Specific Transcription of Homologous Class III Genes in Yeast-soluble Cell-free Extracts*

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Cell-free extracts prepared from whole yeast cells carry out selective and accurate transcription, in vitro, of purified yeast class III genes. Both 5 S rRNA and tRNA genes are specifically transcribed by DNA-dependent RNA polymerase III in these whole cell extracts. These extracts also appear to carry out nucleolytic processing of the in vitro transcribed transcripts. Optimal conditions for specific class III gene transcription in vitro are defined. Initial fractionation of the yeast extract has indicated that multiple chromatographically separable factors (fractions) are required, in addition to RNA polymerase III, for specific in vitro transcription of class III genes.

The utility of soluble in vitro transcription systems for the study of eucaryotic transcription has recently been amply documented (reviewed in Ref. 1). When in vitro transcription studies were coupled with in vitro "genetics," very surprising results were uncovered concerning the regulatory sequence elements of class III genes (transcripts in vivo by DNA-dependent RNA polymerase III). 5 S rRNA (2-4), tRNA (5-8), and adenovirus virus-associated RNA (9, 10) genes all appear to have internal control regions which serve to direct transcription initiation at the 5'-region of these genes. Fractionation of soluble Xenopus laevis ovary extracts has led to the identification and purification of a protein (M. = 37,000) (11) which specifically interacts with the short intragenic control region of the homologous Xenopus 5 S gene (11-14). This protein, termed TFIIIA in concert with Xenopus RNA polymerase III and (at least) two other transcription factors are both necessary and sufficient to reconstitute specific transcription of the Xenopus 5 S gene in vitro. It was our goal to conduct similar experiments with class III gene systems in the lower eucaryote, Saccharomyces cerevisiae, to exploit the unique biochemical and genetic advantages of this organism. In this report, we describe the preparation and properties of a totally soluble transcription system derived from S. cerevisiae. We also describe preliminary chromatographic fractionation studies of these extracts and present data which suggests that there are multiple distinct class III transcription factors required for tDNA and 5 S DNA transcription. Recently, very similar soluble transcription systems have been described.

EXPERIMENTAL PROCEDURES

Strains—Strain 20B-12 (a trp 1 pep 4-3), a proteinase- and ribonuclease-deficient strain (15) of S. cerevisiae, was used in all the studies to be described (kindly supplied by E. Jones).

Growth of Cells and Preparation of Cell-free Extracts—Cells were grown in YEPD media (1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose) to a density measured by absorbance at 650 nm of 5-10. Cells were harvested by centrifugation, washed twice with distilled H2O, and resuspended in solubilization buffer (200 mM Tris-Cl (pH 8.1), 10% v/v glycerol, 10 mM MgCl2, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride) to 2 ml of buffer/g of cells, wet weight. The cell slurry was either used directly for extract preparation or was stored at −80°C. The cell suspension (∼250 ml) was placed in the chamber of a Bead Beater cell disruptor (Biospec Products, Bartlesville, OK). To the chamber was added an approximately equal volume of 0.4-0.5-mm diameter acid-washed glass beads. Sufficient beads were added to exclude all air from the chamber. The unit was then assembled and placed in an ice jacket containing a CaCl2-ice slurry. Cells were lysed by homogenization for 4-5 min total time, in 30-s bursts with 2-3 min cooling time between bursts. The lysate was decanted from the glass beads, and phenylmethylsulfonyl fluoride and β-mercaptoethanol were each re-added to 1 and 10 mM final concentrations, respectively. The lysate was made 0.4 M in ammonium sulfate by the addition of 4 M ammonium sulfate (pH 7.9). The extract was allowed to sit on ice for 10-15 min before centrifugation at 100,000 × g for 60 min. The proteins in the supernatant from this centrifugation (typically a volume of 150-170 ml) were precipitated with ammonium sulfate (0.35 g/ml) and harvested by centrifugation. The precipitated proteins were dissolved in a minimal volume (15-20 ml) of Buffer C (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.9), 20% v/v glycerol, 0.2 mM EDTA, 10 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride) and layered 8-10 h against 100-200 volumes of Buffer C containing 100 mM NaCl. This concentrated extract (average yield ∼25 ml/100 g of cells, 20-30 mg of protein/ml) was divided into aliquots and stored at −80°C. Occasionally, residual nucleic acids were removed by passing the extracts through a DEAE-cellulose column at high salt (0.3 M ammonium sulfate) in Buffer C.

Recombinant Plasmid DNAs—Several recombinant plasmids were used in these studies. pSp44-A, containing a yeast tyrosine suppressor tRNA gene (16), and pFm16, containing a yeast serine suppressor tRNA gene (17), were from M. V. Olson (Washington University, St. Louis, MO). pSc90 containing the S. cerevisiae 5 S rRNA gene was from R. Kramer (Hoffmann-LaRoche). pAd35.11, containing the adenovirus 2 virus-associated RNA II gene on the adenovirus 2 HindIII B fragment, was from H. Shiu-Lok (Cold Spring Harbor, NY). pXlmet, containing a single K. laevis tRNA"tm" gene, was from A. Lassar (Washington University). Plasmids were all propagated in Escherichia coli HB101. Superoxidased plasmid DNA was prepared as described previously (18).

RNA Polymerase Purification—RNA polymerase III was purified through the ion exchange chromatography steps in the procedure of Valenzuela et al. (19).

Fractionation of Extracts—Extracts were absorbed to phosphocellulose (Whatman P-11) columns (10-15 mg of protein/ml of bed volume) equilibrated with Buffer C containing 100 mM NaCl. Bound proteins were step-eluted with Buffer C containing 100 and 1500 mM NaCl. Absorbance was monitored at 280 nm throughout the procedure. Fractions containing protein were pooled, and the samples were desalted into Buffer D (Buffer C + 10 mM MgCl2 + 100 mM NaCl). Following desalting, the phosphocellulose 600 mM NaCl step fraction,
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which contained both transcription factors and endogenous RNA polymerase III was applied to a DEAE-Sephadex A-25 (Pharmacia) column (1-2 mg of protein/ml of bed volume) previously equilibrated with Buffer D + 100 mM NaCl. Bound proteins were step-eluted with Buffer D containing 250 and 1000 mM NaCl. Protein concentration was monitored by absorbance, and appropriate fractions were pooled and desalted prior to assaying for transcription factors. In all the chromatographic steps, fractions equivalent to 10-15% of the column volume were collected. Samples were desalted either by dialysis (8-10 h) or gel filtration on columns of Sephadex G-25 (medium). Following the desalting step, appropriate column fractions were stored at -80°C in small aliquots.

Transcription Assays—Assays were conducted in 50-μl volumes, of which up to 60% of the assay volume was contributed by the extract or column fractionated material. The standard assay contained 12 mM Hepes (pH 7.9), 150 mM NaCl, 10 mM MgCl₂, 12% (v/v) glycerol, 6 mM β-mercaptoethanol, 600 μM each of ATP, CTP, and UTP, 25 μM [α-³²P]GTP (10 Ci/mmol), and 25 μg/ml of supercoiled plasmid DNA. When column fractions were assayed, the reactions were supplemented with 20-50 units (as defined in Ref. 18) of yeast RNA polymerase III. Reactions were incubated 30-60 min at 20°C. Transcription was terminated, and RNAs were purified, fractionated on native polyacrylamide gels, and detected by autoradiography as described (18, 21).

Fingerprint and Oligonucleotide Analysis—³²P-labeled RNAs were synthesized in vitro in scaled-up reactions (500 μl) containing the α-³²P-labeled NTP at 100-200 Ci/mmol. Labeled RNAs were resolved on denaturing polyacrylamide gels and localized by autoradiography. The RNAs were excised from the gel, digested with pancreatic RNAse A or RNAse T₁, and subjected to fingerprint analysis as described by Rubin (30). The RNA fingerprint patterns were compared with those of the corresponding in vivo RNA molecules.

RESULTS

On the assumption that a nuclear extract would be enriched for all the components required for directing selective transcription, we initially used isolated yeast nuclei to prepare a soluble in vitro transcription system. Purified nuclei (31) were lysed, and proteins were solubilized by sonication in the presence of 0.4 M ammonium sulfate. The lysate was subjected to high speed centrifugation and the proteins in the supernatant were concentrated by ammonium sulfate precipitation. The resultant extracts were very concentrated (20-30 mg of protein/ml) and contained significant amounts of RNA polymerase activity (60% of the total cellular RNA polymerase, cf. Valenzuela et al. (19)). These whole cell extracts proved to be much more active than the nuclear extracts in directing the synthesis of discrete-sized low molecular weight RNAs in response to purified S and tRNA genes. Additionally, total yields of extract were much greater. We therefore proceeded to characterize the in vitro synthesized RNAs in more detail. These whole cell extracts were used in all the studies to be described below.

Synthesis of Discrete RNAs by RNA Polymerase III in Vitro—The two homologous templates that we have used in these studies are the yeast S tRNA gene (in the plasmid pSc90) and the serine-ochre suppressor tRNA gene, tRNA⁴⁴⁴ (in the plasmid pPm16). Yeast 5 S tRNA is a very abundant and well characterized molecule. The nucleotide sequence of both the RNA (35, 36) and the gene coding for it (37, 38) have been determined. Mature S tRNA is 121 nucleotides long and contains a polyphosphate terminus at its 5'-end. The structure of the primary transcription product of the S tRNA gene is not entirely certain since molecules containing 3'-extensions of the 5' nucleotides have been reported in vivo (39-41) in yeast and mammalian in vitro systems (42, 43). However, no molecules larger than these have ever been observed in vivo or in vitro. Additionally in vitro mapping experiments (2, 3, 44) have defined the 5' tRNA gene quite precisely to a region ≤130 nucleotides in length.

The serine ochre suppressor tRNA⁴⁴⁴ is present in vivo in only trace amounts. The sequence of the mature tRNA has been determined, and including the -CCA terminus which is added post-transcriptionally, it is 86 bases long (cf. Ref. 17 and references therein). Putative precursor transcripts of this gene 102 and 120 nucleotides long have been detected in vivo (45). The primary transcript of this gene has not yet been characterized though. Approximately 200 base pairs of the plasmid pPm16, which contains tRNA⁴⁴⁴, has been sequenced. The sequence includes the tRNA coding region and 5'- and 3'-flanking sequences. The nucleotide sequence indicates that this tRNA gene does not contain an intervening sequence. Hence, transcription of this gene in vitro should result in a fairly simple transcript pattern, and this is one of the reasons that this gene was chosen for detailed studies. Fig. 1 shows an autoradiogram of a gel which displays the discrete low molecular weight RNAs synthesized in the yeast soluble whole cell extracts in response to the addition of purified DNA templates containing a variety of class III genes (lanes 1, 5, 10, 11, and 12). The synthesis of these RNAs is both DNA-dependent and actinomycin D-sensitive (Fig. 1, Lanes 4, 8, and 9). Consistent with transcription being mediated by DNA-dependent RNA polymerase III, RNA synthesis is not inhibited by low (50 μg/ml, Lanes 2 and 6) or high (1000 μg/ml, Lanes 3 and 7) concentrations of a-amanitin. These concentrations of a-amanitin inhibit yeast RNA polymerases II and I, respectively, but have no effect on enzyme III (46, 47). This resistance to a-amanitin has been observed for both soluble and endogenous nuclear/chromatin bound polymerase III (22, 31).

Three RNA species of approximately 95, 90, and 85 bases in length are observed when transcription is carried out in the presence of the serine tRNA gene template (pPm16) (Fig. 1, Lanes 1, 2, and 3). These RNA species appear to be derived from a common precursor which does not accumulate to any appreciable extent in the nuclear extracts in vitro transcription reaction (see below). In contrast to this gene, transcription of the pSup4 tyrosine tRNA gene, which contains an intervening sequence, leads to a much more complex

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2 The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BT fraction, breakthrough fraction.

3 M. S. Klekamp, unpublished observations.
from the chromatograms and further characterized by nearestneighbor separation methodologies. Oligonucleotides were eluted with either \([\alpha-32P]GTP, ATP, \) or \(UTP\) were resolved in denaturing polyacrylamide gels and purified with either RNase T1 or pancreatic RNase A. The resulting fingerprint analyses. Transcripts labeled "Experimental Procedures." Gel-purified RNAs were digested with the corresponding templates in nondenaturing gels) with the transcripts synthesized from the DNA templates: \(\lambda\) DNA (Lanes 1-4), 5 S DNA (Lanes 5-8) Xenopus tDNA\textsuperscript{ex} (Lane 10), tyrosine SUP-4 tDNA (Lane 11), and adenovirus 2 virus-associated RNA genes (Lane 12). Lane 9 contains the transcription products synthesized when no DNA was added to the extract. \(\alpha\)-Amanitin at a concentration of either 50 \(\mu\)g/ml (Lanes 2 and 5) or 1000 \(\mu\)g/ml (Lanes 3 and 7) was present in some transcription reactions. Actinomycin D (150 \(\mu\)g/ml) was present during transcription of the reactions displayed in Lanes 4 and 8. All DNAs were present at a final DNA concentration of 25 \(\mu\)g/ml. Despite the fact that all these plasmids are of different sizes, there was no more than a 2-3-fold difference in promoter concentration. The labeling nucleotide used was \([\alpha-32P]GTP\).

pattern of transcripts (Fig. 1, Lane 11; see also Refs. 48, 49). We observe the synthesis of only one major RNA species \(\sim 120\) bases in length (Fig. 1, Lane 5) when a yeast 5 S rRNA gene is used to program RNA synthesis by the whole cell extracts. This species is the major \(32P\)-RNA synthesized either at early (10 min) or late (30 min or longer) times of transcription (not shown). In addition, this RNA comigrates on a thin sequencing gel with \(\textit{in vitro}\) 5 S RNA.\textsuperscript{4} This result is somewhat in contrast to what is observed in other class III transcription systems (42, 43) where fairly large amounts of apparent precursor RNAs (3'-extended forms with 8-16 additional residues) accumulate. In general, this is the 5 S DNA transcription profile seen with crude extracts (but see below and Fig. 4). Also shown in Fig. 1 are the RNAs synthesized by yeast extracts when transcription is programmed with heterologous DNA templates: \(X.\ laevis\) methionyl tRNA gene (Lane 10) and adenovirus-2 virus-associated RNA genes (Lane 12). These class III genes direct the synthesis of multiple discrete RNA species which co-migrate (on both denaturing and these nondenaturing gels) with the transcripts synthesized from the corresponding templates in KB cell S-100 extracts (18) and data not shown.\textsuperscript{4}

**Fingerprint Analyses of \textit{in Vitro} Transcripts**—In order to confirm that the discrete-sized \textit{in vitro} synthesized RNAs result from accurate transcription, we characterized the transcripts of the tRNA\textsuperscript{ex} and 5 S rRNA genes in more detail by fingerprint analyses. Transcripts labeled \(\textit{in vitro}\) via synthesis with either \([\alpha-32P]GTP, \) ATP, or \(UTP\) were resolved in denaturing polyacrylamide gels and purified as described under "Experimental Procedures." Gel-purified RNAs were digested with either RNase T1 or pancreatic RNase A. The resulting oligonucleotides were fractionated by standard two-dimensional separation methodologies. Oligonucleotides were eluted from the chromatograms and further characterized by nearest-neighbor and double-digestion techniques.

\textsuperscript{4} M. S. Klekamp and P. A. Weil, unpublished observations.

Fig. 2A represents the autoradiogram of the gel fractionation of RNA synthesized \textit{in vitro} from the 5 S rRNA gene with \([\alpha-32P]GTP\) as the labeled nucleotide. The RNA species marked with an arrow was eluted from this preparative gel and analyzed as described above. The T1 oligonucleotide pattern is shown in Fig. 2B. For comparison, the T1 oligonucleotide pattern of \textit{in vivo} \(32P\)-labeled 5 S RNA is also shown (Fig. 2C). The arrows point to the only major difference between the fingerprint patterns of the \textit{in vivo} and \textit{in vitro} synthesized RNAs, spot 6 (Fig. 2, compare B and C). This oligonucleotide represents the 3'-end of the mature 5 S RNA and has the sequence CppApApUpCpU80 (35). It would not be present in a fingerprint of 5 S RNA labeled \textit{in vitro} with \(\alpha-32P\)GTP. However, this oligonucleotide is present on T1 fingerprints of \([\alpha-32P]ATP\) and \(UTP\)-labeled RNAs (Table 1). Consistent with the known 5'-end sequence (pppGpGp...) of 5 S RNA, 5'-end group analyses of 5 S RNA synthesized \textit{in vitro} in the presence of \(\alpha-32P\)GTP identified only \(3P\)-labeled pppGp, ppGp, and GpG. No other labeled polyphosphates were detectable.\textsuperscript{4} These data, in combination with the fingerprint-sequence data of Table I, indicate that the RNA synthesized by the endogenous RNA polymerase III in the yeast extracts clearly represents a properly initiated and (probably) terminated 5 S tRNA molecule. We cannot rule out, however, the possibility of very rapid nucleolytic processing of 3'-extended forms of 5 S RNA (cf Refs. 39-43). The \(3P\)-labeled products of a preparative \textit{in vitro} transcription of the serine tRNA gene are shown in Fig. 3A. This transcription reaction was conducted for only 10 min. Three major species of RNA, labeled I (\(\sim 95\) nucleotides), II (\(\sim 90\) nucleotides), and III (\(\sim 80\) nucleotides) are observed in roughly equal amounts. RNAs I and III were analyzed in more detail. They were eluted from the preparative gel and digested with either pancreatic or T1 RNases. The T1 oligonucleotide patterns of these RNAs (Fig. 3, B and D, respectively) are identical except for the (almost) total absence of a large T1 oligonucleotide (spot 14; see Table II for oligonucleotide compositions indicated by the arrow in Fig. 3D) from band III RNA. This suggests that band III is derived by processing of band I RNA. The kinetics of appearance of these RNAs is consistent with this idea (see below). All oligonucleotides were eluted and analyzed by nearest-neighbor and double-digestion techniques. Additionally, the larger \([\alpha-32P]GTP\)-labeled T1 oligonucleotides (spots 10-16) were analyzed using partial enzymatic digestions coupled with sizing analyses using polyethyleneimine thin layer chromatography (29). The results of these analyses are summarized in Table II. Comparison of the data obtained for band III RNA with the known sequence for the mature tRNA indicates that this RNA corresponds to the fully processed mature-length, \textit{in vivo} transcript. Oligonucleotides from the 5'-end (spot 13), anticodon (spot 16), and 3' terminus (spot 15) are all present in the fingerprint patterns in equimolar quantities, indicating continuous synthesis throughout the tRNA gene transcription unit. Spot 14, absent from band III RNA and present in band I RNA, is a nona-nucleotide with the sequence ApUpUpApUpUpUpGp. This is the first T1 oligonucleotide expected if transcription initiates upstream from the 5'-terminal G moiety of the mature tRNA\textsuperscript{ex} (17). This nucleotide does not have a polyphosphate terminus (see below). We have never observed significant amounts of larger RNAs (length > 95 bases) accumulating at any stage of the reaction. Correspondingly, there are no detectable amounts of the T1 oligonucleotides that would be expected if the 5' and 3'-flanking DNA sequences of this gene were transcribed. The next two T1 oligonucleotides that would be obtained from a transcript initiating upstream from the spot 14 sequence (bases -9 to -1
upstream from the 5'-end of the mature RNA) are 17 bases long (nucleotides −10 to −26) and 16 nucleotides long (bases −42 to −27). If transcription extended beyond the 3'-end of the transcription unit, two large contiguous T1 oligonucleotides, one 12 bases long (bases +84 to +95) and the other 25 bases long (+96 to +119), would be obtained from the RNA. These large oligonucleotides are clearly not present in the fingerprints. However, we feel that band I RNA probably does not represent the primary in vitro transcription product of this gene since we thus far have been unable to detect polyphosphate termini. It should be noted that putative precursors of 102 and 120 nucleotides have been observed in vivo (45). Our analyses of the 5 S RNA gene transcripts have indicated that polyphosphate termini are at least partially stable in the yeast crude extract and hence should be detectable. However, 5'-end group analysis of RNAs labeled with [α-32P]GTP and experiments using either [β-32P]ATP or [β-32P]GTP (cf. Ref. 50) have failed to reveal polyphosphate termini on the tDNA transcripts. Therefore, it appears that there is very rapid processing of the in vitro synthesized RNA to the partially processed band I form, with slower processing to the mature band III form (through the band II intermediate). Perhaps using fractionated extracts (see below, Fig. 4) or γ-thiol triphosphates, which should inhibit phosphatase activity (51–53), will enable us to identify and isolate the primary transcript of the serine tRNA gene.

Control experiments were conducted in which both the serine tRNA genes and the 5 S RNA genes were transcribed in vitro by purified yeast RNA polymerase III. The resulting RNA and 32P-in vivo labeled 5 S rRNA, respectively. The arrows indicate the only major differences between the in vitro and in vivo 5 S RNAs: spot 6, the 3'-terminal T1 oligonucleotide, CAAUCCUm, B indicates the position of the xylene cyanol FF tracking dye after the running of the second dimension. First and second dimensions were run as indicated in C.

32P-labeled transcripts were then incubated in vitro with the yeast cell-free extract under standard synthesis conditions. No discrete RNAs were generated. These results clearly indicate that neither the discrete serine-tRNA gene transcripts nor the 5 S rRNA transcripts observed above are generated via specific nucleolytic processing of random transcripts.

Taken together, all these results indicate that these two homologous class III genes are transcribed specifically in vitro by endogenous DNA-dependent RNA polymerase III present in the yeast soluble extracts to produce discrete RNAs. These RNAs are not generated via specific processing of random transcripts but rather are the result of accurate and specific initiation, elongation, and termination reactions conducted by the enzymes and factors (see below) present in these extracts. This is the first report of specific transcription of yeast class III genes in vitro in a totally soluble homologous transcription system.

Properties of the in Vitro Transcription System—Extracts prepared as described above are quite stable when stored at −80 °C (full activity retained for greater than 6 months). They can survive several cycles of freeze-thawing with only small decreases in activity. However, storage in small aliquots is still recommended. We have examined the optimal conditions for specific transcription in vitro with the yeast soluble extract. When a crude extract is utilized for transcription, the optimal temperature is 20–25 °C (greater than at 15, 30, or 37 °C). However, if extract passed through DEAE-cellulose at high salt (see "Experimental Procedures") or column-fractionated extract (see below) is used for transcription, the optimal
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TABLE I

Nucleotide sequences were deduced from the following data: mobility in first and second dimension of fingerprint analyses, molar ratios of spots, nearest-neighbor analyses, and known RNA fingerprints and DNA sequences.

| T1 oligonucleotide* | Pancreatic RNase A end products from RNA labeled with | Predicted sequenceb |
|---------------------|-----------------------------------------------|-------------------|
|                     | [α-32P]GTP | [α-32P]ATP | [α-32P]UTP |                       |
| 1                   | G         | G         | G         | CG(G) + CG(A)         |
| 2                   | CG         | G         | AG        | AG(G) + AG(U)         |
| 3                   | AG         | (G)6      | UG(G) + UG(A) + UG(C) |
| 4                   | U, G       | C, (G)    | CUG(G) + CUG(C) |
| 5                   | U          | (U)       | UUG(C)    |                       |
| 6                   | AG         | U, (AU)   | UAG(U)    |                       |
| 7                   | AG         | AAAG, (G) | AAG(A)    |                       |
| 8                   | AG         | AAAG, U   | UAAAG(A)  |                       |
| 9                   | C          | G         | CCUG(A)   |                       |
| 10                  | U + C      | C         | UUCG(A)   |                       |
| 11                  | AG         | AAG, U    | UUAG(A)   |                       |
| 12                  | C          | G         | CACG(U)   |                       |
| 13                  | C          | AAU, C, (G) | AAU, U, (C) | CAAUCU(C) |
| 14                  | AC         | U, C      | AUCAUCAG(C) |
| 15                  | AG         | AAAC, C   | AAACUCCAG(G) |
| 16                  | U          | AAG, C    | AUAACUG(U) |
| 17                  | U          | AG, AU, U, C | ND*       | CCAUAUCUACCAG(A) |

* Oligonucleotide numbering system of Hindley and Page (35).
† Predicted from known DNA/RNA sequence; bases in parentheses represent the nucleotide 3' to the indicated T1 oligonucleotide.
Underlined bases determined from T2 digests of oligonucleotide (nearest-neighbor analysis).
Inconsistent (oligo)nucleotides, either present as extra spots (probably from contaminating oligonucleotides) or missing spots (reasons unknown).
ND, not determined.

Fig. 3. Fingerprints analyses of tDNA7rRNA in vitro transcripts. A preparative transcription reaction templated with tDNA7rRNA, and containing 500 μCi of [α-32P]GTP was carried out as described in “Experimental Procedures.” In vitro transcripts were denatured and fractionated on a 12% acrylamide gel containing 7 M urea. A, an autoradiogram of the 32P-labeled in vitro transcripts. The three major transcripts are indicated by I, II, and III. These three RNAs were eluted from the gel and subjected to RNase T1 digestion, and the resulting oligonucleotides were separated in two dimensions. The fingerprints of RNAs I, II, and III are shown in B, C, and D, respectively. The arrow in D marks the expected position of oligonucleotide 14 (discussed in text), which is missing in the T1 fingerprint pattern of RNA III. B indicates the mobility of the blue dye. First and second dimensions were run as indicated in Fig. 2.
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| Oligonucleotide | bands I, II, and III* |
|-----------------|-----------------------|
|                  | [α-32P]GTP | [α-32P]ATP | [α-32P]UTP | Predicted sequence<sup>a</sup> |
| 1                | G          | G          | G          | G                      |
| 2                | G, U       | G          | G          | UG(U)                  |
| 3                | C          | G          | C          | UG(G)                  |
| 4                | AG         | AG, AC     | AC         | CG(A)                  |
| 5                | G, U       | G          | C          | CUG(C)                 |
| 6                | G, U       | (U)*       | U          | UCG(U)                 |
| 7                | C          | G          | C          | UUG(G)                 |
| 8                | C          | C          | C          | CUCUG(C)               |
| 9                | C          | C          | C          | CCG(A)                 |
| 10               | C          | C          | C          | CCCC(G)               |
| 11               | AG         | AG, AC     | AC         | ACAG(A)               |
| 12               | AAG        | AAG, U     | U          | UUAAG(G)              |
| 13               | AU, G      | U, C       | AC         | CACUAg(G)              |
| 14               | G, U       | U          | AU, U      | AUUAAAGUUG(G)         |
| 15               | U          | AAAU, C    | AAAU, U    | UUCAAAUCCUG(C)        |
| 16               | U          | AAAU, U    | AAAU, AC, G, C | ACUUAAUAACUG(U) |

<sup>a</sup> Data was similar for RNAs I, II, and III except for the absence of oligonucleotide 14 from RNA Band III (see Fig. 3).

<sup>b</sup> Predicted from known DNA/RNA sequence; bases in parentheses represent the nucleotide 3' to the indicated T<sub>I</sub> oligonucleotide.

<sup>c</sup> Inconsistent (oligo)nucleotides, either present as extra oligonucleotides or missing from double digestion analysis.

µg/ml), a fairly strong signal was still detectable. Specific transcription occurs linearly, with little or no lag, for approximately 30-45 min, at which time a steady state between synthesis and random degradation is attained. Under optimal conditions these extracts synthesize from 0.1-10 transcripts/gene/h. The rate of specific transcription is variable, depending on the extract, but approaches that seen in other systems. Pulse-chase experiments indicate a half-life of ~30 min for RNAs in the extract. The apparent processing of the tRNA gene band I transcript to the band III RNA occurs fairly rapidly, within 5-10 min, about 20-30% is processed. We have not yet been able to determine conditions (using whole cell extracts) where we could totally dissociate transcription from RNA processing. The most effective procedure to date has been to carry out transcription reactions in the presence of polyuridylic acid (100 µg/ml) (49). Under these conditions, RNA processing is 35-50% inhibited<sup>4</sup>, while transcription was essentially unaffected (see also Fig. 5A and discussion below). However, we still do not observe the accumulation of the presumed precursor to band I RNA under these conditions.

**Chromatographic Fractionation of Soluble Extracts—** Mammalian and amphibian soluble class III transcription systems have been shown to contain several distinct factors involved in directing selective transcription (11, 54 and references therein). For comparison, we have subjected the soluble yeast extract to ion exchange chromatography. Crude extracts were fractionated on columns of phosphocellulose (P-11) and DEAE-Sephadex by salt-step elution, as outlined in the flow diagram of Fig. 4A. Using phosphocellulose chromatography, three fractions were obtained by the following procedure: A BT fraction, a 600 mM NaCl step-eluted fraction, and a 1500 mM NaCl step-eluted fraction. Each of these fractions were then assayed alone and in combinations to see if specific class III gene transcription could be reconstituted (Fig. 4, B and C). The 600 mM step fraction alone was able to support the specific transcription of both tRNA and 5 S RNA genes (Fig. 4, B and C, Lanes 3). Both 5 S DNA and TDNA transcription reconstitute with high recovery (compare Lanes 1 and 3 in Fig. 4, B and C). Two other class III templates, the tyrosine tRNA and adenovirus virus-associated RNA genes, are also transcribed by the components present in the 600 mM step fraction.<sup>4</sup> RNA processing activity also appears to be present in this 600 mM phosphocellulose step fraction since mature-sized serine tRNA transcripts (Fig. 4B) and partially processed tyrosine tRNA transcripts<sup>4</sup> accumulate in these reactions. Transcription is severely inhibited when the P-11 BT fraction is added to the 600 mM fraction (Fig. 4, B and C, Lane 5). The BT fraction has no qualitative effects on the specificity of transcription, even when very small amounts of this fraction are added to large amounts of the P-11 600 mM fraction.<sup>4</sup> As discussed above, 3'-extended forms of 5 S RNA have been observed both in vitro and in vitro in higher eucaryotes and yeasts. We generally do not observe significant amounts of a 5 S RNA precursor accumulating in vitro when whole cell extracts are used for transcription. However, it is obvious from the autoradiogram shown in Fig. 4C (compare Lanes 1 and 3) that "unprocessed 5 S RNA" apparently does accumulate when P-11 fractionated material is used for in vitro 5 S DNA transcription. This could possibly be due to the removal of inhibitory substances or concentration of a processing activity itself by P-11 chromatography or, alternatively, it could be due to an increase in the amount of incorrect chain termination (i.e. read-through). This longer RNA species has not been characterized further.

All the assays depicted in Fig. 4, B and C, were conducted without the addition of exogenous RNA polymerase III (although the results are identical if exogenous enzyme is added to all the reactions)<sup>4</sup>. Hence, it cannot be determined whether or not the phenomenon of specific transcription observed here is simply due to an "holoenzyme-like" form of RNA polym-
Fig. 4. Fractionation of soluble yeast whole cell extract by ion exchange chromatography. A diagrams the overall strategy for chromatographic fractionation and sequential chromatography on phosphocellulose and DEAE-Sephadex. These columns were eluted by salt step elution yielding six different fractions, indicated by the lower case letters a-f. A 20-ml aliquot of yeast whole cell extract (protein concentration 30 mg/ml), prepared as described in "Experimental Procedures," was applied to an ~80-ml phosphocellulose column (4.5 x 5 cm) that had been equilibrated with Buffer C containing either 600 or 1500 mM NaCl. Fractions of 7 ml were collected and elution was at a flowrate of ~1.5 ml/min. The volumes and protein concentrations of the pooled desalted fractions obtained were 20 ml, 22 mg/ml; 20 ml, 5.9 mg/ml; and 14 ml, 1.4 mg/ml for the BT, 600 mM, and 1500 mM fractions, respectively. The samples were desalted into Buffer D + 100 mM NaCl by chromatography on G-25 (medium). These fractions are indicated by a, b, and c, respectively, in A-E. Protein concentrations were determined by the method of Lowry et al. (57). Aliquots of these fractions were assayed under standard conditions for their ability to promote specific transcription of either tDNA^m (B) or 5 S DNA (C). The assays shown in B and C were identical except for templates. The individual reactions shown in B and C, Lanes 1-8, contained 5 pl of whole cell (WC) extract input to the column, 15 pl of BT, 15 pl of 600 mM fraction, 15 pl of 1500 mM fraction, 15 pl of BT + 15 pl of 600 mM, 15 pl of BT + 15 pl of 1500 mM, 15 pl of 600 mM + 15 pl of 1500 mM, and 10 pl of BT + 10 pl of 600 mM + 10 pl of 1500 mM fractions, respectively. A 4-ml aliquot of the pooled and desalted P-11 600 mM NaCl eluted fraction was applied to an ~20-ml DEAE-Sephadex column (2.6 x 3.6 cm) that was pre-equilibrated with Buffer D + 100 mM NaCl. Unabsorbed proteins were eluted with this same buffer. Bound material was eluted with Buffer D containing either 250 mM or 1000 mM NaCl. Fractions of 1 ml were collected at a flowrate of 0.5 ml/min. The volumes and protein concentrations of the pooled desalted fractions obtained were 4 ml, 3.2 mg/ml; 3 ml, 1.4 mg/ml; and 4 ml, 1.1 mg/ml for the BT, 250 mM, and 1000 mM fractions, respectively. These fractions are indicated by d, e, and f in D and E. The samples were desalted into Buffer D + 100 mM NaCl by chromatography on G-25 (medium). Aliquots of these fractions were assayed under standard conditions for the ability to promote specific transcription of either tDNA^m (D) or 5 S DNA (E). The reactions on the autoradiograms shown in D and E were identical except for the DNA templates indicated above. All reactions contained 20 units of yeast RNA polymerase III except the reactions in Lanes 9 of D and E. The individual reactions shown in Lanes 1-10 D and E contained 5 pl of whole cell extract, 5 pl of P-11 600 mM fraction input to this column, 15 pl of BT fraction, 15 pl of 250 mM step fraction, 15 pl of 1000 mM step fractions, 15 pl of BT + 15 pl of 250 mM fractions, 15 pl of BT + 15 pl of 1000 mM fractions, 15 pl of 250 mM + 15 pl of 1000 mM fractions, 10 pl of BT + 10 pl of 250 mM + 10 pl of 1000 mM fractions, respectively. The reactions shown in B-E were all performed simultaneously with the same reagents. The ^32P-RNAs were analyzed in parallel on 12% acrylamide gels and exposed to the same sheet of x-ray film. Therefore, quantitative comparisons of band intensities can be made between all the lanes.

erase III present in these extracts or due to transcription factors acting in concert with endogenous enzyme III. We thus sought a procedure for fractionating the P-11 600 mM fraction further in order to separate the putative transcription factors from the endogenous RNA polymerase III. This was accomplished by chromatography of the P-11 fraction on DEAE-Sephadex as outlined in Fig. 4A. DEAE-chromatography yielded three fractions: a BT fraction, a 250 mM NaCl step-eluted fraction, and a 1000 mM NaCl step-eluted fraction. All of these fractions were assayed alone and in combinations to determine whether or not specific 5 S DNA and tDNA transcription could be reconstituted. In this set of assays, all reactions were supplemented with exogenous RNA polymerase III (with two exceptions, Fig. 4, D and E, Lane 9).

None of the DEAE-fractions alone are able to support the specific transcription of either tDNA or 5 S DNA (Fig. 4, D and E, Lanes 3-5). In contrast, the combination of DEAE-BT + 250 mM fractions reconstitutes specific transcription of both of these class III gene templates with reasonable recovery (Fig. 4, D and E, Lane 6). This reconstitution of specific transcription is absolutely dependent upon the addition of exogenous RNA polymerase III (not shown). The endogenous
enzyme III binds very tightly to DEAE-Sephadex and is eluted in the 1000 mM step fraction. This endogenous RNA polymerase III can also be used to reconstitute (albeit very poorly) specific transcription effects by the DEAE-BT + 250 mM fractions, as shown in Lanes 9 of Fig. 4, D and E. Simply adding exogenous enzyme III to this combination of components greatly stimulates the amount of specific transcripts (compare Lanes 9 and 10 in Fig. 4, D and E). These results imply that there are minimally three components required to effect specific transcription of yeast class III genes in vitro: DNA-dependent RNA polymerase III and (at least) two chromatographically separable factors (fractons).

Brown and Roeder and their co-workers (11-13, 55) have demonstrated the existence of gene-specific class III transcription factors. One factor termed TFIIIA has been shown to bind specifically to the 5 S DNA internal control region. In addition, this protein will bind specifically to the transcript of the gene 5 S RNA (11-13, 55, 56). In fact, it has been postulated that this dual binding activity (DNA and RNA) could be involved in regulating 5 S RNA gene transcription in vivo. Regardless of the extract mechanisms and role of the nucleic acid binding, this potential property of a 5 S DNA specific transcription factor is quite easily assayed for. If a TFIIIA equivalent exists in the yeast cell-free transcription extracts, then addition of increasing amounts of 5 S RNA should selectively inhibit specific 5 S RNA molecules of 5 S DNA transcription. This experiment was conducted with our whole cell extracts and the results are shown in the autoradiogram depicted in Fig. 5A. tDNAtranscribed (Lanes 1-6) and 5 S DNA (Lanes 7-13) were transcribed in vitro in the presence of increasing concentrations of purified yeast 5 S rRNA (10-10,000 ng of 5 S RNA added/reaction). This represents a molar ratio of 5 S RNA molecules of 5 S DNA to tDNA transcription. These results are presented in quantitative fashion in Fig. 5B. Radioactive bands were excised from the dried gel and counted. The amount of specific transcripts as a function of 5 S RNA concentration is plotted relative to control reactions, which contained no added 5 S RNA. As seen in the autoradiogram, specific tDNA transcription is insensitive to 5 S RNA addition, while 5 S DNA transcription is totally inhibited at a level of ~45 ng/reaction. This represents a molar ratio of 5 S RNA to 5 S DNA “promoter” of about 3. We estimate that there is <5 ng of 5 S RNA added to these reactions by the whole cell extract. Addition of yeast tRNA (at 10 μg/reaction) to tDNA or 5 S DNA transcription reactions has no effect on the amount of specific transcription.

Thus, the 5 S RNA-inhibition effect observed is quite specific and suggests that there does indeed seem to be a TFIIIA-like 5 S RNA-specific transcription factor in S. cerevisiae. Hence, there appears to be at least two (chromatographically) distinct transcription factors present in our yeast extracts. One of these factors is apparently tDNA-specific, while the other factor is 5 S DNA-specific. This notion is corroborated by a series of preliminary experiments which indicate that addition of DEAE-Sephadex BT fraction factor, but not DEAE-Sephadex 250 mM step fraction factor (cf. Fig. 4), will relieve the inhibition of 5 S DNA transcription by 5 S RNA.

The fractionation patterns which we describe for yeast transcription factors are similar to the results of others obtained during the fractionation of transcription extracts from higher eukaryotes. Engelke et al. (11) described the purification of TFIIIA from Xenopus ovary tissue. In this system, TFIIIA (one of the 5 S-DNA specific factors) binds quite tightly to cation exchange columns such as phosphocellulose. Our fractionation pattern is consistent with this. In contrast,
Segall et al. (54) show that when S-100 extracts of human KB cells are applied directly to phosphocellulose at low salt, a 5 S DNA specific transcription factor (presumably TFI1A-like) is found in the BT fraction. However, if nucleic acids are rigorously removed from this phosphocellulose BT fraction containing the KB 5 S DNA-specific factor, it will bind quite tightly to phosphocellulose.5 We have occasionally observed this anomalous chromatographic behavior on phosphocellulose with the yeast 5 S DNA specific factor. We presume that this was due to unusually large amounts of nucleic acids in those particular extracts. The fractionation pattern described above in Fig. 4 is the only reproducible fractionation scheme we currently observe.

**DISCUSSION**

We have described the preparation and characteristics of a totally soluble transcription system derived from yeast. These extracts specifically transcribe both homologous and (probably) heterologous class III genes. This is the first description of such an in vitro DNA-dependent soluble transcription system derived from yeast. These extracts also contain enzymes that accurately process precursor tRNAs. In addition, we have presented preliminary chromatographic fractionation and transcription inhibition studies which demonstrate multiple distinct transcription factors (chromatographic fractions) that are required in addition to DNA-dependent RNA polymerase III for class III gene transcription in vitro. There are apparently different factors for 5 S DNA and tDNA transcription. All of the properties of the soluble yeast extracts are amazingly similar to other previously described soluble eucaryotic transcription systems. This perhaps could not have been predicted with certainty, especially for class III genes (i.e. tRNA and 5 S RNA), since the organization of the S. cerevisiae 5 S rRNA genes (interspersed with the large rRNA gene in an 9-kilobase repeating unit (38)) is so very different from that found in higher organisms.

In comparison to higher eucaryotes, yeast offer many advantages for biochemical and genetic studies. Since many aspects of eucaryotic class III gene structure, transcription, metabolism, and function are highly conserved, we feel confident that detailed studies on class III gene transcription can profitably be pursued in this system. Forthcoming results utilizing soluble yeast transcription systems should therefore provide valuable insights into the mechanisms of regulated eucaryotic gene expression.

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