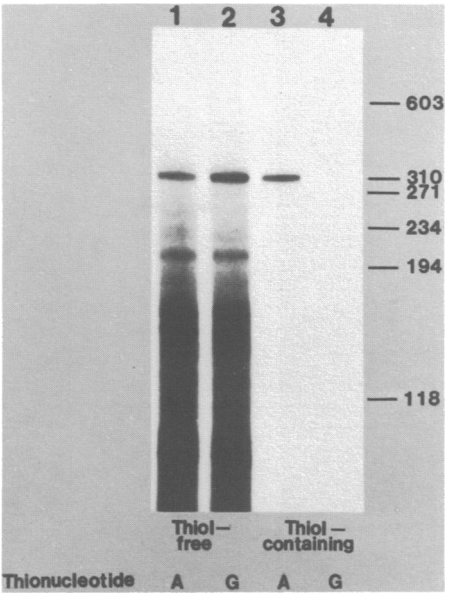


Figure 3. S1 nuclease mapping analysis of $^{32}$P-labeled mouse pre-rRNA initiated in vitro. Transcription reactions contained [$\alpha$-$^{32}$P]UTP and approximately 2 x $10^7$ GR cell nuclei in a total volume of 0.1 ml. 50% of the thiol-containing RNA and 4% of the thiol-free RNA products were analyzed by S1 nuclease mapping, using unlabeled PvuII-cut pI23 DNA as the hybridization probe. Lanes 1-4, same as Fig. 2.

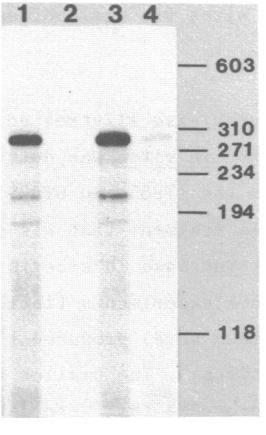


Figure 4. Inhibitor studies for pre-rRNA initiation in vitro. Transcription reactions and S1 mapping analyses were performed as in Fig. 3, using GR (mouse) cell nuclei and ATPβS. Inhibitors added to the transcription reactions were as follows: lane 1, none; lane 2, 20 μg/ml actinomycin D; lane 3, 100 μg/ml α-amanitin; lane 4, 200 μg/ml heparin.

4). These results demonstrate the correct nucleotide specificity for initiation of mouse pre-rRNA and suggest that the apparent low level of initiation with GTPβS shown in lane 4 of Fig. 2 actually reflects contamination with endogenous pre-rRNA. The appearance of a 292-nucleotide band in both types of S1 analyses ([$^{32}$P]DNA/unlabeled RNA and unlabelled DNA/[$^{32}$P]RNA) indicates that our preparations of nuclei initiate pre-rRNA at the correct site. An accompanying experiment (not shown) demonstrated that the amount of labeled pre-rRNA in these reactions is within the linear range of detection for this assay system; when increasing quantities of the Hg-Sepharose-bound fraction from the ATPβS reaction were incubated with a constant amount of unlabeled rDNA hybridization probe, the intensity of the 292-nucleotide band on the autoradiogram increased proportionately.

Three different inhibitors of RNA synthesis were tested for their ability to inhibit initiation of pre-rRNA with ATPβS in the preparations of GR cell nuclei (Fig. 4). Compared with the control reaction (lane 1), 20 μg/ml actinomycin D, a potent inhibitor of elongation, completely inhibited the synthesis of labeled pre-rRNA (lane 2). Initiation was completely resistant to 100 μg/ml α-amanitin (lane 3), consistent with the properties of RNA polymerase I. Heparin inhibits initiation but not chain elongation and, as shown in lane 4, synthesis of thiol-containing pre-rRNA was greatly reduced in the presence of heparin (200 μg/ml). Total RNA synthesis in the same reactions was inhibited 95% by actinomycin D, inhibited 32% by α-amanitin, and increased by 12% by heparin. These results clearly indicate that the synthesis of pre-rRNA in isolated nuclei results from DNA-dependent initiation by RNA polymerase I.

Initiation of pre-rRNA synthesis in nuclei from rat cells

In rat cells GTP is the 5'-terminal nucleotide of 45S rRNA (9). ATPβS and GTPβS transcription reactions were performed with preparations of nuclei from M1.54 cells, a rat hepatoma cell line, using [α-$^{32}$P]UTP as the radioactively labeled substrate. After Hg-Sepharose fractionation, the thiol-containing RNA was analyzed by S1 nuclease mapping. The unlabeled DNA hybridization probe was plasmid pB4-5.1ΔH (Fig. 1B, representing the promoter region and 5'-terminal sequences of rat 45S rRNA), cut with HindIII (+125 relative to the transcription start site), and digested with exonuclease III.

The GTPβS-initiated RNA yielded the predicted 125-nucleotide protected fragment (Fig. 5, lanes 2 and 3), whereas the ATPβS-initiated RNA did not yield a fragment of this size (lane 1). Lanes 1 and 3 contain RNA from

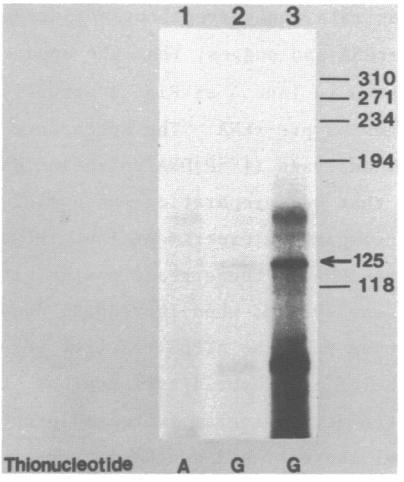


Figure 5. S1 nuclease mapping analysis of $^{32}$P-labeled rat pre-rRNA initiated in vitro. Transcription reactions contained [$\alpha$-$^{32}$P]UTP and approximately 2 x $10^7$ M1.54 cell nuclei in a total volume of 0.1 ml. The thiol-containing RNA fractions were analyzed by S1 mapping, using unlabeled HindIII-cut pB4-5.1$\Delta$H DNA as the hybridization probe. Lane 1, ATP$\beta$S reaction; lanes 2 and 3, GTP$\beta$S reaction. Lanes 1 and 3 represent equal reaction volumes; lanes 1 and 2 represent equal amounts of labeled RNA. The GTP$\beta$S-initiated fraction contained approximately 3 times as much total labeled RNA as the ATP$\beta$S-initiated fraction.

equal reaction volumes and thus illustrate the relative amounts of pre-rRNA initiation with ATP$\beta$S and GTP$\beta$S, respectively. Since the amount of all RNA initiated in vitro with GTP$\beta$S was approximately three times greater than that initiated with ATP$\beta$S, a more stringent control was performed to test the GTP$\beta$S preference for initiation of rat pre-rRNA: equal amounts of labeled RNA from the ATP$\beta$S-initiated and GTP$\beta$S-initiated fractions (lanes 1 and 2, respectively) were compared by S1 mapping. In both comparisons the GTP$\beta$S preference for the 125-nucleotide fragment is clear; however, for quantitative comparison, lanes 1 and 3 (equivalent reaction volumes) provide the best representation of initiation specificity. The band at approximately 90 nucleotides could be a degradation product of the 125-nucleotide 5'-terminal fragment. The origin of the 160-nucleotide band is unknown. Despite the presence of the unexpected bands, these results indicate that initiation of pre-rRNA is occurring at the predicted site and with the predicted nucleotide specificity in the rat cell nuclei. These results were reproduced in an independent experiment. The GTP$\beta$S preference for initiation of rat pre-rRNA provides a useful contrast and control to the

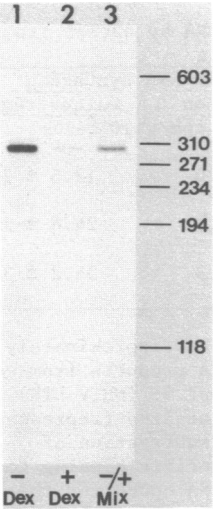


Figure 6. Initiation of pre-rRNA in vitro by nuclei from control and dex-treated P1798 cells. Transcription reactions and S1 mapping were performed as in Fig. 3, using P1798 cell nuclei and ATPβS. Only the results from the thiol-containing RNA fractions are shown. Lane 1, nuclei from control cells; lane 2, nuclei from cells grown for 24 hr in 0.1 μM dex; lane 3, equal numbers of nuclei from control and hormone-treated cells; the total concentration of nuclei was the same for all reactions.

ATPβS preference for initiation of mouse pre-rRNA and thereby further validates the specificity of the transcription initiation assay for nuclear systems.

Glucocorticoids regulate initiation of 45S rRNA synthesis in mouse P1798 T-lymphosarcoma cells

The S1 mapping assay described above was used to measure initiation of pre-rRNA in nuclei prepared from control and glucocorticoid-treated P1798 cells. Nuclei from control cells (Fig. 6, lane 1) initiated 10 times as much pre-rRNA as nuclei from cells grown with 0.1 μM dexamethasone (dex) for 24 hr prior to harvest (lane 2). These results, which have been obtained in a total of three independent experiments, agree with other types of experiments suggesting that glucocorticoids inhibit initiation of transcription of pre-RNA, DNA synthesis and cell division (2, 3, 14). When nuclei prepared from control and hormone-treated cells were mixed in equal numbers and incubated in vitro for transcription (Fig. 6,lane 3), the amount of pre-rRNA initiation was intermediate between the control and dex-treated samples. This indicates that the nuclei from hormone-treated cells did not contain any diffusible inhibitor.

One possible, although trivial, explanation for the loss of pre-rRNA initiation in nuclei from dex-treated cells is a general deterioration of nuclear function. However, previous studies showed that the P1798 cells are still viable even after several days of incubation with dex (1, 14). To confirm that the nuclei isolated from 24-hr dex-treated cells are still

TABLE 2
Synthesis of mouse mammary tumor virus RNA by nuclei from control and dex-treated cells

| dex treatment of cells | total nuclear RNA synthesis | MMTV RNA synthesis | |
|---|---|---|---|
| | | LTR region | Unique region |
| | cpm x $10^{-6}$ | cpm x $10^{-3}$ | |
| none | 10.4 | 3.2 ± 1.0 | 12.5 ± 2.8 |
| 1 hr | 8.8 | 7.8 ± 0.9 | 24.6 ± 2.6 |
| 24 hr | 5.1 | 5.6 ± 1.3 | 35.2 ± 3.6 |

Transcription reactions contained [$^3$H]CTP, GTPβS, and approximately $10^8$ nuclei in 0.5 ml. Approximately 6 x $10^5$ cpm of RNA products from each reaction was hybridized to filters containing M13mp7.95 (MMTV LTR) DNA or M13mp82.55 (MMTV unique) DNA. Total MMTV RNA synthesized (representing each genomic region) was calculated as follows: After subtraction of scintillation counter background (6 cpm) and nonspecific binding to a control DNA filter (2-10 cpm), the specific MMTV RNA bound for the samples from dex-treated cells was in the range 31-81 cpm for LTR filters and 64-319 cpm for unique region filters. The specific MMTV RNA bound was divided by the total radioactivity in the hybridization reaction, corrected for efficiency of hybridization (8-14% for LTR filters and 5-10% for unique region filters), and finally multiplied by the total RNA synthesis. MMTV RNA synthesis is given as the mean and standard deviation from 3 separate filter hybridization reactions.

functional, total and mouse mammary tumor virus (MMTV) RNA synthesis were measured for control and dex-treated nuclei (Table 2). After 24 hr of dex treatment, total RNA synthesis in isolated nuclei decreased by about 50%, compared with nuclei from control or 1-hr dex-treated cells; this decrease reflects the specific decline in rRNA synthesis. MMTV RNA synthesis was measured by hybridization of the RNA synthesized *in vitro* to specific MMTV DNA fragments on nitrocellulose filters. The total amount of MMTV RNA synthesis in the nuclear preparations, which reflects the rate of synthesis in whole cells, was approximately the same in nuclei from cells incubated for 1 hr or 24 hr with $10^{-7}$ M dex. The nuclei from control cells synthesized 2 to 3 times less MMTV RNA than the nuclei from hormone-treated cells. Thus, the decreased initiation of transcription of rDNA is not associated with a general deterioration of the nuclei. These results also emphasize the difference in the time course of dex action on MMTV RNA synthesis and rRNA synthesis.

Whole cell extracts from 24-hr dex-treated P1798 cells are incapable of correctly initiating pre-rRNA chains from rDNA templates *in vitro*; this deficiency can be corrected by the addition of a crude extract (lacking RNA polymerase I) or a partially purified protein fraction from control cells

(3). Nuclei were prepared from control and dex-treated P1798 cells, and initiation of pre-rRNA synthesis was measured in the presence and absence of the crude whole cell extract prepared from control P1798 cells. The amount of extract used in these experiments was sufficient to cause a ten fold stimulation of transcription of the cloned mouse rDNA gene. Nevertheless, the extract did not stimulate initiation when added to nuclei from dex-treated cells (data not shown).

These results indicate that an active RNA polymerase I initiation complex does not reform *in vitro* when the control extract is added to nuclei from dex-treated cells. Perhaps the isolated nuclei are impermeable to the required initiation factor(s), or alternatively RNA polymerase I and/or its accompanying initiation factors simply do not reassociate with the rDNA promoter under these conditions. Failure to form the initiation complex could be due to improper conditions *in vitro* or to modification of the rDNA-associated chromatin in hormone-treated cells. To address these questions, experiments are currently in progress to study pre-rRNA initiation in isolated nucleoli.

## CONCLUSIONS

The experiments described here demonstrate that glucocorticoid-mediated inhibition of pre-rRNA synthesis in P1798 cells is associated with inhibition of initiation upon rDNA-associated chromatin. The data are consistent with the observations that nuclei isolated from hormone-treated cells exhibit 90-95% reduction in elongation of pre-rRNA chains *in vitro* (2) and that soluble extracts from such cells are inhibited >95% in transcribing cloned mouse rDNA *in vitro* (3).

Filter hybridization analyses were used initially to demonstrate initiation of pre-rRNA chains with nucleoside thiotriphosphates in nuclei isolated from *Physarum polycephalum* (15) and from *Xenopus laevis* oocytes (16). In the studies described here, use of the S1 nuclease mapping technique along with the thionucleotides allowed us to determine the precise site of transcription initiation in the isolated nuclei; the data unambiguously demonstrate that authentic initiation of pre-rRNA occurs in nuclei from rat and mouse cell culture lines. The technique employed here provides a means of studying events associated with initiation of transcription upon intact chromatin and may have general applicability in studying regulatory processes in which transcriptional regulation may be affected by modification of chromatin structure.

ACKNOWLEDGEMENTS

The authors thank Dr. L. I. Rothblum for providing the cloned rat rDNA sequences, Dr. A. H. Cavanaugh for providing the P1798 cell extract, and Ms. Betty Branham for preparation of the manuscript. This work was supported by National Institutes of Health Grants CA22394 to E.A.T. and GM28298 to M.R.S. and by American Cancer Society grant SIN-107 to M.R.S. M.R.S. is a recipient of National Cancer Institute Research Career Development Award CA00897.

REFERENCES

1. Thompson, E.A. (1980) Mol. Cell. Endocr. 17, 95-102.
2. Cavanaugh, A.H. and Thompson, E.A. (1983) J. Biol. Chem. 258, 9768-9773.
3. Cavanaugh, A.H., Gokal, P. K., Lawther, R.P. and Thompson, E.A. (1984) Proc. Natl. Acad. Sci. USA 81, 718-721.
4. Smith, M.M., Reeve, A.E. and Huang, R.C.C. (1978) Cell 15, 615-626.
5. Stallcup, M.R. and Washington, L.D. (1983) J. Biol. Chem. 258, 2802-2807.
6. Ringold, G., Lasfargues, E.Y., Bishop, J.M. and Varmus, H.E. (1975) Virology 65, 135-147.
7. Ringold, G.M., Shank, P.R., Varmus, H.E., Ring, J. and Yamamoto, K.R. (1979) Proc. Natl. Acad. Sci. USA 76, 665-669.
8. Arnheim, N. (1979) Gene 7, 83-96.
9. Rothblum, L.I., Reddy, R. and Cassidy, B. (1982) Nucleic Acids Res. 10, 7345-7362.
10. Prewitt, R.S., Washington, L.D. and Stallcup, M.R. (1984) J. Virol. 50, 60-65.
11. Wood, K.M., Bowman, L.H. and Thompson, E.A. (1984) Mol. Cell. Biol. 4, 822-828.
12. Maniatis, T., Jefferey, A. and van de Sande, H. (1975) Biochemistry 14, 3787-3794.
13. Miller, K. and Sollner-Webb, B. (1981) Cell 27, 165-174.
14. Wood, K.M. and Thompson, E.A. (1984) Mol. Cell. Endocrinol., in press.
15. Sun, I. Y.-C., Johnson, E.M. and Allfrey, V.G. (1979) Biochemistry 18, 4572-4580.
16. Hipskind, R.A. and Reeder, R.H. (1980) J. Biol. Chem. 255, 7896-7906.