Nucleoside 5′-[γ-S]Triphosphates Will Initiate Transcription in Isolated Yeast Nuclei†

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ABSTRACT: Transcription was carried out in isolated nuclei by endogenous ribonucleic acid (RNA) polymerase in the presence of nucleoside 5′-[γ-S]triphosphates. The resulting 5′-γ-thiophosphate on the synthesized RNA allows separation of in vitro initiated RNA from bulk RNA. Analysis of this in vitro initiated RNA shows that 5S RNA and pre-tRNA are initiated in vitro by RNA polymerase III. Yeast RNA polymerase III also reinitiates a discrete distribution of RNA species as large as 28 S. The RNA populations initiated with adenosine 5′-[γ-S]triphosphate and guanosine 5′-[γ-S]triphosphate are different.

Nuclear transcription in yeast nuclei has considerable potential for examining the control of gene expression. Genes whose expression is controlled at the transcriptional level are being studied (Zitomer et al., 1979; Hopper et al., 1978). Isolated yeast nuclei perform endogenous transcription (Schultz, 1978; Lohr & Ide, 1979; Tekamp et al., 1979). All four nucleotide triphosphates are required, the reaction is template dependent, and the product is ribonuclease (RNase) sensitive. All three ribonucleic acid (RNA) polymerases are active in the nuclei, and no nuclear lysis is observed during the transcription reaction. Nuclear transcriptional differences can be correlated with structural differences in the template chromatin (Lohr & Ide, 1979). Incorporation of β-^{32}P-labeled ATP and GTP into RNA indicates initiation is occurring in vitro in yeast nuclei (Bennetzen, 1980).

Analogues of nucleoside triphosphates which have a γ-thiophosphate [γ-S] can be incorporated into RNA by RNA polymerases (Reeve et al., 1977; Smith et al., 1978a,b; Sun et al., 1979; Hipskind & Reeder, 1980). Since chain elongation only incorporates the α phosphate, the sulfur is retained only at the 5′ end of RNA transcripts initiated with the γ-S-labeled nucleotides. The transcripts can be labeled throughout their entire length by addition of ^{3}H- or α-^{32}P-labeled nucleotides to the transcription reaction, thus attaining relatively high specific activity. This [γ-S]RNA[1] can be isolated free of bulk RNA by chromatography on mercury–agarose and used for further analysis of transcription initiation.

Nuclear transcription systems with γ-S-labeled nucleotides have shown proper initiation of 5S gene transcripts in mouse myeloma nuclei (Smith et al., 1978a) and of rRNA gene transcripts in *Physarum* nuclei (Sun et al., 1979). *Escherichia coli* polymerase will properly initiate on bacteriophage λ in the presence of γ-S-labeled nucleotides (Smith et al., 1978b). *Xenopus laevis* nuclear homogenate will initiate transcription of rRNA at the correct position and transcribe the correct strand in the presence of γ-S-labeled nucleotides (Hipskind & Reeder, 1980). Vesicular stomatitis virus leader RNA is initiated at the correct position with [γ-S]ATP by an RNA-dependent RNA polymerase (Carroll & Wagner, 1979).

Artifactual transfer of γ-thiophosphate to other nucleotides or nucleic acids occurs in some systems, making identification of the in vitro transcript more difficult (Hipskind & Reeder, 1980; Stallcup et al., 1979). The present report studies the in vitro initiation of RNA polymerase III products with γ-S-labeled nucleotides in yeast nuclei and shows that artifactual transfer of γ-thiophosphate to other nucleotides or nucleic acids does not occur in yeast nuclei.

Materials and Methods

Nuclear Isolation. Mid-log yeast cells (strain Y55) were grown as described (Lohr et al., 1977). Cells were spheroplasted as described (Lohr & Ide, 1979) except that the 0.1 M Tris and 0.1 M EDTA pretreatment was skipped. Spheroplasts were broken in 18% Ficoll as described (Lohr & Ide, 1979), and nuclei were isolated by layering the 18% Ficoll lysate on an isopycnic density gradient of 1 M sorbitol and 0.5 mM $CaCl_2$ dissolved in a solvent of 35% Percoll (Pharmacia) and 65% H_2O, pH 6.5. The gradient was formed before loading by spinning 34 mL of the gradient solution contained in a 50-mL tube in an SS-34 angle rotor at 27000*g* for 50 min. A 6-mL sample of the 18% Ficoll lysate was diluted with 6 mL of 1 M sorbitol and 0.5 mM $CaCl_2$ and then layered on this gradient. Nuclei were banded free from cell debris by a 7500 rpm spin in an HB4 swinging bucket rotor for 15 min. The resulting band of nuclei was washed by dilution with 2 volumes of 1 M sorbitol and 0.5 mM $CaCl_2$, pH 6.5, and pelleted at 4300*g* for 5 min. Nuclei were resuspended for transcription in this buffer. Nuclei isolated by this method will incorporate 20–40 pmol of UTP into RNA per microgram of template DNA in a 15-min synthesis. This nuclear isolation method will be the subject of another publication (G. J. Ide, G. Riedel, and C. A. Saunders, unpublished experiments).[2]

Transcription Assay. RNA syntheses were conducted in a final volume of 50 μL containing the following components: 50 mM Tris-HCl (pH 7.9, 23 °C), 1 mM $MnCl_2$, 10 mM $MgCl_2$, 0.8 M sorbitol, 0.5 mM $CaCl_2$, 5 mM phosphoenolpyruvate, 1 μg of pyruvate kinase, 0.1 M KCl, 50 μM [^{3}H]-

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[1] Abbreviations used: [γ-S]RNA, sulfur-containing RNA synthesized in the presence of nucleoside [γ-S]triphosphates; [γ-S]ATP, adenosine 5′-[γ-S]triphosphate; [γ-S]GTP, guanosine 5′-[γ-S]triphosphate; DTT, dithiothreitol; high molecular weight RNA, RNA larger than 5.8 S; rRNA, ribosomal RNA; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

[2] A more detailed protocol for Percoll isolation of yeast nuclei can be obtained from the author.

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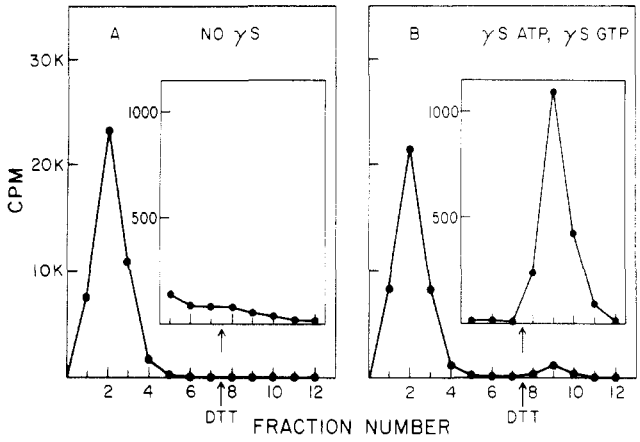

FIGURE 1: Binding of RNA labeled in vitro to mercury–agarose. Duplicate transcription reactions were prepared with unmodified nucleotides (A) or [γ-S]ATP and [γ-S]GTP (B) and chromatographed on a mercury–agarose column. Fractions of 2 mL were collected. Bound RNA was eluted by the addition of 10 mM dithiothreitol (DTT) to the chromatography buffer of the eighth and subsequent fractions.

UTP or [α-^{32}P]UTP (1.6–48 Ci/mmol), and 240 μM ATP (or [γ-S]ATP), CTP, and GTP (or [γ-S]GTP). Reactions are at 25 °C for the times indicated. Acid-precipitable radioactivity in RNA was assayed by using the DE81 filter method (Roeder, 1974). Transcription reactions were terminated with 5 units of DNase I which was repurified to remove RNase (Maxwell et al., 1977), and then 450 μL of 0.5% $NaDodSO_4$, 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 0.1 M NaCl (TNES) was added. Reactions were pronased, phenol extracted, ethanol precipitated, and redissolved in 500 μL of TNES.

Mercury–Agarose Chromatography and RNA Analysis. Affinity chromatography of [γ-S]RNA was as described (Reeve et al., 1977) except the column buffer was TNES containing 0.5% $NaDodSO_4$ rather than 0.1% $NaDodSO_4$. Mercury–agarose column samples were prepared for electrophoresis by ethanol precipitation and redissolution in buffered formamide. Electrophoresis was on acrylamide–urea gels prepared according to Maniatis et al. (1975). Acrylamide concentration is given in the figure legends. Acrylamide concentration was always 30 times the bis(acrylamide) concentration. Gels were fluorographed by the technique of Bonner & Laskey (1974) except the H_2O wash step was for 6 h. I have found artifactual blackening of the film from dimethyl sulfoxide (Me_2SO) with shorter wash times. The film was Kodak XR5 preflashed for quantitative imaging (Laskey & Mills, 1975). The gel in Figure 5 was fluorographed by soaking in 1.0 M sodium salicylate and then drying (Chamberlain, 1979). Gels fluorographed in this manner will occasionally stick to the acetate overlayer sheet upon drying. I have found that a 0.5-mm-thick Teflon sheet cut to fit the gel dryer will eliminate all sticking during the drying.

Results

RNA Synthesis and Chromatography. Isolation of [γ-S]RNA. Substitution of 5′-[γ-S]ATP or 5′-[γ-S]GTP for unmodified nucleotides does not inhibit labeling of RNA during yeast nuclear transcription. Following a 30-min transcription reaction, the purified RNA was separated by chromatography on mercury–agarose. The column profile for a typical experiment with unmodified nucleotides is shown in Figure 1A, with [γ-S]ATP and [γ-S]GTP replacing ATP and GTP in Figure 1B. These data show that γ-S-labeled nucleotides must be present in the transcription reaction for RNA to bind to the mercury–agarose column.

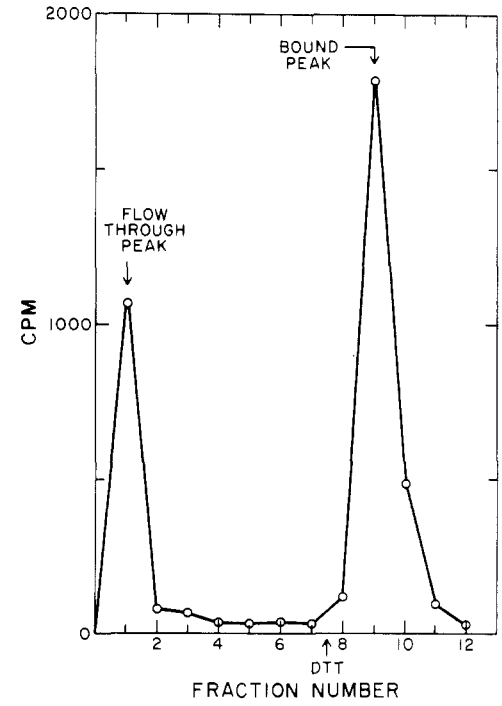

FIGURE 2: Rechromatography of [γ-S]RNA. Bound [γ-S]RNA was isolated as in Figure 1, run over a Bio-Gel P6 column to remove dithiothreitol, and rechromatographed on a mercury–agarose column.

Large Proportion of [γ-S]RNA Will Bind to Mercury–Agarose. If the bound RNA shown in Figure 1B is structurally different than the flow-through RNA, rechromatography of this RNA on mercury–agarose should result in the binding of a large portion of this RNA to the column. Figure 2 shows rechromatography of bound RNA on the mercury–agarose column.

Clearly, the RNA in the bound fraction of Figure 1B is structurally different than the bulk RNA. If the bound RNA is 100% [γ-S]RNA, one would expect 100% of it to bind to the mercury–agarose column in this experiment. However, mercury–agarose does not bind 100% of the [γ-S]RNA in a sample probably because some RNA chain scission occurs during the chromatography (Reeve et al., 1977; Smith et al., 1978a).

Activity That Transfers the γ-S to Elongated RNA Is Not Detectable in Yeast. A possible concern is that RNA kinase activity like that found in mouse L cells (Winicov, 1977) or rat hepatoma tissue culture cell nuclei (Stallcup et al., 1979) may transfer the thiophosphate from the nucleoside [γ-S]-triphosphates to the 5′ end of other RNAs. The following two experiments were performed to show the [γ-S]RNA was newly initiated and not the product of γ-S transfer to an in vitro elongated RNA.

(1) [γ-S]RNA Synthesis Is Template Dependent. Four nuclear transcription reactions (A, B, C, and D) were made (Table I). Three reactions (A, B, and C) received unmodified nucleotide triphosphates and [^{3}H]UTP at zero time and were transcribed for 15 min. At this time, RNase-free DNase I was added to reaction A, and reactions A and B were made 240 μM in [γ-S]ATP and [γ-S]GTP and allowed to transcribe an additional 15 min. RNase-free DNase I was then added to reactions B and C. Reaction D received RNase-free DNase I at zero time and unmodified triphosphates and [^{3}H]UTP at 1 min. The RNA products of all four reactions were then purified and separated by affinity chromatography on mercury–agarose. If thiophosphate transfer is occurring, some

Table I: Control for Transfer of γ-S to Elongated Transcripts[a]

		PREDICTED RESULT IF NO ARTEFACTUAL γS TRANSFER	RESULTS		
			FLOW THROUGH RNA CPM	WASH BACKGROUND CPM	γS RNA CPM
A	Unmodified NTP's → γS NTP's (DI) → Isolate RNA; 0 — 15 16 — 31	NO RNA BOUND	15144	20	20
B	Unmodified NTP's → γS NTP's → Isolate RNA (DI); 0 — 15 — 30 31	SOME RNA BOUND	33755	24	1090
C	Unmodified NTP's → Isolate RNA (DI); 0 — 30 31	NO RNA BOUND	33558	34	20
D	Unmodified NTP's (DI) → Isolate RNA; 0 1 — 31	NO RNA SYNTHESIZED	220	34	24

[a] The isolated RNA from each reaction was dissolved in 0.5 mL of TNES and loaded on a 3-mL mercury-agarose column. The first 4 mL eluted is pooled (flow through). The next 10 mL is discarded, and then a 4-mL fraction (wash background) is collected. The [γ-S]RNA pool is eluted with 4 mL of 10 mM DTT in TNES ([γ-S]RNA). A portion of each pool (0.4) was spotted on DE81 filters; the filters were washed and counted as described under Materials and Methods. A fraction of each sample (0.6) was electrophoresed on a 4% acrylamide gel; values are in ^{3}H cpm incorporated into RNA. The electrophoresed portion of reaction B recovered as [γ-S]RNA displayes the typical banding pattern for reinitiated RNA when fluorographed (data not shown). Total time for each reaction was 31 min.

of the labeled RNA in reaction A should bind to the mercury–agarose.

Reaction A shows that addition of [γ-S]ATP and [γ-S]GTP to ^{3}H-labeled elongated RNA does not produce ^{3}H-labeled [γ-S]RNA in the absence of transcription. Reaction B shows that [γ-S]RNA will be synthesized after a 15-min transcription in the presence of unmodified nucleotides and that the column used for these four reactions will bind [γ-S]RNA. Reaction B also indicates that the DNase I used to degrade the template does not artifactually degrade the [γ-S]RNA. Reaction C demonstrates that RNA will not bind to this mercury–agarose column in the absence of γ-S-labeled nucleotides. Reaction D shows that a 1-min degradation by DNase I (500 units/mL) will almost completely degrade the template DNA.

(II) Size of [γ-S]RNA Increases with Increasing Synthesis Time. The bulk (flow through) RNA from a yeast nuclear transcription shows a minimal increase in size with increasing synthesis time. A 1-min synthesis has RNA as large as 28 S (Figure 3A). This is because most of the label is being incorporated into in vivo initiated RNA which is already quite large. [γ-S]RNA should show a dramatic increase in size with only very short RNA made during short syntheses. As a second experiment to show that the [γ-S]RNA is the product of in vitro initiated transcripts and not the product of γ-S transfer to in vitro labeled RNA, I have displayed [γ-S]RNA which is synthesized in reactions of increasing times on gels.

The [γ-S]RNA shown in Figure 3B shows a dramatic size increase with increasing synthesis time. Notice that 5S and pre-tRNA peaks can be seen after 1 min of synthesis, indicating the elongation rate is greater than 2 bases/s. I have not attempted to calculate a more exact elongation rate.

One would also expect the ratio of [γ-S]RNA to total labeled RNA to increase as synthesis time increases. Flow-through and bound RNA from each time point in Figure 3 were counted on DE81 filters. Results of this experiment show a slight increase in the ratio of [γ-S]RNA to total labeled RNA and that total synthesis is linear to 20 min (not shown).

Transfer of the γ-S to Other Nucleotides Does Not Occur.[3] If [γ-S]RNAs have a unique initiation point with a specific initiating nucleotide and if nonrandom termination is occurring, in vitro initiated [γ-S]RNAs should appear as bands on gels with the banding pattern of [γ-S]RNAs initiated with [γ-S]ATP different from the banding pattern of [γ-S]RNAs initiated with [γ-S]GTP. If, however, significant transfer of γ-S between nucleotides is occurring, the banding pattern of [γ-S]RNA should not be dependent on which nucleotide [γ-S]triphosphate is present in the reaction mix. Fluorographs of [γ-S]RNAs from reactions containing [γ-S]ATP and [γ-S]GTP are shown in Figure 4.

The fluorographs in Figure 4A show an RNA which is initiated with [γ-S]GTP comigrates with yeast 5S RNA. I have positively identified this RNA as 5S RNA by using hybridization techniques (G. Ide, unpublished experiments). All exposures of the RNA initiated with [γ-S]GTP show this band. If the γ-S affinity group from ATP were being transferred to unmodified GTP, one would expect to see the 5S band in the lane marked [γ-S]ATP. A very faint 5S band can be seen in the [γ-S]ATP lane but only when the fluorograph is exposed for 180 days. This may be due to transfer of the γ-S from [γ-S]ATP to unmodified GTP, but I feel it is more likely that the comparatively faint band (compare the 6-day exposure, lane G, with the 180-day exposure, lane A) is due to minor contamination of [γ-S]ATP with [γ-S]GTP since contamination with other nucleotides is common in commercial nucleotide preparations. Note that the large RNAs initiated with [γ-S]ATP have a much more distinct banding pattern than the large RNAs initiated with [γ-S]GTP. This difference is consistently seen on all gels to date.

[3] I have done experiments to show that labeled, unmodified nucleotide triphosphates will artifactually bind to the mercury–agarose column if cochromatographed with nucleotide [γ-S]triphosphates. Addition of EDTA to column buffers, which eliminates the artifactual binding of unmodified nucleic acids (Reeve et al., 1977), does not eliminate the artifactual binding of unmodified nucleotide triphosphates. Thus, quantitative analysis of γ-S transfer to unmodified nucleotide triphosphates is difficult.

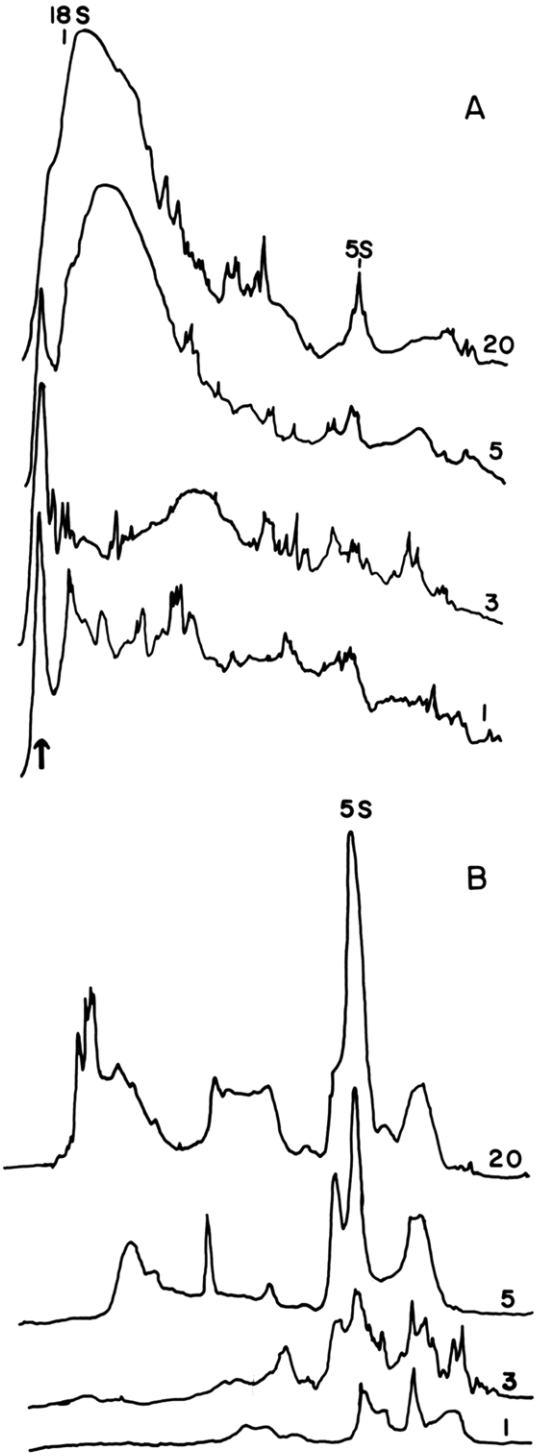

FIGURE 3: Size of bulk and in vitro initiated transcripts with increasing synthesis time. ^{32}P-labeled [γ-S]RNA was synthesized in reactions for the times indicated, isolated on mercury–agarose, and electrophoresed on a 10% acrylamide gel. Data are a scan of an autoradiograph of the dried gel. Electrophoresis is from left to right. Reactions were stopped with 450 μL of NaDodSO$_4$ buffer containing 10 mM EDTA to quench the reactions quickly. Nuclei for this reaction were the washed nuclei used in Figure 5. (A) Flow-through RNA; (B) Bound [γ-S]RNA.

Washing of Nuclei Will Allow Detection of [γ-S]Pre-tRNAs. All pre-tRNAs have a 5′ sequence which is removed as part of processing to mature tRNA. Pre-tRNA can be processed to the mature tRNA by a ribosomal wash fraction (Knapp et al., 1978) or a soluble nuclear fraction (O'Farrell et al., 1978). Thus, processing of yeast tRNA appears to occur by a soluble fraction. The lack of [γ-S]RNA migrating as

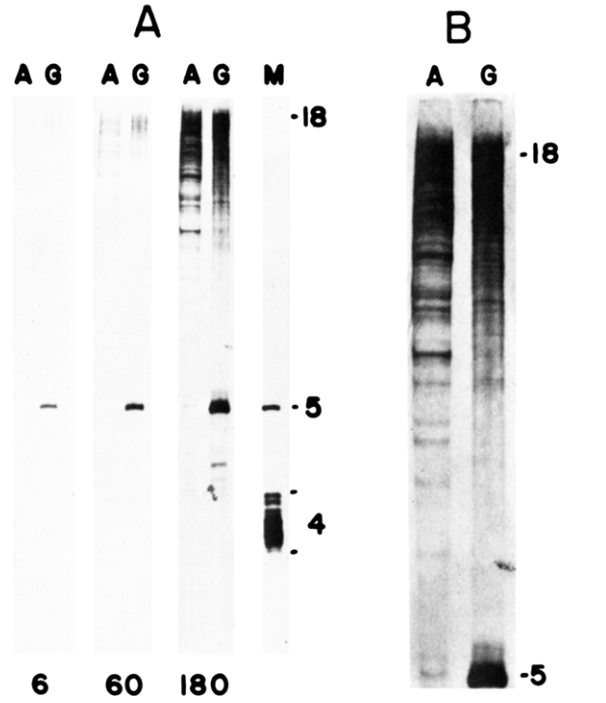

FIGURE 4: Different [γ-S]RNAs are initiated with [γ-S]ATP and [γ-S]GTP. (A) ^{3}H-labeled [γ-S]RNA is isolated from nuclei transcribed 30 min in reactions containing [γ-S]ATP (gel lanes marked A) or [γ-S]GTP (gel lanes marked G), electrophoresed on 6.0% acrylamide gels, fluorographed, and exposed for 6, 60, or 180 days. The lane marked M contains ^{3}H-labeled yeast 5S and 4S RNA. (B) A close-up of the high molecular weight RNA shown in the 180-day exposure of (A).

tRNA precursor molecules (see Figure 4) may be due to rapid processing of the [γ-S]pre-tRNA if the nuclei still contain this fraction. Nuclei which are transcribed after being washed free of soluble processing activity should then allow detection of [γ-S]pre-tRNAs.

To test this hypothesis, I isolated nuclei as described under Materials and Methods, except that the final wash step was repeated. A fluorograph of [γ-S]RNA synthesized by these washed nuclei is shown in Figure 5. Note the broad band of 4.5S RNA and bands between 5 S and 5.8 S which now appear in the [γ-S]ATP lanes (compare with Figure 4A). It is apparent from this exposure (which is equivalent to the 180-day exposure in Figure 4) that washing of nuclei has improved the detection of [γ-S]pre-tRNA.

Distinctly Banded High Molecular Weight [γ-S]RNA Can Be Synthesized in the Presence of High Levels of α-Amanitin. Yeast RNA polymerase I is 50% inhibited at levels of α-amanitin of 300–600 μg/mL whereas yeast RNA polymerase III is not inhibited at α-amanitin levels of 2 mg/mL. Therefore, transcription at levels of α-amanitin greater than 1 mg/mL should yield mainly transcripts by RNA polymerase III. Experiments have shown that nuclei will synthesize RNA as large as 25 S in the presence of 2.4 mg/mL α-amanitin (Schultz, 1978). However, it is not known if these transcripts can be reinitiated in vitro, nor if the transcripts have a specific nucleotide required for initiation.

Figure 6 shows autoradiograms of ^{32}P-labeled [γ-S]RNA transcribed in the presence and absence of 2 mg/mL α-amanitin. RNAs as large as 28 S reinitiated in vitro with either [γ-S]ATP or [γ-S]GTP can be transcribed at these high levels of α-amanitin. Note that the discrete bands present in the [γ-S]ATP (lanes 1 and 2) and [γ-S]GTP (lanes 3 and 4) transcripts are two distinct populations. This difference of [γ-S]ATP and [γ-S]GTP transcripts can always be seen if

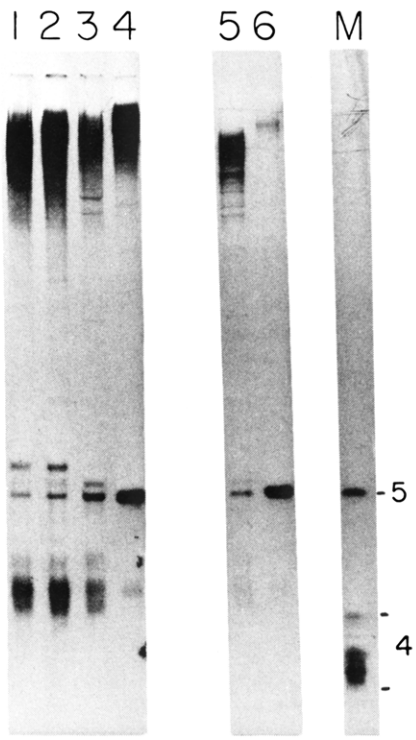

FIGURE 5: Fluorographs of [γ-S]RNA from washed nuclei. Nuclei are prepared as described under Materials and Methods except that the final wash step is repeated. [γ-S]RNAs isolated from duplicate reactions incubated for 5, 10, and 30 min with [γ-S]ATP as the modified nucleotide are shown in lanes 1, 2, and 3. Lane 4 is the [γ-S]RNA from a 30-min reaction with [γ-S]GTP as the modified nucleotide. Lanes 5 and 6 are [γ-S]RNAs synthesized 30 min in the presence of 1 mg/mL α-amanitin with [γ-S]ATP (lane 5) and [γ-S]GTP (lane 6) was the modified nucleotides. The gel is 10% acrylamide. Lane M contains ^{3}H-labeled yeast 5S and 4S RNA.

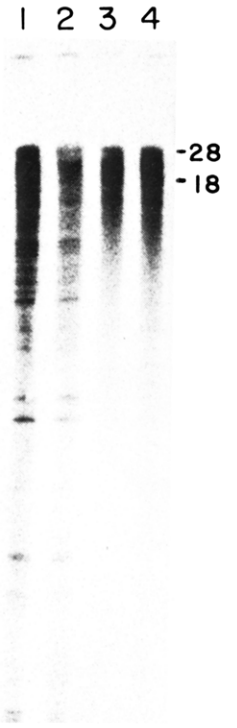

FIGURE 6: High molecular weight RNA initiated with γ-S-labeled nucleotides. ^{32}P-labeled [γ-S]RNA was isolated as described under Materials and Methods, electrophoresed on a 4% acrylamide gel, fixed, dried, and autoradiographed. (Lane 1) [γ-S]ATP present in the transcription reaction, no α-amanitin present. (Lane 2) [γ-S]ATP present in the transcription reaction, 2 mg/mL α-amanitin also present. (Lane 3) [γ-S]GTP present in the transcription, no α-amanitin present. (Lane 4) [γ-S]GTP present in the transcription, 2 mg/mL α-amanitin also present. Marker positions are indicated; the 5S marker was electrophoresed off the gel.

[γ-S]RNA is electrophoresed on gels containing a low percentage of acrylamide.

Discussion

The experiment shown in Figure 1 shows that nonspecific binding of RNA which does not have a γ-S group does not occur in this system. I have found that thorough deproteinization is necessary to eliminate nonspecific binding of RNA. Experiments in which the RNA was not Pronase treated (phenol extracted only) show 0.2% of the labeled RNA will bind to the mercury–agarose column even if no γ-S-labeled nucleotides are present in the reaction mix. The 10 mM EDTA present in the chromatography buffer is also essential to eliminate nonspecific binding of yeast RNA to the mercury–agarose column. If only 1 mM EDTA is present in the chromatography buffer, over 50% of labeled yeast RNA will bind to the column (G. Ide, unpublished experiments).

The control experiments described herein to show that the thiophosphate group is not transferred to other RNA molecules are very important. Experiments that show the RNA populations initiated with [γ-S]ATP and [γ-S]GTP are different are not sufficient to prove the γ-S was not transferred to other RNA molecules. γ-S transfer by kinases could be donor and acceptor molecule specific, giving thiophosphate transfer to different RNA termini, depending on whether the donor thiophosphate was from [γ-S]ATP or [γ-S]GTP. This type of artifact could give the appearance that RNAs "initiated" with [γ-S]ATP and [γ-S]GTP are different when really only the γ-S transfer is specific.

I have analyzed RNA made in the presence of [γ-^{32}P]ATP or [γ-^{32}P]GTP. Either nucleotide will transfer the labeled phosphate to all detectable RNA species present in the nuclei (results not shown), indicating that RNA kinases are active in the yeast nuclei. These data have been confirmed in another laboratory (Bennetzen, 1980). Surprisingly, the data shown herein prove that transfer of γ-thiophosphates does not occur by these kinases. It is apparent that the thiophosphate group is not a substrate for these yeast RNA kinases. Thiophosphate is known to inhibit phosphatases (Goody & Eckstein, 1971; Gratecos & Fischer, 1974), but thiophosphate exchange occurs between nucleotides in a *Xenopus* extract (Hipskind & Reeder, 1980). The almost complete lack of thiophosphate exchange between nucleotides or between nucleotides and nucleic acids in yeast nuclei may be due to thiophosphate inhibition of the yeast phosphate exchange enzymes.

The lack of thiophosphate exchange in yeast nuclei may allow positive identification of the initiating nucleotide for RNA species initiated in vitro. Yeast 5S RNA has a triphosphorylated guanosine at its 5′ end (Hindley & Page, 1972). Hence, that 5S [γ-S]RNA should be initiated with [γ-S]GTP is not surprising. Specificity for a particular initiating nucleotide is not universal, however. Adenovirus VA1 genes show initiation with either of the purine nucleotides, but both gene products are not essential to viral growth (Thimmappaya et al., 1979).

One would not expect high molecular weight RNA to be transcribed by RNA polymerase III since the longest identified polymerase III transcript is smaller than 5.8 S. One cannot rule out the possibility that these high molecular weight RNAs are the result of normal polymerase III transcripts which have transcribed beyond the termination point or are transcribed by an unknown RNA polymerase. However, the flow-through RNA (primarily initiated in vivo) shows α-amanitin-insensitive

high molecular weight transcripts (results not shown) and more than half of the 7S to 25S in vitro synthesized RNA are resistant to 2.4 mg/mL α-amanitin (Schultz, 1978). Thus, it is quite unlikely that this RNA is from improperly terminated small transcripts unless improper termination is prevalent in vivo. The existence of discrete RNA transcripts also argues against improper random termination.

It is important to recognize that the bands of high molecular weight of [γ-S]RNA (>5.8 S) which are seen (Figures 4 and 6) may not be different RNAs but pauses in the transcription of one or a few specific RNAs. This type of behavior has been seen in the transcription of ribosomal genes (Maizels, 1973). It is unlikely that the bands seen here are ribosomal RNA, however, since 2 mg/mL α-amanitin was used for the experiment in Figure 6. This level of α-amanitin should inhibit all polymerase I activity.

The general lack of mature tRNA in the transcription reactions with washed nuclei (Figure 5) may be useful for the isolation of pre-tRNA for tRNA processing experiments.

Acknowledgments

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