Studies on the specificity of preribosomal RNA transcription in nucleoli after selective deproteini-

zation

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ABSTRACT

Fidelity of preribosomal RNA transcription in vitro was studied after selective deproteinization of nucleoli using either sequential salt extraction or sodium deoxycholate treatment. Homochromatography fingerprinting and identification of marker oligonucleotides from a T1 ribonuclease digest of the transcripts were used to evaluate the RNA products. These studies indicated that: (1) nucleoli retained their endogenous RNA polymerase I activity and the specificity of transcription up to 0.6 M NaCl extraction; (2) exogenous RNA polymerase I transcribed nucleolar chromatin only after 1.0 M NaCl extraction and the transcription pattern, like that of totally deprotein-

ized DNA, was completely random; (3) extraction of nucleoli with deoxycholate resulted in a DNP complex in which the endogenous RNA polymerase I transcribed pre-rRNA specifically; however, it also initiated random transcription, producing a "mixed" fingerprint pattern on the homochromatogram. The random transcription was selectively inhibited either by deoxycholate or rifampicin AF/013. These studies indicate that the selectivity of pre-rRNA transcription is due both to the endogenous RNA polymerase I molecules that were involved in transcription in vivo and are tightly bound to the template and to factors in intact nucleoli which prevent random transcription by the released RNA polymerase I molecules.

INTRODUCTION

For an understanding of the mechanism of gene expression in eukaryotes, the complex nuclear transcriptional machinery must be reduced to simpler systems. The localization of ribosomal gene expression and the enzymes responsible for rDNA transcription in nucleoli offers a partial fractionation of components of ribosomal gene expression. Although rDNA comprises only about 0.5% of nucleolar DNA, the only known gene product of nucleolus, either in vivo or in vitro, is preribosomal RNA (1, 2). Hence,
it is likely that there are mechanisms in the nucleolus that would promote only the transcription of rDNA while the bulk of the nucleolar DNA is repressed.

Since DNA-dependent RNA polymerase I is the enzyme that transcribes rDNA in vivo, several attempts have been made to demonstrate the specific transcription of rDNA by purified RNA polymerase I. Holland et al (3) and Van Keulen et al (4) have shown that when deproteinized yeast DNA was transcribed with purified yeast RNA polymerase I, about 6-10% of the transcripts represented rRNA. More recently, Tekamp et al (5) have demonstrated that transcription of acidified (pH 4.5) chromatin by homologous RNA polymerase I produced transcripts which were slightly enriched in rRNA sequences. These studies have shown that a small percentage of the sequences transcribed from deproteinized DNA are ribosomal RNA; but the in vivo conditions wherein RNA polymerase I transcribes only pre-rRNA has not been achieved in vitro.

There are several hundred proteins in the nucleolus which include the proteins of the preribosomal RNP particles and those involved in processing and transport of pre-rRNA (6,7). Most of these proteins were removed when nucleoli were extracted with low ionic strength solutions to isolate chromatin (8,9). Although the isolated chromatin was only about 20% as active as intact nucleoli in its ability to synthesize RNA (8), the transcripts were essentially the same as those of intact nucleoli and closely resemble pre-rRNA by the homochromatography fingerprinting technique (2). Since the factors responsible for maintaining the high fidelity of transcription are present in the nucleolar chromatin, it seemed possible to identify, by selective deproteinization of the chromatin, factors that would either promote specific transcription of ribosomal genes or suppress random transcription of non-rDNA by RNA polymerase I.

In this report, homochromatography fingerprinting analysis was used to assess the specificity of pre-rRNA synthesis in nucleoli after sequential salt extraction and after extraction with sodium deoxycholate. The results suggest that (1) nucleolar chromatin retains its endogenous RNA polymerase activity and the specificity of transcription up to 0.6 M NaCl extraction,
(2) exogenous RNA polymerase I can transcribe nucleolar chromatin only after 1.0 M NaCl extraction and the transcription pattern, like that of totally deproteinized DNA, is completely random and (3) extraction of nucleoli with deoxycholate results in a DNP complex in which the endogenous RNA polymerase I transcribed pre-rRNA specifically and also initiated random transcription on the non-rDNA regions of the nucleolar DNA, producing a "mixed" fingerprint pattern on the homochromatogram. The random transcriptions can be selectively inhibited by either deoxycholate or the initiation inhibitor rifampicin AF/013. These studies indicate that the selectivity of pre-rRNA transcription depends both on the specificity of initiated endogenous RNA polymerase I molecules and on factors present in intact nucleoli, which prevent random transcription by the released polymerase molecules.

MATERIALS AND METHODS

Isolation of Novikoff Hepatoma Nucleoli. Novikoff hepatoma ascites fluid was collected from 6-day tumor-bearing rats. Nuclei and nucleoli were isolated by a modification of the method of Muramatsu et al (10). Nucleoli were suspended in a buffer containing 50% glycerol, 0.05 M Tris-HCl (pH 7.9), 1 mM MgCl$_2$, 0.1 mM EDTA, 2 mM DTT, and 1 mM PMSF and stored at -80°.

Isolation of Nucleolar Chromatin. Nucleoli were extracted twice with 20 volumes (wt/vol.) of 0.075 M NaCl, 0.025 M Na$_4$EDTA pH 8.0, 1 mM PMSF. All operations were conducted at 4°. Extractions were carried out by gentle hand homogenization using a loose fitting Dounce homogenizer and centrifuged at 10,000 xg for 20 minutes. The final gelatinous pellet was suspended in 0.01 M Tris-HCl (pH 8.0), 1 mM PMSF and used within 4 hours.

Salt Extraction of Chromatin. For sequential salt extraction, chromatin was suspended at a concentration of about 1 mg DNA per ml in 0.01 M Tris-HCl (pH 8.0), 1 mM PMSF, and while it was being stirred gently in an ice bath, an equal volume of NaCl solution having a concentration twice that of the needed extractant in 0.01 M Tris-HCl (pH 8.0) 1 mM PMSF was added drop-wise. After stirring for 15 minutes, it was centrifuged at 12,000 xg for 30 minutes. The extraction was repeated after swelling in
0.01 M Tris-HCl. Chromatin was extracted sequentially twice with 0.15 M, twice with 0.35 M and twice with 0.6 M NaCl. The final residue was then extracted with either 1.0 M NaCl or 3 M NaCl, 5 M urea, 0.01 M Tris-HCl, 1 mM PMSF and centrifuged at 225,000 xg for 18 hours in a 60 Ti rotor. The pellet was redissolved in 0.01 M Tris (pH 8.0) - 1 mM PMSF and dialyzed against the same solution.

Sodium Deoxycholate Treatment of Nucleoli. Nucleoli were suspended in a buffer containing 0.01 M Tris-HCl (pH 7.9), 0.5 mM PMSF, 0.2 mM DTT (TPD) at a concentration of 8-10 A260 units per ml and mixed with 0.2 volumes of deoxycholate solution (0.25 M). The contents were mixed gently by inversion in a capped test tube for 5 minutes, layered over 2 volumes of 30% sucrose, 0.01 M Tris-HCl (pH 7.9), 5% glycerol, 0.2 mM DTT, 0.5 mM PMSF, and centrifuged at 225,000 xg for 15 hours in a Beckman 60 Ti rotor. After decanting off the supernatant fluid, the DNP pellet was suspended by gentle homogenization in TPD at a concentration of about 20 A260 units per ml.

Purification of RNA Polymerase I. The 0.075 M NaCl-0.025 M EDTA (pH 8.0) and 0.01 M Tris (pH 8.0) extracts of nucleoli which contained about 80% of the nucleolar soluble RNA polymerase I (8) were used as a source of the enzyme. The pooled extracts were concentrated by ammonium sulfate precipitation (60% saturation) and dissolved in TGEDP (0.05 M Tris, pH 7.9, 25% glycerol, 0.1 mM EDTA, 2 mM dithiothreitol and 0.5 mM PMSF). The enzyme was purified by DEAE-Sephadex and phosphocellulose column chromatography (11). The active fractions from the phosphocellulose column were concentrated by ammonium sulfate precipitation and dissolced in TGEDP.

RNA Synthesis In Vitro. RNA synthesis was carried out in vitro in a solution containing 0.05 M Tris (pH 8.0), 0.05 M KCl, 5 mM KF, 0.6 mM each of CTP, UTP, and ATP, 0.015 mM [3H]-GTP (1 μCi/assay), 5 mM magnesium acetate and 15-20 μg of chromatin or salt extracted chromatin as template. Incubations were performed at 30° and at various time intervals aliquots were taken and placed on DE-81 discs. The discs were washed and processed for counting as described by Blatti et al (12).

In experiments where the RNA transcribed was to be analyzed
by homochromatography fingerprinting, the incubations were scaled up 100 times and the concentrations of the nucleotides were decreased to 0.01 mM each; instead of $[^{3}H]$-GTP, $\alpha^{[32P]}$-labeled ATP, CTP, UTP and GTP (specific activity 100-200 C/mM; 100 µCi per reaction) were used. The in vitro labeled RNA was extracted after 30 min. incubation by the SDS-hot phenol method (13).

Homochromatography of the T₁ Ribonuclease Digest. The RNA transcribed in vitro with $[^{32P}]$-labeled nucleotides was digested with T₁ ribonuclease and electrophoresed on Cellogel strips at pH 3.5 in 7 M urea. It was then transferred to large (45 cm X 40 cm) DEAE cellulose thin layer plates and the homochromatogram was developed with homomixture C-5 as described earlier (14). The autoradiograms were developed by exposing Rp - Royal X-Omat films using DuPont Lightning Plus intensifying screen at -80°.

Chemicals. Ultrapure sucrose, urea and $[^{3}H]$-GTP were obtained from Schwarz-Mann, Orangeburg, NY. $\alpha^{[32P]}$-labeled ATP, UTP, CTP and GTP were obtained from Amersham-Searle and International Chemical and Nuclear Corp., Irvine, CA. T₁ ribonuclease was obtained from Calbiochem. Rifampicin AF/013 was a generous gift from G. Lancini of Gruppo-Lepitit, Italy.

RESULTS

Effects of NaCl Extraction on the Template Activity of Nucleolar Chromatin. When isolated nucleoli from Novikoff hepatoma cells were extracted with saline-EDTA and 0.01 M Tris-HCl (pH 8), the resulting nucleolar chromatin incorporated $[^{3}H]$-GTP into RNA at a rate which was only about 20% that of intact nucleoli (Fig. 1). Although nearly 80% of nucleolar RNA polymerase I was extracted during isolation of chromatin (8,9), addition of purified RNA polymerase I to the nucleolar chromatin did not enhance its transcriptional activity significantly (Fig. 1). Furthermore, sequential extraction of the chromatin with 0.15 M and 0.35 M NaCl did not decrease the endogenous RNA polymerase activity and addition of RNA polymerase I did not increase transcription rates. Extraction with 0.6 M NaCl further decreased the endogenous activity more than 50% and the activity increased
Fig. 1. Transcriptional activities of nucleoli and nucleolar chromatin extracted sequentially with increasing concentrations of NaCl. Shaded bars represent the endogenous RNA synthetic activity and the open bars indicate activity in the presence of exogenously added RNA polymerase I.

only slightly by the addition of exogenous RNA polymerase I. On the other hand, subsequent extraction of the residual chromatin with either 1.0 M NaCl or 3 M NaCl - 5 M urea eliminated all the endogenous activity and exogenous RNA polymerase I transcribed these templates efficiently with a transcriptional capacity nearly 80% that of totally deproteinized nucleolar DNA (Fig. 1).

Homochromatography Fingerprinting Analysis of RNA Transcribed From Nucleolar Chromatin Extracted With NaCl. The RNA transcribed by these salt-extracted chromatins was analyzed by two-dimensional homochromatography fingerprinting (Fig. 2). The presence of marker oligonucleotide fragments found after T1 ribonuclease digestion of in vivo labeled 45S preribosomal RNA in the T, Y and U regions of the map (2,15) is an indication of the fidelity of pre-RNA synthesis. Thus, the nucleolar chromatin retained the high fidelity of transcription (Fig. 2) of intact nucleoli. The addition of RNA polymerase I, did not alter the overall incorporation of [3H]-GTP (Fig. 1) and did not alter the characteristic oligonucleotide pattern (2).

The fingerprinting patterns of the transcripts from NaCl
Fig. 2. Homochromatography fingerprinting pattern of RNA transcribed from nucleolar chromatin. T\textsubscript{1} ribonuclease digest of the transcribed RNA was separated by electrophoresis at pH 3.5 on Cellogel strips in the first dimension and homochromatography on DEAE cellulose thin layer plates in the second dimension. The numbered spots represent some of the marker oligonucleotides from 45S pre-rRNA.

extracted chromatin are shown in Figures 3-5. The transcribed RNA from chromatin extracted with both 0.15 M NaCl (not shown) and 0.35 M NaCl had an oligonucleotide pattern (Fig. 3) identical to that obtained from original chromatin (Fig. 2).

A variable pattern was obtained from the RNA transcribed from chromatin extracted with 0.6 M NaCl. After this extraction, the transcriptional activity decreased to less than 10% of the original nucleoli (Fig. 1); but the transcribed RNA still had many of the large oligonucleotide marker fragments of pre-rRNA (Fig. 4).

Loss of Fidelity of Transcription After Extraction With 1.0 M NaCl. The fingerprinting pattern of RNA transcribed from
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Fig. 3. Homochromatography fingerprinting pattern of RNA transcribed from nucleolar chromatin extracted twice with 0.35 M NaCl, in the presence of purified RNA polymerase I.

chromatin extracted with 1.0 M NaCl is shown in Fig. 5. The pattern differs from those noted above. The RNA transcribed was random inasmuch as none of the marker oligonucleotides of pre-ribosomal RNA in the upper regions of the map (Fig. 2) were detectable. Even in the smaller oligonucleotide regions of the map (Fig. 5, arrows, corresponding to penta- and hexanucleotides), nonspecific stripes were visible instead of distinct spots. This type of random pattern was also characteristic of transcripts from chromatin extracted with 3 M NaCl or from completely deproteinized nucleolar DNA.

Effect of Deoxycholate on Nucleolar Transcription. Extraction of nucleoli with 100 mM deoxycholate extracted 60% of the nucleolar proteins (Table 1). However, unlike extraction with salt, the resulting nucleoprotein complex had nearly 3 times higher endogenous transcriptional activity of intact nucleoli.
Fig. 4. Homochromatography fingerprinting pattern of RNA transcribed from nucleolar chromatin extracted with 0.6 M NaCl, in the presence of purified RNA polymerase I. The arrows indicate the penta- and hexanucleotide regions of the map.

(1). Addition of deoxycholate to the transcription assay inhibited transcription of the complex by about 60% while deoxycholate inhibited the transcription of naked DNA by exogenous RNA polymerase I by 95% (Table 1).

The RNA transcribed by the deoxycholate treated nucleoli was then analyzed by the homochromatography fingerprinting technique. Figure 6 shows the pattern obtained. This was consistently found to be a "mixed" one containing the high molecular weight pre-rRNA specific marker fragments in a background of nonspecific stripes characteristic of random transcription. This evidence for loss of specificity due to increased nonspecific transcription agrees well with the increased A+U/G+C ratio shown in Table 2. When the pellet obtained after 50 mM DOC treatment was transcribed in the presence of added deoxycholate (2.5 mM), the RNA transcribed
Fig. 5. Homochromatography fingerprinting pattern of RNA transcribed from nucleolar chromatin extracted with 1 M NaCl, in the presence of purified RNA polymerase I.

had a fingerprint pattern which did not contain any of the random stripes observed previously (Fig. 7). The map showed the presence of pre-rRNA specific marker fragments and also several additional discrete spots (arrows, Fig. 7). These additional spots were also seen in transcripts of nucleoli extracted with 2% sarkosyl (16). A similar fingerprint to that shown in Figure 7 was obtained when the deoxycholate treated nucleoli were transcribed in the presence of rifampicin AF/013 (see also Table 2).

These changes in the transcriptional patterns were also clearly reflected in the base compositions of the RNAs transcribed. The transcripts from intact nucleoli had a high GC content (A+U/G+C, 0.5) and the transcripts from the pellet obtained after deoxycholate treatment was more DNA-like in base composition with a ratio of 0.89 (Table 2). On the other hand, addition of either deoxycholate or rifampicin AF/013 to the pellet
Table 1. Effect of deoxycholate treatment on the transcriptional activity of nucleoli and purified RNA polymerase I.

<table>
<thead>
<tr>
<th>Deoxycholate Concentration (mM)</th>
<th>3H-GTP (cpm) Incorporated Per μg DNA</th>
<th>Protein/DNA Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleoli</td>
<td>116</td>
<td>4.6</td>
</tr>
<tr>
<td>&quot;</td>
<td>186</td>
<td>3.8</td>
</tr>
<tr>
<td>&quot;</td>
<td>261</td>
<td>3.2</td>
</tr>
<tr>
<td>&quot;</td>
<td>380</td>
<td>1.8</td>
</tr>
<tr>
<td>&quot;</td>
<td>362</td>
<td>1.68</td>
</tr>
<tr>
<td>DOC Pellet</td>
<td>145</td>
<td></td>
</tr>
<tr>
<td>DNA + RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polymerase I</td>
<td>456</td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

aNucleoli, treated with different concentrations of deoxycholate were centrifuged through 30% sucrose as described in Methods and the pellets were assayed for transcriptional activity.

changed the base composition of the RNA transcribed to a high GC content (A+U/G+C, 0.5).

**DISCUSSION**

Homochromatography fingerprinting technique provides a direct analysis of the RNA sequences transcribed in vitro. One can separate approximately 100 marker oligonucleotides obtained after T1 ribonuclease digestion of the 45S preribosomal RNA labeled in vivo. These range up to 30 nucleotides in chain length and include fragments of 18S and 28S rRNA and the transcribed spacer region of pre-rRNA. The appearance of a similar number of spots displayed in the same characteristic fashion on the homochromatogram (Fig. 2) shows that the RNA transcribed in vitro closely resembles the RNA labeled in vivo (2). This technique differentiates specific and random transcripts inasmuch as the transcripts obtained from nucleolar chromatin in
Fig. 6. Homochromatography fingerprinting of RNA transcribed from the DNP pellet obtained after treatment of nucleoli with sodium deoxycholate.

Table 2. Base compositions\(^a\) of RNA transcribed from nucleoli treated with deoxycholate.

<table>
<thead>
<tr>
<th>System</th>
<th>A</th>
<th>U</th>
<th>G</th>
<th>C</th>
<th>A+U/G+C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleoli</td>
<td>13%</td>
<td>20%</td>
<td>35%</td>
<td>32%</td>
<td>0.49</td>
</tr>
<tr>
<td>DOC Pellet</td>
<td>23%</td>
<td>24%</td>
<td>27%</td>
<td>26%</td>
<td>0.89</td>
</tr>
<tr>
<td>DOC Pellet + DOC (2.5 mM)</td>
<td>15%</td>
<td>16%</td>
<td>37%</td>
<td>32%</td>
<td>0.45</td>
</tr>
<tr>
<td>DOC Pellet + Rifampicin AF/013</td>
<td>14%</td>
<td>20%</td>
<td>34%</td>
<td>32%</td>
<td>0.52</td>
</tr>
</tbody>
</table>

\(^a\)Base composition of the RNA transcribed in the presence of [\(\alpha^{32p}\)]-labeled nucleoside triphosphates was determined after alkaline hydrolysis and separation of the mononucleotides by electrophoresis on Whatman 3MM paper at pH 3.45.
Fig. 7. Homochromatography fingerprinting of RNA from the DNP pellet obtained after treatment of nucleoli with sodium deoxycholate, transcribed in the presence of added deoxycholate (2.5 mM).

...vitro by E. coli RNA polymerase have a totally random pattern (2).

The data presented in this study indicate that the specificity of transcription of pre-rRNA by the nucleolar chromatin is retained, although at a reduced level, after 0.6 M NaCl extraction. The specificity is lost after extraction with 1.0 M or higher NaCl concentration. The loss of specificity is coincident with the increase in transcription by exogenous RNA polymerase I. The inability of exogenous RNA polymerase I to transcribe nucleolar chromatin is in agreement with the results of Butterworth et al (17). Howk et al (18) have shown that pretreatment of chromatin with N-ethyl maleimide to inactivate endogenous RNA polymerase makes it transcribable by exogenous RNA polymerase I. However, in our system, either treatment with N-ethylmaleimide or with 1 M urea inactivated the endogenous
activity but exogenous RNA polymerase I was not able to transcribe the resulting chromatin. Recently, Tekamp et al (5) have shown that exogenous RNA polymerase I can transcribe yeast chromatin in which the endogenous activity has been inactivated by prior treatment at acidic pH (<4.5). About 10-13% of the transcripts was ribosomal RNA as determined by competition hybridization, suggesting that there was an enrichment for rRNA transcription. Our studies indicate that homologous RNA polymerase I transcribes yeast chromatin only after 1.0 M NaCl extraction, but the transcripts are random. This indicates that besides a possible inherent specificity of RNA polymerase I, other factors, which are disrupted by the 1 M NaCl treatment, are probably involved in restricting random initiations.

The deoxycholate experiments further substantiate this possibility. In these studies, the endogenous RNA polymerase activity has been shown for the first time to be able to transcribe randomly besides transcribing the genes that the enzymes are bound to. That the random transcription is due to new initiations by the endogenous RNA polymerase is indicated by the fact that the randomness can be specifically suppressed by either rifampicin AF/013 or deoxycholate, both of which inhibit free RNA polymerase activity. This result indicates that RNA polymerase I by itself does not selectively transcribe only rDNA in the absence of specifying mechanisms which are disrupted either by 1.0 M NaCl extraction or by deoxycholate but which are present in the intact nucleolus. Thus, it appears that the specific transcription of pre-rRNA observed in this system is due to elongation by the endogenous RNA polymerase I and reinitiation in vitro, which occurs only after 1.0 M NaCl extraction or deoxycholate treatment, results in random transcription.

Extraction of chromatin with 1.0 M or higher NaCl concentrations removes most of the histones and some tightly bound nonhistone proteins (9). Similarly, deoxycholate also extracts histones (19) and nonhistone proteins23. Thus, extraction of histones and a disruption of the ordered subunit structure of chromatin appears to be coincident with the ability of exogenous RNA polymerase I to randomly transcribe the nucleolar chromatin. Although the existence of normal nucleosomal arrangements of hist-
tones in the active rDNA chromatin is controversial (20,21), it is an interesting possibility that the existence of such an arrangement in the non-rDNA portion of the nucleolar chromatin might restrict random transcription. It is also possible that some of the nonhistone proteins extracted by deoxycholate might be involved in the inhibition of random transcription. Reconstitution experiments under physiological conditions such that the RNA polymerase activity is not inhibited, using the nucleosome forming activity described by Laskey et al (22), may distinguish between these two possibilities.

ACKNOWLEDGEMENTS

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REFERENCES

23. SDS-polyacrylamide gel analysis of the proteins extracted by deoxycholate indicated the presence of bands corresponding to histones and nonhistone proteins. These bands were also present in the pellet fraction suggesting a nonselective extraction by this treatment.
24. PMSF—Phenyl methyl sulfonyl fluoride.