Specific Gene Transcription in Yeast Nuclei and Chromatin by Added Homologous RNA Polymerases I and III*

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When treated at pH < 4.5, yeast nuclei or chromatin lose endogenous RNA synthetic activity. This activity is regained by addition of exogenous RNA polymerases. The specificity of transcription in this system by homologous RNA polymerases I and III has been investigated by gel electrophoresis, hybridization analysis, and RNase T1 mapping. Exogenous RNA polymerase I selectively transcribes rRNA genes. The transcription of these genes by polymerase I is 30- and 8-fold more selective than RNA polymerase III and Escherichia coli polymerase holoenzyme, respectively. Exogenous RNA polymerase III synthesized RNAs similar in size to authentic 5 S RNA, 4.5 S pre-tRNA, and 4 S tRNA. Eleven per cent of this RNA is 5 S RNA as determined by hybridization. Neither polymerase I nor E. coli polymerase synthesizes detectable quantities of RNA in this size range. A T1 ribonuclease digestion of 5 S RNA synthesized by exogenous RNA polymerase III acting on acid-treated chromatin gives a fragment pattern corresponding to that of 5 S RNA. Thus, RNA polymerase III transcribes the entire 5 S gene in this system.

For an understanding of transcriptional control mechanisms in eukaryotes, it is necessary to reconstruct, from appropriate molecular components, in vitro systems that maintain in vivo specificity. Since transcripive selectivity is mediated by three structurally and functionally distinct RNA polymerases (1–3), presumably three independent systems are involved. RNA polymerases I and III have relatively restricted transcriptive functions (large rRNA precursor and 5 S RNA plus tRNA, respectively (1–3)), whereas polymerase II has a more diversified transcriptive function (presumably synthesizes all heterogeneous nuclear RNA (1, 2)).

Analysis of initiation and termination of transcription requires knowledge of the sequence of both the template and the primary transcript. The advent of molecular cloning and rapid DNA and RNA sequencing techniques (4–7) will provide this information for many systems, but as yet it is accessible for only small RNAs like 5 S RNA (8), tRNAs (9, 10), and adenovirus 5.5 S RNA.†

There have been two general approaches to the development of modified transcription systems. One involves the use of nuclei (or nucleoli) or chromatin as template with either endogenous or exogenous RNA polymerases. The rationale for this approach is that these complex systems are likely to retain the necessary components for specific transcription and are therefore a suitable starting material for fractionation. Endogenous polymerase activities in isolated nuclei and chromatin have been shown to transcribe 5 S RNA, tRNA, and certain adenovirus genes (11–16) as well as large rRNA genes (17, 18) with high degree of fidelity. Successful reconstitution of transcriptive specificity has also been reported using chromatin and Escherichia coli RNA polymerase for the globin and ovalbumin genes (19–22). However, the conclusions of these experiments are somewhat tentative due to the complexity of the systems themselves (hence the difficulty of measuring specificity), as well as possible enzymatic artifacts (23–25).

More recent studies on the transcription of the large rRNA, 5 S RNA, and 5.5 S adenovirus RNA genes in nuclei or chromatin by purified homologous RNA polymerases (17, 18, 26–28) do not suffer from these drawbacks. The products are produced in relatively large quantitites and their size and structure are identical or very similar to the natural ones.

A second approach has been the utilization of purified components, namely DNA and RNA polymerases, with the immediate aim of providing a basal level of specificity and an assay for accessory molecules required for reconstitution of specificity. This approach has also experienced some degree of success. We have reported that when yeast DNA is used as a template, purified polymerase I preparations transcribe ribosomal sequences with some degree of selectivity as compared to other sequences transcribed by polymerase II (29). Planta and his colleagues have observed even more impressive selectivity with their polymerase I preparations (30). Unfortunately, the sequence at the site of initiation of transcription of the ribosomal genes is unknown, so that assessment of correct initiation is not feasible in this system. Furthermore, in our hands the experiments have been difficult to reproduce consistently with more characterized templates. Purified polymerase III from higher eukaryotes exhibits no transcriptive specificity on purified DNA templates (26, 31).

We were presently engaged in an analysis of transcription in yeast. For these studies, the yeast ribosomal repeat containing the 5 S RNA and large rRNA genes has been cloned in bacterial plasmids (32). The 5 S RNA gene and its flanking regions have been sequenced and we also have sequence information for the large ribosomal genes especially in the putative initiation and termination regions of the precursor (33, 34). It is now feasible to carry out meaningful transcription experiments with these genes. We describe here the development of a nuclear and chromatin system dependent on exogenous polymerases I and III for the selective transcription of the genes for large rRNAs and 5 S RNA plus tRNA, respectively. Our results indicate that, at least in the case of the 5 S RNA, the gene is accurately transcribed by polymerase III in

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† S. M. Weissmann, personal communication.
vitro. This enzyme apparently initiates and terminates correctly in this system.

EXPERIMENTAL PROCEDURES

Preparation of Yeast RNA—Ribosomal RNA was prepared from Saccharomyces cerevisiae A364A cells as described by Rubin (35). 5 S rRNA was further purified by chromatography on Sephadex G-100 as described by Monier (36). Tritium-labeled rRNA was prepared from A364A cells grown on a minimal medium (37) to which [5,6-3H]Hurdine and [8-3H]adenine (46 and 25 Ci/mmol, respectively, Amersham/Searle) were each added to a final concentration of 50 µCi/ml. 3P-Labeled 6 S, 4.5 S pre-tRNA, and 4 S tRNA markers were prepared from A364A cells grown to midlog on low phosphate YEPD (35) + 300 µCi/ml of carrier free 32P (New England Nuclear). Isolated yeast RNA-dependent RNA Polymerases—Yeast RNA polymerases were isolated from the commercial strain, F1, as described previously (38, 39).

Preparation of Yeast Nuclei and Chromatin—S. cerevisiae 1278b cells (40) were grown at 30°C in YEPD medium (35), harvested in late log by centrifugation, and washed twice with cold distilled water. Spheroplasts and nuclei were isolated by a modification of the method of May as described by Duffus (41). The pellet from 50 g of cells was suspended in 306 ml of phosphate/citrate buffer, (0.037 mM citric acid, 0.126 mM NaHPO₄, 0.6 mM AmSO₄, 0.02 M 2-mercaptoethanol, and 0.6 M trichloroacetic acid by the method of Lowry et al. (43). Nuclei were resuspended in 20 volumes of 1 M NaCl. The ratio for chromatin was 12 to 24. These values are comparable to those reported previously for yeast nuclei (41, 45).

Effect of Acid pH on the Activity of Yeast RNA Polymerases—Polymerases I, II, and III were incubated 5 min at 0°C in buffers of different pH (as indicated in Fig. 1) containing 25% glycerol, 1 mM EDTA, 0.1 M KCl, and 0.02 M 2-mercaptoethanol. Cells were lysed by equilibrating this solution in a Parr bomb at 850 psi for 30 min at 4°C followed by release of the pressure. This modification in the nuclei preparation procedure was developed in this laboratory by Dr. Frank Masiarz. After centrifuging at 7000 rpm for 10 min in Sorvall SS34 rotor, the supernatant containing the nuclei was resuspended at 25,000 × g for 45 min. The nuclear pellet was carefully resuspended in 0.05 M Tris-HCl (pH 8.0), 25% glycerol, 1 mM EDTA, 0.02 M 2-mercaptoethanol, and 1 mM PMSF and centrifuged in conical graduated tubes at 2000 rpm for 20 min in a Sorvall GLC centrifuge. The pellet was resuspended in four volumes of the above buffer, aliquoted, and stored at −70°C until used.

Chromatin was prepared from nuclei by a modification of the method of Reeder (42). Nuclei were resuspended in 20 volumes of 1 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 12.5% glycerol, 0.02 M 2-mercaptoethanol, and 1 mM PMSF. After shaking in ice for 15 min, the chromatin was pelleted by centrifugation for 10 min at 15,000 × g and gently resuspended by homogenization in the same buffer.

DNA concentration was measured by the method of Burton (43) using calf thymus DNA as a standard. Protein was determined after precipitation with 10% trichloroacetic acid by the method of Lowry et al. (43) using bovine serum albumin (Sigma) as the standard. Nuclei prepared as described above had protein/DNA ratios between 20 to 40; the ratio for chromatin was 12 to 24. These values are comparable to those reported previously for yeast nuclei (41, 45).

Acid Treatment of DNA-dependent RNA Polymerases, Nuclei, and Chromatin—Two volumes of 0.05 M formic acid (pH 2.5), 25% glycerol, 0.1 mM EDTA, 0.1 M KCl, 1.0 mM PMSF, were added to 1 volume of nuclei or chromatin on ice, lowering the pH to 3.3 and 2.7, respectively. After 10 min at 0°C, 1 volume of 2 M Tris-HCl (pH 9.4), 25% glycerol, 0.1 mM EDTA, 0.15 M KCl, 1.0 mM PMSF was added, raising the pH to 8.25.

In Vitro Synthesis of RNA in Isolated Nuclei and Chromatin—The standard reaction in a final volume of 0.5 ml contained 100 µCi of [32P]UTP (100 to 200 Ci/mmol; Amersham/Searle); and 1 to 2 µg of DNA, either as nuclei or chromatin. When indicated, RNA polymerases were added to nuclei or chromatin and incubated for 5 min at 0°C followed by addition of a mixture containing nucleoside triphosphates. The reaction was incubated at 25°C for 10 min, and the mixture was added to 10 µl of 5% SDS, 0.1 M EDTA (pH 7.4). Synthesis of RNA was measured by spotting an aliquot of the reaction on Whatman DE81 filter discs (38). The RNA products of in vitro synthesis were isolated by hot phenol extraction (40) and ethanol precipitation. In some cases, when only the products of RNA polymerase III transcription were being analyzed, the procedure was modified since greater than 95% of the transcription products of RNA polymerase III are released from the nuclei and are present in the supernant of the reaction. In this instance, the SDS solution was omitted and the reaction mixture was centrifuged for 5 min at 2000 × g to pellet nuclei or chromatin. The supernatant was phenol-extracted and precipitated with ethanol. The RNA was collected by ethanol precipitation and dissolved in sterile distilled water.

Elution of RNA from Gel—The 3P-labeled spots corresponding to a given RNA were excised from the gel and eluted by grinding the gel pieces followed by washing with 0.02 M 2-mercaptoethanol in 0.1 M Tris/borate/EDTA buffer (pH 8.3) (48) containing 10% glycerol, 0.1% SDS, and 0.1% bromophenol blue and heated 3 min at 65°C before electrophoresis. One-dimensional analysis of low molecular weight RNA was performed on vertical slab gels (30 cm × 14 cm × 3 mm) containing 2% acrylamide and 0.5% agarose (47). Polyacrylamide gel electrophoresis was carried out by a modification of the procedure of Fradin et al. (49). The first dimension was a 10% acrylamide separating gel 30 cm high containing 4 M urea in Tris/borate/EDTA buffer (pH 8.3) (48) containing 10% glycerol, 0.1% SDS, and 0.1% bromophenol blue and heated 3 min at 65°C before electrophoresis. Two-dimensional electrophoresis was carried out by a modification of the procedure of Fradin et al. (49). The first dimension was a 10% acrylamide separating gel 30 cm high containing 4 M urea in Tris/borate/EDTA buffer (pH 8.3) (48) and subjected to electrophoresis for 3 to 4 days at 300 V. In the second dimension, a strip of gel containing RNAs from 4 S to 6 S was embedded in a 20% acrylamide gel containing 4 M urea and Tris/borate, pH 8.3 (48) and subjected to electrophoresis for 3 to 4 days at 300 V.

Hybridization Analysis—Plasmid pBD4 containing the 9.3-kb yeast Xma I rDNA repeat fragment was diluted with Xma I digested yeast chromatin DNA to a ratio of 9:1. Portions of the rDNA repeat containing the 18 S rRNA was prepared by Eco RI digestion of plasmid pBD14 (32). The 2.5-kb fragment of the rDNA containing the 5 S DNA was prepared by Eco RI digestion of plasmid pBD25 (32). Both Eco RI yeast fragments were separated from the plasmid vector by preparative sucrose gradient centrifugation (34). DNA was immobilized on 47-mm diameter nitrocellulose filters (39) from 6.5-mm diameter filters was performed as indicated in various experiments. Each filter contained either approximately 9 µg of plasmid DNA of which 4.5 µg was ribosomal DNA, 7 µg of 18 S rDNA, or 9 µg (Batch I) or 5.5 µg (Batch II) of 5 S DNA, respectively. DNA-containing filters as well as blank filters were prehybridized for 16 h at 50°C in 200 µl of 5 × SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate, 0.1% formamide) for approximate 10 h. After prehybridization, filters were washed twice for 10 min at 50°C in 5 × SSC, 50% formamide, then washed twice at room temperature in 2 × SSC. Hybridization was performed under the same conditions as described above, between 20,000 and 60,000 cpm of in vitro-synthesized 32P-labeled RNA was included per reaction as well as an appropriate 14P-labeled RNA internal standard. All reactions were performed in duplicate. Filters were washed as described above and then incubated for 1 h at room temperature in 2 × SSC + 20 µg/ml RNase A. Filters were then washed again twice in 2 × SSC, dried, and counted in toluene/Omnifluor.

RESULTS

Acid Inactivation of RNA Polymerases—Confirming and extending a previous observation (51), we have found that all three yeast RNA polymerases, as well as the rat liver enzymes3.

3J. Martial and P. Valenzuela, unpublished results.
RNA synthesis in acid-treated nuclei was not affected by the higher concentration of formic acid described under "Experimental Procedures." All reactions were carried out at pH 8, and assays at pH 8 as indicated under "Experimental Procedures." One hundred percent of activity corresponds to 15,000 cpm or 225 pmol of UMP incorporated into RNA in 10 min. Buffers are formate from pH 3 to 4, acetate from pH 4 to 5.5, Tris/maleate from pH 5.5 to 7.5, Tris-HCl from pH 7.5 to 8.5. ○, polymerase I; ●, polymerase II; △, polymerase III.

**Table I**

<table>
<thead>
<tr>
<th>Nuclei acid treatment</th>
<th>RNA polymerase added</th>
<th>Counts per min</th>
<th>Picomoles of UTP incorporated/mg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>None</td>
<td>301,840</td>
<td>16.5</td>
</tr>
<tr>
<td>+</td>
<td>None</td>
<td>3,978</td>
<td>0.18</td>
</tr>
<tr>
<td>+</td>
<td>RNAP I</td>
<td>88,018</td>
<td>4.8</td>
</tr>
<tr>
<td>+</td>
<td>RNAP III</td>
<td>84,462</td>
<td>4.6</td>
</tr>
</tbody>
</table>

are irreversibly inactivated by incubation at acid pH. Fig. 1 shows the activity remaining after incubation of the enzymes with buffers of acid pH for 5 min at 4°C. A sharp decrease in the stability of the enzymes occurs below pH 4.5. The rate of inactivation is dependent on the acidity of the medium; at pH 4, the half-life of enzyme activity is approximately 30 s.

Sucrose gradient centrifugation and SDS gel electrophoresis of the resulting fractions indicate that the acid treatment produces a drastic change in the quaternary structure of yeast RNA polymerase I. The 48,000, 44,000, 36,000, and 24,000 peptides dissociate from the enzyme and the remaining complex self-assembles and sediments as a dimer. A similar phenomenon may occur during acid inactivation of polymerases II and III.

**Acid Treatment of Isolated Nuclei**—The irreversible inactivation of purified yeast RNA polymerases suggested that acid treatment might selectively inactivate the endogenous RNA polymerases in isolated nuclei. As shown in Table I, a brief acid treatment of nuclei reduced endogenous RNA polymerase activities to about 1% of the control level. In other experiments, acid treatment of nuclei resulted in residual RNA polymerase activities 0.5 to 6% of that seen in untreated nuclei. The effect of acid treatment on the structural integrity of the nucleoprotein was checked by staphylococcal nuclease digestion. After nuclease digestion, the expected ladder pattern was seen in both control and acid-treated nuclei; however, the rate of digestion of acid-treated nuclei was significantly faster than that of control nuclei (data not shown), suggesting some alteration in the structure/composition. However, there were no differences in the histone complement of acid-treated and control nuclei as analyzed by acid-urea gel electrophoresis.

**Transcription in Acid-treated Nuclei by Exogenous RNA Polymerases**—Addition of purified homologous RNA polymerases I or III to acid-treated nuclei stimulated total RNA synthesis about 25-fold (Table I). In other experiments stimulations as high as 50-fold have been observed. Addition of either polymerase to untreated nuclei resulted in at most a slight increase (<10%) in RNA synthesis.

The kinetics of RNA synthesis in isolated nuclei, acid-treated nuclei, and acid-treated nuclei to which exogenous RNA polymerase I, II, or III was added were examined (Fig. 2). In all cases, RNA synthesis by endogenous RNA polymerase was linear for at least 15 min of incubation at 25°C. RNA synthesis by exogenous RNA polymerase I or II was linear for at least 60 min. In some cases the reaction with added RNA polymerase I was linear up to 3 h (data not shown).

**Gel Electrophoretic Analysis of RNA Products**—The products of endogenous and exogenous RNA polymerase I were examined by gel electrophoresis as shown in Fig. 3. The RNA transcripts synthesized by endogenous enzyme are of heterogeneous size; however, some bands of discrete size are distinguishable. In particular, a faint band of 2.5 × 10⁶ daltons can be seen in the analysis of short reactions (Fig. 3A). This RNA has the same size as that reported for the ribosomal precursor (37). In longer synthesis reactions, it was replaced by a band of 2.2 × 10⁶ daltons (Fig. 3B). The molecular weight of this RNA does not correlate with any of the reported intermediates in RNA biogenesis. It may represent an as-yet undetected intermediate, a degradation product of the 35 S RNA, or another nonrelated RNA species.

As expected, acid treatment of nuclei greatly reduced endogenous RNA synthesis. Addition of exogenous RNA polymerase I to acid-treated nuclei resulted in the synthesis of a heterogeneous array of products (Fig. 3B), no products of discrete size were observed in the gel electrophoresis analysis. As can be seen in Fig. 3, A and B, an increase in the incubation time resulted in a substantial decrease in the size of the RNA products of both the endogenous and exogenous reactions. Thus the absence of specific bands may be the result of degradation.

The synthesis of low molecular weight RNAs by endogenous and exogenous polymerases was also examined (Fig. 4). Untreated nuclei synthesized RNAs which migrated similarly to a band of 2.2 × 10⁶ daltons. In all cases, RNA synthesis by endogenous RNA polymerase I was linear for at least 15 min of incubation at 25°C. RNA synthesis by exogenous RNA polymerase I or II was linear for at least 60 min. In some cases the reaction with added RNA polymerase I was linear up to 3 h (data not shown).

**Fig. 2.** Kinetics of RNA synthesis in acid-treated nuclei by exogenous RNA polymerases I, II, and III. RNA synthesis was assayed as described under "Experimental Procedures," except 5 μCi of [3H]UTP was used as label and unlabeled UTP was increased to 0.1 mM. RNA polymerase II was assayed in 1.6 mM MnCl₂ instead of MgCl₂. Assays were conducted at 37.5 mM NaCl except the assay with added RNA polymerase III which was carried out at 125 mM NaCl. ○, endogenous activity; ⌐, endogenous activity after acid inactivation; activity of acid-treated nuclei + exogenous RNA polymerase I (▲—▲) + exogenous RNA polymerase II (□—□) + exogenous RNA polymerase III (○—○).
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FIG. 3. Gel electrophoretic analysis of RNA synthesized as a function of time in acid-treated and in control nuclei with exogenous RNA polymerase I. RNA was synthesized under standard conditions at 37.5 mM NaCl. Electrophoresis was in 2.5% acrylamide, 0.5% agarose gels. A, RNA synthesized by endogenous nuclear RNA polymerase activity. A film from a longer exposure of the gel has been inserted to show the discrete bands marked by a and b. B, RNA synthesized in acid-treated nuclei by added yeast RNA polymerase I. 32P-labeled 25 S and 18 S rRNAs were included in each gel as markers.

FIG. 4. Gel electrophoretic analysis of low molecular weight RNA synthesized in acid-treated nuclei by exogenous RNA polymerases. Lane a, 32P-labeled yeast marker RNAs; Lane b, acid-treated nuclei + yeast RNA polymerase I; Lane c, endogenous RNA polymerase activity of acid-treated nuclei; Lane d, Endogenous RNA polymerase activity in yeast nuclei; Lane e, acid-treated nuclei + yeast RNA polymerase III; Lane f, acid-treated nuclei + E. coli RNA polymerase holoenzyme (Miles Laboratories).

to authentic 5 S tRNA, 4.5 S tRNA precursors, and mature 4 S tRNAs (Fig. 4, a and d). The small differences sometimes observed in the migration of 5 S and tRNAs as in Fig. 4, a and d, probably are due to differences in the salt concentration between the two samples. Dissimilarities in the migration patterns of pre-tRNAs and tRNAs may reflect quantitative differences in the composition of these two RNA classes under the different conditions of synthesis. Acid treatment of nuclei almost completely abolished RNA synthesis of these size classes (Fig. 4c). Addition of homologous RNA polymerase I or E. coli RNA polymerase greatly stimulated RNA synthesis (Fig. 4, b and f, respectively) but no discrete transcripts in this size range were detectable in the RNA synthesized by either enzyme. In both instances the majority of the products were >8 S in size and remained at the interface of the stacking and running gel. In contrast, addition of yeast RNA polymerase III to acid-treated nuclei resulted in the synthesis of 5 S, 4.5 S, and 4 S RNAs similar to the products of endogenous RNA polymerase III transcription (Fig. 4e).

The nature of the products in the 4 to 5 S size range synthesized by endogenous RNA polymerase and by exogenous RNA polymerase III in isolated nuclei was further investigated by two-dimensional gel electrophoresis. The array of spots observed is qualitatively similar in both instances (Fig. 5), indicating that endogenous and exogenous RNA polymerase III are recognizing the same sequences. However, there are obvious differences between endogenous and exogenous polymerase III transcripts which may be a result of acid treatment. Certain of the spots were further identified. The 5 S RNA synthesized by the exogenous RNA polymerase III was eluted and shown to selectively hybridize to the 2.5-kb Eco RI fragment containing the 5 S gene. For example, from 17,500 and 14,380 cpm (endogenous and exogenous transcription, respectively), 1980 and 1650 cpm, respectively, hybridized to filters containing 5 S DNA. All counts were competed by cold yeast 5 S rRNA. No counts hybridized to filters containing 18 S and 25 S rRNA genes. We have also shown specific hybridization of particular spots from the 4.5 S region to plasmids containing tRNAAsp (52) and tRNAVal (53) genes (data not shown). The nature of these 4.5 S pre-tRNA has been recently described (64, 55).

Salt Dependence of RNA Polymerase III Transcription in Nuclei—The salt dependence of transcription by endogenous and exogenous RNA polymerases in nuclei and in acid-treated nuclei, respectively, was compared to the salt dependence of polymerase III transcription of calf thymus DNA. As can be seen in Fig. 6, transcription by RNA polymerase III on calf thymus DNA displayed the characteristic double optima previously reported for this enzyme (3). In contrast, only a single salt optimum was seen for endogenous synthesis or for exogenous RNA polymerase III synthesis. Thus, the second salt optimum seen with polymerase III on calf thymus DNA may represent a nonphysiological reaction.

The effect of monovalent salt concentrations on RNA synthesis by endogenous or exogenous RNA polymerase III was analyzed by polyacrylamide gel electrophoresis. As shown in

FIG. 5. Two-dimensional gel electrophoresis of low molecular weight RNA synthesized from yeast nuclei by endogenous RNA polymerases and from acid-treated nuclei by exogenous RNA polymerase III. RNA was synthesized and separated by two-dimensional gel electrophoresis as indicated under "Experimental Procedures." a, exogenous RNA polymerase III; b, endogenous RNA polymerases.
Subsequent experiments showed that nuclei washed with 0.5 M NaCl at concentrations above 0.25 M, Fig. 7, the synthesis of 4 to 5 S products declines precipitously. Activity appears to be sensitive to salt. The RNA products of endogenous RNA polymerase III is detectable at 0.35 M NaCl but not at 0.45 M NaCl. Subsequent experiments showed that nuclei washed with 0.5 M NaCl still retained the capacity to synthesize 5 S RNA and 4 S RNAs with exogenous RNA polymerase at lower salt concentration. Thus enzyme activity rather than template activity appears to be sensitive to salt. The RNA products of endogenous and exogenous RNA polymerase III varied with salt concentration (Fig. 7). At the lowest salt concentration examined, both endogenous and exogenous RNA polymerase III synthesized mainly 4.5 S tRNA precursors and very little mature 4 S RNA was seen. At higher salt concentrations this situation was reversed. Thus the synthesis of 4 S products seems dependent on the action of salt-dependent enzymes (54, 55).

The Nature of the Transcription Products Synthesized by

Exogenous RNA Polymerase I and III in Acid-treated Nuclei—The transcription products of exogenous RNA polymerases were further identified by hybridization to appropriate homologous rDNA (Table II). Fifteen percent of the RNA polymerase III transcripts hybridized to filters containing the 2.5-kb Eco RI fragment containing the 5 S gene. Seventy-five percent of this RNA was competed by added 5 S rRNA. The remaining 25% of the hybridized material has not been identified; it may represent transcription from the other strand or from spacer regions. In contrast, there was no significant hybridization of RNA polymerase III transcripts to the 2.0-kb Eco RI fragment containing 18 S rDNA.

The data obtained from the analysis of the products of RNA polymerase I are more complex. Nineteen percent of the in vitro transcripts hybridized to the Eco RI fragment containing the 5 S gene but none was competed by 5 S rRNA. Thus polymerase I seems to transcribe the opposite strand of the 5 S gene, or the adjacent spacer region, or both.

Twenty-five to thirty-three percent of RNA polymerase I transcripts hybridized to plasmid pBD4 DNA containing the 9.3-kb Xmn I complete ribosomal repeat fragment. This represents only 30 to 75% more hybridization than with the 2.5-kb Eco RI 5 S rDNA containing fragment alone. About 40%

<table>
<thead>
<tr>
<th>Added RNA polymerase</th>
<th>32P input</th>
<th>DNA filters</th>
<th>Competitor RNA</th>
<th>-Competitor +Competitor</th>
<th>Efficiency of hybridization</th>
<th>Input hybridized</th>
<th>Competitor specific</th>
</tr>
</thead>
<tbody>
<tr>
<td>III</td>
<td>23,680</td>
<td>5 S</td>
<td>5 S</td>
<td>172</td>
<td>42</td>
<td>4.6</td>
<td>15.8</td>
</tr>
<tr>
<td>I</td>
<td>49,726</td>
<td>5 S</td>
<td>5 S</td>
<td>867</td>
<td>200</td>
<td>21.6</td>
<td>15</td>
</tr>
<tr>
<td>E. coli</td>
<td>58,970</td>
<td>pBD4</td>
<td>18 S + 25 S</td>
<td>1407</td>
<td>453</td>
<td>54.5</td>
<td>4.4</td>
</tr>
</tbody>
</table>

* Calculated from the efficiency of hybridization of an internal 3H-labeled RNA standard. The 5 S [3H]RNA was used as the internal standard for 5 S DNA hybridizations. 18 S [3H]RNA was the internal standard used in 18 S rDNA hybridizations. Stable [3H]RNA (18 S, 25 S, 5.8 S, and 5 S rRNAs) was used as internal standard in hybridizations to the ribosomal repeat except when only 18 S rRNA was competitor, then only 18 S [3H]RNA was used as internal standard. The difference in hybridization to pBD4 DNA filters with RNA polymerase I transcripts may reflect differences in the hybridization efficiency of the different internal standards utilized.

* The 5 S DNA filters each contained 5.5 μg of the 2.5-kb rDNA Eco RI fragment containing the 5 S gene except for the second hybridization to 5 S DNA with RNA polymerase III transcripts; the filters used in this experiment each contained 9 μg of 5 S DNA. 18 S DNA is the 2.0-kb rDNA Eco RI fragment containing sequences which code for 18 S rRNA. pBD4 is a plasmid containing the 9.3-kb Xmn I repeat fragment.
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of the RNA hybridized to the ribosomal repeat was competed by 18 S rRNA and no additional competition was noted with 25 S rRNA. Similar results were obtained after 1 h transcription reaction except that significant RNA (6%) was competed by 25 S rRNA above that competed by 18 S rRNA (40%). The identity of the nonribosomal RNA sequences synthesized by exogenous RNA polymerase I was not investigated further.

The sequences of the RNA synthesized by E. coli RNA polymerase were also investigated (Table II). E. coli RNA polymerase transcripts showed insignificant hybridization to the Eco RI 5 S DNA-containing fragment and very low hybridization to plasmid DNA containing the Xma I ribosomal repeat fragment. In both cases, the amount of observed hybridization is consistent with random transcription of the yeast genome by E. coli RNA polymerase.

Selective Transcription of tRNA and 5 S RNA Genes in Acid-treated Chromatin by RNA Polymerase III—In order to test whether the degree of transcriptional selectivity shown by RNA polymerase III in acid-treated nuclei was maintained in chromatin, acid-treated chromatin was transcribed by exogenous RNA polymerases I and III and by E. coli holoenzyme. As shown in Fig. 8, RNA polymerase I and E. coli RNA polymerase synthesized only large molecular weight products while RNA polymerase III synthesized discrete 5 S, 4.5 S, and 4 S RNAs. The in vitro transcripts of exogenous RNA polymerase III transcription were further analyzed by two-dimensional gel electrophoresis (Fig. 9). Despite a higher background than that observed in nuclei transcription, the pattern typical for polymerase III can be seen (compare Figs. 9 and 5). The 5 S RNA synthesized in vitro from chromatin by

![Fig. 8. Gel electrophoretic analysis of RNA synthesized from yeast chromatin by endogenous and exogenous RNA polymerases. a, RNA products of endogenous RNA polymerase(s) in yeast chromatin; b, RNA products of endogenous RNA polymerase(s) after acid treatment of chromatin; c, RNA products synthesized from acid-treated chromatin upon addition of (c) E. coli RNA polymerase; d, yeast RNA polymerase I; e, yeast RNA polymerase III; and f, α-32P-labeled yeast marker RNAs. RNA appearing at the top of the gel, particularly in a, may be the result of incomplete phenol extraction.](http://www.jbc.org/)

![Fig. 9. Two-dimensional electrophoresis of RNA synthesized by RNA polymerase III on acid-treated yeast chromatin. RNA was synthesized and separated by two-dimensional gel electrophoresis as indicated under “Experimental Procedures.”](http://www.jbc.org/)

![Fig. 10. RNase T1 oligonucleotides from 5 S RNA synthesized by yeast RNA polymerase III with acid-treated chromatin as template. The spot corresponding to 5 S tRNA from Fig. 9 was excised from the gel and eluted as described under “Experimental Procedures.” The RNA was digested by RNase T1, and the resulting oligonucleotides were separated by the standard fingerprint method (56). From their position in the fingerprint, the following assignments were made: Spot 1, G; Spot 2, AG; Spot 3, CACCG; Spot 4, CAACU; Spot 5, UG; Spot 6, CUG; Spot 7, CCUG; Spot 8, YAG and UAG; Spot 9, ACG; Spot 10, AACUCUG; Spot 11, AUCAACUG; Spot 12, CCAUACUACCAG; Spot 13, UUCUCG; Spot 14, UUAG; Spot 15, UUG. The identity of the following oligonucleotides was confirmed by digestion with RNase A and subsequent electrophoresis at pH 3.5 on DE81 paper (56): Spot 4 gives the expected labeled products A, U and C; Spot 5 gives as expected labeled AU; Spot 12 gives as expected labeled AU, A, C, and G; Spot 13 gives labeled G and C; Spot 14 gives no radioactive products; Spots 13 and 14 should also give labeled U which was not detected in our experiment since it runs out of the paper.](http://www.jbc.org/)
Sixteen labeled spots were obtained (Fig. 10) as expected from filters containing 5 S rDNA. All of the hybridized counts were competed by cold yeast 5 S rRNA. No counts hybridized to filters containing 18 S and 25 S rRNA genes.

To test the fidelity of 5 S RNA transcription from chromatin by exogenous RNA polymerase III, the 5 S RNA spot was eluted from the gel (Fig. 9) and digested with RNase T1. Sixteen labeled spots were obtained (Fig. 10) as expected from the sequence of this RNA (57) when it is synthesized from [α-32P]UTP (Spot 8 is a doublet). The position of the spots in the T1 map as well as the composition of some spots determined by RNase A digestion correspond to those predicted from yeast 5 S RNA (57). This indicates the specific transcription of the entire gene in chromatin by exogenous yeast RNA polymerase III.

**Discussion**

The experimental analysis of the components required for specific transcription requires the simplification of the transcribing system. Isolated nuclei, and in some cases, chromatin are capable of specific synthesis of certain RNA species, via endogenous RNA polymerase activities. 5 S and 4.5 S pre-tRNA genes as well as certain adenosine genes are accurately transcribed in isolated nuclei and chromatin from HeLa and adenovirus-infected KB cells (11-16). There is also evidence for the synthesis of large rRNA sequences by isolated nuclei and nucleoli (17, 18). We report here a nuclear and chromatin system in which endogenous polymerase activity is destroyed and exogenous polymerase is required for specific transcription.

**Selective Inactivation of Endogenous Polymerases—**The inactivation of endogenous polymerase in nuclei and chromatin and the requirement for exogenous polymerase III for 5 S transcription has been previously reported by Jaehning and colleagues who used N-ethylmaleimide (28). Although obviously useful in their studies, this reagent has the disadvantage of interacting indiscriminately with all available —SH groups. Thus it may not be generally applicable for the inactivation of polymerases and may inactivate other protein systems as well. Our own initial experiments in yeast indicated N-ethylmaleimide was not a suitable reagent for inactivating endogenous polymerases. Acid treatment may be a relatively useful reagent for inactivating of proteins, especially large multisubunit molecules. Preliminary attempts to reconstitute polymerase activity from its subunits led to the observation of acid inactivation of these enzymes not only from yeast but from higher eukaryotes as well. This occurs at moderately low pH, <4.5, about the same range that is required for the dissociation of aldolase subunits, for example (58). In contrast to aldolase, however, the effect on polymerases seems irreversible. Sedimentation experiments and subsequent analysis of molecular composition indicate that some of the subunits are lost and suggest the formation of a stable dimer, or oligomer. Whatever the mechanism of polymerase inactivation, however, acid treatment renders nuclei and chromatin dependent on exogenous enzymes for significant levels of transcription. In all such inactivation experiments, the question whether exogenous enzyme acts independently of the subunits of the endogenous enzyme cannot be answered conclusively. The fact that the added polymerases I and III have subunits in common yet nevertheless function independently to transcribe their target genes supports the view that the template (and perhaps ancillary factors) interacts with the exogenous polymerase themselves.

**Specific Transcription by Polymerase I—**With exogenous RNA polymerase I, acid-treated nuclei synthesize RNA with linear kinetics for periods of at least 1 h. Twenty-five to thirty-five percent of the total RNA hybridizes with the ribosomal repeat DNA. Much of the hybridizable counts are competed by 18 S RNA. The remainder of the counts bound to the ribosomal repeat may be sequences of the transcribed spacer of the large ribosomal precursor. The transcripts not hybridized by the ribosomal repeat may represent specific transcription of other RNAs (59), or nonspecific transcription due to alterations of a certain fraction of the enzyme molecules or of the template.

Exogenous polymerase I maintains a high degree of transcriptional selectivity as compared to RNA polymerase III (no 18 S RNA transcribed as measured by competition with 18 S RNA) or E. coli RNA polymerase holoenzyme (3.2% competed by 18 S + 25 S rRNA versus 2.4% calculated for random transcription). The transcription of rDNA sequences by RNA polymerase I is at least 6 to 10 times greater than random (more if the selective transcription of the 5' end of the gene occurs). The products of exogenous RNA polymerase I transcription in nuclei are heterogeneous in size. The size decreases with incubation time, as if the transcripts were destroyed enzymatically. Whether this is nonspecific ribonuclease activity or the activity of a processing enzyme is not known.

The primary transcript of RNA polymerase I in vivo is ribosomal precursor RNA which is thought to have a molecular weight of at least 2.5 x 10^6 (37, 60). The 5.8 S, 18 S, and 25 S rRNAs are derived from this precursor by processing. A 2.6 x 10^6 dalton RNA was, in fact, detected at early times among the products of endogenous but not exogenous polymerase I. This may represent the completion of chains initiated in vivo. At later times this RNA disappeared and was replaced by an RNA of 2.2 x 10^6 daltons. The latter RNA may be an as yet unrecognized intermediate in the processing of rRNA (37, 60) or it may be a degradation product.

Considerations of the amount of DNA required to code for precursor rRNA, the known size of the yeast rDNA repeating unit and the known polarity of transcription of yeast 18 S and 25 S rRNA (32), suggests that the 2.5-kb Eco RI rDNA fragment containing the 5 S rRNA gene also includes the coding region for the 5' end of the rRNA precursor. Our finding that approximately 50 to 60% of the sequences hybridizing to the rDNA repeating unit are specific for the 2.5-kb Eco RI fragment is consistent with this hypothesis.

Hybridization analysis of exogenous RNA polymerase I products synthesized during the first 15 min of incubation indicates that the transcription continues into the 18 S rRNA coding region (competition with 18 S rRNA) but that no transcription occurs in the region containing 25 S rRNA sequences. (Little additional competition by 25 S plus 18 S rRNA over that observed with 18 S rRNA alone.) However, the transcripts formed in 60 min by polymerase I contained a small amount of 25 S rRNA. These results suggest that rDNA transcription by exogenous RNA polymerase begins in the 2.5-kb Eco RI fragment and terminates prematurely.

**Specific Transcription by Polymerase III—**The analysis of the transcription with polymerase III is much more straightforward than with polymerase I. As has been reported previously for other systems (11-13, 15, 16), isolated yeast nucleoli and chromatin synthesize RNAs that co-migrate with authentic 5 S RNA, 4.5 S pre-tRNA, and 4 S tRNA. The fidelity of synthesis of low molecular weight RNAs in nuclei and chromatin was further confirmed by selective hybridization to plasmids containing the 5 S ribosomal RNA, tRNA^15'5', and tRNA^16'5' genes.
Following acid treatment, there is no synthesis of low molecular weight RNA in nuclei and chromatin. Upon addition of yeast RNA polymerase III, RNA synthesis was stimulated at least 25-fold. Only 5 S RNA, 4.5 S RNA, and 4 S RNA were made by exogenous RNA polymerase III. In contrast, neither exogenous RNA polymerase I or E. coli polymerase produced RNAs smaller than 8 S. Transcriptional selectivity was maintained, as evidenced by the similarity in the two-dimensional patterns of products of exogenous RNA polymerase I or E. coli polymerase III. The results of this study suggest that 5 S RNA is a primary gene transcript. We have correctly initiated and terminated the synthesis of 5 S rRNA by T, ribonuclease digestion. The nucleotide sequence of T1 oligonucleotides is that predicted from the entire genome of yeast. We propose that exogenous RNA polymerase III displays a high degree of selectivity and that reconstitution of specificity is possible.

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