Accurate transcription of homologous 5S rRNA and tRNA genes and splicing of tRNA *in vitro* by soluble extracts of *Neurospora*

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ABSTRACT

We have developed soluble extracts from *Neurospora crassa* capable of accurately and efficiently transcribing homologous 5S rRNA and tRNA genes. The extracts also appear to quantitatively end-process and splice the primary tRNA transcripts. Although the extracts could not transcribe a heterologous (yeast) 5S rRNA gene, they did transcribe a yeast tRNA^{Leu} gene and slowly process the transcripts. In addition, we have developed a novel strategy for rapidly sequencing uniformly labelled RNAs using base-specific ribonucleases. We have used this procedure to verify the identity of the *in vitro* transcripts and processing products.

INTRODUCTION

The dissection of eukaryotic gene regulation at the molecular level requires *in vitro* systems which faithfully transcribe cloned genes. In a wide range of organisms, from yeast to mammals, *in vitro* transcription systems have been developed which at least partially reproduce the *in vivo* transcription of homologous cloned genes by RNA polymerase I (1-4), RNA polymerase II (5-7) and RNA polymerase III (8-12). These systems have enabled the identification of many of the proteins and DNA sequences required for accurate transcription initiation by the three RNA polymerases (13-20). In the fungi, extensive information about mechanisms of gene regulation has also been obtained by genetic analysis. In *Neurospora crassa*, for instance, the expression of the *qa* genes (21-25), transcribed by RNA polymerase II, and the 5S rRNA genes (26), transcribed by RNA polymerase III, have been analyzed in particular detail.

In order to combine the power of these two approaches, we have developed soluble extracts of *Neurospora* capable of directing the accurate *in vitro* transcription of homologous cloned 5S rRNA and tRNA genes by RNA polymerase III. The extracts also appear to accurately trim and splice primary tRNA transcripts produced in this system. We have developed a novel strategy for rapidly sequencing uniformly labelled RNAs using base-specific ribonucleases

and have used this procedure to demonstrate the fidelity of the transcription and processing reactions.

MATERIALS AND METHODS

Strains and Plasmids.

Neurospora strains used were either 105C (A qa-1S^C) or 336-3-3-79A (A arom-9 qa-3 qa-4). Both strains were wild-type with respect to nuclease and protease production. Of the recombinant plasmids used, pFSPT6, containing a Neurospora α 5S rRNA gene, was derived from pKD52 which, along with pMF3, containing a Neurospora tRNA^{Phe} gene (27), was a gift of R.L. Metzenberg (University of Wisconsin). pSc5S containing a yeast 5S rRNA gene was a gift of P.A. Weil (University of Iowa). YEp13 containing a yeast tRNA^{Leu} gene (28) and pLH40 containing a Neurospora tRNA^{Leu} gene (29) were gifts of L. Huiet (University of Georgia). Plasmids were propagated in SK3430 (23) and DNA was prepared as described (23), except that the supercoiled DNA was purified 2 or 3 times using CsCl/EtBr gradients and was sometimes purified additionally by chromatography on Sephacryl S-1000.

Preparation of Transcription Extracts.

The procedure basically derives from those for preparation of yeast transcriptional extracts (8,9). Conidia were prepared by growth on solid media consisting of 1.5% sucrose, 1% casamino acids, Fries' Salts, 1.5% agar (21) then harvested and germinated at 25°C for 4 hours in liquid 1.5% sucrose, Fries' Salts medium at a concentration of 10^6 - 10^7 per ml. The germinated conidia were rapidly chilled to 0°C, harvested by centrifugation and then washed once in ice cold water and once in extraction buffer. 5-10 ml packed volume of washed conidia were suspended in 15-20 ml of extraction buffer [100 mM Tris acetate pH 7.9, 50 mM potassium acetate (KOAc), 10 mM MgSO₄, 20% glycerol, 3 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM ethylenediamine tetraacetic acid (EDTA)], then 30 μ l of 1 mg/ml Pepstatin A (Sigma) was added and the suspension placed in the 60 ml chamber of a Bead Beater cell disrupter (Biospec Products) together with sufficient ice cold 0.5 mm glass beads to exclude all air. The conidia were disrupted with four 15 second bursts, separated by 45 seconds each for cooling by an ice/water jacket. The lysate was decanted from the beads and the beads washed gently several times with more extraction buffer. The lysate and washes were combined to give a final volume of 58 ml. All subsequent steps were carried out as close as possible to 0°C. The lysate was made 0.9 M in (NH₄)₂SO₄ by dropwise addition of 4 M (NH₄)₂SO₄ pH 7.9. The

lysate was gently stirred for 15 min then centrifuged for 2 hours at 50,000 rpm in a Ti 60 rotor (Beckman). Proteins were precipitated from the supernatant by slow addition of solid $(\text{NH}_4)_2\text{SO}_4$ (0.35 g/ml supernatant). The $(\text{NH}_4)_2\text{SO}_4$ was neutralized by addition of 10 μl 1 M KOH per g $(\text{NH}_4)_2\text{SO}_4$. The precipitate was either collected by centrifugation from 3 ml aliquots of suspension or the entire precipitate was collected as a single aliquot. The precipitate was frozen in liquid N_2 and stored at -80°C . Aliquots of precipitate or the entire precipitate were redissolved in 50 μl or 1.5 ml, respectively, of dialysis buffer [20 mM potassium 4-(2-hydroxyethyl)-1-piperazine ethane sulfonate (K Hepes) pH 7.9, 10 mM potassium ethyleneglycol bis (2-amino ethyl ether) N,N,N',N' tetra acetate (K_2EGTA), 10 mM MgSO_4 , 20% glycerol, 5 mM DTT] containing 1 mM PMSF and 20 μM Pepstatin A. The dissolved extracts were then dialysed in 100 μl aliquots against 2.5 l dialysis buffer for 2 hours in a microdialyser (Bethesda Research Laboratories) at 0°C using a 12,000-14,000 MW-cutoff membrane, then quick-frozen and stored at -80°C . Yield was usually 2.0-2.5 ml of extract with a protein concentration of 20-40 mg/ml.

Many of the experiments presented in this paper were carried out using extracts produced by treating the initial homogenate with 0.4 M $(\text{NH}_4)_2\text{SO}_4$ instead of 0.9 M $(\text{NH}_4)_2\text{SO}_4$. These earlier extracts produced considerably more non-specific transcription.

Transcription and Splicing Reactions. Transcription reactions (25 μl) typically contained 1-8 μl extract, 10 mM K Hepes pH 7.9, 5 mM K_2EGTA , 10 mM $\text{Mg}(\text{OAc})_2$, 103 mM KOAc (total $[\text{K}^+] = 120\text{ mM}$), 2-3% (v/v) glycerol (from the extract), 2.5 mM DTT, 400 μM each GTP, CTP and ATP, 50 μM UTP, 4 mM potassium phosphoenol pyruvate (PEP), 0.6 μg supercoiled plasmid DNA, 0.1-0.5 μCi [$\alpha\text{-}^{32}\text{P}$] UTP. Reactions were incubated 20-30 min at 30°C . For processing and splicing of tRNA, after an initial 20 min transcription reaction, ATP was added to 4 mM, triton X-100 to 0.5% and UTP to 400 μM , and incubation continued for 20-40 min at 30°C . Reactions were stopped by addition of 100 μl of 100 mM sodium acetate pH 5, 10 mM EDTA, 4% sodium dodecyl sulfate (SDS), 4 M urea and then were extracted once with 150 μl phenol/chloroform/isoamyl alcohol (50:50:2). The aqueous phase was collected and the RNA precipitated by addition of a half volume of 7.5 M ammonium acetate and then two volumes of ethanol. The RNA pellet was washed with 80% ethanol, redissolved in 10 μl formamide/dye and the RNA resolved by electrophoresis on 8% acrylamide/ 7 M urea gels (30).

Sequence Analysis of Transcription Products. ^{32}P -labelled RNAs were synthe-

sized in 2-fold scaled up reactions containing 100 μCi of $[\alpha\text{-}^{32}\text{P}]$ UTP for 1 hour and were purified by electrophoresis on 0.3 mm 7 M urea/5% acrylamide gels. The transcription products were localized by autoradiography and eluted from the gel slices by soaking overnight at 4°C in 0.5 M NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 0.2% N-lauryl sarcosine (sarkosyl). For sequence analysis of the 5' ends of the RNAs, the RNA was ethanol precipitated, washed with 80% ethanol then redissolved in 10 μl of 10 mM Na_3EDTA pH 7.4, 30% recrystallized formamide containing 5 μg of M13 phage virion DNA complementary to the 3' half of the RNA (see Results). The RNA and DNA were hybridized for 3 hours at 37°C. The hybrids were then divided into aliquots and partially digested with base-specific ribonucleases. The base-specific ribonucleases were purchased from Bethesda Research Laboratories and the digestion conditions were modified from the strongly denaturing conditions suggested by the manufacturer. These modifications were necessary to preserve the integrity of the RNA·DNA hybrids while maintaining partially denaturing conditions. The modified digestion conditions were: G-specific reactions: 1 μl RNA·DNA hybrids were digested with 0.05 or 0.1 units T_1 ribonuclease for 15 min at 55°C in 5 μl of 25 mM Na citrate pH 5, 200 $\mu\text{g}/\text{ml}$ tRNA; A-specific reactions: 1 μl hybrids digested with 0.2 or 0.4 units U_2 ribonuclease for 15 min at 37°C in 5 μl 50 mM Na acetate pH 4.5, 4 M urea, 200 $\mu\text{g}/\text{ml}$ tRNA; A+U-specific reactions: 1 μl hybrids digested with 2 or 4 units Phy M ribonuclease for 15 min at 37°C in 5 μl 25 mM Na citrate pH 5, 4 M urea, 200 $\mu\text{g}/\text{ml}$ tRNA; U+C-specific reactions: 1 μl hybrids digested with 1 or 2 unit B. cereus ribonuclease for 15 min at 55°C in 5 μl 25 mM Na citrate pH 5, 200 $\mu\text{g}/\text{ml}$ tRNA; C-specific reactions: 1 μl hybrids digested with 1 or 1.5 units CL3 ribonuclease for 15 min at 37°C in 5 μl 25 mM Na phosphate pH 6.5, 200 $\mu\text{g}/\text{ml}$ tRNA. Reactions were stopped by addition of 5 μl of 10 M urea, 2% SDS, dye and freezing to -80°C, then denatured and concentrated at 90°C for 3 min. The resultant oligonucleotides were resolved on 8 or 12% acrylamide/7 M urea gels. M13 clones were constructed in vectors mp10 or mp11 (30) and the M13 phage virion DNA for the hybridization step was isolated as described (30).

For T_1 ribonuclease "fingerprinting" the eluted RNA was precipitated together with 1 μg of tRNA, redissolved in 30 μl 10 mM Tris pH 7.4, 1 mM EDTA, and digested 6 hours with 10 units T_1 ribonuclease. The oligonucleotides were then lyophilized, dissolved in formamide/dye, and fractionated on a 20% acrylamide/7 M urea gel alongside DNA size markers generated by chemical cleavage (44) of a ^{32}P end-labelled DNA fragment.

RESULTS

The strategy adopted for obtaining transcriptional extracts from Neurospora was essentially the same as for higher organisms and for yeast. The mycelia or conidia were lysed so as to preserve the nuclei, extracted with high salt (0.9 M $(\text{NH}_4)_2\text{SO}_4$) and a high speed supernatant of the extract concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation. Germinated conidia were chosen as in preference to mycelia because conidia contain a higher concentration of nuclei (32) and because a burst of RNA polymerase III activity occurs during conidial germination (45). Indeed, conidial extracts reproducibly yielded 10-20 fold greater synthesis of specific transcripts and less non-specific end-labelling and synthesis of RNA, than mycelial extracts (B. Tyler, unpublished). In all of the work presented here, conidial extracts were utilized. Although there are no efficient pleiotropic mutants of Neurospora deficient in nuclease or protease production, analogous to the pep4-3 mutation of yeast (33), many of the abundant nuclease and protease activities present in Neurospora grown on synthetic medium are sensitive to chelating agents such as EDTA and EGTA (34-36), to PMSF (34), or to pepstatin A (an acid protease inhibitor) (37). Therefore, rapidly growing wild-type Neurospora strains were used as sources for the transcriptional extracts.

Specific Transcription of a Homologous 5S rRNA Gene.

To test the extracts for specific RNA polymerase III transcription, the extracts were incubated with supercoiled plasmid DNA pFSPT6 containing a cloned Neurospora α 5S rRNA gene (26) in the presence of nucleoside triphosphates, including $[\alpha\text{-}^{32}\text{P}]$ UTP, and an ATP regenerating system. Figure 1, track b shows that a 120 nucleotide (nt) RNA was synthesized. This RNA was not synthesized if the 5S rDNA plasmid was omitted (track c) or replaced with the vector plasmid pBR322 (track d), or if actinomycin D (track e) or cordycepin triphosphate (an ATP analogue) (track f) were included in the reaction, or if GTP and CTP were both omitted from the reaction (track g). Small amounts of the RNA were produced if either CTP alone (track h) or GTP alone (track i) was omitted, presumably due to small amounts of GTP and CTP contamination in the commercial preparations of the other NTP's. Since the RNA was the same size as 5S rRNA and required 5S rDNA and all four NTP's for its synthesis, the 120 nt RNA was presumably 5S rRNA synthesized *in vitro* from the 5S rDNA template by enzymes present in the Neurospora extract. The inhibition of synthesis of the RNA by actinomycin D confirmed that the RNA was in fact synthesized from the DNA template and the inhibition by cordycepin triphosphate confirmed that the RNA was synthesized by an RNA

Figure 1. Specific Transcription of 5S rDNA In Vitro. Transcription reactions in tracks b and e-i contained 0.6 μ g supercoiled pFSPT6 DNA (α 5S rRNA gene). Tracks c and d contained no DNA or 0.6 μ g pBR322 DNA, respectively. Reactions in tracks e and f contained in addition, 30 μ g/ml actinomycin D or 1 mM cordycepin 5' triphosphate (3' dATP), respectively. In the reactions in track g, CTP and GTP were both omitted; in h, CTP alone was omitted; and in i, GTP alone was omitted. Track a contains a 118 nt size marker.

polymerase (38), presumably RNA polymerase III. 5S rRNA synthesis was not inhibited by 1 mg/ml α -amanitin (not shown).

Sequence Analysis of In Vitro Synthesized 5S rRNA.

To prove that the 120 nucleotide RNA was indeed 5S rRNA and to determine if the RNA was initiated at the same nucleotide in vitro as in vivo, we directly determined the nucleotide sequence at the 5' end of the RNA. Procedures are available for rapidly sequencing RNA in which end-nested sets of fragments are produced by partial digestion of the RNA with base-specific ribonucleases (31). However, these procedures require end-labelled RNA in order to identify the nested fragments. Since in vitro synthesized RNA is uniformly labelled, and since existing procedures for sequencing uniformly labelled RNAs are very laborious, we developed a novel strategy for generating end-nested fragments from uniformly labelled RNA. As outlined in figure 2, the set of fragments is produced by protecting one end of the RNA from ribonuclease digestion by prior hybridization to single-stranded DNA complementary to that end of the RNA. In the case of the 5S rRNA, the single stranded DNA was obtained by cloning the 3' end of the 5S rRNA gene into an M13 single-stranded DNA phage vector using a BamHI site located at nucleotide

Figure 2.
Strategy for Generating End-Nested Fragments from Uniformly Labelled RNA.

30 within the 5S rRNA gene. Therefore, when the *in vitro* synthesized 5S rRNA was hybridized to the single-stranded truncated 5S rDNA and partially digested with ribonucleases, a nested set of fragments 90 to 120 nucleotides long was produced with a common 3' end. When several different base-specific ribonucleases were used and the nested fragments resolved side by side as shown in figure 3A, the sequence of the 5' end of the RNA from nucleotide +1 to +28 could be determined. When, as in this case, the DNA hybridizes to more than 50% of the length of the RNA, the end-nested fragments are all larger than the free oligonucleotides produced from digestion of the unhybridized end of the RNA. Therefore it is not necessary to first isolate the DNA•RNA hybrids bearing the end-nested fragments. If necessary, such isolation can be achieved by high salt precipitation (our unpublished data). As shown in figure 3B, the sequence of the *in vitro* RNA exactly matches that of the *Neurospora* α 5S rRNA gene (26) from nucleotides +1 to +28. In addition to the 120 nt 5S rRNA, a 122 nt 5S related RNA (arrowed in figure 3A) was also synthesized by the extracts in smaller amounts. The nucleotide sequence analysis shown in figure 3A indicates that this RNA results from premature initiation of a 5S rRNA transcript at position -2. For instance, the strong band corresponding to C₂₆ in the C track does not have a faint satellite band 2 nucleotides up the ladder, as it would if the 122 nt RNA contained two additional residues at the 3' end. At positions -2 and -1, the first two nucleotides (AC) of the bona fide α 5S rRNA are repeated (figure 3B).

We were unable to determine whether or not the *in vitro* 5S rRNA tran-

Figure 3. Sequence Analysis of the 5' End of In Vitro Synthesized 5S rRNA. Panel A shows a sequencing ladder of the 5' end of *in vitro* 5S rRNA (see text). Base-specific ribonuclease digestions were as described in the Materials and Methods, using 0.05 units T₁ (G), 3 units PhyM (A+U), 2 units *B. cereus* (C+U) or 1 unit CL3 ribonuclease (C). Panel B compares the sequence of the *in vitro* 5S rRNA with the 5S rRNA sequence. The initiation sites of the 120 nt 5S rRNA and 122 nt 5S related RNA are arrowed. The M13 clone used for the sequence analysis shown in A commences at the *Bam*HI site. The RNA sequence shown in B was derived from the autoradiograph shown in A and from shorter and longer exposures of it.

script initially bore a 5' polyphosphate group because reconstruction experiments indicated that 5' phosphate groups had a half-life of less than one minute in the transcription reaction, even in the presence of 60 mM potassium phosphate. Indeed, we frequently observed phosphatase-resistant labelling of the transcript (at internal α -phosphate positions) with [γ -³²P]ATP and [γ -³²P]GTP, presumably due to abundant phosphatases and nucleoside kinases in the extracts. Consequently, we cannot rule out that the 5' end of the *in vitro* transcript is generated by 5' end-processing.

Properties of the Extracts.

The transcriptional extracts are stored at -80°C either as ammonium sulfate precipitates or in 20% glycerol after dialysis. The dialysed extracts are very stable and survive many cycles of freeze/thawing with little loss of activity, provided they are thawed on ice and maintained at 0°C for as short a time as possible while thawed. However, the ammonium sulfate precipitates were somewhat more stable for long term storage.

Figure 4. Optimization of Extract Concentrations. Reactions were as described in the Materials and Methods except that 1 μ l, 2 μ l, 4 μ l, 6 μ l or 8 μ l of extract were used as indicated. The extract was produced by treatment of the homogenate with 0.4 M $(\text{NH}_4)_2\text{SO}_4$ (see text). The template was 0.6 μ g pFSPT6 (5S rDNA) in each case.

The optimal extract concentration for 5S rDNA transcription varied from extract to extract, and was determined for each new extract. Typically 1-8 μ l of extract (25-40 mg/ml protein) were required for a 25 μ l reaction. Figure 4 shows the optimization of an extract produced by extraction with 0.4 M $(\text{NH}_4)_2\text{SO}_4$. At low extract concentrations 5S rRNA synthesis is weak, steadily increasing with increasing extract. Above the optimum, synthesis begins to be inhibited. At the highest extract concentrations (not shown) there is extensive end-labelling of endogenous RNA and much non-specific transcription occurs. Different extracts also vary in the amount of non-specific synthesis and end-labelling of RNA. In particular, extracts produced by extraction with 0.9 M $(\text{NH}_4)_2\text{SO}_4$ showed 5-10 fold less non-specific transcription than those produced by extraction with 0.4 M $(\text{NH}_4)_2\text{SO}_4$.

The salt optima for 5S rRNA synthesis by the extracts depended greatly on the particular anion used (figure 5A). Sodium acetate and potassium acetate produced greater than 70% maximal activity from 60 mM to 150 mM cation concentration with 120 mM being optimal. However, sodium chloride and potassium chloride were optimal at 60-90 mM and inhibited transcription 50 fold when present at 150 mM. Ammonium sulphate was very similar to the

Figure 5. Optimization of Salt Concentration. In A, monovalent cation concentration was varied. The reactions contained 17 mM K^+ as Hepes and EGTA plus 5 mM $(NH_4)_2SO_4$ from the extract, giving an initial cation concentration of 27 mM. Sodium acetate (o), potassium acetate (□), sodium chloride (●), potassium chloride (■), or ammonium sulphate (▲) were then added to increase the cation concentration to the indicated level. Transcripts were quantified after a 20 minute reaction by Cerenkov counting of reaction products precipitated from ethanol in the presence of 2.5 M ammonium acetate. The reaction products were subsequently fractionated by gel electrophoresis to confirm that >95% of the radioactivity was present as 5S rRNA. The solid line indicates the combined data for the chloride salts; the dotted line, for the acetate salts; and the dashed line, for ammonium sulphate. In B, Mg^{2+} concentration (as acetate) was optimized. Reactions contained 60 mM K^+ as buffer and acetate, plus 20 mM $(NH_4)_2SO_4$. The transcription rate was measured as in A.

chlorides, being optimal at 60 mM. All five salts produced very similar maximum activities (figure 5A) but ammonium acetate produced only 76% of the activity produced by the other salts (not shown). The sensitivity of the *Neurospora* extracts to chloride ions distinguishes them from yeast RNA polymerase III transcriptional extracts which have an optimum of 150 mM sodium chloride (8). The Mg^{2+} optimum was 10-12.5 mM (figure 5B) similar to the yeast extracts (8,9).

The optimal DNA concentration for specific 5S rRNA transcription was 25 μ g/ml of supercoiled plasmid. Below that, transcription decreased in proportion to the amount of DNA present. Above the optimum, non-specific transcription increased in proportion to the amount of DNA present while 5S rRNA synthesis either remained constant or declined. 5S rRNA synthesis was strongly inhibited by naked RNA and single-stranded DNA. Hence, contamination of plasmid DNA preparations with small amounts of RNA produced lower DNA optima and inhibition at high DNA concentrations, compared with highly purified DNA preparations. Therefore, we routinely purified plasmid DNA twice by centrifugation in CsCl/ethidium bromide and frequently also by

chromatography on Sephacryl S-1000.

Synthesis of 5S rRNA was optimal at 30°C compared to 20°C, 25°C and 35°C and continued to increase for 1 hour after commencement of incubation. The initial net rate of synthesis in the most active extracts was approximately 5-10 transcripts/gene/hour. 5S rRNA levels decreased after 2 hours presumably due to degradation. Pulse chase experiments indicated that the 5S rRNA had a half-life of approximately 10 min under the normal reaction conditions. If EDTA was also added in excess of the Mg^{2+} concentration, the RNA was stable for at least an hour indicating that virtually all of the nuclease activity was Mg^{2+} -dependent (35,36). Although one of the major proteases of *Neurospora* is sensitive to PMSF (34), we do not include PMSF even to 0.1 mM in the dialysis buffer because all of the solvents for PMSF we have tried (methanol, ethanol, isopropanol or dimethylsulfoxide) significantly inhibited transcription. Also, PMSF covalently and stably modifies the proteases that it inhibits (39). Similarly, the acid protease inhibitor pepstatin A, which is a substrate analogue, binds stably to the *Neurospora* acid protease (37) and hence is added only during isolation of the extract. 5S rRNA synthesis was stimulated 5-10 fold by the addition of an ATP-regenerating system (4 mM phosphoenolpyruvate (PEP) or 4 mM creatine phosphate) though addition of neither pyruvate kinase (12 units/ml) nor creatine kinase (12 units/ml) produced any additional stimulation. Presumably the exogenous enzymes were inhibited by the crude extracts or else the extracts contained sufficient endogenous pyruvate kinase and creatine kinase. We routinely use PEP because it can be used to rephosphorylate all of the NDP's to some extent. Addition of nucleoside diphosphate kinase produced no additional stimulation.

Transcription of tRNA Genes.

tRNA genes are also transcribed by RNA polymerase III and the mature size tRNA is produced by end-processing of a precursor transcript and removal of the intervening sequence, if present, by splicing (40). Figure 6 shows that both a *Neurospora* tRNA^{Phe} gene (27) and a *Neurospora* tRNA^{Leu} gene (29) are transcribed by the extracts to produce a complex set of transcripts. The cloned tRNA^{Phe} gene yielded a major transcript of about 93 nt and minor transcripts of 107 nt and 77 nt (figure 6, lane b) while the tRNA^{Leu} gene yielded major transcripts of 108 nt and 128 nt (figure 6, lane c). The major tRNA^{Phe} transcript (93 nt) is close in size to that predicted for the end-processed but unspliced tRNA^{Phe} (91 nt; ref 27) while the 77 nt transcript is close in size to the mature tRNA^{Phe} (78 nt). The major tRNA^{Leu} transcript (108 nt) is close in size to that expected for unspliced tRNA^{Leu} (107 nt;

Figure 6. Specific Transcription of tRNA Genes
 Reactions contained 0.6 μ g pBR322 DNA (track a), 0.6 μ g pMF3 DNA (tRNA^{Phe} gene) (track b), or 0.6 μ g pLH40 DNA (tRNA^{Leu} gene) (track c). Incubation was for 20 min.

ref 29). The largest tRNA^{Phe} and tRNA^{Leu} transcripts presumably represent the primary tRNA transcripts or intermediate processing products of them. Interestingly, while all of our extracts strongly transcribed the tRNA^{Leu} gene, transcription of the tRNA^{Phe} gene varied greatly. Whereas some extracts transcribed the tRNA^{Phe} gene as strongly as the tRNA^{Leu} gene, other extracts were quite inactive for tRNA^{Phe} transcription. Therefore, transcription of these two different tRNA genes may involve distinct proteins with different stabilities or different solubilities during extraction.

Processing and Splicing of tRNA Transcripts.

We have used the tRNA^{Leu} gene to further characterize tRNA gene transcription by the *Neurospora* extracts. Whereas small amounts of tRNA^{Phe} were apparently spliced during transcription of the tRNA^{Phe} template, no splicing of tRNA^{Leu} transcripts could be detected. Therefore, we set out to establish conditions which favored more complete splicing. In yeast, the ribonuclease which initiates tRNA splicing is a membrane-associated enzyme and is stimulated by non-ionic detergents such as triton X-100 (41). In addition, the RNA ligase which completes the splicing requires ATP (41). Therefore, we incubated the tRNA^{Leu} gene with *Neurospora* extract under normal transcriptional conditions for 20 min, then added triton X-100 to 0.5%, ATP to 4 mM, and unlabelled UTP to 500 μ M, then continued the incubation for 30 min at

30°C. As shown in figure 7A track C, the tRNA^{Leu} transcripts were quantitatively processed and spliced under these conditions to produce a tRNA^{Leu} measuring 81 nt compared to the mature size of 83 nt. If both the triton X-100 and the additional ATP were omitted (track b) there was little or no splicing, and the processed but unspliced 108 nt form accumulated. Track a shows the transcripts present immediately after the initial 20 min transcription reaction (see above).

To verify that the 81 nt band was derived from the 108 nt band by removal of the 27 nt intervening sequence, we took advantage of the fact that the largest oligonucleotide (24 nt) produced by T₁ ribonuclease digestion of unspliced tRNA^{Leu} transcripts should derive from the intervening sequence (29). Therefore, we isolated pure preparations of the 128 nt, 108 nt and 81 nt tRNA^{Leu} transcripts by elution from gel slices, digested them completely with T₁ ribonuclease, and fractionated the resultant oligonucleotides by size on a 7 M urea/20% acrylamide gel. Figure 7B shows that the largest T₁ oligonucleotide produced from the 128 nt and 108 nt transcripts migrates at about 26 nt and is not produced from the 81 nt transcript, consistent with (but not proving) accurate splicing of the 108 nt transcript.

To verify that the 108 nt tRNA^{Leu} transcript was produced by end-processing (trimming) of the 128 nt transcript (see above), and to verify that the 108 nt and 81 nt transcripts had the same 5' ends, we determined the nucleotide sequences of the 5' ends of the three transcripts by the strategy outlined in figure 2. The single-stranded DNA complementary to the 3' end of the RNAs was obtained by cloning into phage M13 the 3' end of the gene commencing at a NarI site at nucleotide 26. Each transcript was hybridized to single-stranded DNA from the M13 3' end clone, partially digested with base-specific ribonucleases, and the end-nested fragments resolved on a sequencing gel. Figure 8B shows the sequencing of the 5' end of the 108 nt transcript. The sequence matches precisely the sequence from the 5' end of tRNA^{Leu} gene to the NarI site (figure 8D). The sequencing ladder from the 128 nt transcript (figure 8A) however could not be clearly read due to apparent 3' heterogeneity of the transcript. Nevertheless, certain frequently cleaved nucleotides, e.g., C₃, A₇, A₁₄, C₁₆, and C₂₀, could be identified as strong doublet bands by comparison to the sequencing ladder of the 108 nt transcript. By counting the number of bands from these identifiable residues to the top end of the 128 nt ladder, it was possible to identify the 5' end of the 128 nt transcript. Accordingly, the 5' end was localized to nucleotide -11 of the tRNA^{Leu} gene (figure 8D). In addition,

Figure 7. Processing and Splicing of tRNA^{Leu} Transcripts. Panel A: Transcription and Splicing Reactions. 0.6 μ g pLH40 DNA was incubated for 20 min with 4 μ l of extract. Then either the reaction was terminated (track a) or it was continued for 40 min at 30°C after addition of UTP to 400 μ M (track b) or of ATP to 4 mM, triton X-100 to 0.5% and UTP to 400 μ M (track c). Panel B: Complete T₁ ribonuclease digests of the three different tRNA^{Leu} transcripts were resolved on a 7 M urea/20% acrylamide gel and detected by autoradiography. IVS indicates the oligonucleotide derived from the intervening sequence in the unspliced transcripts. Approximate size markers (indicated on the right) were obtained by chemical cleavage (44) of ³²P end-labelled DNA.

the difference in mobility of these characteristic bands in the 128 nt ladder as compared to the 108 nt ladder indicated that the 128 nt transcript consisted of two distinct species which differed at their 3' ends from the 108 nt transcript by 8 nt and 9 nt, respectively. For instance, the fragments from the 128 nt species corresponding to C₁₆ co-migrate with the fragment from the 108 nt species corresponding to A₇. Therefore, by reference to the sequence of the gene, these transcripts must include 2 and 3 U residues, respectively, transcribed from the T₁₀ transcription terminator sequence of the gene (29). The 11 nt difference at the 5' end and the 8 nt or 9 nt difference at the 3' end between the 128 nt and 108 nt tRNA^{Leu}

Figure 8. 5' Sequence Analysis of tRNA^{Leu} Transcripts. A, B and C show 5' sequencing ladders of the 128 nt, 108 nt and 81 nt transcripts respectively. The ladders in A and B are from the same experiment and their relative mobilities are shown exactly as in the original autoradiograph. Numbering of the nucleotides follows the gene sequence (29) which corresponds to the standard tRNA numbering until nucleotide 20. Gene residue 21 in the D loop is 20.1 by the standard system so that G₂₇ and G₃₇ are standard residues G₂₆ and G₃₆, respectively. X denotes positions at which the nucleotide could not be determined. In A and B, ribonucleases used were 0.1 units T₁ (G), 0.5 units U₂ (A), 4 units PhyM (A+U), 1 unit *B. cereus* (C+U), 1.5 units CL3 ribonuclease (C). In C, ribonucleases were: 0.1 units T₁ (G), 0.2 or 0.4 units U₂ (A), 2 or 4 units PhyM (A+U), 1 or 2 units *B. cereus* (C+U), 1 or 2 units CL3 ribonuclease (C). Panel D compares the tRNA sequences with the tRNA^{Leu} gene sequence (29). Double-underlined residues indicate positions of strong cleavage (see text). The M13 clone used for the sequence analyses commenced at the NarI site. The 5' end of the 128 nt transcript was identified as described in the text. The RNA sequences shown in D were derived from the autoradiographs shown in A, B and C and from shorter and longer exposures of them.

transcripts accurately account for the measured size difference between these two transcripts.

The 5' sequence of the spliced 81 nt transcript (figure 8C) matched that of the 108 nt transcript exactly (figure 8D). However, the sequence of the 81 nt transcript could be clearly read from the ladder past the NarI site at

Figure 9. Transcription of Yeast Genes by *Neurospora* Extracts. Transcription reactions (20 min) contained 0.6 µg each of: a, pSc5S (yeast 5S rDNA); b, pFSPT6 (*Neurospora* 5S rDNA); c, YEP13 (yeast tDNA^{Leu}); d, pLH40 (*Neurospora* tDNA^{Leu}).

+26 through to G₃₇, one nucleotide prior to the presumptive splice site in this RNA. Presumably the 13 nt sequence between the *NarI* site and the splice site does not hybridize to the single-stranded tDNA under the partially denaturing digestion conditions when the intervening sequence has been spliced from the RNA. In addition, within this region, one G residue, G₂₇, was not cleaved at all by ribonuclease T₁ during the sequencing reaction (figure 8C), whereas the surrounding G residues were very strongly cleaved. Since this residue is modified to 2,2-dimethyl guanosine in the other seven nuclear tRNA^{Leu}'s sequenced (42), it is possible that the *Neurospora* extracts have quantitatively modified this residue *in vitro*, perhaps as part of the normal maturation process.

Species Specificity of the *in vitro* Transcription Reactions.

To determine the extent to which the *Neurospora* transcriptional extracts could direct the transcription of heterologous genes, a yeast 5S rRNA gene and a yeast tRNA^{Leu} gene were incubated with *Neurospora* extracts under conditions in which the corresponding *Neurospora* genes were transcribed. Figure 9 shows that the yeast 5S rRNA was not transcribed at all (track a). Track b shows transcription of the *Neurospora* gene. In contrast, the yeast tRNA^{Leu} gene (track c) was strongly transcribed. However, processing of the yeast transcripts was much slower than that of the corresponding *Neurospora* tRNA^{Leu} transcripts (track d). Whereas comparable amounts of the *Neurospora* 128 nt precursor and 108 nt unspliced RNAs were produced, the great majority

of the yeast transcripts were of a 133 nt precursor RNA. This RNA corresponds to the Neurospora 128 nt RNA due to a 7 nt difference between the sizes of the intervening sequences and V loops of the two tRNA genes (28,29). Relatively little of the yeast 116 nt unspliced RNA accumulated. Moreover, an additional 145 nt transcript of the yeast template accumulated which had no detectable counterpart with the Neurospora template. It is possible that the 145 nt transcript is the primary transcript of the yeast tRNA^{Leu} gene, but that the corresponding transcript from the Neurospora template (which would be ~138 nt) is processed too rapidly to be detected.

DISCUSSION

We have developed a procedure for producing soluble extracts from conidia of Neurospora crassa which accurately and efficiently transcribe homologous 5S rRNA and tRNA genes. The extracts also accurately end-process the primary tRNA transcripts and appear to remove the intervening sequence by splicing. We have used these extracts to show that the primary in vitro transcript of a Neurospora tRNA^{Leu} gene bears at least 11 and possibly up to 21 additional 5' residues, as well as 8 to 9 additional 3' residues. The extracts should also enable the expression of the Neurospora 5S rRNA and tRNA genes to be examined at the molecular level. The protein and DNA sequence requirements for 5S rRNA gene expression are of particular interest since in Neurospora, these genes are dispersed throughout the genome and exist in a number of different sequence families (26).

Although the DNA sequences required for transcription of Neurospora 5S rRNA and tRNA genes are unknown, the inability of the Neurospora extracts to transcribe the yeast 5S rRNA gene would be consistent with the poor conservation of nucleotides 55-80 (7 differences) between the Neurospora and yeast genes (26), if this region was required for 5S rDNA transcription in Neurospora as it is in Xenopus (16,17). Conversely, the ability of the Neurospora extracts to transcribe the yeast tRNA^{Leu} gene would be consistent with the complete conservation of the 9-18 nt and 53-61 nt regions of the two genes (28,29) if, as in Xenopus (18), these regions were required for tDNA transcription in Neurospora. The 5' flanking sequences of the Neurospora and yeast tRNA^{Leu} genes (28,29) are however very different, indicating that although the 5' region is highly conserved among different yeast tRNA^{leu} genes (43), 5' sequences do not appear to be necessary for in vitro transcription by the Neurospora extracts. The poor conservation of the 5' sequences may explain the slow end-processing of the yeast tRNA transcripts

by the *Neurospora* extracts. This slow processing may also provide experimental access to the earliest steps in tRNA processing (40) and provide information as to the precise role of the conserved 5' sequences (43).

We have also developed a novel strategy for rapidly sequencing uniformly labelled RNA by digestion with base-specific ribonucleases. The procedure has been used here to verify the identity of the products of the *in vitro* transcription system and to identify the 5' ends of the different tRNA transcripts. Although the procedure requires cloned DNA homologous to the RNA, the strategy should also be useful for determining differences between the sequences of the RNA and DNA due, for instance, to splicing or mutation, or where the RNA and DNA are from different organisms. The procedure may also be useful for sequencing particular RNA species which cannot be obtained in pure form.

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