Nuclear Transcription in Vitro. Sensitivity to Inhibition by Ribosyldichlorobenzimidazole and Rifamycin AF/013†

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ABSTRACT: L cell nuclear preparations were shown to transcribe RNA for periods up to 1 h at 37 °C. Nearly 70% of the transcription products were sensitive to inhibition by 1 μg/mL of α-amanitin, indicating that they were transcribed by RNA polymerase II. Analysis of polyphosphorylated termini of in vitro synthesized RNA showed the presence of a phosphatase activity which prevents quantitative recovery of these termini. The finding of in vitro labeled polyphosphorylated termini in RNA greater than 12 S after short periods of incubation shows initiation in vitro for this size class. The labeling of these polyphosphorylated termini is decreased in the presence of rifamycin AF/013. The use of two apparent inhibitors of initiation, rifamycin AF/013 and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), has allowed detection of in vitro initiated transcripts of heterogeneous nuclear RNA. Both of these inhibitors act primarily at later times of incubation, in contrast to α-amanitin which acts on elongation and inhibits in vitro RNA synthesis immediately. The selective pattern of DRB inhibition on hnRNA is retained in vitro and some accumulation of large-size molecules is observed. It can be estimated that about 30% of the >12S hnRNA sequences transcribed in vitro are sensitive to DRB and 48% of >12S RNA are sensitive to rifamycin AF/013 inhibition.

The study of eukaryotic gene expression in vitro requires the ability of isolated nuclei to carry out transcription for an extended period of time. It also requires the ability to correctly initiate transcription and subsequently process the products of transcription to the mature RNA species.

Prolonged RNA transcription has been shown in nuclei from a variety of cells (Reeder & Roeder, 1972; Marzluff et al., 1973; Wu & Zubay, 1974; Busiello & DiGirolamo, 1975; Ernest et al., 1976) and the in vitro synthesis of RNA has been demonstrated by all three classes of RNA polymerases. Ribosomal RNA transcription of RNA polymerase I was described by Zylber & Penman (1971) in HeLa cell nuclei and Reeder & Roeder (1972) in nuclei from *Xenopus laevis*. Furthermore, the *X. laevis* rRNA transcripts were shown to be specific for the correct strand of DNA and the portion of DNA transcribed in vivo. RNA polymerase II products have been examined in HeLa (Zylber & Penman, 1971), mouse myeloma (Marzluff et al., 1973), oviduct (Ernest et al., 1976), and slime mold (Jacobson et al., 1974) nuclei. Specific sequences of immunoglobulin light chain (Smith & Huang, 1976) and silk fibroin (Suzuki & Giza, 1976) have been identified. Transcription of transfer RNA and 5S RNA genes by polymerase III also has been identified (McReynolds & Penman, 1974; Weinman & Roeder, 1974; Marzluff et al., 1974). In most of these studies, identification of the specific enzyme activities has been greatly facilitated by α-amanitin since the three classes of eukaryotic RNA polymerases exhibit differential sensitivity to inhibition by this toxin (Balatti et al., 1970).

Several of the RNA processing reactions have also been observed in isolated nuclei. Cleavage of rRNA precursors occurs (Marzluff et al., 1973; Busiello & DiGirolamo, 1975) and polyadenylation of RNA has been demonstrated in nuclei from a variety of sources (Banks & Johnson, 1973; Jelinek, 1974; DePomerai & Butterworth, 1975). The guanylation and methylation reactions for cap formation at 5′ termini of nuclear RNA molecules have been demonstrated in HeLa nuclei (Groner & Hurwitz, 1975) and L cell nuclei (Winicov & Perry, 1976) and the occurrence of internal methylation reactions has been shown in both systems. Other nuclear modifications have included a phosphatase activity reported by Marzluff et al. (1974) and a previous report from this laboratory of polynucleotide kinase activity on endogenous RNA (Winicov, 1977).

The question of whether initiation of transcription occurs in isolated nuclei has not been resolved. Although evidence exists for initiation of low molecular weight products by RNA polymerase III (Marzluff et al., 1974, 1975; Gilboa et al., 1977; Egyházi, 1974), Gilboa et al. (1977) have reported less than 5% of initiation by RNA polymerase II. Because of the inherent difficulties in quantitating γ- or β-^{32}P-labeled 5′-polyphosphate termini in the presence of the nuclear phosphatase (Marzluff et al., 1974) and polynucleotide kinase (Winicov, 1977) activities, the question of initiation of transcription was approached by experimental use of inhibitors which have been shown to act on or near initiation. The nucleoside analogue 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB)[1] has been shown to be a specific inhibitor of hnRNA and mRNA synthesis in *Chironomus tentans* (Egyházi, 1974, 1975) and HeLa cells (Sehgal et al., 1976a,b), without appreciable effect on the elongation of growing chains (Egyházi, 1975). Similar activity has been shown by the rifamycin SV derivative AF/013 on isolated eukaryotic RNA polymerases I and II (Meilhac et al., 1972). This inhibitor was also shown to be active in nuclear preparations of a lower eukaryote (Davies & Walker, 1977).

The use of these two inhibitors in the L cell nuclear system has allowed detection of a sizeable proportion of in vitro transcripts which appear to be initiated in vitro by endogenous RNA polymerase II. The results also indicate that the phosphatase activity reported by Marzluff et al. (1974) for 5S RNA greatly influences the recovery of polyphosphorylated termini from RNA greater than 12 S.

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[1] Abbreviations used: DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; Me_2SO, dimethyl sulfoxide; SAH, *S*-adenosylhomocysteine; Rif, rifamycin.

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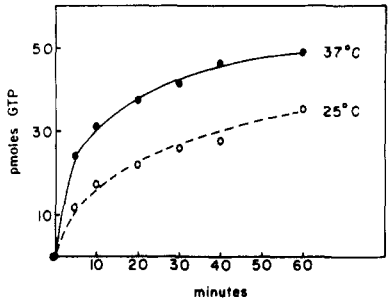

FIGURE 1: Time course of [^{3}H]GTP incorporation in nuclear preparation at 25 and 37 °C. Measurements of acid-insoluble counts are expressed as pmol of GTP incorporated per 3.5 × 10^7 nuclei.

Experimental Section

Materials. ^{3}H-Labeled GTP and UTP, as well as [α-^{32}P]GTP, were purchased from New England Nuclear Corp. DRB was a gift from Merck Sharp & Dohme Research Laboratories and was dissolved in water at 90 °C, cooled, and diluted in the assay mixture. Rifamycin SV AF/013 originated from Gruppo Lepetit SPA. The α-amanitin was obtained from Sigma Corp.

Preparation of L Cell Nuclei and RNA Transcription. Mouse L cells were grown and the nuclei prepared in hypotonic sucrose solution by Dounce homogenization as reported previously (Winicov & Perry, 1976). The nuclear preparation was incubated in vitro at 1 to 1.5 × 10^8 nuclei/mL at 37 °C for the indicated time periods. RNA synthesis appeared to be linear for longer time periods at 37 °C using lower nuclear concentrations (1 × 10^7 nuclei/mL). The conditions of RNA synthesis were essentially as described previously (Winicov & Perry, 1976) in the presence of 3 μM *S*-adenosylmethionine, 1 mM ATP, 0.4 mM CTP and UTP, and 0.1 or 0.4 mM GTP. In experiments using [^{3}H]UTP, the UTP concentration was lowered to 0.1 mM. To compensate for increased nucleotide triphosphate concentrations, the $MgCl_2$ concentration was adjusted to 4 mM. Concentrations of inhibitors were used as indicated for individual experiments.

Total acid-insoluble incorporation was measured by precipitation of an aliquot of the assay mixture with ice-cold 5% Cl_3CCOOH, 30 mM pyrophosphate. The precipitate was collected on Whatman GF/C filters and dried and the radioactivity measured by liquid scintillation counting.

RNA Extraction and Fractionation. RNA was extracted and separated from mononucleotides on a Sephadex G-50 column as described previously (Winicov & Perry, 1976). RNA was then extensively denatured in 80% dimethyl sulfoxide (Me_2SO) for 2 min at 60 °C and separated on a sucrose–sodium dodecyl sulfate gradient according to size class (Winicov & Perry, 1976).

Analysis of in Vitro Synthesized RNA Digests. Extensive enzymatic digestion of the gradient purified RNA was carried out in two steps. RNA was first digested with RNase T_2 (Winicov, 1977) at pH 4.5 followed by digestion with ribonuclease A and T_1 at pH 7.4 as described by Schibler et al. (1977). The mononucleotides, oligonucleotides, and 5′ termini were separated by DEAE-Sephadex column chromatography in 7 M urea (Perry et al., 1975) using a partial digest of yeast RNA as absorbance markers for column elution of the various charge peaks. P_1 nuclease digestions were carried out at pH 5.3 as described (Winicov, 1977).

Results

Characterization of the in Vitro RNA Synthesis. To characterize L cell nuclear RNA synthesis, acid-insoluble GTP incorporation was measured as a function of time as shown in Figure 1. At the same time a comparison was made between incorporation at 37 and 25 °C since a number of other laboratories had reported increased synthesis with time at the lower temperatures (Marzluff et al., 1973; Ernest et al., 1976). The results in Figure 1 show an initial burst of synthesis followed by continued GTP incorporation at 37 °C for time periods up to 60 min, but synthesis at 25 °C continued at a slower rate without increased accumulation of RNA.

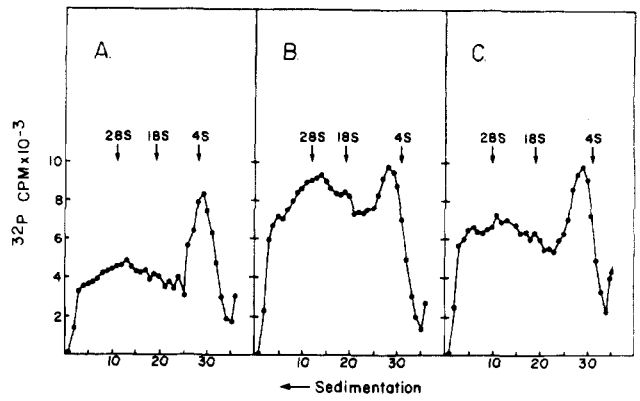

FIGURE 2: Sucrose gradient analysis of RNA synthesized in vitro at different times of incubation at 37 °C. RNA was denatured in 80% Me_2SO at 60 °C before gradient sedimentation: (A and B) 6- and 20-min incubation; (C) 20-min incubation (α-amanitin was added at 1 μg/mL after 6 min at 37 °C).

Since the cells are pretreated in vivo with 80 ng/mL of actinomycin D before preparation of nuclei, most ribosomal RNA synthesis continues to be inhibited during the in vitro incubation as previously reported (Winicov & Perry, 1976). However, in that report about 30% of the in vitro synthesized RNA was shown to be smaller than 12 S by centrifugation after exposure to Me_2SO at 60 °C. To determine the proportion of in vitro nuclear transcripts synthesized by RNA polymerase II in the L cell system, incubations were carried out at 37 °C in the presence of 1 μg/mL α-amanitin. The results are summarized in Table I and show 60–73% inhibition of UTP incorporation by 1 μg/mL α-amanitin at the times tested. Since about 30% of total incorporation can be recovered in RNA smaller than 12 S, which is α-amanitin insensitive (Table V), these results indicate that most of the RNA synthesized in vitro larger than 12 S in this system is transcribed by RNA polymerase II. These findings agree with our previous observation (Winicov & Perry, 1976) of α-amanitin sensitivity of methylation products in RNA greater than 10 S since the substrate for those methylations appeared also to be the product of polymerase II.

Analysis of in Vitro Synthesized RNA as a Function of Time. The time course of GTP incorporation in Figure 1 shows a short burst of synthesis followed by a slower rate of synthesis for the remainder of the incubation. To further investigate the type of RNA made in each time period, the nuclei were labeled with [α-^{32}P]GTP for either 6 or 30 min, and the RNA was extracted as described in the Experimental Section, denatured with 80% Me_2SO, and separated according to size by gradient centrifugation. Figure 2 shows that all size classes of RNA are synthesized in the initial time period as depicted in Figure 2A, but the major increase in newly synthesized RNA in the subsequent time period (30 min) is primarily in RNA greater than 12 S (Figure 2B). The third panel in Figure 2 shows that 1 μg/mL α-amanitin inhibits primarily the greater than 12S RNA class, even when added at 6 min after the start of the incubation. Presumably, it requires a few minutes for the inhibitor to equilibrate with the nuclear compartment since only 25% inhibition is obtained

Table I: α-Amanitin Inhibition of [^{3}H] UTP Incorporation into RNA in Nuclear Preparations[a]

time after addition of α-amanitin (min)	incorpn of [^{3}H] UTP (cpm)		
	control	+α-amanitin	% inhibn
5[b]	3004	880	71
5	3004	1207	60
10	4598	1250	73
20	5673	2176	62

[a] Nuclei were incubated at 37 °C with or without 1 μg/mL of α-amanitin added at zero time of incubation. Duplicate aliquots were removed from each assay and acid precipitable radioactivity was determined. [b] Nuclei were preincubated with α-amanitin in ice for 5 min before addition of isotope and start of incubation at 37 °C.

Table II: Recovery of in Vitro [α-^{32}P] GTP-Labeled Di- and Triphosphorylated 5′ Termini as a Function of Time of Incubation at 37 °C

RNA size class	time (min)	[^{32}P] GTP incorpn (cpm)		5′ termini[c]/ no. of nucleotides
		total[a]	−5 to −6 region[b]	
>12 S	5	46 442	214	870
	20	93 314	78	4785
12–3 S	5	23 118	393	235
	20	31 736	382	332

[a] Total cpm recovered from a DEAE-Sephadex column, greater than 95% as mononucleotide. [b] The sum of cpm in peaks in the −5 to −6 region of Figure 3. [c] Calculated as total number of nucleotides incorporated (total cpm × 4) ÷ cpm in −5 to −6 region.

when added during the course of the experiment, as compared with 60–70% inhibition shown in Table I. This explanation is supported by the increased level of inhibition after preincubation of nuclei in presence of α-amanitin (as shown in Table I).

An attempt was made to detect in vitro synthesized 5′-phosphorylated termini in RNA greater than 12 S despite reports of phosphatase activity (Marzluff et al., 1974), which had made recovery of in vitro initiated 5S RNA termini difficult. L cell nuclei were labeled in vitro with [α-^{32}P]GTP (specific activity 0.8 Ci/nmol) for either 5 or 20 min, and the RNA was extracted, denatured, and separated on sucrose gradients according to size class as described. The α-^{32}P-labeled RNA was then extensively digested with ribonucleases T_2, T_1, and A and the digest analyzed by DEAE-Sephadex–urea column chromatography. RNA of greater than 12 S and 12–3 S was analyzed for each time point and the results are shown in Figure 3 and summarized in Table II. Figure 3 shows the column elution profile in the region of −4 to −6 charge markers. Diphosphorylated (ppXp) and triphosphorylated (pppXp) termini elute from these columns with −5 and −6 charges, respectively. In vitro formed cap I and cap II structures also elute in this region (Winicov & Perry, 1976), but their formation was inhibited by the presence of *S*-adenosylhomocysteine. *S*-Adenosylhomocysteine effectively prevents methylation and cap formation in isolated nuclei (Winicov, in preparation) and was therefore included in the assay mixture at a concentration of 360 μM to eliminate the cap contribution of ^{32}P to peaks −5 and −6. As shown in Figure 3A, phosphorylated 5′ termini can be detected in RNA greater than 12 S after 5 min at 37 °C but decrease markedly in concentration at 20 min of incubation (Figure 3B). In contrast, low molecular weight RNA (12–3 S), Figures 3D and 3E,

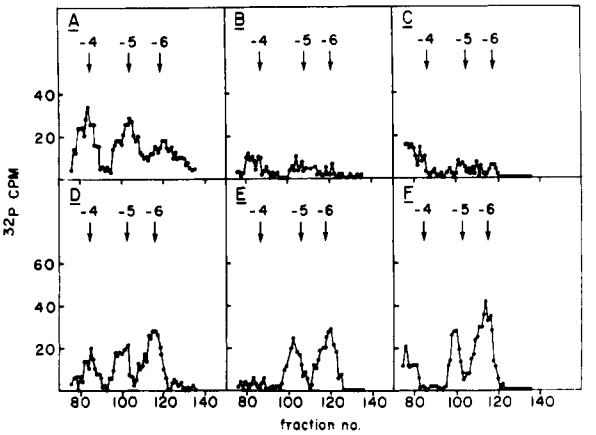

FIGURE 3: Analysis of in vitro labeled polyphosphorylated 5′ termini in RNA >12 S and 12–3 S as a function of time of incubation at 37 °C. Equal counts of [α-^{32}P]GTP-labeled RNA synthesized for 6 or 20 min in the presence of 360 μM SAH, in each size class, were extensively digested with RNases T_2, T_1, and A. The digest was analyzed by DEAE-Sephadex column chromatography in 7 M urea and data from the appropriate region of the column are shown. Each sample was counted to at least 7% accuracy: [>12S RNA] (A) 6-min incubation; (B) 20-min incubation; (C) with 1 μg/mL α-amanitin. [12–3S RNA] (D) 6-min incubation; (E) 20-min incubation; (F) with 1 μg/mL α-amanitin.

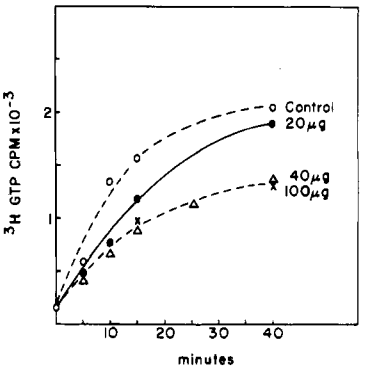

FIGURE 4: Rifamycin AF/013 inhibition of [^{3}H]GTP incorporation in L cell nuclei. Nuclei were incubated 5 min at 4 °C in the complete incubation mixture with [^{3}H]GTP at the indicated rifamycin AF/013 concentration, shifted to 37 °C, and acid-insoluble incorporation was measured at the indicated time periods.

shows no appreciable change in the concentration of phosphorylated termini during the time of incubation. A considerable proportion of the counts appears to be in diphosphorylated termini even after a 5-min incubation. These findings indicate that the relative recovery of initiation termini in greater than 12S RNA after a prolonged period of incubation can be a qualitative process at best and is not a reliable measure of initiation by RNA polymerase II.

Effect of Rifamycin AF/013 on Nuclear RNA Synthesis. To circumvent the instability of 5′-phosphorylated termini in vitro, as shown in Figure 3B, the nuclei were incubated in the presence of eukaryotic RNA polymerase inhibitor rifamycin AF/013, which has been shown to act at the initiation step by isolated RNA polymerases I and II (Meilhac et al., 1972). Use of this inhibitor would be expected to give a more accurate indication of the amount of RNA synthesis which depends on in vitro initiation than quantitation of intact 5′-phosphorylated termini.

L cell nuclei were preincubated in vitro at 4 °C with increasing concentrations of rifamycin AF/013, shifted to 37 °C, and the time course of GTP incorporation in acid-insoluble counts was followed. The results of Figure 4 show that rifamycin AF/013 is a good inhibitor of RNA synthesis in the

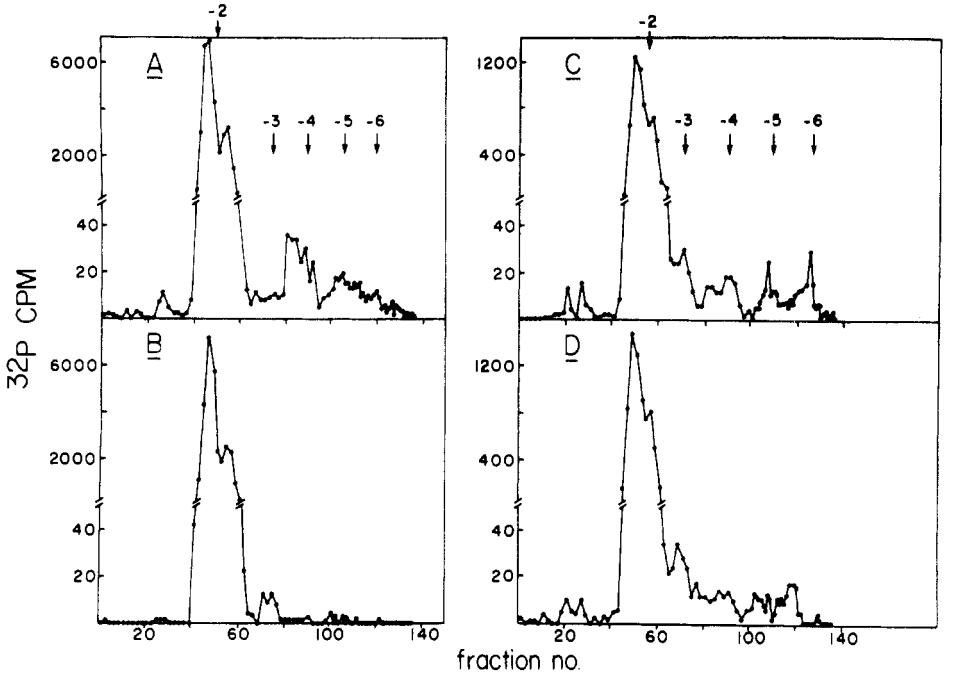

FIGURE 5: Rifamycin AF/013 inhibition of [α-^{32}P]GTP in vitro labeling of polyphosphorylated 5′ termini. Nuclear RNA was synthesized for 15 min at 37 °C ± 40 μg/mL rifamycin AF/013. The denatured RNA was separated according to size and equal number of counts from each fraction were analyzed by DEAE-Sephadex chromatography after extensive digestion with T_2, T_1, and A RNases. Each sample was counted to at least 7% accuracy. [>12S RNA] (A) Control; (B) 40 μg/mL rifamycin AF/013. [12–3S RNA] (C) Control; (D) 40 μg/mL rifamycin AF/013.

nuclear system. Maximum inhibition of endogenous RNA synthesis is observed at 40 μg/mL which is a concentration comparable to that effective with isolated RNA polymerases (Meilhac et al., 1972). After a 40-min incubation (Figure 4), 36% of total GTP incorporation is inhibited by 40 μg/mL of rifamycin AF/013. Unlike the results reported by Rose et al. (1975) with Rif AF/013, isolated polymerase, and denatured DNA, the Rif AF/013 inhibition of nuclear RNA synthesis is not competitive with GTP concentration (data not shown). In fact, increased inhibition can be observed at GTP concentrations up to 0.40 mM (Winicov, in preparation).

Further analysis was carried out on RNA greater than 12 S and RNA 12–3 S which had been synthesized in the presence of rifamycin AF/013. The RNA was extensively digested and approximately equal number of counts for each size class were analyzed by DEAE-Sephadex column chromatography in 7 M urea. Figure 5 shows column elution profiles for RNA >12 S (Figures 5A and 5B) and 12–3 S (Figures 5C and 5D). As in Figure 3, radioactive material eluting with a −5 to −6 charge would represent polyphosphorylated 5′ termini. Although lower counts were obtained in this region because of the increased time of incubation, a small, but significant peak of radioactivity can be seen in this region from control RNA >12 S synthesized in vitro (Figure 5A), which is undetectable in similar RNA synthesized in the presence of 40 μg/mL of rifamycin AF/013 (Figure 5B). These results augment the inhibition data by rifamycin AF/013 in Figure 4, suggesting that, in these nuclei, the inhibitor is acting by preventing initiation of polymerase(s) which transcribe this class of RNA.

To achieve better identification and quantitation of the phosphorylated termini of in vitro synthesized RNA, the analysis was carried out on [α-^{32}P]GTP-labeled RNA after sequential digestion with multiple nucleases (T_2, T_1, A, and P_1). Complete digestion (as shown by absence of a dinucleotide peak at −3 in Figure 5 and −1 in Figure 6) was achieved and three peaks of ^{32}P radioactivity were eluted from DEAE-

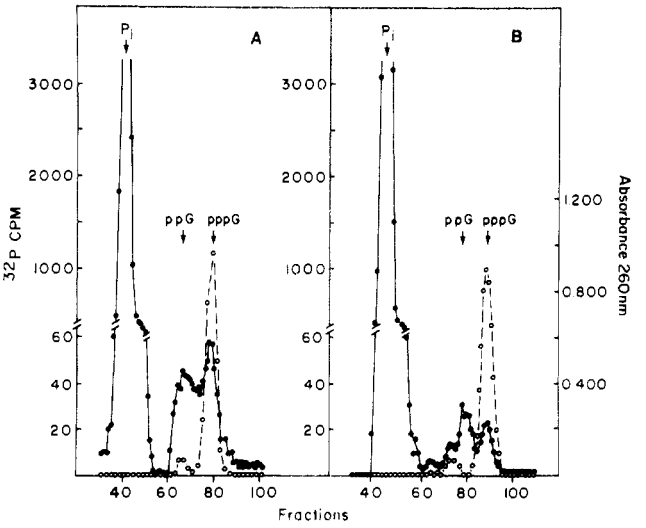

FIGURE 6: Rifamycin AF/013 inhibition of [α-^{32}P]GTP in vitro labeling of di- or triphosphorylated 5′ termini. Nuclear RNA was synthesized for 10 min at 37 °C ± 40 μg/mL rifamycin AF/013 in the presence of 360 μM SAH and 0.4 mM GTP. The denatured >12S RNA was treated as described for Figure 5, except for additional digestion with P_1 nuclease. GTP and GDP absorbance markers were monitored at 260 nm. (A) Control; (B) 40 μg/mL rifamycin AF/013.

Sephadex–urea columns. Most of the radioactivity eluted as inorganic phosphate, but two other peaks could be quantitated, which coeluted with GTP and GDP absorbance markers. No ^{32}P counts were recovered from the column in the region where cap cores could be expected to elute, consistent with the inhibition of cap formation in the presence of SAH. The elution profiles for the digestion products of RNA greater than 12 S, synthesized in the presence and absence of Rif AF/013, are shown in Figures 6A and 6B, respectively. Since equal number of counts were applied to each column, it is readily apparent that Rif AF/013 had considerably reduced the number of polyphosphorylated termini in RNA greater than 12 S. The

Table III: Effect of Rif AF/013 on ^{32}P Distribution from [α-^{32}P]GTP in T_2, T_1, A, and P_1 Nuclease Digest

RNA	Rif AF/013 (μg/mL)	GTP (cpm)[a]	% inhibn	GDP (cpm)	% inhibn
>12 S	0	303		374	
	40	109	64	148	60
12-3 S	0	874			
	40	647	26		

[a] RNA fractions were labeled and analyzed as in Figure 6. Equal numbers of counts of each fraction were applied on a DEAE-Sephadex-urea column, the eluate fractions analyzed, and counts coeluting with GTP and GDP summed across the peak. Total counts: RNA > 12 S, 85 500 cpm; RNA 12-3 S, 51 700 cpm.

Table IV: [^{3}H]GTP Incorporation in L Cell Nuclei in the Presence of DRB

	DRB (μM)	% incorpn[a]
expt I	0	100
	30	106
	60	95
	100	76
expt II	0	100
	100	76
	200	76

[a] Acid-insoluble radioactivity was measured after incubation of duplicate samples for 40 min at 37 °C. One hundred percent incorporation represents 660 cpm in experiment I and 900 cpm in experiment II.

data are summarized in Table III for RNA greater than 12 S and 12–3 S and show inhibition of polyphosphorylated termini for both size classes, indicating that the inhibition occurs at the level of RNA chain initiation.

Inhibition of Nuclear RNA Synthesis by DRB in Vitro. Recent studies in several laboratories have shown inhibition of nuclear hnRNA synthesis and appearance of mRNA by DRB (Egyházi, 1975; Sehgal et al., 1976a; Sehgal et al., 1976b), a specific inhibitor of RNA polymerase II, which has been thought to act at the level of initiation. Therefore, if the rifamycin AF/013 inhibition of RNA synthesis is due to inhibition of initiation, similar results may be expected from use of DRB.

Initial measurements of DRB effects on in vitro RNA synthesis in L cell nuclei showed maximum inhibition at concentrations of 100 μM DRB, in 40 min of incubation at 37 °C, as shown in Table IV. A more careful analysis of the time course of inhibition revealed a minimal effect of DRB on in vitro RNA synthesis as shown in Figure 7 for the first 10 min of incubation, but a low level (24%) of inhibition becomes apparent at later time points. This type of pattern of inhibition might be expected if most of the initial synthesis was due to chain elongation and the continued synthesis depended on a slower rate-limiting step, that of initiation.

To verify this interpretation, in vitro synthesized RNA was extracted from control and DRB-treated nuclei at early (5 min) and late (30 min) times during incubation, denatured, and analyzed for size distribution and inhibition patterns of DRB. The results in Figure 8 show typical sucrose gradient sedimentation profiles of RNA synthesized during a 5-min incubation in control and DRB-treated nuclei (Figures 8A and 8B, respectively) as well as during a 30-min incubation (Figures 8C and 8D, respectively). RNA synthesized in 5 min shows approximately equal amounts of incorporation into RNA >12 S with a slight shift toward larger molecules in the

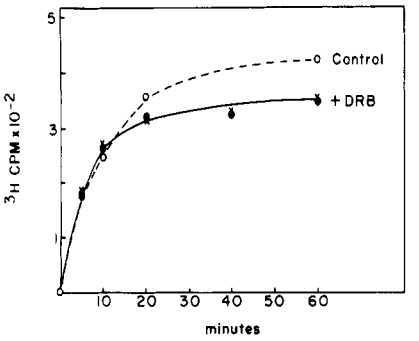

FIGURE 7: Time course of DRB inhibition of [^{3}H]GTP incorporation in L cell nuclei. Nuclei were incubated at 37 °C in the presence of DRB, and acid-insoluble incorporation was measured at the indicated time periods. DRB, 100 μM (●); DRB, 200 μM (X).

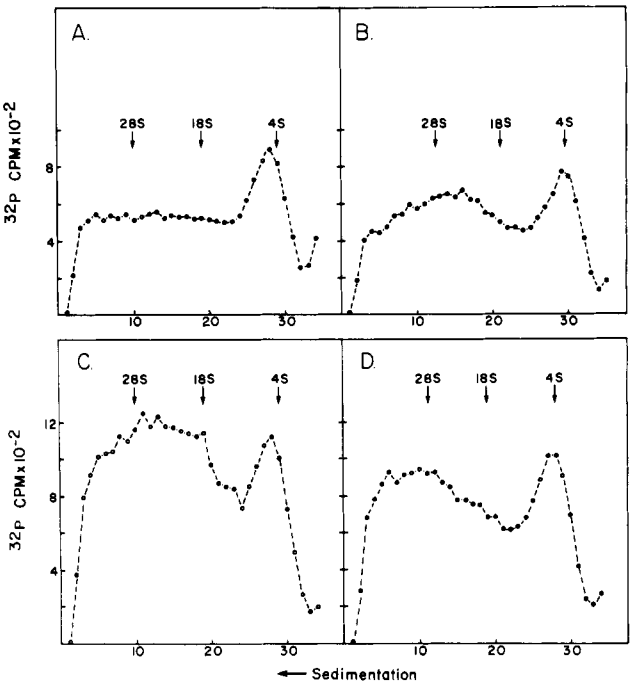

FIGURE 8: DRB inhibition of RNA transcription in vitro in L cell nuclei. RNA synthesis using [α-^{32}P]GTP was allowed to proceed at 37 °C for 5 or 30 min in nuclei ± 100 μM DRB. The extracted RNA was denatured with 80% Me_2SO at 60 °C and separated by gradient sedimentation. Control: (A) 5-min incubation; (C) 30-min incubation. DRB (100 μM): (B) 5-min incubation; (D) 30-min incubation.

DRB-exposed nuclei (Figure 8B). The inhibition becomes even more pronounced in the >12S RNA after 30 min and the RNA size distribution in DRB-treated nuclei remains shifted toward the larger size classes. These results are qualitatively similar to those obtained from DRB studies in HeLa cells in vivo (Sehgal et al., 1976a) and are presented quantitatively in Table V. While most of the inhibition by DRB, as well as α-amanitin, can be seen in the >12S RNA size class, a comparison of percent inhibition in >28S and 28S–12S size classes between α-amanitin and DRB reveals a greater percent inhibition for 28–12S RNA by DRB, while α-amanitin inhibits elongation in both classes equally. Significant inhibition of incorporation is also seen in the 12–3S RNA size class. This interpretation is supported by a total lack of inhibition in the lower molecular weight class of RNA by α-amanitin. In fact, a slight increase is observed in the accumulation of RNA transcripts for this size class in the presence of 1 μg/mL α-amanitin.

The interpretation of these experiments largely depends on the model by which the initial burst of in vitro transcriptional

Table V: Inhibition of RNA Synthesis in Relation to Size by α-Amanitin and DRB

expt	inhibitor	time of incubation (min)	>12S RNA cpm incorp control	>12S RNA cpm incorp + inhibitor	>12S RNA % inhibn total	>12S RNA % inhibn >28 S	>12S RNA % inhibn 28–12 S	12–3S RNA cpm incorp control	12–3S RNA cpm incorp + inhibitors	12–3S RNA % inhibn
1	α-amanitin[a] (1 μg/mL)	6	64 227	ND				42 835	ND	
		20	187 130	141 620	25	25	24	62 890	68 180	0
2	DRB (100 μM)	5	45 040	47 368	0	0	10	25 723	22 372	15
		30	90 560	64 464	29	8	38	30 816	28 600	7
3	DRB (100 μM)[b]	30	17 772	12 288	31			18 164	15 244	16

[a] α-Amanitin was added 6 min after start of incubation at 37 °C in the presence of the isotope. [b] [^{3}H]UTP was added after a 5-min incubation at 37 °C in the presence of cold nucleoside triphosphates. Nuclear preparations in experiments 1 and 2 were labeled with [α-^{32}P]GTP and [^{3}H]UTP in experiment 3 for the indicated time periods; the RNA was extracted, denatured with Me_2SO, and separated according to size by gradient centrifugation. Absorbance at 260 nm was used to measure RNA recoveries in each experiment. ND, not determined.

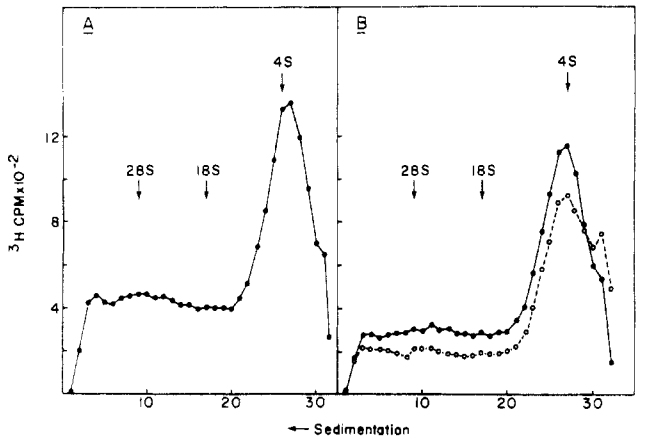

FIGURE 9: Inhibition of in vitro RNA transcription by 100 μM DRB after a 5-min incubation at 37 °C with unlabeled nucleoside triphosphates. Nuclear preparations ± 100 μM DRB were incubated at 37 °C for 5 min with unlabeled nucleoside triphosphates in the complete incubation mixture. After 5 min in the unlabeled medium [^{3}H]UTP was added and the incubation continued for either 5 or 25 min. The nuclear RNA was extracted, denatured, and separated on sucrose gradients. (A) Control; 25-min labeling; (B) 100 μM DRB; 5-min labeling (○); 25-min labeling (●).

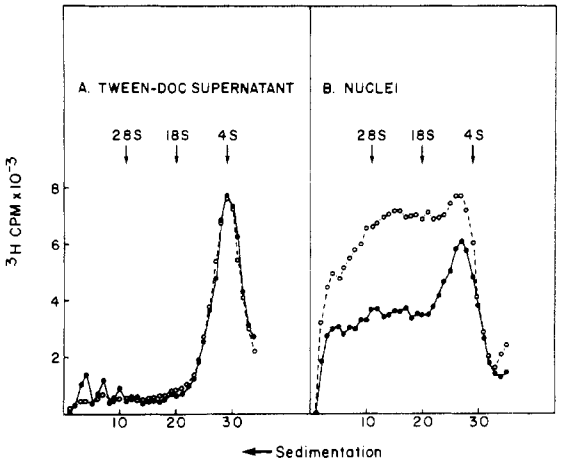

FIGURE 10: DRB inhibition of RNA transcription in vitro in nuclear and Tween 40–deoxycholate wash fractions. RNA synthesis using [^{3}H]UTP (and 0.4 mM GTP) was allowed to proceed at 37 °C for 30 min. The nuclei were washed with the detergent solution at 2 °C, and RNA was extracted, denatured with Me_2SO, and analyzed by sucrose gradient sedimentation as described (Winicov & Perry, 1976). (A) Detergent wash fraction. (B) Nuclear fraction. RNA from control nuclei (○); DRB treated nuclei (●).

activity is primarily due to elongation, and the subsequent synthesis due to both initiation and elongation. The shift toward larger size molecules in the presence of DRB would then imply the finishing of those RNA chains without reinitiation. Therefore, if the in vivo initiated chains were completed in the absence of isotope, subsequent synthesis in the presence of DRB should show inhibition of incorporation, but no shift in distribution of RNA chains, since DRB resistant chains should continue to be synthesized normally, as shown in *Chironomus* and in HeLa cells (Egyházi, 1974; Tamm et al., 1976; Sehgal et al., 1976b). This was found to be the case in an experiment shown in Figure 9. Nuclei were incubated for 5 min with or without DRB at 37 °C in the complete unlabeled assay mixture to finish most of the RNA chains initiated in vivo; the isotope was added after this 5-min incubation period, and the in vitro synthesized RNA classes were analyzed in control and DRB-treated nuclei after further incubation. As shown in Figure 9B, accumulation of large size RNA (as shown in Figure 8D) in the presence of DRB is prevented by preincubation under conditions of unlabeled RNA synthesis; only DRB-resistant synthesis occurs under these conditions, supporting the mechanism of DRB inhibition of initiation by RNA polymerase II for a subpopulation of hnRNA.

Recently, premature termination has been suggested as a mode of action for DRB inhibition, with subsequent accumulation of RNA molecules in the 400–800 nucleotide range (Tamm, 1977; Fraser et al., 1978). Analysis of the 12–3S in vitro transcription products in the L cell nuclear system is complicated by the variable synthesis of tRNA and 5S RNA in different nuclear preparations. Therefore to investigate the effect of DRB on nuclear RNA accumulations in the 12–3S region, the nuclei were washed quickly with a Tween 40–deoxycholate–buffer wash (Winicov & Perry, 1976) after in vitro incubation to remove the 4–5S RNA. Sucrose gradient analysis of denatured RNA from the wash and nuclear fractions is depicted in Figure 10. It is quite clear that, while no inhibition of RNA synthesis can be discerned in the wash fraction containing the 4–5S RNA, all size classes of RNA are inhibited in the nuclear fraction by DRB. However, differential susceptibility to DRB inhibition does seem to exist between >12S RNA and 12–3S RNA size classes as shown by the 50 and 25% inhibition, respectively.

Discussion

The extended period of RNA synthesis at 37 °C in L cell nuclei initially suggested the possibility of in vitro initiation occurring in these nuclei. This observation, together with the finding of up to 73% inhibition of in vitro RNA synthesis by 1 μg/mL α-amanitin, indicated that most of the synthetic activity was due to RNA polymerase II. The size distribution of RNA synthesized in vitro at early and late periods of in-

cubation, shown in Figure 2, supports this conclusion, since most of the counts incorporated at later time periods are found in RNA greater than 12S.

Although the rigorous proof of 5′ termini in initial nuclear transcription product relies on identification of the di- and triphosphorylated termini (Verbert et al., 1974; Schibler & Perry, 1976; Schmincke et al., 1976; Gilboa et al., 1977), actual quantitation of initial transcription products appears to be more difficult. Schibler & Perry (1977) have demonstrated that the di- and triphosphorylated termini in hnRNA are metabolically less stable than cap structure in vivo and Marzluff et al. (1974) have been unable to quantitate 5S RNA initiation in vitro, because of the instability of phosphorylated termini due to phosphatase activity. Such a phosphatase activity is also present in L cell nuclei since after a 5-min incubation with [α-^{32}P]GTP, labeled 5′ termini of RNA greater than 12 S (Figure 3A) can be seen to contain not only pppXp structures eluting with a −6 charge but also ppXp (−5) and pXP (−4) under conditions where cap formation is prevented by SAH. Although the pXp structures could have arisen from internal cleavage of a growing chain, the ppXp and pppXp structures could only arise from 5′ termini of in vitro initiated chains. Since even after a 5-min incubation at least half of the ^{32}P counts of the polyphosphorylated termini are in the diphosphate peak, this indicates the presence of an active phosphatase which attacks these termini very soon after their formation. It should be noted that, since the RNA was labeled with [α-^{32}P]GTP, the 3′ phosphates in these termini would be labeled by any penultimate G residue. Counts in the 3′-phosphate position as well as the α-phosphate of the di- and triphosphorylated guanosine terminus result in an overestimate (in Table II) of 5′ termini per number of nucleotides incorporated; however, this reservation is not necessary in experiments where the RNA was digested with P_1 nuclease. The decreased detectability of polyphosphorylated termini with time in greater than 12S RNA may also indicate that, although initiation of RNA polymerase II products occurs at early times of incubation, the rate of initiation must decrease significantly so that the phosphate cleavage reactions prevent subsequent 5′-terminal group detection.

It is therefore more convenient to consider the inhibition data from studies with rifamycin AF/013 and DRB to assess the contribution of initiation by polymerase II to in vitro nuclear RNA synthesis. This value ranges from 24% (in the presence of DRB) to 36% (in the presence of rifamycin AF/013) of total nuclear sequences synthesized in vitro, under conditions where most ribosomal RNA synthesis has been suppressed. The mechanism of action of these two inhibitors on the initiation step of RNA polymerase II in the nuclear system can be inferred from their major inhibitory effect at late times during the in vitro incubation, rather than during the initial burst of transcriptional activity involved in completion of RNA chains initiated in vivo. This time course of inhibition is in marked contrast to that observed with α-amanitin, which most effectively inhibits transcription through elongation and is found to be effective from the very start of the in vitro incubation. Both rifamycin AF/013 and DRB are effective inhibitors in the nuclear system at concentrations used in vivo for DRB (Egyházi, 1974, 1975; Sehgal et al., 1976a,b) and on isolated RNA polymerases in vitro (Meilhac et al., 1972), which is consistent with their proposed mode of action in the nuclei. Also, the lower level of inhibition by DRB is consistent with interpretation of in vivo data, which showed only a partial sensitivity of hnRNA to DRB and postulated a DRB-insensitive class of hnRNA transcripts (Egyházi, 1974; Sehgal et al., 1976b). And finally, the inhibition of polyphosphorylated termini in greater than 12S RNA as well as 12–3S RNA by rifamycin AF/013 links the activity of this inhibitor to the actual appearance of 5′-terminal structures.

Therefore, it can be concluded that the L cell nuclear system initiates in vitro about a third of its >12S RNA transcribed sequences. Although phosphatase activity prevents quantitation of in vitro formed triphosphorylated termini, the use of the two inhibitors has allowed detection of the presence of these in vitro initiated sequences and may prove to be useful in the characterization of some initial transcripts. It is also useful to note that the L cell nuclear system has retained at least some of its transcriptional specificity. This is indicated by the more limited inhibition with DRB, a specific inhibitor for a fraction of hnRNA, than with rifamycin AF/013, a broad category inhibitor due to the bulky hydrophobic side chain (Meilhac et al., 1972) of this molecule.

The question regarding premature termination of cellular transcription products by DRB remains unclear. The differential susceptibility of >12S RNA and 12–3S nuclear RNA as seen in Figure 10 does not rule out such a mechanism. However, it is not possible to discern at this time whether the DRB-resistant transcripts are prematurely terminated hnRNA molecules or a naturally occurring subclass of nuclear RNA. Experiments are in progress to differentiate between these possibilities.

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Purification and Preparation of Antibody to RNA Polymerase II Stimulatory Factors from Ehrlich Ascites Tumor Cells†

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ABSTRACT: An improved method was developed for purification of the protein termed S-II that specifically stimulates RNA polymerase II of Ehrlich ascites tumor cells. The specific activity of the final preparation was 400 000 units/mg of protein, which is about 30-fold higher than that of the previous preparation [Sekimizu, K., et al. (1976) *Biochemistry 15*, 5064]. The final preparation gave a single band on both sodium dodecyl sulfate and nondenaturing gel electrophoresis, and the protein extracted from the band on nondenaturing gel had stimulatory activity. S-II is a basic protein with a molecular weight of 40 500. The fundamental characteristics of S-II determined with the previous preparation were confirmed with completely purified S-II. A specific antibody to S-II was prepared. This antibody inhibited only the stimulatory activity of S-II and did not affect the activity of RNA polymerase II itself. Thus, S-II is probably not a component of the multimeric proteins of RNA polymerase II.

It is likely that many proteins participate in regulation of eukaryotic transcription, but little is known about them, because the transcriptional machinery of eukaryotic cells cells much is much more complicated than that of prokaryotic cells and has not yet been well characterized. In studies on the proteins regulating eukaryotic transcription, attempts have been made to isolate proteins that affect the activity of RNA polymerase II in vitro. Proteins that stimulate homologous RNA polymerase II have been isolated from various organisms (Stein & Hausen, 1970; Seifart, 1970; Natori, 1972; Lentfer & Lezius, 1972; Sugden & Keller, 1973; Lee & Dahmus, 1973), but at present it is difficult to evaluate their significance, because most of them are still impure and it is unknown how they stimulate RNA synthesis. One crucial question is whether these proteins are subunits of RNA polymerase II or not, but to answer this question these proteins must be purified. So far, three groups have reported the purifications from different sources of proteins that stimulate RNA polymerase II (Lee & Dahmus, 1973; Seifart et al., 1973; Benson et al., 1978). However, the criteria of purification used by these groups were different, and it seems difficult to compare the characteristics of these proteins from different sources, because their grades of purity are different.

Previously we reported two proteins from Ehrlich ascites tumor cells, named S-I and S-II, that specifically stimulate the activity of homologous RNA polymerase II in vitro (Natori et al., 1973a), and we described the purification of S-II to near homogeneity (Sekimizu et al., 1976). These protein factors seemed to be candidates for the regulatory protein of transcription, because they showed distinct template specificity, and S-II was found to enhance the formation of the initiation complex with RNA polymerase II and homologous DNA in the presence of nucleoside triphosphates (Sekimizu et al., 1977). However, further study showed that S-II was still not pure, although it gave a single band on sodium dodecyl sulfate gel electrophoresis in Weber and Osborn's system (Weber & Osborn, 1969).

This paper describes an improved method for purification of S-II. We adopted the following criteria for judging the purity of the stimulatory factor. (1) It should give a single band on both sodium dodecyl sulfate and nondenaturing polyacrylamide gel electrophoresis. (2) The protein migrating as a single band on nondenaturing polyacrylamide gel should have activity for stimulating RNA polymerase II. We es-

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