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Products of RNA Polymerases in HeLa Cell Nuclei

(α-amanitin/actinomycin D/ionic strength)

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ABSTRACT RNA polymerase activities in intact HeLa cell nuclei have been examined and compared to activities investigated in previous studies of the purified enzymes. The RNA synthesized by the mammalian polymerases while still in nuclei is identified. The polymerases are tentatively identified by location, sensitivity to α-amanitin, and response to manganese and altered ionic strength. Polymerase I is located in the nucleolus and labels partially complete precursor molecules of ribosomal RNA. Polymerases II and III are in the nucleoplasm and both label giant nuclear heterogeneous RNA.

The transcription of DNA in the cells of higher organisms (eukaryotes) appears more complex than in prokaryotes. Major problems in this area concern the role of the giant nuclear heterogeneous RNA of metazoan cells (1–4), the origin of the cytoplasmic messenger RNA, and the relationship between RNA metabolism studied *in vivo* and RNA polymerase (EC 2.7.7.6) activities examined *in vitro*.

In vitro studies of the transcription process of mammalian cells began with the examination of the "aggregate enzyme" described by Weiss (5). The complexity of the mammalian transcription systems was suggested by Widnell and Tata, who examined RNA synthesis by intact mammalian cell nuclei (6). They demonstrated two distinct enzymatic activities: ribosomal-like RNA synthesis, that is strongly dependent on Mg^{++}, and DNA-like RNA synthesis, which is active in the presence of Mn^{++}.

Stable, solubilized RNA polymerase from mammalian nuclei, free of template, were obtained by Ramuz, *et al.* (7) and Jacob, Sajdel, and Munro (8). Most recently, a comprehensive detailed study of the isolated RNA polymerases from rat liver and sea urchin embryo nuclei was reported by Roeder and Rutter (9–11). This work showed that three types of polymerases may be separated on the basis of chromatographic properties and sensitivity to ions. The polymerases were designated I (nucleolar), and II and III (nucleoplasmic). Until now, however, the products made by these polymerases while still in the nuclei, have not been described.

MATERIALS AND METHODS

Isolation of the nuclei. HeLa cells S-3 were grown in suspension at a concentration of 4×10^5 cells per ml as described (12). The cells were washed twice with Earle's saline, allowed to swell in hypotonic lysis buffer [$MgCl_2$ 5 mM–KCl 6mM–Tris·HCl (pH 7.6) 10 mM], and then broken by mechanical shearing (Potter homogenizer) or by addition of a nonionic detergent (NP 40) to a concentration of 0.5%. Dithiothreitol (DTT) was added to 1 mM. The nuclei were separated by centrifugation at 2000 rpm for 2 min, washed once in lysis buffer and resuspended in the incubation medium ($MgCl_2$ 5 mM or $MnCl_2$ 1.6 mM–KCl 6 mM–Tris·HCl (pH 7.6) 50 mM– DTT 1 mM–glycerol 25%). Where indicated, $(NH_4)_2SO_4$ was added.

Assay for RNA Polymerase Activity. Nuclei derived from 2×10^7 cells were incubated in 0.5 ml. Three nonradioactive nucleoside triphosphates were added to a final concentration of 40 μM, and [^{3}H]UTP or [^{3}H]CTP to 2 μM (25 μCi). The suspensions were incubated for 5 min at 26°C. The reactions were stopped by addition of cold lysis buffer. The ionic strength was increased to that of [0.5 M NaCl, 0.05 M $MgCl_2$, 0.01 M Tris·HCl (high salt buffer, HSB pH 7.6)] and the resulting gel was digested with 100 μg of DNase for 1 min at 26°C. The nucleoli were separated by sedimentation through a 15–30% sucrose gradient in HSB for 15 min at 17,000 rpm as described (12, 13), or by differential centrifugation for 5 min, at 8000 rpm (crude nucleoli). In this case, the total radioactivity was measured; the nucleolar pellet was resuspended in 2 ml of SDS (sodium dodecyl sulfate) buffer (0.5% SDS, 0.1 M NaCl, 0.001 M EDTA, 0.01 M Tris, pH 7.4); sodium pyrophosphate (pH 7.0) was added to a concentration of 0.04 M, and UMP to 5 mM; 3 ml of a cold solution containing 20% trichloroacetic acid and 0.04 M sodium pyrophosphate was added. The precipitate was collected on Millipore filters and washed ten times with 5% trichloroacetic acid containing 0.04 M sodium pyrophosphate; the radioactivity was measured as described (15). The nucleoplasm supernatant was processed in a similar way.

Analysis of the RNA Molecules Produced. The nuclear digest was fractionated by sedimentation in a sucrose gradient in HSB. The pellet contains the nucleolar fraction and was redissolved in SDS buffer. The supernatant was concentrated by precipitation with 2 volumes of cold 95% ethanol and then redissolved in SDS buffer. This corresponds to the nucleoplasmic fraction.

From both fractions, the RNA was extracted at room temperature by the phenol–sodium dodecyl sulfate procedure as described (16), and analyzed by sucrose gradients in SDS buffer.

Chemicals. Tetrasodium salts of [5-^{3}H]UTP (21 Ci/mmol) and [5-^{3}H]CTP (27.5 Ci/mmol) were obtained from New England Nuclear Corp.

RESULTS

In these experiments, partially purified nuclei are obtained from cultures of HeLa cells by induction of swelling of the cells in hypotonic buffer, then disruption either by mechanical

shearing or detergent treatment. After incubation, the nuclei are further fractionated into nucleoplasmic and nucleolar fractions, and the labeled RNA is examined.

The sedimentation profile of *in vitro* labeled RNA that is associated with the nucleolar and nucleoplasmic fractions is shown in Fig. 1. The ^{3}H-labeled nucleolar RNA in Fig. 1*A* is distributed between 18S and 45S, and appears to be composed of partially completed nucleolar RNA chains. For comparison, nucleolar RNA labeled with ^{14}C for 70 min *in vivo* before nuclear isolation and incubation is shown (Fig. 1*A*, broken line). Both the 45S precursor and the 32S intermediate are radioactive. The prelabeled RNA has apparently not been significantly degraded during *in vitro* incubation. The distribution of *in vitro* labeled RNA is not expected to resemble the prelabeled RNA, in this case, since the latter had undergone considerable processing (17). The *in vitro* distribution resembles the partial chains seen after *in vivo* pulses of 2 min or less (18). The leading edge of the sedimentation profile of *in vitro* labeled nucleolar RNA corresponds to a sedimentation value of 45 S, this is consistent with the assumption that partially completed ribosomal precursor molecules of all sizes are labeled.

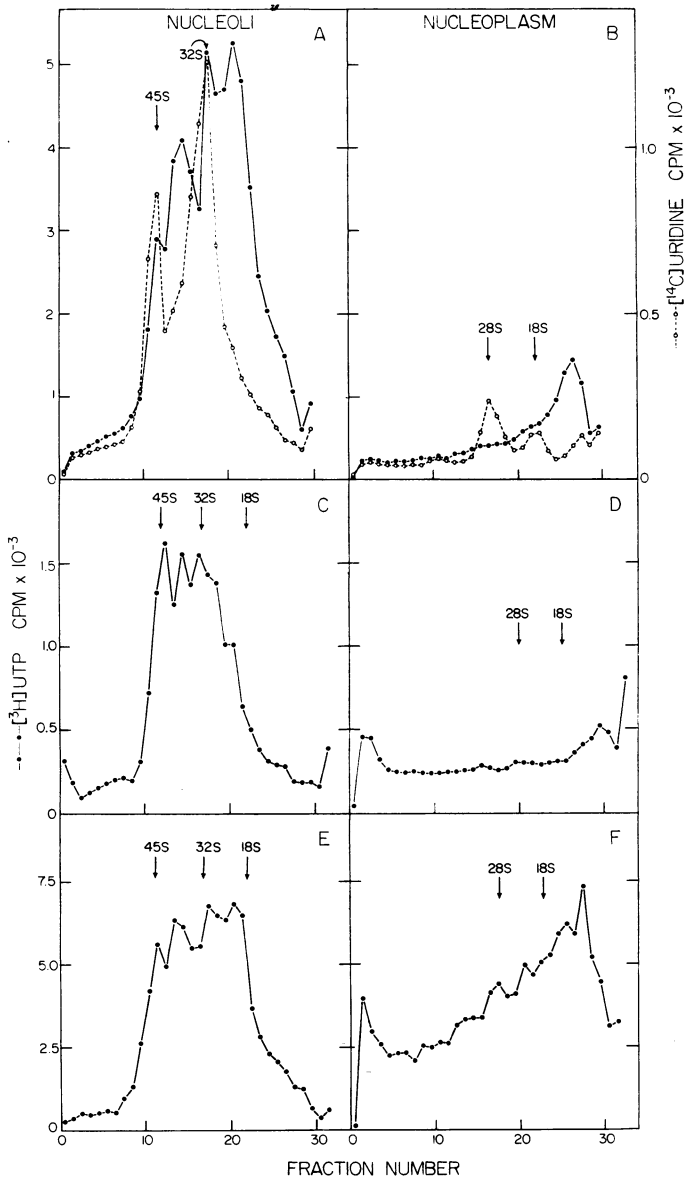

FIG. 1. Sucrose density gradient analysis of the products of the RNA polymerases from HeLa cell nuclei. (*A* and *B*) The incubation was performed in 5 mM $MgCl_2$. (●——●) In this case, the cells were prelabeled with 0.2 ml of [^{14}C]uridine (53 Ci/mol) for 70 min at 37°C before isolation of the nuclei (O– –O). The preparation and incubation of the nuclei and the RNA extraction were as described in *Methods*. The extracted RNA was centrifuged on 15–30% SDS–sucrose gradients in SW25.3 rotor, for 10.5 hr at 24,000 rpm at 23°C. (*C* and *D*) Incubation was in 1.6 $MnCl_2$ and low ionic strength. The centrifugation was for 15 hr at 20,000 rpm and 23°C in SW25.3 rotor. (*E* and *F*) The incubation was in 1.6 mM $MnCl_2$–0.1 M $(NH_4)_2SO_4$. The centrifugation was for 11 hr at 24,000 rpm and 23°C in SW25.3 rotor. The S values were obtained from the absorbance tracings (not shown in the figures).

RNA from the nucleoplasmic fraction, labeled *in vitro*, is shown in Fig. 1*B*. It sediments from 6 S to greater than 100 S in a comparatively flat distribution that resembles nuclear heterogeneous RNA, prelabeled *in vivo* (also Fig. 1*B*). The prelabeled RNA also has two peaks corresponding to 18S and 28S RNA, which are produced in the nucleolus and exported during the relatively long prelabeling period (70 min) (19).

When Mn^{++} is substituted for Mg^{++}, the labeling of nucleolar RNA is significantly inhibited (Fig. 1*C*), while the nucleoplasmic incorporation remains relatively unaffected (Fig. 1*D*). This is in agreement with (6). It has been shown previously that the total activity is increased in buffers of higher ionic strength (20). Figs. 1*E* and *F* show that incubation in 0.1 M $(NH_4)_2SO_4$ increases the labeling of nucleolar RNA about 5-fold, while nucleoplasmic incorporation is enhanced about 12-fold.

Identification of the RNA in the nucleolar fraction as ribosomal precursor is further substantiated by taking advantage of the fact that synthesis of ribosomal RNA in the nucleolus, *in vivo*, is completely inhibited by concentrations of actinomycin D (21, 22), which have little effect on nuclear heterogeneous RNA synthesis (23). Cells were treated with a low concentration of actinomycin D for 1 hr before isolation of the nuclei. The results in Fig. 2 show that *in vitro* incorporation of the label by the nucleolar fraction is completely suppressed, while the radioactivity in the nucleoplasm is depressed by about 30%. Thus, the polymerase activities from cells treated with a low concentration of actinomycin D are similar to the *in vivo* activities.

An important criterion for distinguishing polymerases is their sensitivity to α-amanitin, a cyclic polypeptide that is a major active agent in poisoning by the mushroom *Amanita phalloides* (24). It was purified and its structure was elucidated by Weiland (25). The profound effect of the drug on RNA metabolism in rat liver was described by Fiume and Stirpe (26), who then described inhibition of RNA synthesis in isolated nuclei (27). More recently, the target of α-amanitin was found to be specifically polymerase II (28).

The effect of α-amanitin on nuclei incubated at low ionic strength is shown in Fig. 2 and at high ionic strength in Fig. 3. The nucleolar activity is essentially insensitive to the drug, while the nucleoplasmic activity is extensively inhibited. Fig. 3 shows that maximum inhibition is achieved by 0.1 μg of the drug per ml. There is, however, an amanitin-resistant activity, amounting to 50% at low ionic strength (Fig. 2) and 5% at high ionic strength (Fig. 3). We tentatively identify this amanitin-resistant activity with polymerase III.

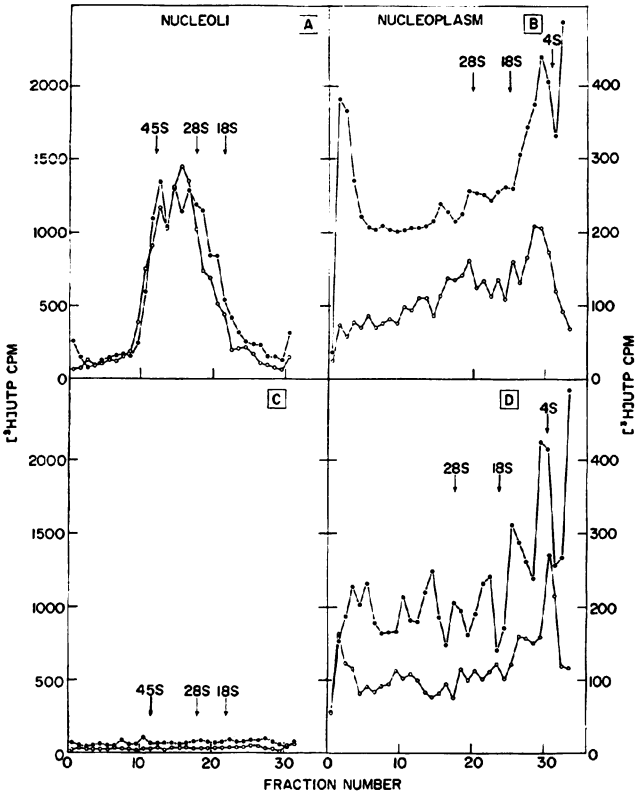

FIG. 2. Effect of prior treatment with a low concentration of actinomycin and of α-amanitin on polymerase activities. 2×10^7 cells were used in each experiment. The cells were incubated for 60 min at 37°C without (*A*, *B*) and with actinomycin D (0.04 μg/ml) (*C*, *D*). The nuclei were prepared as described in *Methods*. Incubation was in 1.6 mM $MnCl_2$ and low ionic strength. α-amanitin was added to a final concentration of 2.5 μg/ml and kept for 10 min at 0°C before the addition of the nucleoside triphosphates. The incubation and RNA extraction were as described in *Methods*. ●——●, without α-amanitin O——O, with α-amanitin.

The resistance of the nucleolar activity to α-amanitin, coupled with its preference for magnesium ion indicates that it is due to polymerase I. The amanitin sensitive activity in the nucleoplasm appears to be due to polymerase II. It should be noted that polymerase II is very sensitive to ionicstrength; thus, the amount of amanitin sensitive incorporation is highly dependent on the conditions of incubation.

The data in Figs. 2*B* and *D* show the sedimentation distribution of amanitin-sensitive and resistant nucleoplasmic RNA. The concentration of the drug used in these experiments is in excess of that necessary for maximum inhibition (see Fig. 3). In both cases, the RNA produced *in vitro* resembles the giant nuclear heterogeneous RNA that is labeled *in vivo*. Thus, it appears that both polymerase II and polymerase III synthesize giant heterogeneous RNA. These incubations are performed at low ionic strength and the incorporation by polymerase II is relatively low.

The amanitin-resistant nucleoplasmic activity might arise from some portion of polymerase II that remains inaccessible to the drug. However, the next experiments suggest that the amanitin-sensitive and resistant activities are, in fact, due to distinct enzymes. Fig. 4 shows that the effect of ionic strength on the amanitin-sensitive and resistant

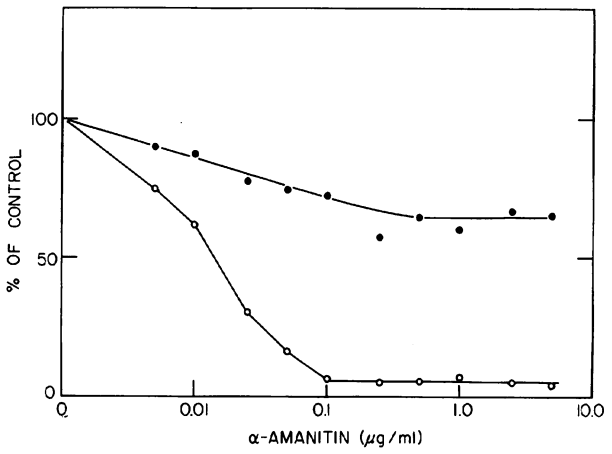

FIG. 3. Dose response to α-amanitin of the nucleoli and nucleoplasmic RNA polymerases at high ionic strength. Each sample contained nuclei from 8×10^6 cells that were incubated in 0.2 ml of 1.6 mM $MnCl_2$–0.1 M $(NH_4)_2SO_4$ as described in *Methods*. The nucleoli were separated by differential centrifugation, and the radioactivity was measured as described in *Methods*. ●——●, nucleoli: O——O, nucleoplasm.

activities is very different. Nuclei were incubated in 0.1 M $(NH_4)_2SO_4$. The nucleolar activity shown in Fig. 4*A* is stimulated several-fold at the high ionic strength and remains insensitive to α-amanitin. Nucleoplasmic incorporation of the label is greatly stimulated in the absence of amanitin. In contrast, with amanitin, increased ionic strength has little or no effect on the labeling of RNA in the nucleoplasm. This result is interpreted to mean that the amanitin-sensitive activity (polymerase II) is stimulated by increased ionic strength, while the amanitin-resistant activity (polymerase III) is unaffected by the altered ionic environment.

A more detailed examination of the effect of ionic strength on the nucleoplasmic activities is shown in Fig. 5. Nucleolar polymerase activity was inhibited by prior treatment of the cells with low concentrations of actinomycin D, so that the laborious separation of nucleolar and nucleoplasmic fractions was avoided. The amanitin-sensitive activity is greatly altered

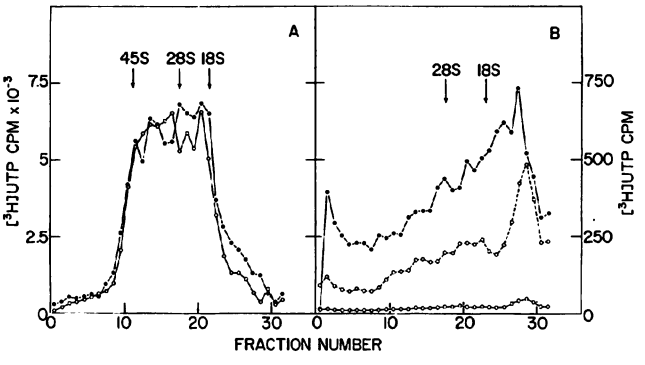

FIG. 4. Effect of ionic strength on the α-amanitin-sensitive and resistant polymerase activities. Nuclei from 2×10^7 cells were used in the incubations. The incubation medium was 1.6 mM $MnCl_2$–0.1 M $(NH_4)_2SO_4$, 2.5 μg/ml α amanitin was added 10 min before the incubation. The fractionation and RNA preparation was as indicated in *Methods*. In *B*, the α-amanitin results are plotted on an expanded scale shown on the right side. O——O, with α-amanitin; ●——●, without α amanitin; *A*, nucleoli; *B*, nucleoplasm.

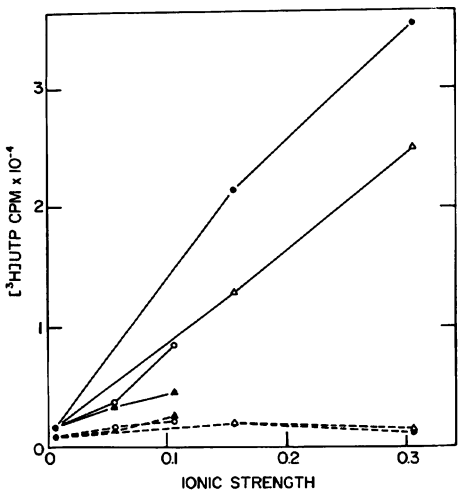

FIG. 5. Effect of ionic strength on the nucleoplasmic polymerase activities. Cells were incubated for 30 min in 0.04 μg/ml of actinomycin D, before harvesting. Nuclei from 4 × 10^6 cells were used in each incubation and were obtained and incubated as in *Methods*. The incubation medium was 1.6 mM $MnCl_2$ and the different salts were added as follows: ●——●, $(NH_4)_2SO_4$; Δ——Δ, K_2SO_4; O——O, NH_4Cl; ▲——▲, KCl. *Broken lines* indicate the addition of α-amanitin (0.25 μg/ml) with the salt.

by ionic strength while the amanitin resistant activity is unaffected. The effect on the amanitin-sensitive activity is relatively independent of the particular ions used (29, 20). The amanitin-sensitive nucleoplasmic enzymatic activity resembles polymerase II in sensitivity to ionic strength, while the amanitin-resistant activity resembles polymerase III (9).

The results presented in Table 1 show that high concentrations of actinomycin D strongly inhibit nucleoplasmic incorporation. Separate experiments have shown that the nucleolar polymerase is inhibited by more than 90% under the same conditions. Since actinomycin D specifically inhibits DNA-directed RNA synthesis, it would appear that the major portion of the activity is due to DNA-directed RNA polymerases. The actinomycin-resistant activity will be described in detail in another report.

TABLE 1. *Effect of actinomycin D and α-amanitin on [³H]CTP incorporation*

Expt.	[³H]CTP cpm incorporated	%
Complete	22,605	100
+ α-amanitin	2,835	12.5
+ Actinomycin D	2,656	11.7
+ α-amanitin + Actinomycin D	347	1.5

1.6 × 10^7 cells were incubated in 8 ml of medium with actinomycin D, to a final concentration of 0.04 μg/ml, for 30 min at 37°C. The nuclei were prepared as described in *Methods;* identical aliquots were used for each incubation. Incubation was in 5 mM $MgCl_2$–50 mM $(NH_4)_2SO_4$, the other components were as described in *Methods*. The incubation was performed for 10 min at 26°C. α-amanitin was added to a final concentration of 0.5 μg/ml. Actinomycin D was added at a concentration of 50 μg/ml of medium. 10 μCl of [³H]CTP (27.5 Ci/mM) was used in each incubation.

Table 2 shows that the nuclear product is completely sensitive to RNase and that the activities are dependent on the presence of all four nucleotide triphosphates. Similar results are obtained when labeled CTP or ATP are substituted for labeled UTP.

DISCUSSION

The amount of synthesis performed by the intact nuclei studied here is limited. The incorporation reaction is essentially terminated after 10 min of incubation at 26°C. Polymerase activities add in the order of 100 nucleotides to previously existing molecules. Whether any new RNA molecules are initiated is unknown.

The relative amounts of activity observed *in vitro* may not be simply related to amounts of synthesis occurring *in vivo*. Thus, it is not certain at present which of the activities is responsible for the bulk of the nucleoplasmic labeling, although it seems likely that the amanitin-sensitive activity that is the most active at moderate ionic strength *in vitro* is also most active *in vivo*.

The major discrepancy between the behavior of the purified polymerases and those described here is the difference in response to Mg^{++} and Mn^{++} ions. The purified polymerases obtained from rat liver that are described by Roeder and Rutter (9) are more active in the presence of Mn^{++} than Mg^{++}. This is especially true of polymerase II, which is 10 times more active in Mn^{++}, while the corresponding ac-

TABLE 2. *Effect of nucleotide triphosphates on [³H]UTP incorporation*

	Expt.	Time (min)	[³H]UTP cpm incorporated	%
1	Complete	10	15,572	100
	+α-amanitin	10	2,467	15.8
	Complete	0	139	0.9
	−GTP, CTP, ATP	10	1,983	12.7
	−GTP, CTP, ATP + α-amanitin	10	752	4.8
	−GTP, CTP, ATP	0	101	0.6
2	Complete	6	13,701	100
	+ RNase	6	522	3.8

In Expt. 1, cells were incubated with low concentration of actinomycin D (final concentration 0.04 μg/ml) for 30 min at 37°C. Nuclei were prepared as described in *Methods*. The nuclei derived from 6 × 10^6 cells were used for each incubation; the incubation was in 1.6 mM $MnCl_2$–50 mM $(NH_4)_2SO_4$ for the times indicated. α-amanitin was added to a final concentration of 0.5 μg/ml. The radioactivity was measured as described in *Methods*.

In Expt. 2, nuclei derived from 4 × 10^7 cells were incubated in the complete mixture with 1.6 mM $MnCl_2$ and low ionic strength for 6 min, the RNA was extracted as described in *Methods*, and divided in two aliquots, one of which was incubated in 1.5 mM $MgCl_2$–10 mM Tris·HCl (pH 7.6)–10 mM NaCl, with 100 μg/ml of pancreatic RNase at 37°C for 90 min. The radioactivity was measured as described in *Methods*.

tivity in these nuclei is depressed. The nucleolar activity described here is strongly inhibited by Mn^{++}, in agreement with previous results by Tata, Jacob, and Munro and others (8, 20), while the soluble polymerase I of Roeder and Rutter appears indifferent to the cation used (9). Preliminary results suggest that the differences are due in part to an earlier termination of the *in vitro* reaction in Mn^{++} when nuclei are used.

The results reported here suggest two distinct systems of synthesis in the nucleoplasm, both of which produce nuclear heterogeneous RNA. The existence of two classes of this puzzling RNA species would resolve several apparent paradoxes. Very different lifetimes have been reported for nuclear heterogeneous RNA, depending upon the method of measurement. Thus, continuous labeling, which emphasizes the contribution of long-lived molecules, indicates a half-life in excess of 1 hr for the nuclear heterogeneous RNA (23). A very different method was used by Soeiro, Warner, and Darnell (30), which emphasizes the contribution of short-lived molecules and which indicates a lifetime of 10 min or less. Two or more classes of RNA that had different lifetimes would account for these apparently disparate results.

If nuclear heterogeneous RNA serves as a precursor to cytoplasmic messenger RNA, as has been suggested (31), the two types of nuclear heterogeneous RNA might account for different classes of messenger RNA molecules.

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