Specific transcription of mouse ribosomal DNA in a cell-free system that mimics control *in vivo*

(recombinant DNA/ribosomal genes/cell-free extracts/RNA polymerase I/transcription control)

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ABSTRACT   Cloned ribosomal DNA (rDNA) from mouse, which contains the initiation site of 45S pre-rRNA transcription and 5' flanking sequences, has been used as the template in an *in vitro* transcription system. In the presence of extracts from rapidly growing Ehrlich ascites cells, RNA polymerase I initiates specifically in that region of purified rDNA where the 5' end of 45S rRNA has been mapped. This is shown by electrophoretic analysis of the length of run-off transcripts synthesized from truncated templates, by S1 nuclease mapping, and by hybridization analysis of the *in vitro* products. The ability of the crude extracts to promote faithful transcription of mouse rDNA correlates with the proliferation rate of the cells. Only extracts prepared from exponentially growing mouse cells contain the factor(s) required for the faithful transcription of mouse ribosomal genes. Extracts from nongrowing or slowly growing mouse cells show very little activity. Thus, the cell-free system somehow reflects the rRNA synthetic activity of the cell and will prove valuable for the identification and purification of the various factors that are involved in the specific read-out of rDNA and may play a central role in the regulation of transcription of the ribosomal genes.

The production of rRNA in eukaryotes is very efficiently regulated at the transcriptional level (1–5). The dramatic alterations in the rate of rRNA synthesis according to the proliferation rate of the cell provide a suitable system for investigating gene regulation in eukaryotes. Virtually nothing is known yet about the signals and components required for the accurate initiation of rRNA synthesis by RNA polymerase I and about additional factors that may play a role in the expression of the genes coding for rRNA.

One approach to the study of these problems would be the use of a cell-free system that mirrors natural transcription events. Such soluble transcription systems, containing purified RNA polymerase and crude cytoplasmic or whole cell extracts, have been recently reported to selectively transcribe cloned genes that are transcribed by RNA polymerase II or RNA polymerase III (6–10). However, no analogous system is working for class I polymerase genes yet. In this paper, the applicability of such a crude *in vitro* system for the transcription of ribosomal genes is investigated. It is shown that extracts prepared from rapidly growing mouse cells contain the factor(s) necessary for the specific initiation of mouse ribosomal DNA (rDNA) transcription. Extracts prepared from cells after nutritional shift-down or from human cells do not promote correct transcription of mouse rDNA.

MATERIALS AND METHODS

DNA Templates. The cloning of mouse rDNA in bacteriophage λ has been described (11, 12). For the present experiments this 11.35-kilobase (kb) fragment was inserted into the 2.7-kb plasmid vector pUR2, a plasmid pBR322 derivative (13).

In some cases, the *Sal* I-B fragment cloned in pBR322 (plasmid pSalB) was used. To assay for specific run-off transcripts, the recombinant plasmids were truncated with different restriction endonucleases, extracted with phenol, chloroform, and ether, and dissolved at 500 μg/ml in 20 mM Tris·HCl (pH 7.5)/0.1 mM EDTA. When isolated rDNA fragments were used as templates, the DNA was purified by preparative electrophoresis, electroelution, and DEAE-cellulose chromatography (11).

For the transcription of the adenovirus 2 (Ad2) major late gene the *Sma* I-F fragment of Ad2 DNA cloned in the *Sma* I site of pBR313 (plasmid pSmaF) was used (8, 9).

Recombinant DNA experiments were conducted in accordance with the German guidelines for recombinant DNA research.

Preparation of S-100 Extracts. Ehrlich ascites tumor cells were cultivated at a density of 8×10^5 cells per ml in RPMI medium supplemented with 5% fetal calf serum. The crude cell extracts were prepared according to Weil *et al.* (7). The culture was rapidly cooled, and the cells were collected by low-speed centrifugation and washed with phosphate-buffered saline. The cells were allowed to swell for 10 min in a hypotonic buffer containing 10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, and 0.5 mM dithiothreitol. After centrifugation the cells were suspended in 2 vol of the original packed cell volume in the hypotonic buffer and homogenized in a Dounce homogenizer, made isotonic by addition of 0.1 vol of 300 mM Hepes (pH 7.9)/1.4 M KCl/30 mM MgCl₂, and the homogenate was spun at $100,000 \times g$ for 1 hr. The supernatant was carefully removed and dialyzed for 6–8 hr against a buffer containing 20 mM Hepes (pH 7.9), 20% (vol/vol) glycerol, 100 mM KCl, 0.2 mM EDTA, and 1 mM dithiothreitol before storage in portions at −70°C. The protein concentration of the resulting S-100 extracts was 25–30 mg/ml.

In Vitro **Synthesis and Analysis of RNA.** The final volume of the standard RNA synthesis reaction mixture was 50 μl, half of the volume being contributed by the S-100 extract. The final concentrations of components in the assay were: 10 mM Hepes (pH 7.9), 10% (vol/vol) glycerol, 75 mM KCl, 0.5 mM dithiothreitol, 10 mM creatine phosphate, 600 μM each of ATP, CTP, and UTP, 25 μM unlabeled GTP, as well as 5 μCi of $[\alpha^{-32}P]$GTP (specific activity 10–25 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels). To most reactions α-amanitin was added at 200 μg/ml. The assay mixture contained 0.75–1.25 μg of template DNA and *ca.* 750 μg of S-100 protein, including 20–30 units of RNA polymerase I as measured with denatured calf thymus DNA. When more than 30 units of exogenous RNA polymerase I was added, the specificity of the reaction was reduced. The reaction

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Abbreviations: kb, kilobase(s); bp, base pair(s); rDNA, ribosomal DNA, the DNA coding for rRNA; Ad2, adenovirus 2; ETS, external transcribed spacer; NTS, nontranscribed spacer.
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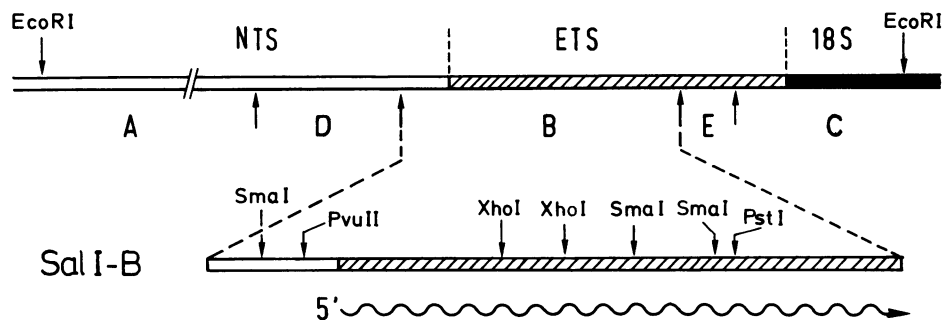
FIG. 1. Initiation region of mouse rDNA, showing the cloned *Eco*RI fragment of mouse rDNA and its *Sal* I cleavage sites (upward arrows). NTS, nontranscribed spacer; ETS, external transcribed spacer. An expanded *Sal* I-B fragment and its transcript (wavy line) are shown at the bottom.

mixture was incubated at 30°C for 45 min. Synthesis was terminated by the addition of 50 mM sodium acetate, 0.5% NaDodSO₄, and 25 μg of *Escherichia coli* tRNA. RNAs were extracted with phenol and chloroform, precipitated with ethanol, and fractionated on 4% acrylamide gels as described by Weil *et al.* (7). For size determination, the transcripts and ^{32}P-labeled DNA size markers were denatured by incubation for 2 hr at 37°C in a solution containing 1 M glyoxal, 50% (vol/vol) dimethyl sulfoxide, and 100 mM Hepes (pH 7.9) according to McMaster and Carmichael (14).

Hybridization Analyses. Samples to be hybridized were treated with RNase-free DNase (Worthington) at 40 μg/ml for 5 min at room temperature. After deproteinization, they were run over a Sephadex G-50 column to remove the excess of unincorporated nucleotides. For a Southern blot analysis (15), the ethanol-precipitated RNA was dissolved in 0.3 M NaCl/0.03 M sodium citrate/0.2% NaDodSO₄/1 mM EDTA and hybridized for 20 hr at 68°C to an *Eco*RI/*Sal* I digest of pMr974 or λMr974 DNA that had been fractionated on agarose gels and transferred to a nitrocellulose sheet.

The S1 nuclease mapping of the *in vitro* transcripts was performed essentially according to Berk and Sharp (16).

RESULTS

We have previously reported the cloning and characterization of an *Eco*RI rDNA fragment from mouse that contains 6.15 kb of the nontranscribed spacer and 5.2 kb of the transcribed gene region (11, 12). The recombinant bacteriophage carrying this *Eco*RI fragment of mouse rDNA has been designated λMr974; the corresponding plasmid is pMr974. Fig. 1 shows a schematic map of the structural organization of this rDNA fragment as well as some restriction endonuclease sites within the *Sal* I-B fragment that will be used for truncation of the template.

Recently, we have shown that nucleolar 45S RNA represents the primary transcript of rDNA and have mapped and determined the sequence of the initiation site of pre-rRNA synthesis (unpublished data). For the identification of specific run-off products generated in the cell-free system it was necessary to locate the 5' end of 45S RNA relative to one restriction site, which is to be used for truncation of the template. In Fig. 2 a nuclease S1 protection experiment is shown that uses the 990-base pair (bp) *Pvu* II/*Xho* I fragment derived from *Sal* I-B and an excess of 45S RNA for hybridization.

Trimming the hybrid with S1 nuclease generated two smaller fragments 840 and 640 bp long. This indicates that the 45S RNA as isolated from sucrose gradients represents a mixture of at least two RNA species differing at their 5' ends by 200 nucleotides. Because the S1 mapping procedure cannot distinguish downstream promoters from RNA processing sites, it is uncertain whether the heterogeneity of the rRNA precursor is the

result of rapid cleavage at the 5' end or whether two tandem promoter sites are located in front of the coding region. In any case, this heterogeneity is likely to complicate the interpretation of transcription experiments *in vitro*.

For the *in vitro* transcription studies a cell-free system similar to that described by Wu and Zubay (17) was used. It consisted of a soluble cell extract (designated S-100 fraction) prepared by high-speed centrifugation of whole cell homogenates that had been supplemented with purified RNA polymerase I. The assay for selective transcription is the appearance of distinct RNA bands. Because on a circular template the synthesis of a discrete-sized RNA requires both initiation and termination to occur at specific sites, we have transcribed linear "truncated" templates to allow termination to occur at DNA ends. If the 5' end of the 45S RNA corresponds to the pre-rRNA starting site, one or two transcripts 840 or 640 nucleotides long should be produced after cleavage of the rDNA template with *Xho* I, provided that the RNA polymerase I initiates *in vitro* at the same site(s) as *in vivo* and continues to the end of DNA at the *Xho* I site.

In the initial experiments whole pMr974 DNA in the circular form and after cleavage with *Xho* I was employed as a template in the S-100 system. Fig. 3 shows that, after extraction of RNA from *in vitro* reaction mixtures and gel electrophoresis, RNA of discrete sizes is synthesized. The majority of RNAs are high molecular weight transcripts in the upper parts of the gel. On the basis of their hybridization to the vector DNA (see Fig. 6) most of these transcripts are presumed to be generated from the vector (lane b). When the template was not digested with *Xho* I, very long molecules were observed on the top of the gel (lane c). After cleavage with *Xho* I a distinct new RNA band appeared; the appearance was sensitive to actinomycin D and resistant to high concentrations of α-amanitin. Omission of exogenous purified enzyme from the assay did not alter qualitatively or quantitatively the pattern of the transcription products.
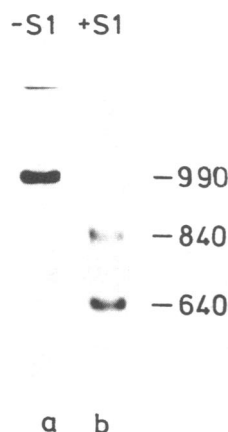

FIG. 2. S1 nuclease mapping of 45S pre-rRNA. Ten micrograms of 45S RNA purified from sucrose gradients was hybridized to 0.2 μg of the 990-bp *Pvu* II/*Xho* I fragment labeled at the 5' end by polynucleotide kinase and treated with S1 nuclease. Lane a, no S1 nuclease; lane b, 45 units of S1 nuclease (Sigma).
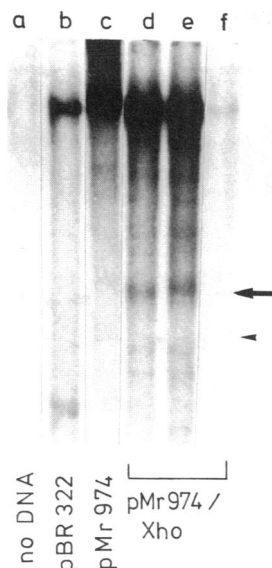
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end of which is about 250 nucleotides upstream from the *Xho* I site, was used for hybridization the protected region was shorter—i.e., 590 and 380 bp long (not shown). This means that the 640-nucleotide-long transcript must posses a different 5′ end than the 840-nucleotide-long RNA and does not result from premature termination. Furthermore, there are two smaller products—345 and 310 nucleotides long—that hybridize to the *Sal* I-B fragment as well.

The S1 experiment suggested that the majority of the specific *in vitro* transcript has been initiated 840 bp upstream from the *Xho* I site. To confirm this and to more accurately map the *in vitro* initiation site, we have used *Sal* I-B DNA truncated with *Pst* I, *Sma* I, *Xho* I, or *Taq* I in the cell-free system and analyzed the size of the resulting transcripts after denaturation with glyoxal (not shown here). After denaturation high molecular weight RNA was still observed that probably resulted from end-to-end transcription of the restriction fragments by RNA polymerase I. In addition to those unspecific transcripts, specific RNA products whose length decreased as the 3′ end of the template approached the presumptive initiation site could be identified. The length of the major run-off transcripts has been estimated to be about 1900 nucleotides after truncation with *Pst* I, 1550 for *Sma* I, 840 for *Xho* I, and 535 for *Taq* I. The length of these products corresponds to the sizes expected if the 5′ end of the *in vitro* transcript is the same as that of 45S pre-rRNA.

For analysis of the specificity of the initiation reaction at the rDNA promoter the labeled transcript was hybridized to restriction fragments obtained by cleaving pMr974 or λMr974, respectively, with *Eco*RI and *Sal* I. As shown in Fig. 5, *Sal* I cuts the ribosomal insert into five fragments designated *Sal* I-

FIG. 3. Electrophoretic analysis of RNAs synthesized *in vitro* by RNA polymerase I. The autoradiogram shows the gel analysis of RNAs synthesized in the S-100 system in the presence of different templates. Lane a, no DNA added; lane b, pBR322 DNA at 20 μg/ml; lane c, uncleaved pMr974 DNA at 25 μg/ml; lane d, pMr974 DNA cleaved with *Xho* I at 30 μg/ml; lane e, same as lane d plus α-amanitin at 200 μg/ml; lane f, same as lane d plus actinomycin D at 20 μg/ml. No exogenous RNA polymerase I has been added to the assays. The arrow indicates the specific run-off transcript at the *Xho* I site; the arrowhead marks the position of the 560-base Ad2 *Sma* I-F specific transcript.

When, however, more than 30 units of exogenous RNA polymerase I was added, the specificity of the reaction was abolished. This means that the factor(s) in the S-100 extract are not present in large excess compared to RNA polymerase I. A proof for the accuracy of the cell-free transcription system would be the demonstration that RNA polymerase I has initiated transcription at a unique point on the DNA that corresponds to the initiation site *in vivo*. Therefore, an S1 protection experiment analogous to that described in Fig. 2 was performed. ^{32}P-Labeled RNA synthesized *in vitro* on *Xho* I-truncated pMr974 DNA in the presence of S-100 was hybridized to an excess of unlabeled *Sal* I-B fragment. After S1 nuclease treatment some discrete hybrid bands appeared, the major one being approximately 840 bp long (Fig. 4).

In addition, a transcript 640 nucleotides long hybridizes to the *Sal* I-B or *Pvu* II/*Xho* I fragment, too. This hybridization pattern of the *in vitro* transcript strongly resembles that of 45S pre-rRNA (see Fig. 2). When a 940-bp *Taq* I fragment, the 3′
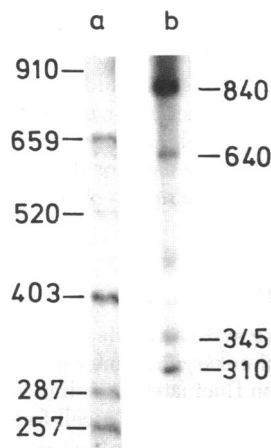

FIG. 4. S1 nuclease mapping of *in vitro* transcripts. [^{32}P]RNA was synthesized in the S-100 system in a 5-fold enlarged standard reaction mixture containing 100 μCi of [^{32}P]GTP and treated with RNase-free DNase at 40 μg/ml for 5 min at room temperature in the presence of *E. coli* tRNA at 200 μg/ml. After deproteinization it was hybridized to 0.4 μg of *Sal* I-B DNA and treated with 45 units of S1 nuclease (lane b). Lane a shows the positions of size markers from ^{32}P-labeled *Alu* I-digested pBR322 DNA.
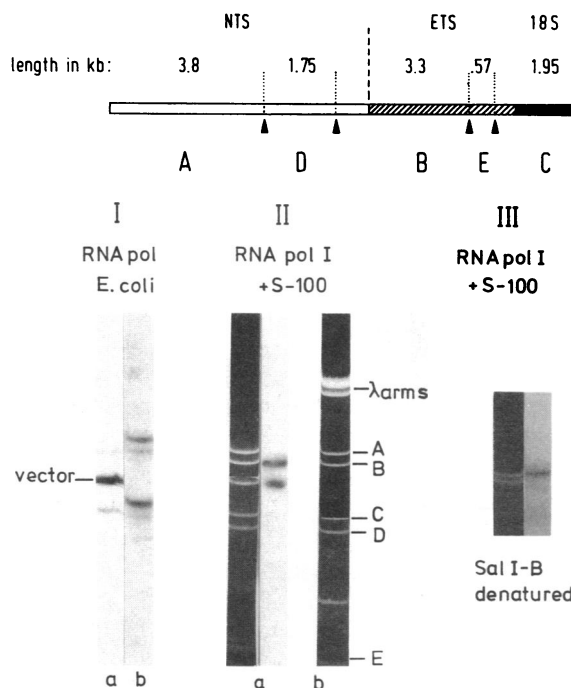

FIG. 5. Hybridization analysis of RNA products. pMr974 (lanes a) and λMr974 (lanes b) were digested with *Eco*RI and *Sal* I as shown schematically at the top (the arrows indicate the positions of *Sal* I cleavage sites). The DNA fragments were separated on agarose gels (ethidium bromide fluorescent bands are shown) and transferred to nitrocellulose strips (15). (*I*) Hybridization with ^{32}P-labeled RNA extracted from *E. coli* HB101 transformed with pMr974. (*II*) RNA synthesized in a standard reaction mixture containing RNA polymerase I (pol I) and *Xho* I-truncated pMr974 DNA (1.5 μg/50 μl) as template. (*III*) Hybridization of *in vitro* transcripts to separated strands of fragment *Sal* I-B.

A–E. Transcripts produced by *E. coli* RNA polymerase either *in vivo* or *in vitro* hybridize to a different extent to all ribosomal sequences, both to the transcribed regions B, C, and E and to the NTS fragments A and D. In contrast, the RNA transcribed by RNA polymerase I in the presence of S-100 hybridizes almost exclusively to *Sal* I-B, the fragment that contains the pre-rRNA initiation site. Significant labeling of neither the NTS fragments A and D nor C and E, the regions located downstream from the *Xho* I site, was observed. In addition to the selective transcription of the *Sal* I-B fragment, plasmid sequences are read by RNA polymerase I in the S-100 system (Fig. 3, lane b). This results in a strong hybridization of the transcription products to the 2.7-kb vector (Fig. 5, lanes a). When the *Xho* I-truncated pMr974 transcript was hybridized to separated strands of the *Sal* I-B fragment, the great majority of the *in vitro* synthesized RNA hybridized to the slowly moving strand, indicating that asymmetric transcription is occurring in the cell-free system.

In an attempt to characterize the components that promote specific *in vitro* transcription of mouse rDNA, the S-100 extract was incubated at different temperatures before the template and RNA polymerase I were added. Preincubation for 10 min at more than 30°C resulted in an inactivation of the active component(s) (not shown). This means that at least one factor is probably a protein, because it is nondialyzable and heat-labile. More interesting, the factor(s) seem to exhibit a certain species specificity. S-100 extracts from cultured human KB cells do not promote specific transcription of mouse rDNA genes (Fig. 6). Extracts from frog oocytes or eggs inhibited transcription markedly. As a control, the KB and mouse extracts were used to transcribe the *Sma* I-F fragment of the major Ad2 late gene (8, 18). It is evident that the KB extract contains the factor(s) essential for the selective initiation on the Ad2 late promoter, yielding a 560-nucleotide-long run-off transcript. However, the mouse extracts were absolutely inactive in the polymerase II system.

FIG. 7. Correlation of the activity of S-100 extracts with the proliferation rate of the cells. (*I*) S-100 extracts were prepared from logarithmically growing mouse cells (a), stationary cells (b), and cells that had been starved for serum for 3 hr (c) and assayed in the standard reaction mixture containing *Xho* I-truncated pSalB as template and 25 units of RNA polymerase I. (*II*) Mixing experiment between extracts from growing and stationary cells. The experiment was similar to that of *I*, with extract from logarithmically growing cells (a), extract from stationary cells (b), and a mixture of equal amounts of the two extracts (c). Arrowheads indicate the position of the specific run-off transcript.

The same result was obtained when a cloned lysozyme gene was used as a template. Only in the presence of KB cell extract, not in the presence of mouse extract, was the lysozyme gene transcribed faithfully (unpublished observation). This indicates either a certain species specificity of the factors required for both polymerase I and polymerase II genes or the presence of inhibitory components for RNA polymerase II in the mouse extracts.

The factor(s) required for the accurate transcription of rDNA genes is present only in S-100 extracts derived from very rapidly growing cells. When the extracts were prepared from stationary cells or after nutritional shift-down, very little specific transcript was observed (Fig. 7*I*). In this respect the cell-free system mirrors the rRNA-synthesizing capacity of the cell and implies that rRNA synthesis *in vivo* is regulated by factors operating at the level of transcription. The inability of extracts from non- or slowly growing cells to promote specific transcription is probably due to a low level of essential factor(s) that exert a positive regulatory function on rDNA transcription rather than being due to the accumulation of an inhibitor that overcomes the initiation of polymerase I on the rDNA promotor. This conclusion can be drawn from mixing experiments between extracts from growing and stationary cells. As shown in Fig. 7*II*, addition of an active S-100 extract from growing cells to an inactive extract mediates accurate transcription initiation. This ability of factor(s) to complement inactive extracts can be used for the isolation of proteins necessary for specific transcription of the ribosomal genes.
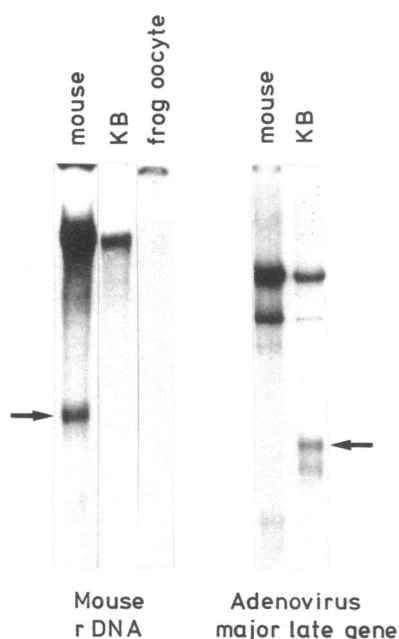

FIG. 6. Species specificity of the S-100 extracts. Mouse rDNA (*Xho* I-truncated pSalB) and the Ad2 major late gene (*Sma* I-truncated pSmaF) were used in a transcription assay mixture containing equal amounts (on protein basis) of S-100 extracts obtained from cultured mouse cells, human KB cells, or frog oocytes. The assays were supplemented with 25 units of RNA polymerase I or 50 units of RNA polymerase II, respectively. Arrows indicate specific run-off transcripts from mouse rDNA and the Ad2 major late gene, respectively.
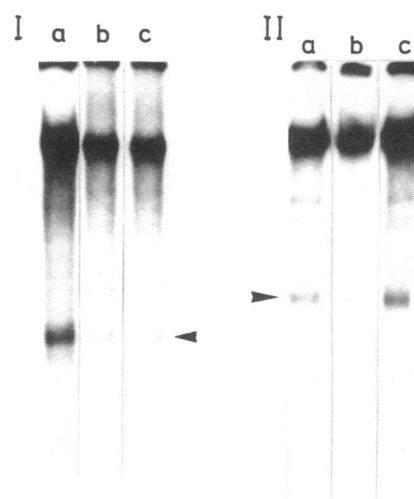
DISCUSSION

Ribosomal genes provide an attractive system for the analysis of factors involved in the regulation of eukaryotic gene expression because their rate of transcription fluctuates according to the growth rate of the cells. This paper describes a cell-free system that contains those components necessary for the selec-

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tive transcription of ribosomal genes from mouse. The *in vitro* system used is essentially the one that has been established for the transcription of viral or cellular genes transcribed by RNA polymerase II or III (6–9). The present studies were facilitated by the availability of cloned mouse rDNA that contains the start of the 45S rRNA transcription unit as well as a large part of spacer sequences that flank the transcribed region. This cloned DNA fragment as well as smaller subclones served as template for RNA polymerase I in the presence of S-100 extracts prepared from rapidly growing Ehrlich ascites cells. The run-off transcripts obtained with this rDNA truncated a few hundred nucleotides after the presumptive initiation site with a restriction enzyme clearly showed that, in the presence of cellular extracts, RNA polymerase I recognizes the initiation site for pre-rRNA and selectively transcribes the rRNA coding sequences. This conclusion is based on several independent methods of analysis. First, transcripts synthesized with truncated templates containing different well-defined lengths of the pre-rRNA coding region had discrete sizes corresponding to the distance between the 5' sequence of 45S pre-rRNA and the respective restriction site. Second, the transcripts derived from the large cloned rDNA fragment were analyzed by hybridization to restriction endonuclease fragments and separated DNA strands. It was shown that the RNA polymerase I transcribed the cloned rDNA asymmetrically, starting at that restriction fragment where the 5' end of pre-rRNA has been mapped.

Unfortunately, so far I have been unable to compare the 5' sequences of the *in vitro* transcripts with the sequence of pre-rRNA. This is due to the enormous size, fragility, and probable heterogeneity of 45S RNA. Other workers as well failed to find a unique 5'-terminal triphosphate on mammalian pre-rRNA (19, 20). Probably very rapid processing at the 5' end of the precursor molecules occurs *in vivo*, so that only very few primary transcripts can be found (21, 22). This is consistent with our finding (unpublished) that only 10% of mouse 45S rRNA molecules could be capped *in vitro*, which is an indication for the presence of di- or triphosphate termini at the 5' end (23, 24). Therefore, the heterogeneity of both the *in vivo* and *in vitro* transcripts that is evident in the S1 nuclease mapping experiment and after gel electrophoresis under denaturing conditions could be explained either by processing at the 5' end or by initiation at two tandem promoters. Indeed, reduplication of ribosomal promoters has been demonstrated in both bacterial and eukaryotic rDNAs (25–28).

To date, DNA sequences from the NTS/ETS boundary have been determined for ribosomal genes from yeast (29, 30), *Drosophila melanogaster* (31), three frog species (refs. 27, 32; B. Allet, R. Bach, and M. Crippa, personal communication), and mouse (unpublished data). A comparison of those sequence data did not reveal significant homology in the region of transcription initiation. Though the organisms compared are evolutionarily widely distant and it cannot be ruled out that conservation of regulatory sequences is observed in more closely related species, we suggest that the specificity and regulation of RNA polymerase I might be brought about by species-specific factors. This is based on the observation that we have obtained faithful transcription of mouse DNA only in S-100 extracts prepared from cultured mouse cells. Heterologous extracts prepared from human KB cells or frog oocytes inhibited transcription of pMr974 DNA. In this respect the RNA polymerase I genes obviously differ from RNA polymerase II genes, for which specific transcription of mRNA-coding genes from different species, such as adenovirus early and late genes (8–10), chicken conalbumin, ovalbumin (9) and lysozyme genes (unpublished data), as well as mouse globin genes (33) has been observed with extracts from human KB or HeLa cells. In addition to the species specificity, the amount or activity of the component(s) that confer selectivity to RNA polymerase I seems to fluctuate according to the growth rate of the cells. Only extracts prepared from exponentially growing cells catalyze the synthesis of the specific ribosomal transcripts, whereas extracts from nongrowing cells are virtually inactive. Thus the extracts reflect the rDNA transcriptional activity *in vivo*, which is correlated with the proliferation rate of the cells (1–5). Because the activity of the extracts from nongrowing cells can be restored by the addition of extracts from growing cells, the cell-free system will prove valuable for the further investigation and purification of the various factors that regulate the transcription of rDNA.

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