AN ABSTRACT OF THE THESIS OF

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Title: IN VITRO TRANSCRIPTION IN THE YEAST: SACCHAROMYCES CEREVISIAE

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Abstract approved: _____
K. E. Van Holde

The structure and transcriptional activity of intranuclear and isolated chromatin from logarithmically growing yeast cells has been compared to chromatin from cells which have entered the stationary phase and ceased growing. Both chromatins show a similar nucleosomal repeat pattern and a 160 bp repeat size when digested with staphlococcal nuclease. The rate of DNase I digestion of growing phase is greater than in stationary. Growing phase nuclei are also 5 to 20 times as active as stationary in the amount of endogenous transcription. Analysis of elongating transcripts indicates the transcriptional differences between growing and stationary are due to differences in *in vivo* initiation. The DNase I susceptability and transcriptional differences noted in nuclei are maintained in sucrose gradient isolated oligonucleosomes and mononucleosomes from the two states.

As an adjunct to structural and transcriptional studies of yeast, a rapid technique for isolation of yeast nuclei has been developed. Briefly, the method consists of layering of the 18% ficoll lysate prepared by the method described in Lohr and Ide (1979), on an isopycnic density gradient of 1M sorbitol, 0.5mM $CaCl_2$ dissolved in a solvent of 35% Percoll (Pharmacia) 65% H_2O, pH 6.5. The gradient is pre-formed before loading by spinning 34ml of the gradient solution contained in a 50ml tube in an SS-34 angle rotor at 37,000 xg for 50 minutes. Six ml of the 18% ficoll lystate is diluted with 6ml 1M Sorbitol 0.5mM $CaCl_2$ and then layered on this gradient. Nuclei are banded free of cell debris by a 7,500 rpm spin in an HB4 swinging bucket rotor for 15 minutes. The resulting band of nuclei is washed by dilution with 2 volumes 1M Sorbitol, 0.5mM $CaCl_2$ pH 6.5 and pelleted at 4300 xg for 5 minutes. Nuclei isolated by this method will incorporate 20 to 40 picomoles UTP into RNA per ug template DNA in a 15 minute synthesis. The nuclei are substantially free of cytoplasmic contamination as measured by alcohol dehydrogenase activities.

Transcription initiation in isolated yeast nuclei by endogenous RNA polymerase has been studied using nucleoside 5'-[γ-S] triphosphates as affinity probes. <u>In vitro</u> initiated RNA can be separated from bulk RNA on a mercury agarose affinity column. Activity that transfers the [γ-S] group to other nucleotides or other RNA molecules (often

troublesome in other systems) cannot be detected. Analysis of the *in vitro* initiated RNA shows that 5S and pre t-RNA are initiated *in vitro* by endogenous RNA polymerase III. Endogenous RNA polymerase III also initiates a discrete distribution of RNA species as large as 28S. The RNA populations initiated with 5'-[γ-S] adenosine 5' triphosphate and 5'[γ-S] guanosine 5' triphosphate are different.

In vitro Transcription in the Yeast: *Saccharomyces cerevisiae*

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in charge of major

Redacted for Privacy

Chairman of Department of Biochemistry
and Biophysics

Redacted for Privacy

Dean of Graduate School

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Typed by Deanna L. Cramer for Gregory James Ide

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Introduction

Much effort has been directed toward determining the structural basis of differential gene expression in eukaryotes and, recently, its relationship to the nucleosomal organization of chromatin (Felsenfeld, 1978). Because of the limited amount of transcription in most cells, systems generally used (for example, chicken reticulocyte-erythrocyte, hen oviduct) probe the structure of a very small amount of active DNA (one or a few genes) in a high background of non-active DNA. Also, several fractionation schemes to separate euchromatin from heterochromatin have been devised (Gottesfeld and Butler, 1977; Gottesfeld and Partington, 1977; Sanders and Hsu, 1977; Itzhaki *et al.*, 1978). At best these approaches will enrich for actively transcribed chromatin, usually in a large background of non-transcribed chromatin.

As another system to approach the question of differential gene expression, the transition of yeast chromatin as cultures grow from the very active, logarithmically

growing phase into the stationary phase is studied here. As cells cease growing, DNA replication certainly ceases and transcription should decrease. In fact, Gross and Pogo (1974) have shown that starvation of yeast spheroplasts produces a striking decrease in transcription rate which is reflected in rates of incorporation in isolated nuclei. This system has several advantages for studying the relation of transcription to chromatin structure: (1) the changes are reversible; upon addition of fresh medium, cell growth resumes after a brief lag phase; (2) the different states can be produced from the very same inoculum without addition of exogenous, potentially perturbatory substances; (3) in growing yeast at least 40% of the rather small yeast genome is transcribed into poly A containing cytoplasmic RNA (Hereford and Rosbash, 1977) and obviously 100% of the genome is replicated. It can also be argued that the proportion of growing chromatin which behaves like active chromatin is very high. Yeast DNA sequences which are transcribed into cytoplasmic poly A^+ mRNA have the same accessibility to DNase I digestion as the remainder of the DNA in the yeast genome (Lohr and Hereford, 1979b). It has been shown in chicken DNA that sequences which are transcribed or transcribable have an elevated DNase I sensitivity when compared to non-transcribed sequences (Weintraub and Groudine, 1976; Garel and Axel, 1976). If this analogy extends to yeast the

DNase I results and the Mg^{++} solubility results indicate that in growing yeast the entire genome possesses a conformation associated with transcriptionally active chromatin. Thus, chromatin studies on growing yeast should give information about the structure of active chromatin.

To look for observable structural differences between the two states of yeast chromatin, nuclease probes have been used (staphylococcal nuclease, DNase I). Endogenous transcription studies show that functional activity differences between the two states are maintained in isolated nuclei. Staphylococcal nuclease digests of intranuclear chromatin have been fractionated on sucrose gradients to show that the observed structural and functional differences noted in nuclei are preserved in isolated chromatin fragments. Finally, experiments are discussed which attempt to determine the basis for the functional and the structural differences noted between growing phase and stationary chromatin.

Experimental Procedures

Growth of Cells, Isolation and Nuclease Digestion of Nuclei

Cells (Y55) for "growing" and "stationary" cultures were inoculated from the same inoculum, allowed to grow as described (Lohr *et al.*, 1977b), for the same number of doublings and harvested at a density of 4-5 x 10^7 (growing)

and 20-30 x 10^7 (stationary). Both sets of cells were washed with H_2O, pretreated in 0.1M Tris - 0.1M EDTA pH = 8.0, 0.5% β-mercaptoethanol for 30 min at 4°C (growing) and 32°C (stationary), spun at 10,000 xg for 10 minutes, washed in S buffer (1.1M sorbitol, 24 mM KH_2PO_4 pH = 6.5) and spun again as before. Cells were resuspended in S buffer plus 0.4 mM Ca^{2+} at 0.25 g/ml, zymolyase Z-5000 (Kirin Breweries Ltd.) was added to 1.5 mg/ml. Excellent spheroplasts are obtained in 30 minutes to 1 hour at 32°C. Spheroplasts were isolated by centrifugation at 3000 xg for 10 minutes. Growing and stationary nuclei were isolated as before (Lohr et al., 1977b) except: PMSF was added to 1 mM at the 18% Ficoll step, to 0.5 mM at the HM resuspension and to 0.5 mM in the digestion buffer; the lysate in 18% Ficoll was scrubbed by 5-10 up and down strokes with a Teflon pestle in a test tube. Staphylococcal nuclease digestion was done at 200-400 μg/ml DNA, 200 U/ml enzyme for 15-20 minutes, EDTA was added to 4 mM and the chromatin dialyzed according to Nelson et al., 1977 (for sucrose gradients) or the reaction stopped and DNA isolated and acid solubilities done as per Lohr et al., 1977a) (for time courses of digestion). DNase I digestions were done at 200-400 μg/ml DNA, 50-60 U/ml enzyme for varying periods of time, the DNA isolated and acid solubility determined as described (Lohr et al., 1977b).

Isolation and Analysis of Nucleoprotein Particles

Staphylococcal nuclease digests, after dialysis, were concentrated and put on 15-30% isokinetic sucrose gradients (McCarty *et al.*, 1974), and spun for 16-18 hours at 38,000 RPM in an SW40 rotor. Fractions were collected dropwise, through the bottom of the tube.

Samples for DNA gels were treated with RNase (Lohr *et al.*, 1977b), made 2.0% in Sarkosyl, 0.15M NaCl, pronase added to 50 μg/ml and put on gels. Samples for determining RNA/DNA ratios were hydrolyzed in 0.3N KOH for 1 hour at 37°C, $HClO_4$ added to 0.3N on ice, spun at 10,000 xg for 10 minutes, the supernatant (S_1) removed and the pellet hydrolyzed by 0.5N $HClO_4$ at 70°C for 20 minutes, and spun at 10,000 xg for 10 minutes and the supernatnat removed (S_2). The A_{260} values of S_1 (RNA) and S_2 (DNA) were read and compared to known concentrations of RNA and DNA subjected to the same procedure.

Redigestion of Oligomeric Nucleoprotein

Samples were made 0.5 mM in Mg^{2+}, DNase I added to 15-50 U/ml and digestion done for various times. Reactions were stopped, DNA extracted and acid solubilities

determined as per Lohr *et al*. (1977b) using the modified DABA procedure of Lohr *et al*. (1977a). Three percent non-denaturing gels were run as in Lohr *et al*. (1977b), 8% polyacrylamide-urea denaturing gels were run according to Maniatis *et al*. (1975).

Nuclear and Oligomeric and Monomeric Nucleoprotein Endogenous RNA Synthesis

RNA syntheses were conducted in a final volume of 37.5λ containing the following components: 10 mM Tris-HCl (pH 7.9, 23°C); 100mM KCl; 10 mM $MgCl_2$; 240 μM ATP, CTP and and GTP; 10 μM ^{3}H-UTP (1.6 Ci/mmole). Nuclear assays also contained 1M Sorbitol; 0.5 mM $CaCl_2$; 5 mM phosphoenolpyruvate and 20 μg of pyruvate kinase per ml (Schultz, 1978). Isolated nucleoprotein assays contain 0.55 μg DNA (by DABA) as chromatin. Nuclear assays contain 20λ nuclei (∿200-400 μg DNA/ml), isolated as described. Assays were incubated at 25°C for 15 min and terminated with 2 ml ice, cold TCA and 10 μg calf thymus DNA (GF/C assays) or by addition of 5 volumes 1% Sarkosyl containing 50 mM pyrophosphate (DE81 assays). Acid precipitable radioactivity in RNA was assayed on Whatman GF/C filters as described (Adman *et al*., 1972) or by the DE81 filter method (Roeder, 1974). Calculation of the number of picomoles UMP incorporated is by the method outlined in Appendix I. Analysis of ^{3}H uridine and ^{3}H uridine monophosphate is done by using the method of

Cox *et al*. (1973b). Experiments to test the Mg^{++} solubility of yeast nucleoprotein were done using the method of Gottesfeld and Butler (1977).

Results

Isolation of Stationary Phase Nuclei

Nuclei are isolated by a commonly used procedure (Wintersberger *et al*., 1973). In one of the spins, in 18% Ficoll, in which growing phase nuclei pellet most of the nuclei from stationary phase float (Duffus, 1975)! This is due to the attachment to the nucleus of a vesicular-like sphere which is probably the vacuole. The attachment can be broken by a few strokes homogenization with a Teflon pestle in a test tube (Wintersberger, private communication) with no apparent damage to the nuclei. Homogenization is now used in the growing phase nuclear isolation also, after lysis in Ficoll, so that the two kinds of nuclei are isolated by the same procedure.

Nuclei isolated from the two states appear slightly different in the light microscope. Both kinds are basically spherical objects but the growing phase nuclei are somewhat larger (presumably more swollen) and the nuclear surface has a rougher texture compared to the smoother, smaller stationary nuclei.

The Basic Nucleosomal Structure

Digestion of chromatin by the enzyme staphylococcal nuclease (E.C. 2.1.4.7) provides a measurement (Kornberg, 1977) of the length of the repeating unit of chromatin (core + spacer DNA: Axel, 1975; Sollner-Webb and Felsenfeld, 1975; Van Holde *et al.*, 1975). The DNA produced by staphylococcal nuclease digestion shows the same repeat size and the same course of size decrease with digestion for the stationary and growing phase chromatin (Figure 1).

DNase I Digestion

DNase I digestion of intranuclear chromatin from growing cells produces a series of regularly spaced, discrete bands up to at least 300 b (Lohr *et al.*, 1977c). Studies of chromatin from stationary cells show the digestion goes significantly slower than the digestion of chromatin from growing cells. The ladder (<110 b) and the extended ladder (>140 b) appear quite distinctly in both growing and stationary nuclei (Figure 2).

That the DNase I digestion proceeds more rapidly in the growing phase nuclei may be explained by differences in chromatin structure or in some other aspect of nuclear structure, such as permeability of the nuclei. However, DNase I digestion of isolated chromatin fragments suggests

Figure 1. The solid lines are best fits of the double-strand DNA fragment sizes from staphylococcal nuclease digests of nuclei from growing cells, plotted as a function of the % DNA rendered acid soluble. The circles (O) show the double strand sizes of DNA fragments from a digestion of nuclei from stationary cells. All sizes were determined in comparison to PM2-HaeIII restriction fragments run in parallel tracks of the same gel. I, II, III, IV refer to the DNA size classes 1, 2, 3, and 4 times the basic repeat size class.

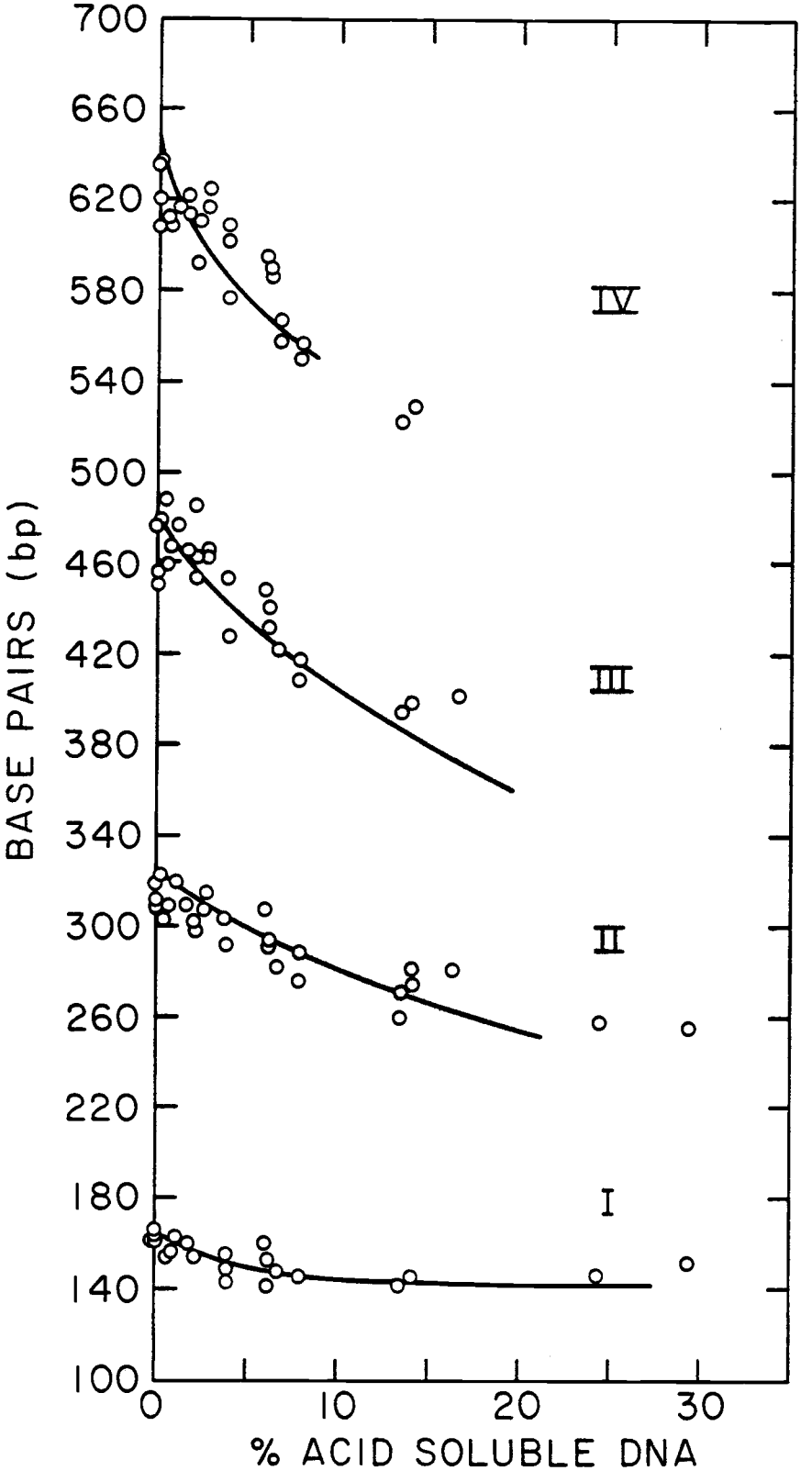
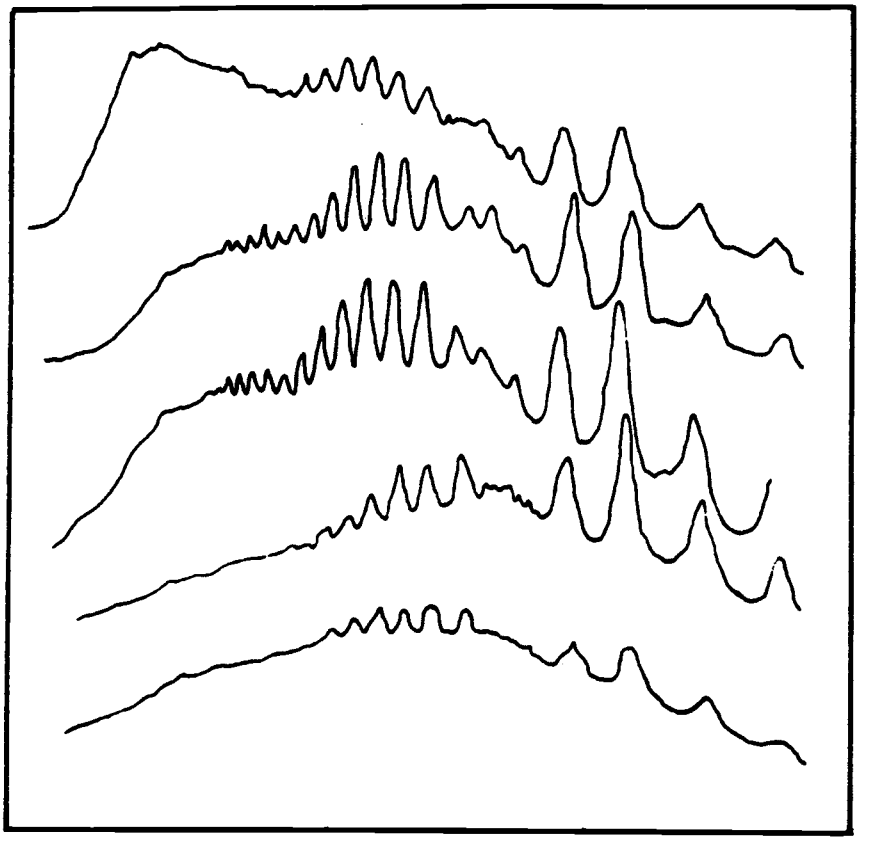

700
660
620
580
540
500
460
420
380
340
300
260
220
180
140
100
BASE PAIRS (bp)
0
10
20
30
% ACID SOLUBLE DNA
IV
III
II
I

Figure 2. Scans of DNase I digests of growing and stationary nuclei from yeast strain, A364a, a haploid, run on an 8% polyacrylamide-urea denaturing gel. Migration is from left to right. The scans show from top to bottom: stationary, 1 min digestion; growing, 1 min digestion; stationary, 3 min; growing, 2 min; stationary, 7.5 min of digestion all done at the same DNA and DNase I concentrations.



that the differences are due to chromatin structural differences (see Figure 3B in Lohr and Ide, 1979a).

Nuclear Transcription

Yeast nuclei can perform endogenous transcription (Schultz, 1978). As shown in Table I our nuclei from growing cells incorporate ^{3}H-UTP into an acid insoluble form at several times (5-20 times) the rate of incorporation in nuclei from stationary cells. All four ribonucleoside triphosphates are required for this process and the product is RNase sensitive as expected for DNA dependent RNA synthesis. These differences are due to differences in RNA synthesis and not differential degradation because 1) the incorporated counts are stable in the absence of continued incorporation (Table I), and 2) a mixture of stationary and vegetative chromatin (obtained from lysed nuclei) maintains a high level of incorporation and does not show a drastic decrease which would be expected if increased endogenous RNase were the explanation for the low level of incorporation in stationary phase (Table I).

Yeast possesses three classes of RNA polymerases (Schultz and Hall, 1976; Valenzuela *et al.*, 1976); which vary in their sensitivity to the mushroom toxin, α-amanitin. Based on α-amanitin sensitivity determined *in vitro* (Schultz and Hall, 1976; Valenzuela *et al.*, 1976), all three polymerases are probably active in nuclei (Schultz,

Table 1. Endogenous transcription in nuclei.

	Endogenous	+ RNase	+ ^{3}H-UTP only
Growing	8500	0	0
Stationary	600	0	0
	Endogenous	**+ 30 min in EDTA**	**Endogenous chromatin**
Growing	10000	13700	10400
Stationary	800	1100	1300
Growing and Stationary	-	-	9800

Endogenous transcription was done in isolated nuclei as described in Experimental Techniques. Values are in cpm ^{3}H-UTP incorporated/μg DNA (background subtracted).

(+ RNase) - RNase (300 μg/ml, 15 min) added subsequent to incorporation.

(^{3}H-UTP only) - all incorporation conditions as usual except only 1 nucleotide triphosphate (UTP) was added.

(+ 30 min. in EDTA) - EDTA was added to the endogenous incorporation and the sample remained at incorporation conditions for 30 minutes.

(Endogenous chromatin) - aliquots of nuclei were pelleted, lysed in low ionic strength buffer and endogenous transcription performed on growing chromatin alone, stationary chromatin alone and a mixture of the two (mixed at nuclei stage).

1978; Bennetzen and Hall, private communication), from both growing and stationary cells (Figure 3A). The relatively small percentage of II activity compared to higher I + III activities is characteristic of the yeast cell (Sripati and Warner, private communication). Certainly I observe some transcription by polymerase II, which is inhibited by α-amanitin concentrations of 10 μg/ml, and by III which also may transcribe some single copy genes in yeast (Schultz, 1978) and is α-amanitin resistant.

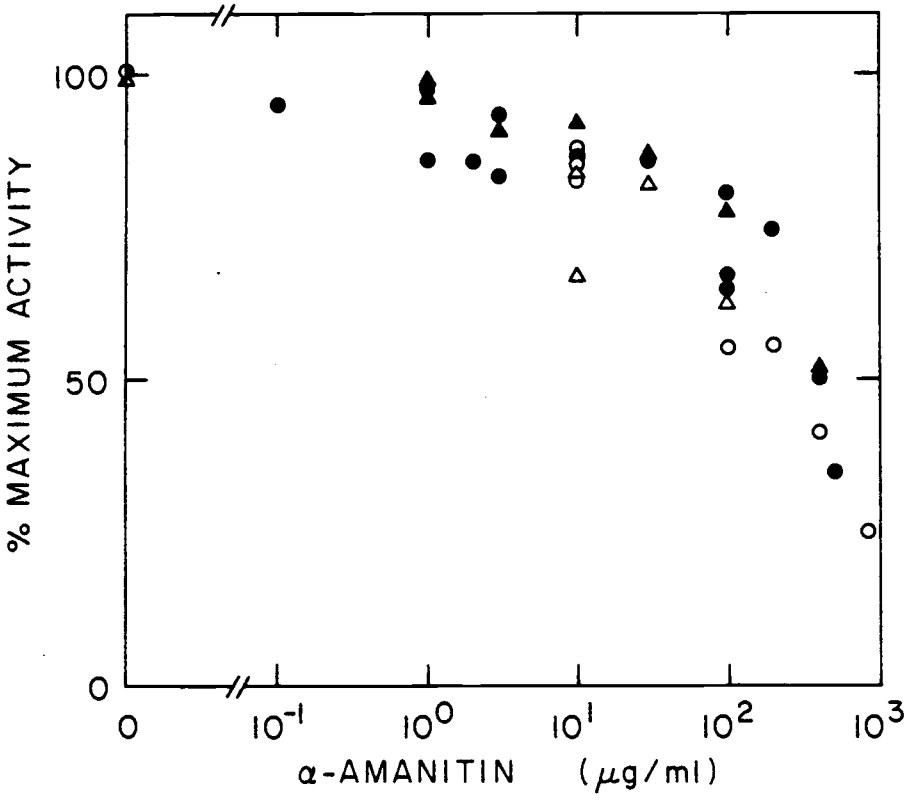
The relatively small percentage (10-30%) of II activity compared to higher I + III activities is characteristic of the relative amounts of transcription observed in the intact yeast cell itself (Sripati and Warner, private communication). Thus all three RNA polymerase activities are present in our isolated nuclei with no apparent substantial enrichment or loss of any particular polymerase fraction. Profiles of transcriptional activity as a function of salt are shown in Figure 3B.

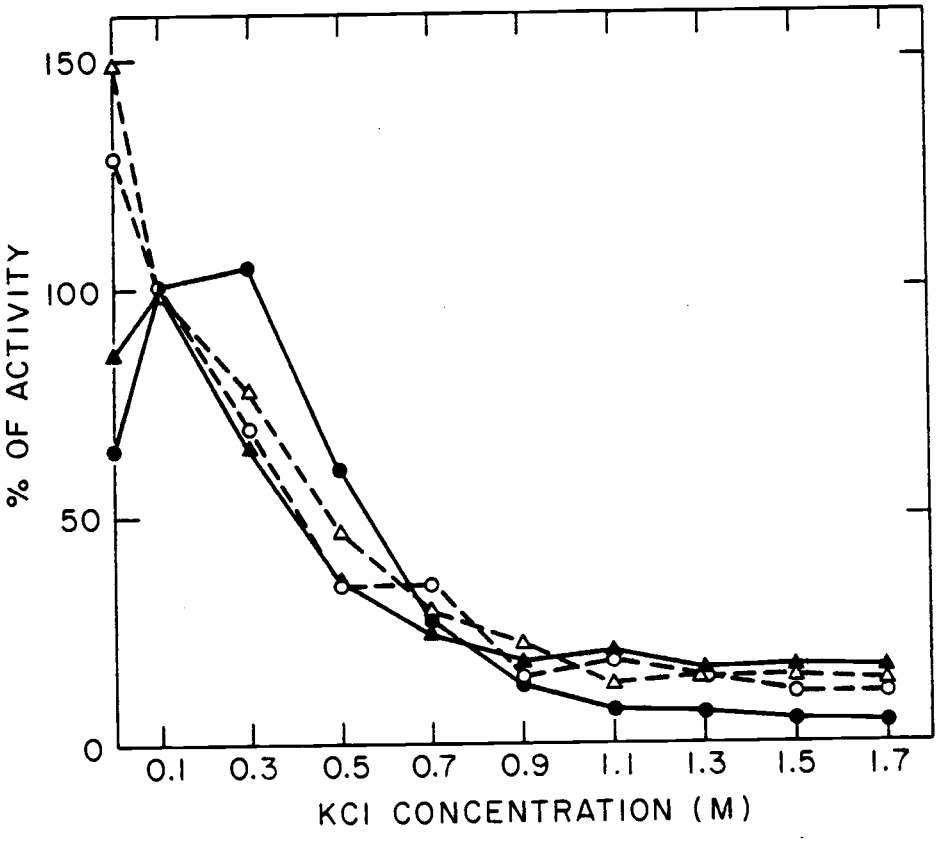
Compared to nuclei from other eukaryotes, growing yeast nuclei synthesize RNA at a very high rate. For example, the growing phase nuclei are even more active than these incorporations show because there are cold UTP pools in the nuclei which dilute the label. This can be shown by increased incorporation using higher levels of radioactivity. Nevertheless, rates of 35-40 pmole/μg DNA in a 15 minute incorporation have been measured. Thus, yeast is an

Figure 3. Effect of α-amanitin and KCl concentrations on endogenous RNA synthesis.

A. Conditions are as given in experimental procedures with α-amanitin varied as shown. (● and ▲) growing and stationary oligomeric nucleoprotein, the "oligo" fraction (see Table II), (O and Δ) growing and stationary nuclei.

B. Aliquots of nuclei or chromatin were assayed as in experimental procedures, varying KCl concentration as shown. Data was normalized to 100% at 0.1M KCl. The data is an average of a number of preparations. (● and ▲) growing and stationary oligomeric nucleo protein, the "oligo" fraction (see Table II), (O and Δ) growing and stationary nuclei.


100
50
0
% MAXIMUM ACTIVITY
0
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
α-AMANITIN (μg/ml)


150
100
50
0
% OF ACTIVITY
0.1
0.3
0.5
0.7
0.9
1.1
1.3
1.5
1.7
KCl CONCENTRATION (M)

uniquely active system in which to study transcription and the structure of transcriptionally active chromatin.

Isolation of Oligomeric Nucleoprotein Particles

Can the kinds of differences noted in stationary vs. growing nuclei be maintained in isolated, fractionated chromatin? To this end, staphylococcal nuclease intranuclear chromatin digests have been fractionated by sedimentation in isokinetic sucrose gradients (Nelson *et al.*, 1977). Recently another report suggesting that transcriptionally active chromatin can be thus obtained has appeared (Tata and Baker, 1978).

Compared to nucleosomes from organisms with less active genomes, there are very large amounts of RNA relative to the amounts of DNA on both kinds of nucleosomes. For example, the "oligo" (refer to Table IIA) fraction from growing phase can have RNA/DNA ratios of 1.5/1, while even monomers have a ratio of 1.3/1. If special care is taken to prevent RNase activity (for example, use of diethylpyrocarbonate), these ratios can be even higher. As might be expected, there is generally less RNA associated with a given amount of DNA on oligonucleosomes from the inactive state than from the active (for example, stationary "oligo" RNA/DNA = .9).

Table II. Endogenous transcription in isolated oligomeric and monomeric nucleoprotein particles.

A) Oligomeric and Monomeric Nucleoprotein

	(1) Endogenous	(2) +DNase I	(3) +RNase	(4) ^{3}H-UTP only
Stationary				
"Huge"	1,000	<100	100	100
"Oligo"	1,000	100	<100	200
"Mix"	500	100	<100	200
"Mono"		-	100	100
Growing				
"Huge"	33,500	250	200	100
"Oligo"	16,100	300	250	200
"Mix"	5,800	200	200	200
"Mono"	2,000	-	100	100

Nucleoprotein fractions from sucrose gradients were pooled into the following designations: "Huge", containing mainly DNA >1000 bp (>6-7 nucleosomes); "Oligo", containing mainly DNA 500-1500 bp (3-10 nucleosomes); "Mix", containing DNA 140-600 bp (1-4 nucleosomes); "Mono", composed almost entirely of 160-140 bp DNA (1 nucleosome). "Mono" shown in this table is from a different (more active) preparation, so numbers are not directly comparable to the Huge, Oligo, and Mix incorporations shown. However, I have observed the same trends in incorporation levels in

Table II (continued)

each of the 11 experiments done to date. (1) Endogenous transcription as described in Experimental Techniques. (2) Endogenous transcription after (RNase free) DNase I treatment (130 U/ml; 15 minutes). (3) Endogenous transcription followed by pancreatic RNase A treatment (300 μg/ml, 15 minutes). (4) Endogenous transcription as in (1) but using only the labeled nucleotide, in this case ^{3}H-UTP. Values are in cpm ^{3}H UTP incorporated per μg DNA per 15 minute assay.

B) Oligomeric Nucleoprotein Compared to DNA

Huge growing	21,400
Huge stationary	2,300
Calf thymus DNA + Pol II	10,200

Endogenous transcription of the "Huge" nucleoprotein fraction containing DNA, mainly 10-20 x repeat size, were done as described (Methods). High molecular weight, denatured calf thymus DNA was transcribed by wheat germ polymerase II under template limiting conditions, using the same buffers and nucleotide levels as for endogenous transcription. Values are in cpm/μg DNA.

Table II (continued)

C) Oligomeric and Monomeric Nucleoprotein

	Growing Phase		Stationary Phase	
	Endogenous	+Pol II	Endogenous	+Pol II
Huge	40,500	43,000	3,000	3,500
Oligo	26,900	24,600	3,400	3,100
Mix	4,700	5,400	1,040	810
Mono	1,800	1,500	110	130

Endogenous transcriptions of the Huge (containing DNA mainly 10-20 x repeat size), Oligo (containing DNA mainly 4-8 x repeat size), Mix (containing DNA mainly 2-4 x repeat size) and Mono (containing DNA mainly 1 x repeat size) nucleoprotein fractions from sucrose gradients were transcribed as described (Methods) in the absence (endogenous) and presence of wheat germ polymerase II.

DNase I Digestion of Isolated Oligomeric Nucleosomal Particles

As with nuclei, isolated nucleoprotein particles from growing cells show increased DNase I susceptibility compared to particles isolated from stationary cells. The gels in Lohr and Ide (1979a, Figure 3) show that the course of size decrease for digestion of oligomeric and monomeric nucleosomal particles goes visibly faster for growing particles even though DNA, DNase I and Mg^{++} concentrations are the same for both digestions. Thus some structural aspect of growing phase oligonucleosomes and mononucleosomes renders them preferentially DNase I sensitive.

The differential rate of transcription between stationary and growing chromatin is also seen in isolated chromatim (Table IIA-C). ^{3}H-UTP is incorporated into RNase sensitive material in growing chromatin fragments at 10-40 times the rate in fragments from stationary. Growing phase chromatin is also more active than naked DNA (Table IIB) transcribed by wheat germ Pol II under template limiting conditions. Incorporation of label is prevented by prior digestion of the chromatin by RNase free DNase I (Table IIA); there are quite low levels of background incorporation when only the labeled nucleotide is added (Table IIA). Furthermore, the same sort of differences are observed when other ^{3}H nucleotides or α-labeled ^{32}P-ATP is used as the incorporated isotope instead of ^{3}H-UTP (not shown) Thus

the ^{3}H-UTP incorporation must reflect DNA dependent RNA polymerase activity, in these isolated nucleosomes.

Not all chromatin fragments from growing nuclei are equally active; generally speaking, the larger the size of the chromatin fragments, the greater the transcription rate (Table II). Furthermore, the ratio of the transcription rates between the various pools remains constant throughout a time course of incorporation from 10 seconds through 15 minutes (Ide and Lohr, unpublished observations), so it must reflect a true rate difference. Thus some aspect necessary for transcription is diminished in smaller oligo or mononucleosome sized particles.

The α-amanitin and salt profiles (Figure 3) of transcription in these fragments resemble the nuclear patterns. There is clearly some transcription (10-30% depending on the preparation) by polymerase II, the most sensitive to α-amanitin. RNA polymerase must be rather firmly attached to the chromatin fragments, for it remained bound through a dialysis and sucrose gradient preparation in the presence of EDTA and the salt profiles show that fairly high levels of salt are required to remove it. Interestingly, (1) the maximum incorporation for nuclei occurs at 0 salt but at 0.1-0.3M KCl for the isolated nucleosomes; (2) the maximum ratio of activity (growing/stationary nucleosomes) occurs at .3-.5M KCl as if it is easier to dissociate some part of the endogenous polymerase from the

stationary chromatin at these lower salts. Increasing levels of salt cause the ratio of incorporated counts (growing/stationary) to decrease (Figure 3).

There is little initiation, as measured by rifamycin AF/103, γ-SH RNA isolation (Chapter 3 of this thesis) or by addition of exogenous DNA, so that the main activity must be chain elongation as others have noted (Cox, 1973a) for chromatin. Addition of exogenous RNA polymerase II from wheat germ makes little difference in incorporation (Table IIC) in either growing or stationary. Thus the chromatin template must not be readily accessible to exogenous polymerase.

Addition of Sarkosyl to the Transcription Mix Does Not Change the Relative Activity of Growing vs. Stationary

Why are the growing phase chromatin fragments so much more active in transcription? One can suggest several explanations. Perhaps there are fewer RNA polymerases bound to the stationary particles. On the other hand, there may be conformational states of the chromatin strand (for example, supercoiled states) which favor (or disfavor) transcription such that the polymerases remain bound to both, but are more active on growing phase chromatin. Treatment of chromatin with the detergent Sarkosyl has been shown to cause the dissocation of chromosomal proteins but leave RNA polymerase (Green and Brooks, 1976; Green *et al.*,

1975), which can still synthesize RNA. If comparable amounts of RNA polymerase were bound to growing and stationary chromatin fragments but the stationary fragments are in a conformation which inhibits transcription, treatment with Sarkosyl should release this inhibition and growing and stationary incorporation levels should become more comparable. Table III shows that this is not the result.

Low Sarkosyl (0.22%) or amounts which have been shown to release most of the chromosomal proteins (0.45%) (Scheer, 1978) caused the level of incorporation in stationary to decrease as fast or faster than for growing phase chromatin. Thus we conclude that the most likely explanation for the differences in incorporation levels between growing and stationary chromatin is that there are different amounts of RNA polymerase bound to the chromatin fragments in the two states. It is also possible that the polymerase bound to stationary chromatin is a less active enzyme form than the polymerase on growing chromatin. Experiments to quantitate the number of growing RNA chains indicate the differences in incorporation are due to different amounts of RNA polymerase bound to the chromatin.

Solubility Studies of Isolated Nucleosomal Particles

Gottesfeld and coworkers (Gottesfeld and Butler, 1977; Gottesfeld and Portington, 1977) have described a method for separating transcriptionally active (soluble in Mg^{2+})

Table III. The effect of Sarkosyl on endogenous transcription.

	Growing Phase		Stationary Phase	
	0.22%	0.44% Sarkosyl	0.22%	0.44% Sarkosyl
Huge	$(\frac{13700}{38900}) = 0.35$	$(\frac{11000}{38900}) = 0.28$	$(\frac{850}{2600}) = 0.33$	$(\frac{550}{2600}) = 0.21$
Oligo	$(\frac{8300}{13900}) = 0.6$	$(\frac{4600}{13900}) = 0.33$	$(\frac{900}{3500}) = 0.26$	$(\frac{950}{3500}) = 0.27$
Mix	$(\frac{4500}{7100}) = 0.63$	$(\frac{3400}{7100}) = 0.48$	$(\frac{450}{1700}) = 0.26$	$(\frac{450}{1700}) = 0.26$

Endogenous transcription of the fractions shown (see Table II for sizes) were done in Methods except that in some cases the incorporation mix also contained the indicated % Sarkosyl. The numbers in parenthesis are the cpm ^{3}H-UTP incorporated per μg expressed as

$$\frac{\text{cpm/}\mu\text{g DNA indicated Sarkosyl}}{\text{cpm/}\mu\text{g DNA 0 Sarkosyl}}$$

to give the ratio shown to the right of each parenthesis.

from inactive chromatin (insoluble in Mg^{2+}) in rat liver. When we apply this separation technique to isolated yeast chromatin fragments ("oligo", refer to Table II) we find that 85-95% is Mg^{2+} soluble (Table IV) compared to only 15-20% for rat liver (Gottesfeld and Butler, 1977a; Gottesfeld and Portington, 1977b). Brief, RNase treatment causes 85-90% of the fragments to become insoluble in this procedure (not shown).

Both growing and stationary chromatins behave similarly. Since Gottesfeld and Butler suggest that it is the presence of RNA on the chromatin which accounts for the solubility differential and since stationary clearly has RNA, this result is not surprising. Stationary phase chromatin exists in a depressed state of transcriptional activity but perhaps since the state must be readily reversible, profound changes, such as total loss of nascent RNA, do not readily occur. Rather, the rate of transcription is most affected.

Measurement of the Number of Growing RNA Chains and the Rate of Synthesis

The number and length of radioactive RNA chains synthesized by stationary and growing chromatin preparations can be determined by alkaline hydrolysis of the labelled RNA. Nucleotide units from 3'-ends, chain interiors and 5'ends of RNA chains are hydrolysed to ribonucleosides,

Table IV. Mg^{++} solubility of nucleoprotein particles.

	Control	2mM $MgCl_2$
Growing "Oligo"	100	97
Stationary "Oligo"	100	99

Samples of chromatin were dialyzed with 25mM Tris-acetate buffer, pH 6.6. Aliquots were then allowed to sit on ice 20 min, then spun at 4300 xg (6000 rpm in SS34 rotor). Supernatants and pellets were separated and assayed for DNA content by a modified DABA assay. Values are the percentage of the chromatin remaining in supernatant after treatment.

Table V. Comparison of synthesis rate and number of growing chains in stationary and growing chromatin.

		UMP (cpm)	U (cpm)	UMP/U
Stationary Oligo	control	5,920	60	99
Stationary Oligo	chase	5,980	0	
Growing Oligo	control	45,200	576	78
Growing Oligo	chase	42,400	0	

Aliquots of "oligo" preparations were transcribed for one minute (control), or transcribed for one minute and chased with cold UTP for one minute (chase). RNA was isolated, alkaline digested and PEI chromatographed as in Cox _et al_. (1973b).

ribonucleoside monophosphates and ribonucleoside tetraphosphates, respectively. The hydrolysis products can be separated by thin layer chromatography on PEI plates (Barry and Gorski, 1971; Cox et al., 1973b). The number of labeled ribonucleosides indicates the number of growing RNA chains, the ratio of labelled monophosphates to ribonucleosides gives an estimate of the average rate of synthesis.

The results of these experiments (Table V) indicate the rate of endogenous RNA synthesis is approximately the same in stationary and growing chromatin as evidenced by the U/UMP ratio. However the number of elongating chains in the growing chromatin is almost ten fold that seen in the stationary chromatin, suggesting the number of bound RNA polymerases is much higher in the growing chromatin. The chase experiment is added to demonstrate the RNA chains are elongating at the time the reaction is stopped.

Discussion

Since almost half the yeast genome is transcribed (Hereford and Rosbash, 1977) in growing cells, one would expect that studies of bulk yeast chromatin would include much (but not only) information about the structure of genetically active as well as information about inactive chromatin. However, there is evidence suggesting that in fact a much higher proportion of growing chromatin behaves

like active chromatin. Yeast DNA sequences which are transcribed into cytoplasmic poly A^+ mRNA have a DNase I susceptibility which is identical to the remainder of the (presumably untranscribed) DNA of the yeast genome (Lohr and Hereford, 1979b). Weintraub and Groudine (1976) and Garel and Axel (1976) have shown that there is a particularly DNase I sensitive conformation associated with transcribed and transcribable chromatin. If the analogy extends to yeast, the DNase I results and the Mg^{2+} solubility results suggest that in growing yeast, the entire genome possesses the same conformation, a conformation associated with transcriptionally active chromatin by two criteria. Thus, bulk chromatin studies in growing yeast should yield mainly information about the structure of active chromatin.

The endogenous transcription results are quite important because they suggest that one can isolate in nuclei and more surprisingly, even in nuclease produced oligonucleosomes, chromatin which maintains functional characteristics of the *in vivo* state. Thus nuclei and oligonucleosomes from growing cells incorporate ^{3}H-UTP into RNase sensitive material at an order of magnitude higher rate than the same material from stationary cells. α-Amanitin sensitivity suggests that all three polymerases are involved. One cannot be sure that the transcription enhancement is not due to only a small subfraction of the chromatin which could be invisible to bulk studies. However, if it is only a

subfraction, this subfraction must include all three RNA polymerase activities (Figure 3). Thus there is reasonable confidence that the nuclei and even the isolated chromatin maintains its *in vivo* conformation and so constitutes a valuable material upon which to perform structural studies comparing growing and stationary chromatin.

Structurally speaking, the basic nucleosomal architecture, as elicited by nucleases, seems at least similar in both states. Staphylococcal nuclease digestion of both active and inactive states of yeast chromatin produces size classes of DNA reflecting the presence of nucleosomal repeating units, spaced at an average of about 160 bp along the chromatin strand.

Nucleosome phasing, which refers to the presence of discrete, and possibly quantized, spacer lengths between core particles along the chromatin strand, producing the observable result that the ladder of DNA fragments produced by DNase I extends to 300 bases or more in size, seems to be present in inactive as well as active chromatin. On the other hand, although producing the same pattern, digestion by DNase I does proceed faster on the more active chromatin as measured by the rate of the size decrease of the DNA fragments with digestion.

Interestingly, the behavior of the active state of chromatin in yeast compared to the stationary closely parallels the results obtained on multicellular eukaryotic

chromatin when the structure of the subset of active genes in a genome is compared to the structure of the bulk (inactive) chromatin. Transcriptionally active genes can be isolated as core particle nucleosomes (Lacy and Axel, 1975), so presumably contain histones; the nucleosomal repeat size reflects the bulk nucleosomal repeat size (Bellart et al., 1978). Digestion by DNase I produces fragments of the usual submonomer DNase I ladder (Camerini-Otero et al., 1978), yet the digestion of active chromatin by DNase I proceeds at a distinctly elevated rate (Weintraub and Groudine, 1976; Garel and Axel, 1976). Thus these results in yeast may relate to the structure of active chromatin in multicellular eukaryotes also. The powerful advantage of the yeast system is that the structural changes affect enough of the genome to be visibly detectable.

One could postulate that growing chromatin has a more "open" conformation which allows more re-initiation of RNA polymerases on growing chromatin than on stationary, this being the basis for the differences in transcription. There are four pieces of evidence that this is not the case however: 1) transcriptions done in the presence of Rifamycin AF/013, an inhibitor of initiation, show little decrease in transcription (G. Ide, unpublished); 2) exogenous wheat germ RNA polymerase II does not increase template activity (Table IIC); 3) isolation of newly initiated RNA indicates that less than 10% of the labelled RNA is

initiated *in vitro* (Chapter III, this thesis); 4) addition of denatured calf thymus DNA to the transcription does not increase incorporation indicating there is little free RNA polymerase (G. Ide, unpublished). The experiments measuring the number of growing RNA chains and the Sarkosyl experiments demonstrate the transcriptional differences between the two states are due to differences in the amount of bound and/or active RNA polymerase in chromatin from the two states. Thus the differences in transcription are not the result of differences of *in vitro* initiation.

In summary, growing *vs*. stationary yeast chromatin should be a very useful system for studying transcriptional control. One can isolate chromatin fragments which are active in transcription, contain all the RNA polymerases and seem to maintain functional and structural differences of the *in vivo* state.

II. RAPID ISOLATION OF YEAST NUCLEI

Introduction

Published methods for isolation of yeast nuclei are of two major approaches. One is through lysis of normal yeast cells using various pressure methods (Duffus, 1969; Bhargava and Halvorson, 1971; Wintersberger et al., 1973; Sajdel-Sulkowska et al., 1974), the other is to produce yeast protoplasts by enzymatic digestion of the yeast cell wall and subsequently lyse the protoplasts by osmotic or pressure shock releasing the nuclei (May, 1971; Wintersberger et al., 1973; Roziijn and Tonino, 1964; Schultz, 1978; Tekamp et al., 1979). The former method has the advantage that the nuclei are from physiologically normal cells, however the yield is quite low and the microscopic appearance of the nuclei would indicate they are damanged during isolation. The method of nuclear isolation from protoplasts gives high yields of yeast nuclei, however the nutritional state of the nuclei during the protoplasting step, and the relatively long isolation procedure may adversely affect the physiological state of these nuclei. Almost all yeast nuclei used for biochemical studies involve production of yeast protoplasts intermediate to nuclear isolation.

The method of nuclear isolation used previously in this laboratory for all experiments (the differential

centrifugation method) is an adaptation of the method of Wintersberger (1973) and was designed by D. Lohr (Lohr and Van Holde, 1975; Lohr *et al.*, 1977a). It gives very clean nuclei but suffers from the fact that many tedious and time-consuming centrifugations are required. A search of the literature yields a method in which nuclei are very rapidly isolated from the yeast *S. pombeii* by centrifuging lysed spheroplasts through 3M sorbitol onto a cushion of 4M sorbitol (Duffus, 1969). However, this method has two problems: 1) the nuclei are contaminated with unbroken cells, cell walls and membranes; 2) these levels of sorbitol are hyperosmatic and tend to shrink the nuclei. Obviously what is needed is a centrifugation medium which can be used in a continuous gradient rather than the step gradient of Duffus (1969) but having the high density of 3M sorbitol without being hyperosmotic. Colloidol silica is a centrifugation medium which has a very low osmolality but allows one to attain relatively high densities. Colloidal silica has been used widely for the isolation of cells and subcellular organelles (see reviews by Schmitt and Herrmann, 1977; Wolff, 1975), viruses (Pertoft, 1970ab; Klingeborn and Pertoft, 1972), plant cell nuclei (Hendriks, 1972), spermatid nuclei (Loir and Wyrobec, 1972), chloroplasts (Tokabe *et al.*, 1979), and pancreatic islets (Buitrago *et al.*, 1977). Viability of several cell types is maintained after isolation on colloidal silica gradients (Pertoft *et al.*,

1977). Since colloidal silica has a usage for isolation of such a wide range of biologically interesting particles, it is a logical choice for isolation of yeast nuclei.

The osmolality contribution from colloidal silica is negligible and it has a high density. Thus iso-osmotic solutions of widely varying density can be made by changing the ratio of colloidal silica to H_2O in the solution. The osmolality experienced by biological particles as they pass through density changes during centrifugation is constant and they will not swell, contract or change density as they move through the gradient.

The goal of these experiments is to design a reproducible, rapid isolation procedure which uses the protoplasting techniques previously designed in the laboratory (Lohr and Ide, 1979) through the breakage of protoplasts in 18% ficoll but which replaces the numerous subsequent centrifugations with a single density gradient of colloidal silica.

Experimental Procedures

The preparative technique used in all of these experiments is to layer the 18% ficoll protoplast lysate (or some dilution of this lysate) from the nuclear isolation procedure of Lohr and Ide (1979) on colloidal silica gradients of varying compositions and spin these gradients at 7,500

rpm for 15 minutes in an HB4 rotor. Nuclei are isolated directly from the gradients. All gradient solutions contain 0.5mM $CaCl_2$, 1M sorbitol, 0.5mM PMSF and are at pH 6.5 (S buffer). Yeast nuclei are known to be very stable to lysis in this osmotically stabilized buffer. Gradient densities are varied by changing the ratio of colloidal silica to water as the solvent for the gradient solutions. Two types of colloidal silica are used for this purpose. Ludox AM (DuPont) was used for preliminary experiments, however it inhibits endogenous transcription, and since endogenous transcription was expected to be one use for these nuclei all subsequent experiments were done using Percoll (Pharmacia), polyvinyl pyrollidone coated colloidal silica which does not inhibit endogenous transcription. Dialysis of Ludox or purifying it on an activated charcoal column, known to remove impurities which interfere with other experiments (Price and Dowling, 1977), did not remove this transcription inhibiting effect from Ludox.

A final preparative technique which can be used for all strains of yeast is shown in Appendix III. Copies of this technique have been sent to over sixty laboratories.

Results

Nuclei isolated by the Lohr and Ide technique were layered on Ludox AM gradients containing steps of known density. From this experiment it is shown that yeast

nuclei have apparent density between 1.116 and 1.152 (i.e. the nuclei banded between steps of these densities).

Nuclei were then isolated by making continuous gradients where the top of the gradient is 10% colloidal silica, 90% water and the bottom is 60% colloidal silica, 40% water (both containing 0.5mM $CaCl_2$, 1M sorbitol, 0.5mM PMSF, pH 6.5). When layered on these gradients the 18% ficoll protoplast lysate sinks into the gradient slightly. However, the nuclei sediment to midway in the gradient and are noticeably more homogenous than nuclei isolated on step gradients. This experiment was duplicated with either dialyzed Ludox AM or Percoll as the colloidal silica and a portion of the nuclei were assayed for endogenous transcription levels by the method of Lohr and Ide (1979a). In each case the nuclei isolated using Percoll were 3 to 4 times more active than those isolated using Ludox. Subsequent experiments where Ludox or Percoll were added to nuclei (isolated by the differential centrifugation technique) show that Ludox inhibits endogenous transcription but Percoll does not (data not shown). Percoll was used for all experiments described from here foreward.

Noticing that the load sunk slightly in the previous gradients (10% to 60% colloidal silica) and that the nuclei were banding approximately in the middle of the tube, several preparations were done using continuous gradients varying the percent colloidal silica at both the top and

bottom of the gradient. Dilution of the 18% ficoll protoplast lysate with one volume S buffer lowers the density of the load so it does not sink. Ten to fifty percent Percoll continuous gradients give the most uncontaminated nuclei, as judged by microscopic analysis.

Gradients of Percoll can be generated by centrifugation of a single solution of Percoll in an angle head rotor (Pertoft et al., 1978). Using various percentages and times of centrifugation it is found that a solution of 35% Percoll, 65% water (containing 0.5mM $CaCl_2$, 1M sorbitol, 0.5mM PMSF, pH 6.5) spun 15,000 rpm (27,000 xg) in a Sorvall SS-34 rotor gives a satisfactory gradient with nuclear resolution as good or better than the 10% to 50% continuous gradients. The 18% ficoll protoplast lysate still sinks slightly on loading so dilution with one volume of S buffer before loading the gradient is needed. These nuclei are tested by several biochemical criteria to see if this is a generally useful yeast nuclear preparation.

Cytoplasmic Contamination

The assay for alcohol dehydrogenase, a soluble cytoplasmic enzyme is used as an assay for cytoplasmic contamination. The assay is based on the increase in absorbance at 340nm which results from the reduction of NAD in the presence of alcohol and alcohol dehydrogenase (Vallee and Hoch, 1955).

Table VI shows the results of this assay on three preparations of nuclei. Note that in all three preparations the nuclei contain between 5% to 9% of the alcohol dehydrogenase found in the cytoplasm (load fraction). These samples were taken directly from the Percoll gradient and assayed. In preparation #3 nuclei were washed by dilution with two volumes S buffer and sedimented at 4300 xg for five minutes. These nuclei were re-suspended in a volume of S buffer equal to that which was taken from the gradient and assayed for alcohol dehydrogenase activity (washed nuclei). It is obvious from the data that this wash lowers cytoplasmic contamination greatly and thus this wash step has been added to all Percoll nuclei preparations.

Nuclease Digestion

One of the major uses for yeast nuclei in many laboratories is for nuclease digestion studies of chromatin structure. Percoll nuclei have been digested with DNase I or micrococcal nuclease and the products analyzed on gels.

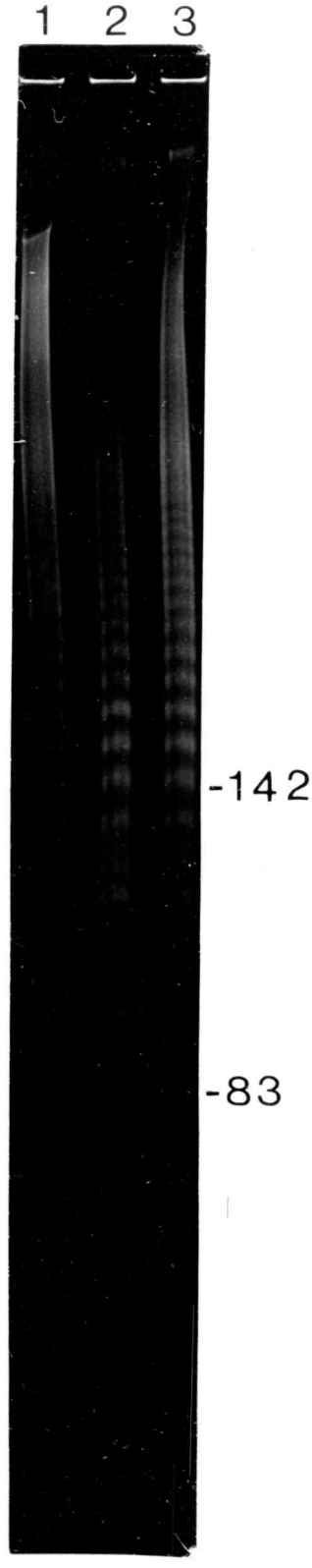
Digestion of chromatin or nuclei with pancreatic DNase I produces an array of DNA fragments differing in size by approximately 10 bases which can be detected on denaturing gels. This "ladder" extends to approximately 200 bases (Noll, 1974) unless high resolution denaturing gels are used for detection in which case the "extended ladder" can be seen to extend to 300 bases or more (Lohr *et al.*, 1977b),

Table VI. Alcohol dehydrogenase assay for cytoplasmic contamination.

	Preparation #1	Preparation #2	Preparation #3
Cytoplasm (load fraction)	1.08	1.18	0.87
Nuclei (direct from gradient)	0.10	0.06	0.06
Washed nuclei			<0.01

The spectrophotometer is zeroed at 340nm with each cuvette containing 3ml of the following solution: 16mM sodium pyrophosphate pH 8.8, 0.33M ethanol, 8.33mM NAD. At zero time 20λ of the gradient fraction is diluted to 100λ and added to the sample cuvette. Data are the resulting increase in absorbance per minute taken from the initial linear portion of the spectrophotometer output.

Figure 4. Electropherograms of DNase I digests of Percoll nuclei. DNA was isolated and electrophoresed on 8% acrylamide, 7M urea gels as described (Lohr *et al.*, 1977c). Lanes 1, 2, 3 were digested for 1, 5 and 30 minutes respectively. Size of DNA in bases is indicated.


1 2 3
-142
-83

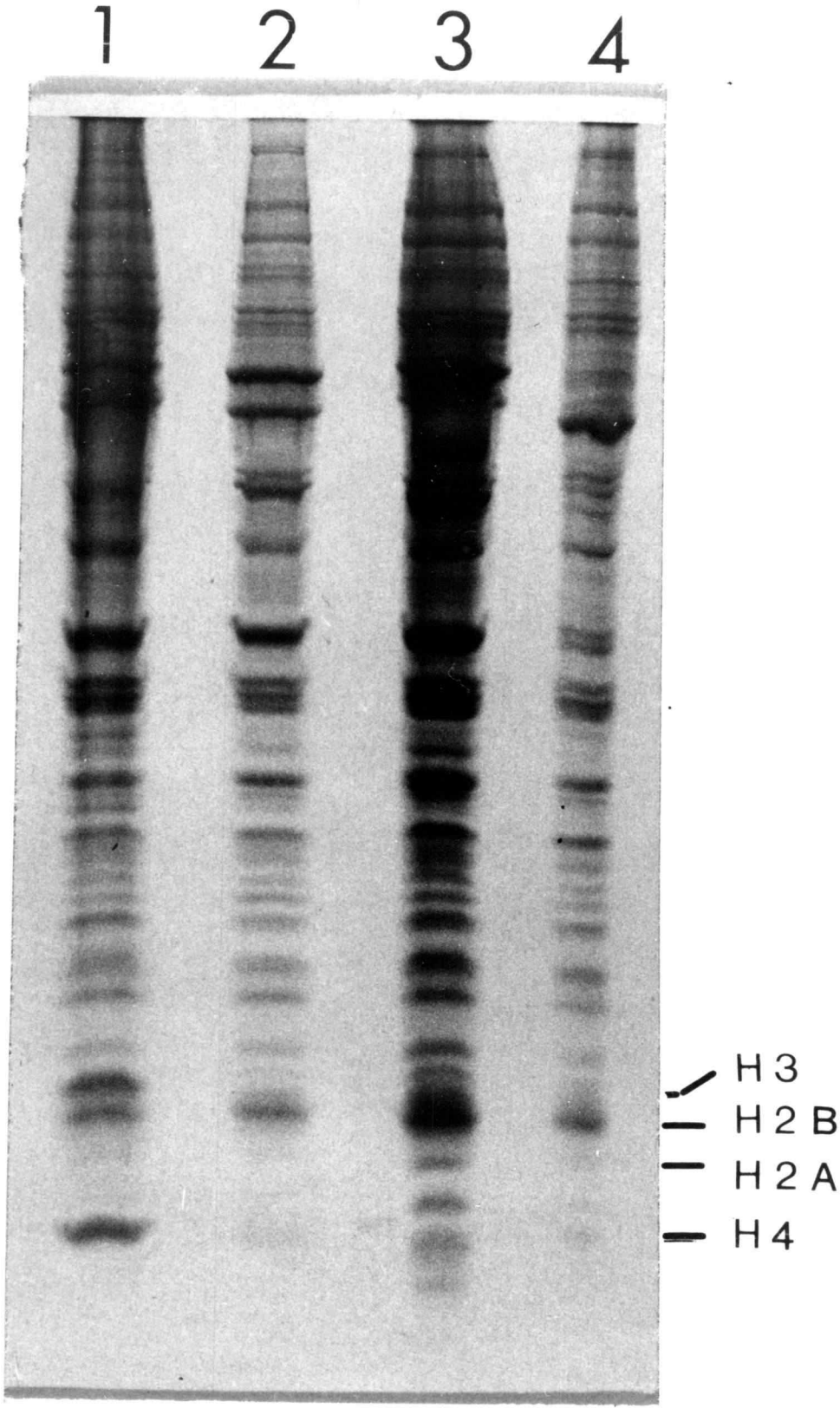
indicating phasing of nucleosomes with respect to each other. The extended ladder is particularly clear in DNase I digests of yeast. Figure 4 shows DNase I digests of Percoll nuclei electrophoresed on an 8% acrylamide-urea denaturing gel as described (Lohr et al., 1977c). Note the clarity of the bands above 200 bases indicating Percoll nuclei retain the phasing detected by DNase I.

As a further test to show that changes in chromatin structure do not occur upon isolation of Percoll nuclei, the repeat length of the DNA resulting from digestion with micrococcal nuclease was measured. When compared with the published repeat lengths for yeast (Lohr et al., 1977; isolated by the differential centrifugation method), the same repeat length is found.

Proteins

The nuclei isolated on Percoll gradients are primarily intended for studies of yeast chromatin structure and transcription. Any studies of chromatin structure rely on the isolation of "native" chromatin structure with minimal proteolysis of nuclear proteins. Studies on yeast show that Histones H3 and H4 are very susceptible to proteolysis. Therefore, an experiment was done to compare the nuclear proteins of yeast co-migrating with chicken core histones on SDS gels. Figure 5, lane 1, shows the total proteins from isolated Percoll nuclei (total nuclear proteins); lane 2

Figure 5. SDS gel electrophoresis of nuclear proteins. Gradient or nuclei samples were added to 2X SDS buffer, boiled one minute, then frozen until running. Gel formulation is that of Laemmli (1970). Lane 1, Percoll nuclei; lane 2, material which does not enter Percoll gradient; lane 3, protoplasts; lane 4, nuclei isolated by the differential centrifugation technique.


1
2
3
4
H3
H2B
H2A
H4

shows the proteins remaining at the top of the gradient (mainly cytosol), lane 3 shows proteins from protoplasts (total yeast proteins), lane 4 shows nuclear proteins from nuclei isolated by the differential centrifugation technique. Note that only the Percoll nuclei and the protoplasts show appreciable Histone H3 and H4. The nuclei isolated by the more time consuming procedure have almost no intact Histone H3 or H4. Note also that the material from the load portion of the Percoll gradient contains few proteins co-migrating with the histones indicating little loss of chromatin to the load fraction.

Transcription

Nuclei isolated using the Percoll technique were used exclusively for the work detailed in the third chapter of this thesis. These results prove Percoll nuclei will perform endogenous transcription and re-initiation of transcription.

Discussion

The nuclei isolated by this procedure appear less contaminated and more homogenous than nuclei isolated by differential centrifugation techniques. The alcohol dehydrogenase assay also shows that the nuclei are quite free of cytoplasmic contamination. This laboratory has shown Percoll nuclei to be usable for isolation of non-proteolyzed histones (J. Davie, personal communication), for nuclease

digestion studies (see Figure 4) and endogenous transcription (see Chapter III of this thesis, all of the work therein was done with Percoll nuclei).

Laboratories using any yeast nuclear isolation procedure which utilizes 18% ficoll for lysis of the yeast protoplasts can easily adopt the Percoll isolation procedure. Simply dilute the 18% ficoll lysate one fold with S buffer and load the material on a preformed Percoll gradient, spin and harvest (as delineated in Appendix II).

There are few transfer steps involved in this nuclear isolation, thus labeled nuclei can be isolated with little contamination danger. Isolation of nuclei from multiple cultures can also be easily handled by this procedure.

Polyvinylpyrollidone (PVP) has been shown to inhibit transcription in yeast nuclei (Sajdel-Sulkowska et al., 1974). Since Percoll is PVP coated silica there is danger that unbound PVP present in Percoll could inhibit transcription. No evidence of such inhibition is seen in any Percoll nuclei preparations. Pertoft et al. (1978) have shown that very little of the PVP is unbound to the Percoll. They have also demonstrated that polyethylene glycol, known to desorb polyesters adsorbed to silica surfaces (Dietz and Hamann, 1976) will not desorb PVP from silica.

The nutritional state of nuclei during the protoplasting step may adversely affect the endogenous transcription

of these nuclei. Incubation of protoplasts in growth media increases nuclear transcriptional activity (DeKloet and Beltz, 1975). These protoplasts are still alive as evidenced by fermentation bubbles. Percoll nuclei with high transcriptional activity (including RNA Polymerase I) can be made from these protoplasts (Ide and Lohr, unpublished). Thus, the nutritional deficiency effects on nuclei isolated with an intermediate protoplasting step may be remedied.

The rapidity of the Percoll technique may be its most important feature. Since the differential centrifugation technique procedure also uses 0.5mM PMSF as a protease inhibitor in all preparative steps, one would expect the nuclear proteins to be relatively free of proteolysis. However, Figure 5 indicates that the degree of proteolysis is less in Percoll nuclei than in nuclei isolated by the differential centrifugation technique. The rapidity of the Percoll technique may also make it very useful for the isolation of other nuclear biomolecules.

Another yeast nuclear isolation technique using Percoll has been developed (J. Thorner, personal communication). In this technique the nuclear isolation also takes under three hours. Soluble enzyme markers show a contamination level of less than 2% of the total cellular activities of phosphoglycerate kinase and thymidylate synthetase. However, the nuclei are stabilized by 10mM $MgCl_2$, known to condense chromatin, and the preparation uses the detergent

Triton X-100 during the lysis step, subsequent washes or the Percoll gradient itself. Since condensation of chromatin is undesirable in any work studying chromatin structure and since the effect of detergents on chromatin structure or transcription is still unknown, the Thorner technique is not useful for isolation of nuclei to be used for subsequent chromatin structural or transcriptional studies.

It should be noted that gradients of Percoll can be formed simply by freezing and slowly thawing solutions of Percoll (Haff, 1979). This method may be adaptable to making a large number of Percoll gradients if nuclear isolation is done regularly in the laboratory.

III. NUCLEOSIDE 5'-[γ-S] TRIPHOSPHATES WILL INITIATE TRANSCRIPTION IN ISOLATED YEAST NUCLEI

Introduction

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 and Ide, 1979; Tekamp *et al.*, 1979). All four nucleotide triphosphates are required, the reaction is template dependent and the product is RNase sensitive. All three 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 and 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 nucleotide 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 and 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] nucleotides. The transcripts can be labeled throughout their entire length by addition of ^{3}H or α-^{32}P nucleotides to the transcription reaction thus attaining relatively high specific activity. This [γ-S] RNA can be isolated free of bulk RNA by chromatography on mercury agarose and used for further analysis of transcription initiation.

Nuclear transcription systems using [γ-S] 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). *E. coli* polymerase will properly initiate on bacteriophage λ in the presence of [γ-S] 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] nucleotides (Hipskind and Reeder, 1980). Viscular stimatitis virus leader RNA is initiated at the correct position with [γ-S] ATP by an RNA dependent RNA polymerase (Carroll and Wagner, 1980).

Artifactual transfer of γ-thiophosphate to other nucleotides occurs in some systems making identification of the *in vitro* transcript more difficult (Hipskind and Reeder, 1980; Stallcup *et al.*, 1979). The present report studies the *in vitro* initiation of RNA polymerase III products with [γ-S] nucleotides in yeast nuclei and shows

that artifactual transfer of γ-thiophosphate to other nucleotides or nucleic acids does not occur in yeast nuclei.

Experimental Procedures

Nuclear Isolation

Mid log yeast cells (strain Y55) were grown as described (Lohr *et al.*, 1977). Cells were spheroplasted as described (Lohr and Ide, 1979) except that the 0.1 M tris 0.1 M EDTA pretreatment was skipped. Spheroplasts were broken in 18% ficoll as described (Lohr and Ide, 1979) and nuclei were isolated by layering the 18% ficoll lysate on an isopycnic density gradient of 1 M sorbitol, 0.5 mM $CaCl_2$ dissolved in a solvent of 35% Percoll (Pharmacia), 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 27,000 xg for 50 minutes. Six ml of the 18% ficoll lysate was diluted with 6 ml 1M sorbitol, 0.5 mM $CaCl_2$ and then layered on this gradient. Nuclei were banded free from cell debris by a 7,500 RPM spin in an HB4 swinging bucket rotor for 15 minutes. The resulting band of nuclei was washed by dilution with 2 volumes of 1 M sorbitol, 0.5 mM $CaCl_2$, pH 6.5 and pelleted at 4300 xg for 5 minutes. Nuclei were resuspended for transcription in this buffer. Nuclei

isolated by this method will incorporate 20 to 40 picomoles UTP into RNA per μg template DNA in a 15 minute synthesis.

Transcription Assay

RNA syntheses were conducted in a final volume of 50λ containing the following components: 50 mM Tris-HCl (pH 7.9, at 23°C), 1 mM $MnCl_2$, 10 mM $MgCl_2$, 0.8 M Sorbitol, 0.5 mM $CaCl_2$, 5 mM phosphoenolpyruvate, 1 μg pyruvate kinase, 0.1 M KCl, 50 μM ^3H UTP or α-^{32}P-UTP (1.6 to 48 Ci/mmole), and 240 μM ATP (or [γ-S]ATP), CTP and GTP (or [γ-S] GTP). Reactions are at 25° for the times indicated. Acid precipitable radioactivity in RNA was assayed using the DE81 filter method (Roeder, 1974). Transcription reactions were terminated with 5 units DNase I which was repurified to remove RNase (Maxwell et al., 1977), then 450 λ 0.5% SDS, 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.1 M NaCl (TNES) was added. Reactions were pronased, phenol extracted, ethanol precipitated and re-dissolved in 500λ 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% SDS rather than 0.1% SDS. Mercury agarose column samples were prepared for electrophoresis

by ethanol precipitation and re-dissolution 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 bisacrylamide concentration. Gels were fluorographed by the technique of Bonner and Laskey (1974) except the H_2O wash step was for 6 hours. I have found artifactual blackening of the film from DMSO with shorter wash times. Film was Kodak XR5 pre-flashed for quantitative imaging (Laskey and 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 minute 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 6A; with [γ-S] ATP and [γ-S] GTP replacing ATP and GTP in Figure 6B. This data shows that [γ-S] nucleotides must be present in the transcription reaction for RNA to bind to the mercury agarose column.

Large Proportion of [γ-S] RNA will Bind to Mercury Agarose

If the bound RNA shown in Figure 6B is structurally different than the flow through RNA, re-chromatography of this RNA on mercury agarose should result in the binding of a large portion of this RNA to the column. Figure 7 shows re-chromatography of bound RNA on the mercury agarose column.

Clearly the RNA in the bound fraction of Figure 6B 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).

Figure 6. 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. Two ml fractions were collected. Bound RNA was eluted by the addition of 10 mM dithiothreitol (DTT) to the chromatography buffer of the eighth and subsequent fractions.

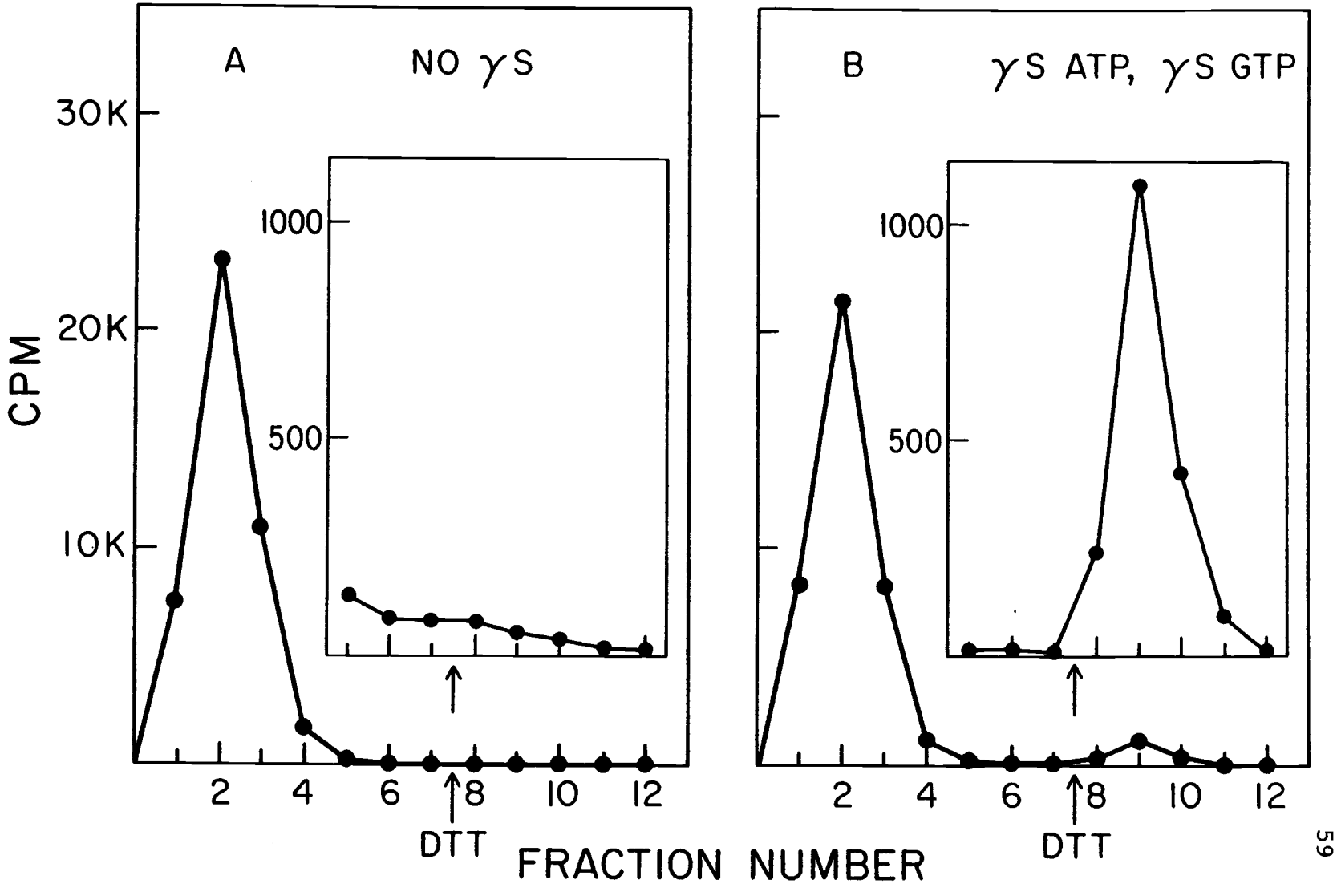
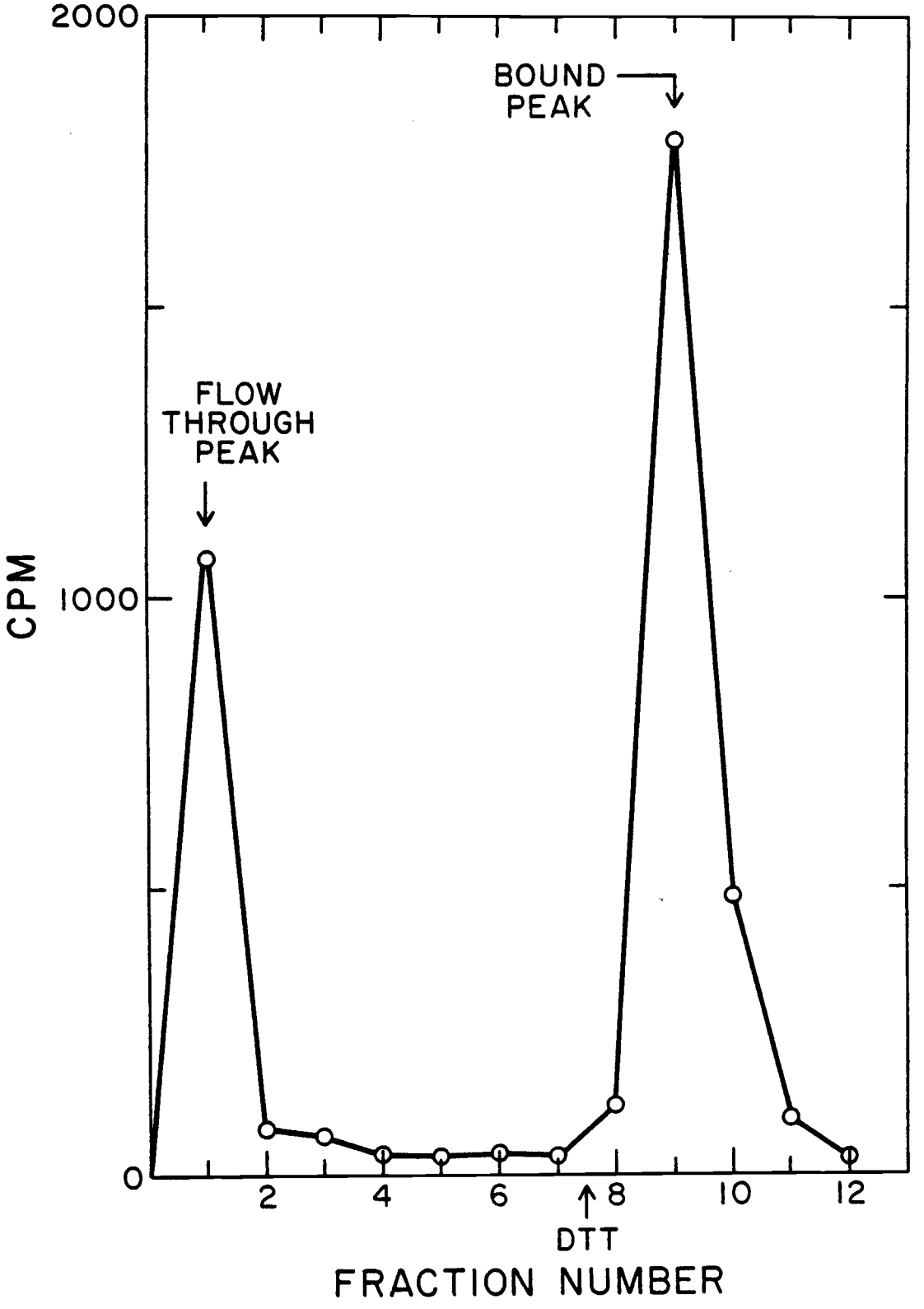

A
NO γS
B
γS ATP, γS GTP
CPM
30K
20K
10K
1000
500
1000
500
2
4
6
8
10
12
2
4
6
8
10
12
DTT
DTT
FRACTION NUMBER

Figure 7. Re-chromatography of [γ-S] RNA. Bound [γ-S] RNA was isolated as in Figure 6, run over a Bio-Gel P6 column to remove dithiothreitol and re-chromatographed on a mercury agarose column.


2000
1000
0
CPM
BOUND
PEAK
FLOW
THROUGH
PEAK
2
4
6
8
10
12
DTT
FRACTION NUMBER

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 [γ-S] nucleotide triphosphates to the 5' end of other RNAs. The following 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.

[γ-S] RNA Synthesis is Template Dependent

Four nuclear transcription reactions (A, B, C, and D) were made (Table VII). Three reactions (A, B and C) received unmodified nucleotide triphosphates and ^{3}H-UTP at zero time and were transcribed for 15 minutes. 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 minutes. 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 minute. The RNA products of all four reactions were then purified and separated by affinity chromatography on mercury agarose. If thiophosphate transfer is occurring, some of the labeled RNA in reaction A should bind to the mercury agarose.

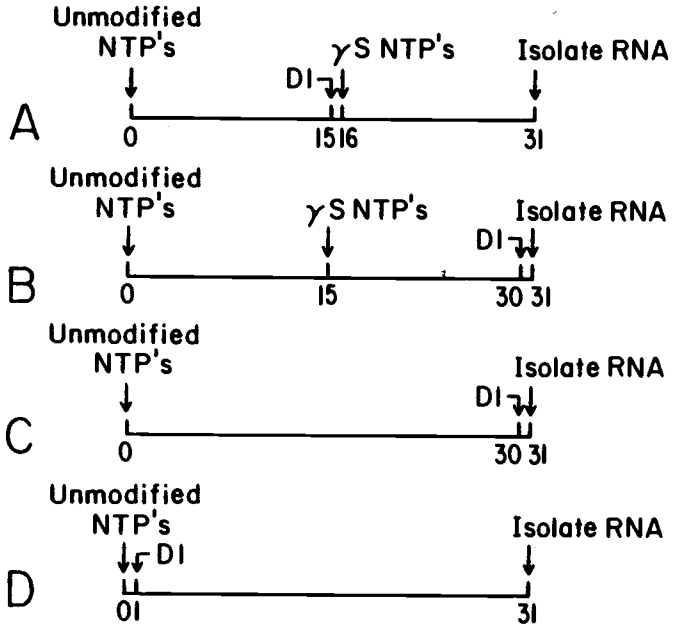
Reaction A shows that addition of [γ-S] ATP and [γ-S] GTP to ^{3}H labeled elongated RNA does not produce ^{3}H [γ-S] RNA in the absence of transcription. Reaction B shows that [γ-S] RNA will be synthesized after a 15 minute transcription in the presence of unmodified nucleotides and that the column 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] nucleotides. Reaction D shows that a one minute degradation by DNase I (500 units/ml) will almost completely degrade the template DNA.

The 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 one minute synthesis has RNA as large as 28S (Figure 8A). 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*

Table VII. Control for transfer of [γ-S] to elongated transcripts.

The isolated RNA from each reaction was dissolved in 0.5 ml TNES, and loaded on a 3 ml Hg 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 Dithiothreitol in TNES ([γ-S] RNA). Four tenths of each pool was spotted on DE81 filters, the filters washed and counted as in experimental techniques. Six tenths of each sample 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 displays the typical banding pattern for re-initiated RNA when fluorographed (data not shown). Total time for each reaction was 31 minutes.



PREDICTED RESULT IF NO ARTEFACTUAL γS TRANSFER	RESULTS		
	FLOW THROUGH RNA CPM	WASH BACKGROUND CPM	γS RNA CPM
NO RNA BOUND	15144	20	20
SOME RNA BOUND	33755	24	1090
NO RNA BOUND	33558	34	20
NO RNA SYNTHESIZED	220	34	24

vitro labeled RNA, I have displayed [γ-S] RNA which is synthesized in reactions of increasing times on gels.

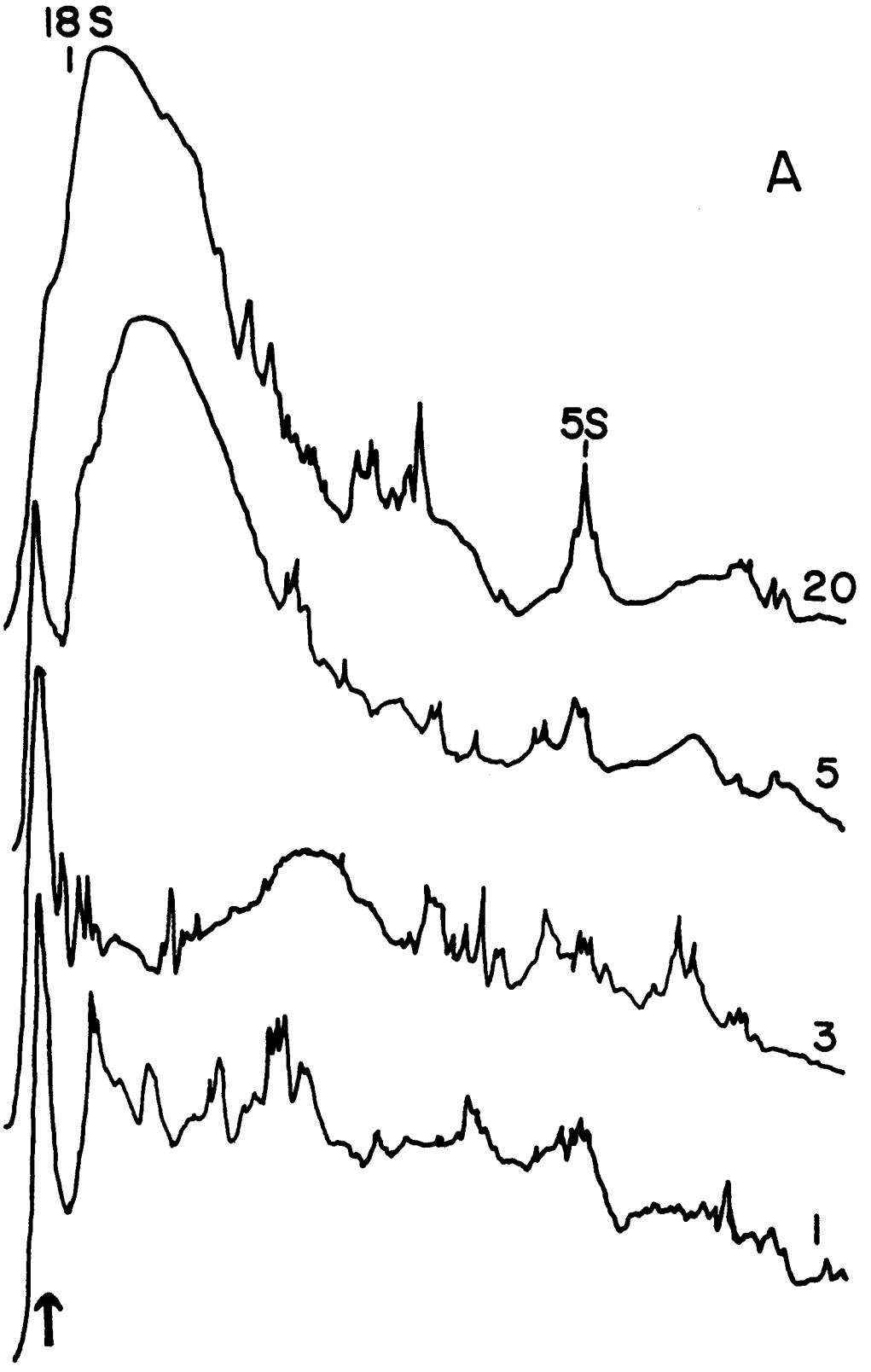
The [γ-S] RNA shown in Figure 8B shows a dramatic size increase with increasing synthesis time. Notice that 5S and pre-tRNA peaks can be seen after one minute of synthesis indicating the elongation rate is greater than 2 bases/second. I have not attempted to calculate a more exact elongation rate.

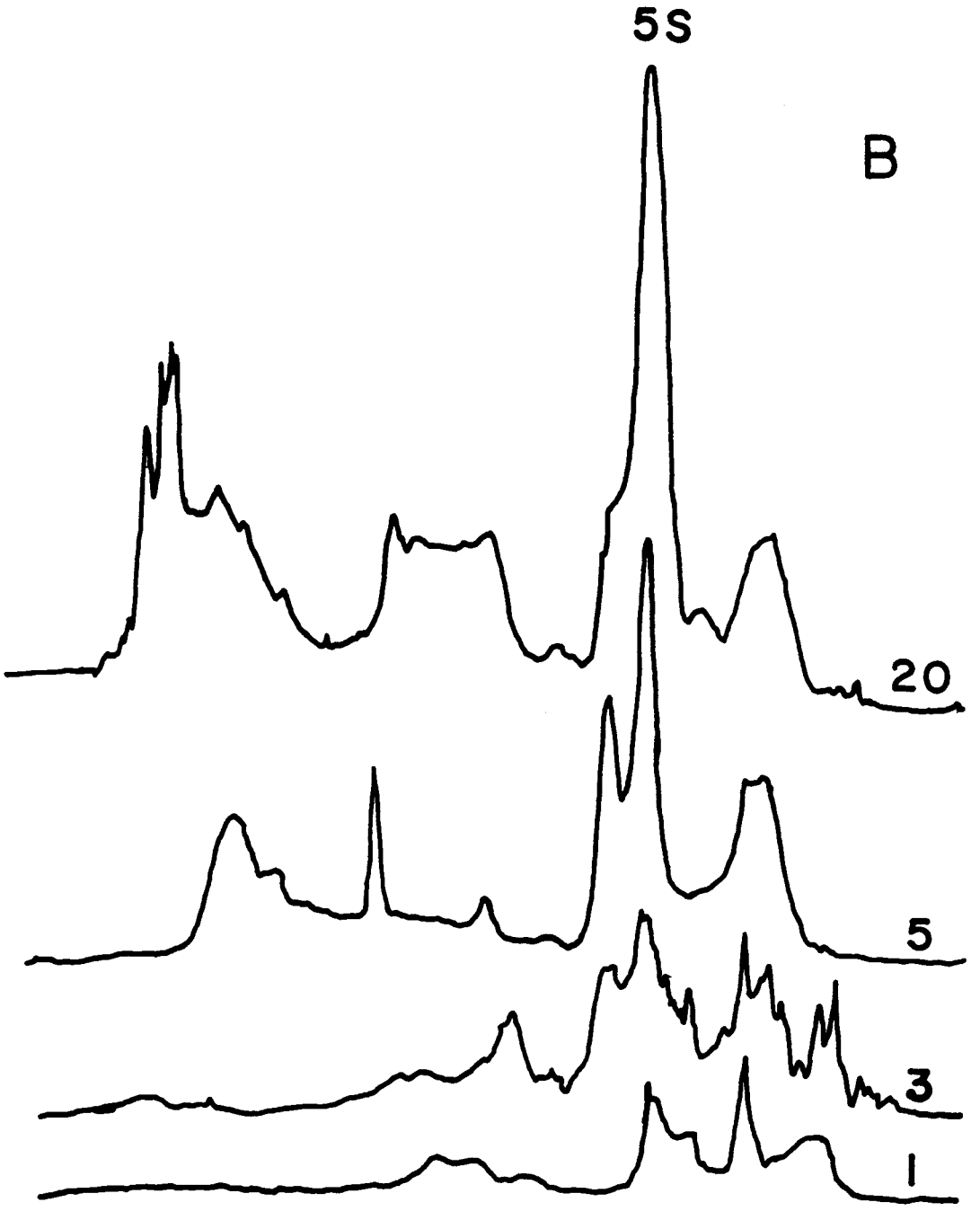
One would also expect the ratio of [γ-S] RNA to total labeled RNA to increase as synthesis time increases. Flow through and bound RNA from each time point in Figure 8 were counted on DE81 filters. Results of this experiment show a slight increase in the ratio of [γ-S] RNA to total labeled RNA and that total synthesis is linear to 20 minutes (not shown).

Transfer of the [γ-S] to Other Nucleotides Does Not Occur

If [γ-S] RNAs have a unique initiation point with a specific initiating nucleotide and if non-random 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

Figure 8. Size of bulk and *in vitro* initiated transcripts with increasing synthesis time. ^{32}P-[γ-S] RNA was synthesized in reactions for the times indicated, isolated on mercury agarose and electrophoresed on a 10% acrylamide gel. Data is a scan of an autoradiograph of the dried gel. Electrophoresis is from left to right. Reactions were stopped with 450λ SDS buffer containing 10 mM EDTA to quench the reactions quickly. Nuclei for this reaction were the washed nuclei used in Figure 10. Figure 8A is the flow thru RNA, Figure 8B is bound [γ-S] RNA.

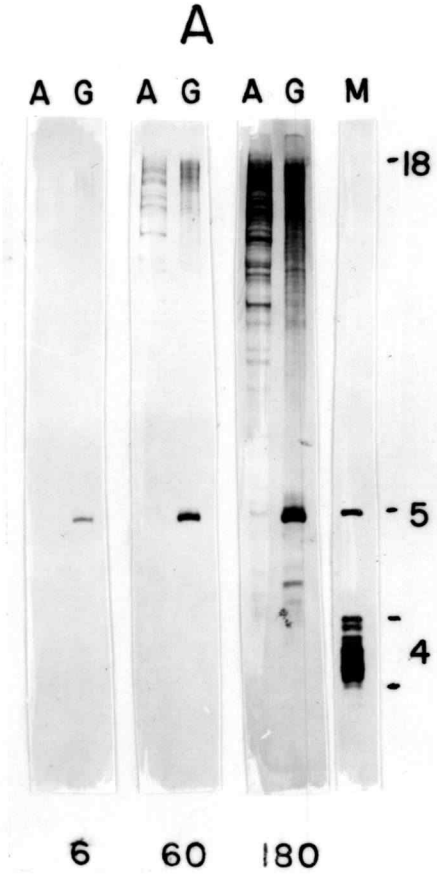

18S
A
5S
20
5
3
1

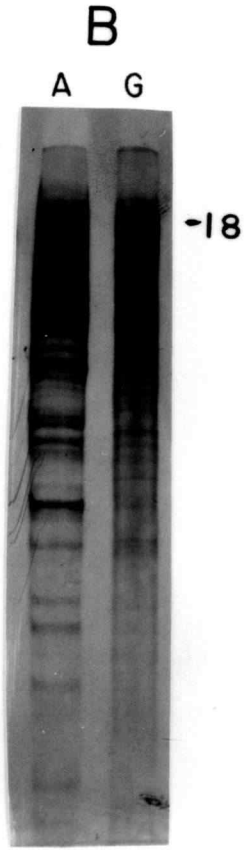

5S
B
20
5
3
1

dependent on which [γ-S] nucleotide 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 9.

The fluorographs in Figure 9A show an RNA which is initiated with [γ-S] GTP and co-migrates with yeast 5S RNA. I have positively identified this RNA as 5S RNA using hybridization techniques (G. Ide, unpublished). 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 six 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.

Figure 9. Different [γ-S] RNAs are initiated with [γ-S] ATP and [γ-S] GTP. (A) ^{3}H [γ-S] RNA is isolated from nuclei transcribed 30 minutes in reactions containing [γ-S] ATP (gel lanes marked A) or [γ-S] GTP (gel lanes marked G), electrophoresed on 60% acrylamide gels, fluorographed and exposed for 6, 60 or 180 days. The lane marked M contains ^{3}H yeast 5S and 4S RNA. (B) A close up of the high molecular weight RNA shown in the 180 day exposure of Figure 9A.


A
A G A G A G M
-18
-5
-4
6 60 180

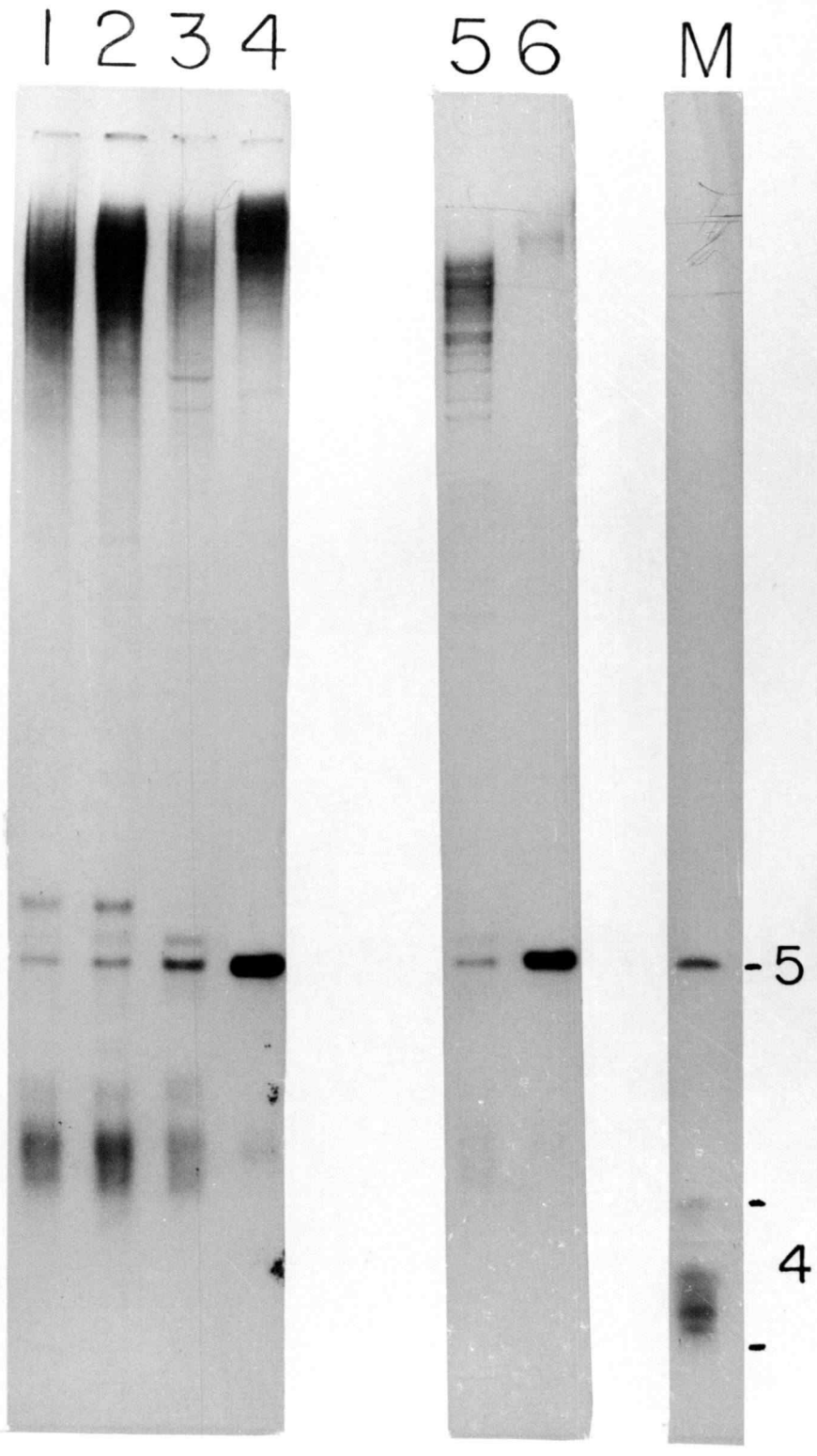

B
A G
-18

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 tRNA precursor molecules (see Figure 9) 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 in experimental procedures, except that the final wash step was repeated. A fluorograph of [γ-S] RNA synthesized by these washed nuclei is shown in Figure 10. Note the broad band of 4.5S RNA and bands between 5S and 5.8S which now appear in the [γ-S] ATP lanes (compare with Figure 9A). It is apparent from this exposure (which is equivalent to the 180 day exposure in Figure 9) that washing of nuclei has improved the detection of [γ-S] pre tRNA.

Figure 10. Fluorographs of [γ-S] RNA from washed nuclei. Nuclei are prepared as in methods except that the final wash step is repeated. [γ-S] RNA isolated from duplicate reactions incubated for 5, 10 and 30 minutes with [γ-S] ATP the modified nucleotide are shown in lanes 1, 2 and 3. Lane 4 is the [γ-S] RNA from a 30 minute reaction with [γ-S] GTP the modified nucleotide. Lanes 5 and 6 are [γ-S] RNAs synthesized 30 minutes in the presence of 1 mg/ml α amanitin with [γ-S] ATP (lane 5) and [γ-S] GTP (lane 6), the modified nucleotide. Gel is 10% acrylamide. Lane M contains ^{3}H yeast 5S and 4S RNA.

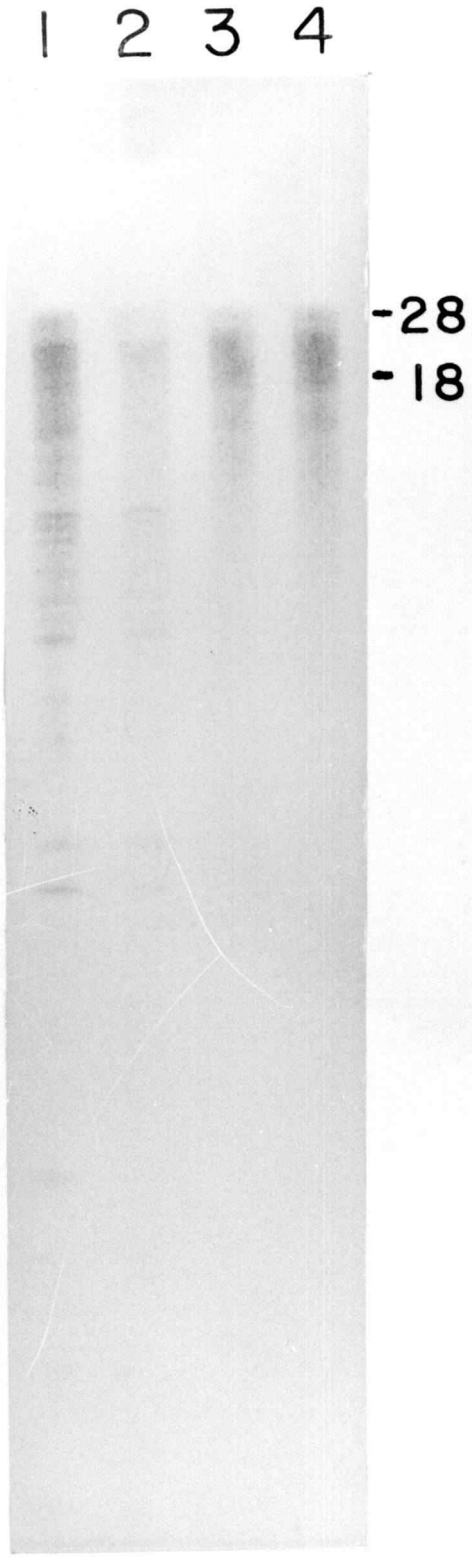

1 2 3 4
5 6
M
5
4

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 to 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 25S in the presence of 2.4 mg/ml α amanitin (Schultz, 1978). However, it is not known if these transcripts can be re-initiated *in vitro*, nor if the transcripts have a specific nucleotide required for initiation.

Figure 11 shows autoradiograms of ^{32}P-[γ-S] RNA transcribed in the presence and absence of 2 mg/ml α amanitin. RNAs as large as 28S re-initiated *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 [γ-S] RNA is electrophoresed on gels containing a low percentage of acrylamide.

Figure 11. High molecular weight RNA initiated with [γ-S] nucleotides. ^{32}P [γ-S] RNA was isolated as in experimental procedures and 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, 5S marker was electrophoresed off the gel.


1 2 3 4
-28
-18

Discussion

The experiment shown in Figure 6 shows that non-specific 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 non-specific 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] nucleotides are present in the reaction mix. The 10 mM EDTA present in the chromatography buffer is also essential to eliminate non-specific 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).

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 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. This data has been confirmed in another laboratory (Bennetzen, 1980). Surprisingly, the data shown herein proves 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 and Eckstein, 1971; Gratecos and Fischer, 1974), but thiophosphate exchange occurs between nucleotides in a _Xenopus_ extract (Hipskind and Reeder, 1980). Again, the almost complete lack of thiophosphate exchange between nucleotides 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 and Page, 1972). Hence, that [γ-S] 5S 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 of RNA to be transcribed by RNA Polymerase III since the longest identified Polymerase III transcript is smaller than 5.8S. 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 thru 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 is 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 (>5.8S) which are seen (Figures 9 and 11) 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 11. 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 10) may be useful for the isolation of pre-tRNA for tRNA processing experiments.

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APPENDICES

APPENDIX I. Calculation of the RNA Synthetic Specific Activity of Nuclei.

This is usually done by calculating the number of picomoles of UTP incorporated into RNA per microgram DNA template in a 15 minute reaction. To correlate the number of cpm with the dpm, assays where a known amount of UTP is spotted on α DE81 filter and counted. However, as pointed out by Roeder (1974), ^{3}H UTP and ^{3}H UMP incorporated into RNA do not count with the same efficiency on DE81 filters. Thus, to correlate the counting efficiency the following experiment is done. RNA from transcription reactions and known amounts of ^{3}H UTP are spotted on DE81 filters and counted in PPO, POPOP Toluene cocktail in the usual manner. These filters are then removed from the cocktail and incubated in 250λ of 6:1, NCS:H_2O (NCS is a quaternary ammonium tissue solubilizer from Nuclear Chicago) overnight. This incubation hydrolyses the RNA and UTP on the filters. Four ml of the Toluene PPO-POPOP cocktail is then added to each sample and it is re-assayed by scintillation counting. By comparison of the average of seven experiments, it was found that solubilized ^{3}H has a counting efficiency of 22.55 ± 1.48%. Using a comparison of the ^{3}H RNA assay before and after solubilization, a counting efficiency of 7.34 ± .36% was found for ^{3}H RNA counted on DE81 filters in the Toluene PPO, POPOP cocktail. Using this counting efficiency one can then easily

Appendix I (continued)

calculate the number of cpm which correspond to an incorporation of 1 picomole of UTP into RNA. The nucleotide triphosphate containing transcription substrate is always made to a known specific activity and nucleotide concentration when the substrate is made. For instance, if a substrate has a specific activity of 4.8 Ci/millimole, solution of the following equation will give

$$4.8 \text{ nanoCi/picomole} \times 2{,}220 \text{ dpm/nanocurie}$$

$$\times \frac{73.4 \text{ cpm}}{1000 \text{ dpm}} = 782 \text{ cpm/picomole}$$

Knowing the input amount of DNA (as chromatin or nuclei) then allows calculation of the transcriptional activity of the system in terms of picomole UMP incorporated per ug DNA.

APPENDIX II. Percoll R Yeast Nuclei Prep

Materials Needed

Zymolyase 5000	or	Lyticase
Available from Kirin Brewery Research Lab Takasaki, Gumma Prof. Japan (45.00/gram)		Prepared by method of J. Scott and R. Scheckman U.C. Berkeley (Biochemistry)

500 ml Bottles for GS-3 rotor

50 ml polycarbonate tubes for SS-34 rotor

Rounded End Glass Rod ~20 cm long x ~0.7 cm in diameter

Teflon Potter - Elvehgem pestle, loose fitting in the 50 ml polycarbonate tubes

Solutions Needed

PMSF Stock - 100 mM in 100% isopropanol (PMSF to be added to each buffer just before use [<15 min.] by pipetting below the surface of the buffer while rapidly stirring).

S buffer
- 1.1 M Sorbitol
- 20 mM KH_2PO_4 pH 6.5
- 0.5 mM $CaCl_2$

0% Percoll
- 1 M Sorbitol
- 0.5 mM $CaCl_2$
- to volume with H_2O, pH to 6.5 (little buffering).

100% Percoll
- 1 M Sorbitol
- 0.5 mM $CaCl_2$
- to volume with Percoll®, pH'd to 6.5 (pHing will take considerable HCl but add slowly to avoid precipitating the Percoll®). Percoll® is available from Pharmacia or Sigma.

18% Ficoll (w/w)
- 18 grams Ficoll
- 0.465 ml 0.1 M $CaCl_2$
- up to 100 grams with 20 mM KH_2PO_4, pH to 6.5

Pre-treatment stock (optional)
- 0.1 M EDTA
- 0.1 M Tris-HCl pH 8.0

Before Beginning Prep

Begin pre-spin of 34 ml 30 or 35 percent Percoll® 1 mM PMSF gradients. Spin @27,000x g (15,000 RPM, Sorvall SS-34) 4° for 50 minutes to form gradient. We use 50 ml Polycarbonate tubes for the gradients.

Percoll of various percentages is made by mixing appropriate volumes of 100% Percoll® and 0% Percoll. Gradient is stable for many hours. PMSF is added to inhibit proteases. We use 30% gradients for haploids, 35% for diploids. It is very important to use an angle rotor for gradient formation.

Growth of Cells and Harvest

Grow cells to mid-log (~5 x 10^7 cells/ml). Harvest cells @3,400 xg for 1 minute (GS-3 rotor, 4000 RPM). Wash cells free of media by re-suspension in distilled H_2O and re-harvest (again @3,400 xg for 1 minute).

Replacement of the H_2O in the wash with 200 mM EDTA, 0.5% β mercaptoethanol can be used instead of the next step which is pretreatment. The short time of pretreatment with this 200 mM EDTA, 0.5% β mercaptoethanol seems adequate for most strains of yeast growing logarithmically.

Dissolve the amount of Zymolyase that will be needed for Spheroplasting. 6 mg. Zymolyase per gram wet weight should be dissolved in a small volume of S buffer.

We harvest in pre-weighed 500 ml polycarbonate bottles so by weighing the wet pellet after harvest one can determine the "wet weight" of the cells. This wet weight is needed to determine volumes of reagents used later in the prep. (1 liter of cells at 5 x 10^7 cells/ml is about 5 grams "wet weight".)

Pre Treatment (optional step)

Add per gram wet weight cells
- 1.4 ml 0.1 M EDTA, 0.1 M Tris HCl pH 8.0
- 24λ β-Mercaptoethanol

Bring to a final volume of 3.5 ml/gram wet weight with H_2O.
Incubate 30 min on ice with gentle stirring. Harvest pre-treated cells @2000 xg/min (4000 rpm, SS-34 rotor) at 4°.

Pretreatment is necessary to spheroplast cells grown into stationary phase (Nucleic Acids Research, 6, 1909 (1979)) but may not be necessary for all strains harvested in log phase. When designing a prep for a particular strain I would suggest doing the 4° pretreatment if cells are log phase, 32° if stationary.

Wash to remove pretreatment solution. Re-suspend pretreated cells in 5 ml/gram wet weight S buffer. Harvest @2000 xg, 1 minute (4000 rpm, SS-34).

This step is not needed except in experiments where removal of the EDTA or the mercaptoethanol is desired.

Spheroplasting

Re-suspend in 4ml S buffer + 0.5mM PMSF per gram wet weight cells. Add pre-dissolved Zymolyase 5000 (Kirin brewery) to a concentration of 1.5 mg/ml (6 mg/gram wet weight cells) or add the proper amount of Lyticase (this is batch dependent).

Since the amount of Zymolyase needed for a prep is based on wet weight, it is best to dissolve it immediately after cell harvest. (Since wet weight is known at that time). Zymolyase will take 15 minutes to dissolve.

Incubate @32°C (with very gentle rocking) until spheroplasted. Begin checking to see if the cells are spheroplasted at 20 minutes and check thereafter every 5 minutes. With recent batches of Zymolyase 5000 the usual spheroplasting time has been 40 minutes. Spheroplasting is complete when all buds are gone or when Spheroplasts lyse in 18% Ficoll.

The spheroplasting solution should be agitated only enough to keep the spheroplasts from settling out. Rapid agitation will prematurely lyse some spheroplasts giving subsequent clumping and heterogeneous spheroplasting. Correct spheroplasting is the most important step of the preparation.

Harvest spheroplasts by spinning at 4300xg for 5 minutes (6,000 rpm, Sorvall SS-34) at 4°C.

Be careful to keep the solutions colc from this point on. All procedures at 4° or on ice.

Re-suspend spheroplasts in 5 ml S buffer per gram wet weight and harvest at 4300xg for 5 minutes (6,000 rpm, Sorvall SS-34).

This step washes the spheroplasts free of Zymolyase.

Lysis of spheroplasts

Resuspend spheroplasts in 100λ/gram wet weight 18% Ficoll, 1mM PMSF using a round tip glass rod. When this looks homogeneous dilute with 2.9 ml 18% Ficoll, 1 mM PMSF per gram wet weight cells.

We do this by laying the tube to about horizontal and resuspending by rotating the rod in a circular motion.

Homogenize

Use a loose fitting Teflon Potter-Elvehjem pestle to homogenize in a 50 ml polycarbonate tube.

Check lysis microscopically, more than 80% of the spheroplasts should be lysed. Eight to ten strokes is usually needed. Stationary phase nuclei float if this step is omitted.

Dilution for gradient

Dilute with an equal volume 0% Percoll® 1 mM PMSF, mix.

This is done to lower the density before loading on the gradient. It also will minimize the swelling of nuclei that occurs in 18% Ficoll. The lysis, homogenization and dilution steps should take a total of about 5 minutes. Work quickly to prevent degradation of nuclear protein and nucleic acids.

Loading the Percoll Gradient

Load the material from 2 g wet weight cells (~12 ml total volume of load) on a pre-spun 30 or 35% Percoll gradient.

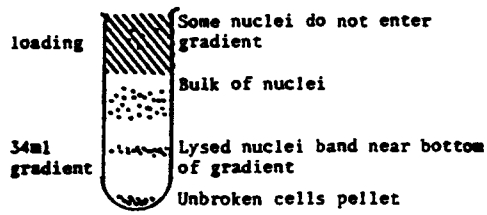
30% for haploid strains
35% for diploid strains

I do the loading by tipping the gradient tube to about 45° and gently layering the sample using a 5 ml disposable pipet which has had the last 1 cm of the tip cut off.

Spinning Gradient

After loading, mark the position of the bottom of the load on the outside of the tube. Put tubes in an HB4 swinging bucket rotor. Spin at 7,500 rpm for 15 minutes.

Resulting gradient should look as such:



Fractionate Gradient

Fractionate crudely by pulling off the load with a 5 ml disposable pipet. To remove Percoll from the nuclei and to wash nuclei, dilute nuclei band with 2 volumes 0% Percoll and pellet at 4300 xg for 5 minutes (4000 rpm in SS-34). Resuspend in desired buffer.

If re-suspended in 0% Percoll nuclei will shrink in size.

APPENDIX III. Isolation of [γ-S] RNA

Transcription Reactions

Transcriptions are routinely done in a final volume of 50λ. 5λ of a stock solution containing the following are put in a microfuge tube: 500 mM Tris-HCl (pH 7.9 @ 23°C), 10 mM $MnCl_2$, 100 mM $MgCl_2$, 50 mM phosphoenolpyruvate, 10 μg/ml pyruvate kinase, and 1.0 M KCl. 5λ of an experimental variable solution such as α amanitin, KCl, NaCl, $(NH_4)_2SO_4$, Rifamycin Af/013 etc. is then added. If no experimental solution is used, 5λ of H_2O is added instead. 35λ of isolated nuclei or chromatin are then added to the microfuge tube and allowed to equilibrate for 5 minutes with the previously added components. The transcription reaction is begun by adding 5λ of a 10X nucleotide triphosphate stock, mixing, and incubating at 25° for the desired time

Termination and Deproteinization

Transcription reactions are terminated by adding 5 units of DNase I which has been re-purified to remove RNase (Maxwell *et al.*, 1977). A DNase I digestion of one minute will degrade the DNA enough for subsequent steps, however, a five minute digestion is routinely used. 450λ of 10 mM Tris pH 7.5, 10 mM EDTA, 100 mM NaCl, 0.5% SDS (TNES

buffer) is then added to the transcription reaction and Pronase or Proteinase K to a final concentration of 200 μg/ml is added. The reaction is then incubated a minimum of 3 hours at 37° to degrade proteins prior to phenol extraction.

Phenol Extraction

Buffered redistilled phenol is prepared by adding 50λ of 0.1 M Tris base to 200λ melted phenol. Add 250λ of this buffered phenol to the microfuge tube and vortex. Then add 250λ of 24:1, Iso-amyl alcohol: chloroform (IAC) and 50λ of 5 M sodium perchlorate. The microfuge tube is vortexed for five minutes and then centrifuged for 15 minutes. 475λ of the upper layer after centrifugation is removed with a pipetman and placed in a new tube. This phenol-IAC extraction is then repeated with 250λ of buffered phenol and 250λ of IAC. The aqueous layer from this second phenol-IAC extraction is then added to 500λ of IAC, vortexed and centrifuged in the microfuge for five minutes. The aqueous layer (now 900λ) is then precipitated by the addition of 2 ml of 95% ethanol. Mix well and store in a -80° freezer for at least one hour. Harvest the nucleic acid by centrifuging 30 minutes in the microfuge. The nucleic acid pellet will loosen unless the supernatant is removed immediately when the centrifuge stops.

Column Preparation

Ten milliliter disposable syringes are used as chromatography columns. The bed support is a polyethylene disk (Bio-Rad catalog number 734-5008) which is cut to the proper size with a cork borer. Attaching a 3-way stopcock to the syringe allows convenient control of the column flow. If the bed volume of the column to be used is small, polypropylene Econo-Columns™ (Bio-Rad catalog number 731-1110) are an alternative to disposable syringes.

Exclusion Chromatography (Optional)

The [γ-S] RNA sample is desalted on a 10 ml. Bio-Gel® P-6 (Bio-Rad catalog number 150-0740) column equilibrated with TNES buffer. This is done to remove unincorporated [γ-S] which compete with [γ-S] RNA for affinity sites on the mercury agarose column. The ethanol precipitation step of sample preparation removes enough of the unincorporated [γ-S] nucleotides that the P-6 chromatography step is usually not needed.

Mercury Agarose Chromatography

Mercury agarose chromatography is done by applying the [γ-S] RNA sample to a new or regenerated mercury agarose column. The column is washed with TNES buffer to remove unbound RNA and the bound RNA is displaced by

elution with TNES buffer containing 20 mM diothiothreitol Using a three ml column of mercury agarose the following volumes are used: wash with TNES buffer collecting seven fractions of two ml each to remove unbound RNA then wash with TNES buffer containing 20 mM dithiothreitol collecting five fractions of two ml each. The [γ-S] RNA is usually eluted by the first four ml of the TNES buffer containing 20 mM dithiothreitol.

Electrophoresis

RNA can be ethanol precipitated by adding 2.5 volumes of 95% ethanol to the fractions. Addition of sonicated *E. Coli* DNA to 50 μg/ml will increase the yield in ethanol precipitates. After centrifugation (12,000 xg for 30 minutes), to harvest the RNA it is dissolved in buffered formamide as the electrophoresis sample buffer.

Electrophoresis on acrylamide-urea gels prepared according to Maniatis *et al*. (1975) works well for high resolution of the RNA. Eight percent acrylamide gels, 20 cm x 20 cm x 0.1 cm, are used for separation of 5S and pre-tRNAs, 20 cm x 20 cm x 0.2 cm 4% acrylamide gels are used for separation of larger RNAs.

Gel Fluorography

Although the Bonner and Laskey (1974) procedure gives very clear fluorographs, the procedure is lengthy and

poses safety hazards to the user. The procedure given by Chamberlain (1979) is the only one now routinely used in this laboratory. The gel is simply soaked in 1.0 M sodium salicylate and then dried. Gels fluorographed in this manner will occasionally stick to the acetate overlayer sheet suggested by Chamberlain. A 0.5 mm thick Teflon sheet cut to fit the gel dryer is an overlay which never sticks to the dried gel. Kodak X-Omat XR5 film is used to record the fluorograph image at -80°. The film should be removed for developing while the dried gel is very cold. Allowing the gel to equilibrate to room temperature will make the film stick to the gel. Alternatively, a piece of plastic wrap can be placed between the gel and the film.

Notes

Stocks of nucleotide triphosphates are dissolved in H_2O. To get a very accurate determination of concentration use the spectral constants found in the CRC Handbook of Biochemistry (ATP, ε_{259} = 15,400; CTP, ε_{271} = 9,000; GTP, ε_{253} = 13,700; UTP, ε_{262} = 10,000). Stocks should be stored frozen and discarded when six months old.