Proceedings of the National Academy of Sciences
Vol. 65, No. 3, pp. 675–682, March 1970

Specific Nucleolar and Nucleoplasmic RNA Polymerases*

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Communicated by Paul Berg, September 5, 1969

Abstract. The DNA-dependent RNA polymerase activity present in rat liver nuclei has been solubilized and purified from whole nuclei and from subnuclear fractions. As reported earlier (Roeder, R. G., and W. J. Rutter, *Nature*, **224**, 234 (1969)), two major chromatographically distinct enzymatic species (I and II) are present in whole nuclei. Subfractionation of whole nuclei into nucleolar and nucleoplasmic fractions had little effect on the total recovery of activity. Purified nucleoli contain predominantly polymerase I, whereas the nucleoplasmic fraction is greatly enriched for polymerase II. A third minor peak of activity has also been resolved in the nucleoplasmic preparations. We conclude that the RNA polymerases are specifically localized within the nucleus and may, therefore, play specific roles in the regulation of genetic transcription.

Multiple forms of RNA polymerase have recently been detected in the nuclei of two eukaryotic organisms.[1] Three components, each with different catalytic properties, were resolved by chromatographic means from soluble extracts derived from the nuclei of sea urchin embryos. Two major RNA polymerase species with catalytic and chromatographic properties similar to their respective counterparts in the sea urchin were also found in rat liver nuclei. These observations suggested the possibility of distinct functional species of RNA polymerase in eukaryotic nuclei.

Polymerase I shows maximal activity at low ionic strength, whereas polymerase II exhibits optimal activity at a higher ionic strength.[1] Furthermore, both polymerases I and II display greater activity with Mn^{++} than with Mg^{++}, although the Mn^{++}/Mg^{++} activity ratio (at the optimal concentration of each ion) is greater for polymerase II.[1] In studies with isolated nuclei, others[2–4] have shown that at low ionic strength (in the presence of Mg^{++}) RNA synthesis occurs primarily in the nucleolus, producing a guanosine-cytidine-rich (ribosomal-like) RNA while at higher ionic strengths (in the presence of Mn^{++}) RNA synthesis in the nucleoplasm is elevated, producing a more DNA-like RNA. These facts led us to propose that RNA polymerases I and II were restricted to the nucleolus and nucleoplasm, respectively. We have now tested this supposition by determining the distribution of the polymerases in the nucleolar and nucleoplasmic fractions of purified rat liver nuclei. As predicted, polymerase I was concentrated in the nucleolus and polymerase II in the nucleoplasm.

Materials and Methods. Preparation of nuclei: Nuclei were isolated from the livers of adult Sprague-Dawley male rats by a modification of the procedures described by Blobel and Potter[5] and by Busch *et al.*[6] The livers were homogenized at 0° in 2 vols of 0.34 *M* sucrose, 15 mM magnesium acetate, and 0.25 mM spermine in a glass-Teflon motor-driven homogenizer. The homogenate was diluted with 2 vols of a 2.3 *M* sucrose solution. Aliquots (50 ml) were placed in centrifuge tubes and each underlaid with 10 ml of 2.3 *M* sucrose. After centrifugation at 25,000 rpm (Spinco 25.2 rotor) for 1 hr, the supernatants were discarded, and the nuclear pellets were resuspended in 0.34 *M* sucrose.

Fraction of nuclei: Nucleolar and nucleoplasmic fractions were obtained by procedures described by Busch and collaborators.[6-8] The nuclear suspension was sonicated in 20-ml aliquots for 10-sec periods with a Branson S-125 sonifier (microtip attachment, setting 3) until nearly all the nuclei were disrupted (about 1 min total sonication time). The sonicates were layered over equal volumes of 0.88 *M* sucrose and centrifuged at 2000 × *g* for 30 min in a horizontal rotor. The upper layers, constituting the nucleoplasmic fraction, were carefully removed. The pellets were resuspended in 0.61 *M* sucrose by mild sonication and spun for 30 min at 2000 × *g*. The nucleolar pellet was then resuspended in 0.34 *M* sucrose. Nucleolar preparations obtained by these procedures appeared microscopically similar to those obtained by Busch.[8] There was no obvious contamination with other nuclear or cytoplasmic components, except for a slight contamination with intact nuclei. However, the nucleoli to nuclei ratio was always greater than 50. (At an average ploidy of 4, this would indicate $\leq 8\%$ nucleoplasmic contamination.)

RNA polymerase solubilization and initial fractionation: Each nuclear, nucleoplasmic, or nucleolar suspension was adjusted to 0.01 *M* Tris-HCl, pH 7.9, 1.0 *M* sucrose, 0.005 *M* $MgCl_2$, 0.005 *M* dithiothreitol. Ammonium sulfate (4 *M*, adjusted to pH 7.9 with ammonia) was added to bring the concentration to 0.3 *M*. The viscous solution was sonicated in 20-ml aliquots for 10-sec periods with a Branson S-125 (microtip attachment, setting 3) until the viscosity decreased to a point at which the solution readily formed drops at the tip of a Pasteur pipet (about 1 min total sonication time). The suspension (fraction 1) was then rapidly mixed with 2 vols of 0.05 *M* Tris-HCl, pH 7.9, 25% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetate (EDTA), 0.5 mM dithiothreitol (TGMED). The precipitate was removed by centrifugation for 1 hr at 105,000 × *g* and discarded. The clear supernatant (fraction 2) was brought to near saturation with ammonium sulfate by the addition of 0.42 g of solid ammonium sulfate per milliliter of solution. The precipitate was collected by centrifugation for 1 hr at 105,000 × *g* and resuspended in TGMED (fraction 3). After

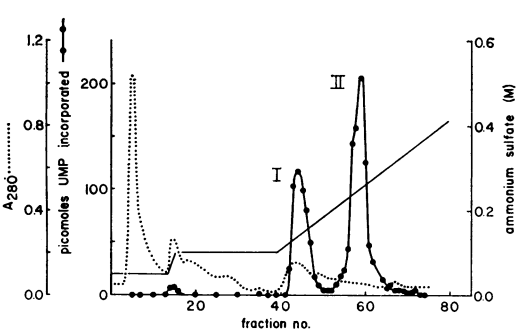

FIG. 1.—DEAE-Sephadex chromatography of total nuclear RNA polymerase: A sample of total nuclear fraction 4 polymerase (3.7 mg protein) from Expt. 1, Table 1, was chromatographed on a 0.7 × 11 cm DEAE-Sephadex (A-25) column as described elsewhere.[1] 0.6-ml fractions were collected and 50-μl aliquots assayed for polymerase activity at about 0.005 mM UTP as described in *Materials and Methods*. The UMP incorporation represents total picomoles UMP incorporation in 10 min per fraction. The activity values for the peak tubes from I and II were 5.8 and 5.9 times greater, respectively, where reassayed at 0.12 mM UTP.

dialysis for several hours against TGMED containing 0.05 *M* ammonium sulfate, the dialysate was centrifuged at 160,000 × *g* for 1 hr. The precipitate, containing little activity, was discarded. The supernatant (fraction 4) was either stored at −90°C or immediately subjected to DEAE-Sephadex chromatography. The chromatography procedure is described in the legend of Figure 1.

Assay for RNA polymerase activity: The components present in the reaction mixtures (125 μl) were as described previously.[1] The concentrations of UTP and ammonium sulfate in the incubation mixtures are given in the appropriate figure or table legend for each experiment. After incubation for 10 min at 37°C, the reactions were stopped by adding 0.10 ml of cold 0.10 *M* sodium pyrophosphate (adjusted to pH 7 with HCl) containing 2 mg/ml RNA, 2 mg/ml bovine serum albumin, 5 mM UTP and quick cooling. Then 0.1 ml of 5% sodium dodecyl sulfate was added and while mixing gently (vortex), 2 ml of a cold solution containing 10% trichloroacetic acid, 0.04 *M* sodium pyrophosphate was added. After collecting on Whatman GF/C filters and washing eight times with 5 ml of 5% trichloroacetic acid containing 0.04 *M* pyrophosphate, the filters were hydrolyzed in 0.5 ml of 5% trichloroacetic acid (95°, 15 min.) and the hydrolysate counted in a dioxane based scintillation solution containing 5 gm Omnifluor (New England Nuclear) and 100 g napthalene/liter of solution.

DNA and protein determinations: DNA was assayed by the diphenylamine procedure of Burton.[9] Protein was measured by the method of Lowry *et al.*[10] For the determination of protein in the dilute DEAE-Sephadex fractions, the samples were concentrated with Diaflo UM-20E ultrafiltration membranes (Amicon Corp.) and subsequently dialyzed versus water prior to assay. Alternatively, the dilute samples were dialyzed, freeze-dried, and subsequently assayed.

Results. Isolated nuclei as well as the nucleolar and nucleoplasmic subfractions derived from nuclei were assayed for the RNA polymerases by first solubilizing the polymerase activity from each and subsequently analyzing the preparations by DEAE-Sephadex chromatography. In order to make a quantitative estimate of the relative amounts of the multiple polymerases in each of these fractions, it was necessary to obtain a high yield of polymerase activity from whole nuclei and show that the subfractionation of the whole nuclei did not appreciably change the yield. As shown in Table 1, the majority of the polymerase activity is recovered directly from intact nuclei or from the fractionated nuclei. Throughout the early phases of the purification procedure, the activity recovered in the nucleolus plus that recovered in the nucleoplasm is about 90 per cent of that originally detected in the nucleus. Calculation of the yields of activity based upon the activity present in the original nuclear suspension (designated 100%) must be made with some caution since this original value is not markedly influenced by exogenous DNA, while after high salt treatment most of the activity is dependent on added DNA. Thus, the template for the polymerase changes from the natural nucleoprotein complex to a denuded DNA and, therefore, the transcriptive efficiency of each template by the polymerase might differ. The correspondence between the activity of the high ionic strength sonicate (fraction 1) and the initial nuclear suspension suggests template effects may be small in this instance, and we assume the recoveries are not misleading. The activity of fraction 4 (which contains less than 1% acid insoluble DNA) is nearly completely dependent upon exogenous DNA (20- to 100-fold stimulation by native DNA). Furthermore, the activity in the nuclei and in the partially purified fractions is dependent upon the presence of all four nucleoside triphosphates, is sensitive to actinomycin-D and DNase, and yields an RNase

TABLE 1. *Yields of soluble RNA polymerase from nuclei and from nucleolar and nucleoplasmic fractions.*

Expt.	Fraction	Specific activity (units/μg protein)	Total activity (units)	Per cent initial activity
1	Nuclear suspension	0.058	6700	100
	F1	0.054	6250	92
	F2	0.060	5780	86
	F3	0.116	6280	94
	F4	0.198	5820	87
2	Nuclear suspension	0.042	8750	100
	Sonicate (0.34 *M* sucrose)	0.048	9750	112
	Nucleoplasm	0.039	5650	65
	F1	0.048	6910	78
	F2	0.054	5420	62
	F3	0.106	5500	63
	F4	0.222	4890	55
	Nucleoli	0.332	4400	50
	F1	0.240	3470	40
	F3	0.280	3380	39
	F4	0.425	3100	35

In expts. 1 and 2, initial nuclear suspensions contained 25 and 48 mg DNA, respectively. The various fractions are those described in *Materials and Methods*. For the nucleolar polymerase solubilization, F1 was subjected directly to ammonium sulfate fractionation, thereby eliminating F2. Aliquots of all the fractions were adjusted to 0.05 *M* Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 25% (v/v) glycerol prior to assay. RNA polymerase activity was determined on 50-μl samples as described in *Materials and Methods* at 0.086 mM UTP (Expt. 1) or 0.12 mM UTP (Expt. 2). The assay concentrations of ammonium sulfate varied from 0.025–0.045 *M* for the nucleolar fractions and from 0.08–0.10 *M* for all others. One unit of activity is that amount of enzyme which catalyzes the incorporation of one picomole of UMP into RNA per minute.

sensitive product (Roeder and Rutter, unpublished observations). We thus conclude that the activity measured is indeed RNA polymerase and that the recovery during the initial fractionation procedures is sufficiently high to allow quantitative analysis of the polymerases by the DEAE-Sephadex chromatography procedure reported elsewhere.[1] The recovery of activity from this procedure is nearly quantitative (greater than 90%). Thus, the total yield of polymerase activity throughout the entire procedure of isolation of the nuclei and nuclear subfractions, and the solubilization, partial purification and chromatographic resolution of the polymerases is of the order of 75 to 90 per cent.

The DEAE-Sephadex elution profiles for the soluble enzyme preparations derived from the nuclear, nucleoplasmic, and nucleolar preparations (described in Table 1) are shown in Figures 1, 2, and 3, respectively. The nucleolar and nucleoplasmic fractions contain predominantly polymerases I and II, respectively. As summarized in Table 2, the ratio of polymerase I to polymerase II activity is about 3.5 times lower in the nucleoplasmic fraction than in whole nuclei. On the other hand, in the nucleoli as isolated, the ratio of I:II is 23- and 80-fold greater than the ratio in whole nuclei and in the nucleoplasmic fraction, respectively.

A third minor peak of RNA polymerase activity eluting at about 0.3 *M* ammonium sulfate was detected in the nucleoplasmic preparations (Fig. 2).

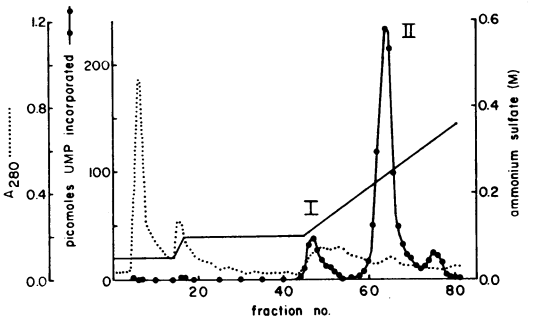

FIG. 2.—DEAE-Sephadex chromatography of nucleoplasmic RNA polymerase: A sample of nucleoplasmic fraction 4 polymerase (2.8 mg protein) from Expt. 2, Table 1, was chromatographed as described in Fig. 1. The activities for the peak tubes from I and II were 4.2 and 5.6 times higher, respectively, when reassayed at 0.12 mM UTP.

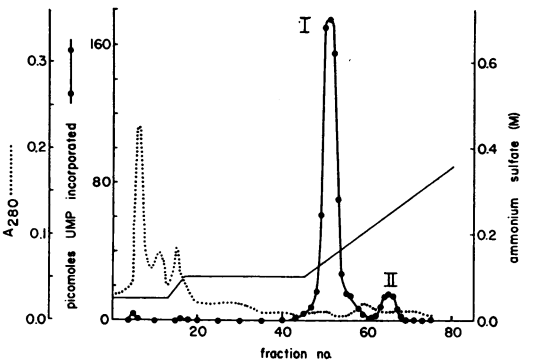

FIG. 3.—DEAE-Sephadex chromatography of nucleolar RNA polymerase: A sample of nucleolar fraction 4 polymerase (1.0 mg protein) from Expt. 2, Table 1, was chromatographed as described in Fig. 1. The activities for the peak tubes from I and II were 3.9 and 3.7 times higher, respectively, when reassayed at 0.10 mM UTP.

We have also detected this activity, in addition to polymerases I and II, in polymerase preparations solubilized from rat liver nuclei according to the procedures of Goldberg *et al.*[11] and Liao *et al.*[12] and subsequently analyzed by our procedures. In the present experiments, however, this form was not detected in polymerase preparations from whole nuclei (Fig. 1). The activity might have been lost during the polymerase solubilization from whole nuclei or not resolved during subsequent DEAE-Sephadex chromatography. (There is, of course, also the possibility that polymerase III is not a natural nuclear constituent and is produced during the analysis by some means from polymerase I and/or II.)

The relatively simple procedure utilized for the analysis of the polymerase results in substantial purification of the several activities. The specific activities for each of the peak fractions eluted from DEAE-Sephadex are reported in Table 2. For the whole nuclei preparation (Fig. 1) the specific activities (pmoles UMP incorporated/min/μg protein) were 3.7 and 17 for polymerases I and II, respectively. For the nucleoplasmic preparation (Fig. 2), the values were 0.5 and 20 for polymerases I and II, respectively, whereas polymerase I from the nucleolar preparation (Fig. 3) had a specific activity of 27. The specific activities reported here are higher than those reported elsewhere[1] by us for rat nuclei. These values were based on 280/260 absorbance ratios, and we believe they were artificially high because of nonprotein materials absorbing in this region (glycerol, or components therein, and dithiothreitol). The values reported here should also be considered as approximations since the low concentrations of protein in some of the purified fractions were virtually at the limits

of detectability of the assay. The specific activities of our polymerases compare favorably with the highest value (47) reported for a mammalian RNA polymerase preparation, obtained recently by Goldberg *et al.*[11] (predominantly polymerase II?) and approach the specific activity (approximately 100) reported by Chamberlain and Berg[16] for the *E. coli* RNA polymerase.

TABLE 2. *Relative concentrations of RNA polymerases I and II in nuclei and in nucleolar and nucleoplasmic fractions.*

Soluble enzyme preparation	Ratio of I:II on DEAE-Sephadex	Specific Activity (pmoles/min/μg protein)	
		I	II
Nucleolar	0.56	3.7	17
Nucleoplasmic	0.16	0.5	20
Nucleolar	13	27	1.5

The ratios of I:II for the nuclear, nucleoplasmic, and nucleolar enzyme preparations were calculated from the data in Figs. 1, 2, and 3, respectively, after summation of the activities (at 0.005 mM UTP) in each peak. The method used for assay of the column fractions (dilution of 50 μl of the fraction to a final assay volume of 125 μl) resulted in assay concentrations of ammonium sulfate which were nearly optimal for peak II fractions but which were slightly suboptimal for peak I fractions. The specific activity values were calculated for the peak fractions using the activity at 0.1 mM UTP and the protein values determined as described in *Materials and Methods*.

Discussion. The present studies demonstrate that RNA polymerases I and II are specifically localized within the rat liver nucleus: form I is present primarily within the nucleolus and form II in the nucleoplasm. The small amount of form II in the nucleolar preparation may result from the contamination of the nucleolar preparation with intact nuclei (up to 8%, see *Materials and Methods*). Similarly, the presence of form I in the nucleoplasmic fraction might result from some nucleolar destruction during the fractionation of the nuclei. Muramatsu *et al.*[7] have shown that the sonication procedure used to fractionate the nuclei can result in the destruction of some nucleoli. These considerations make it likely that forms I and II are restricted, respectively, to the nucleolus and the nucleoplasm.

The third RNA polymerase activity detected in rat liver nucleoplasm in these experiments resembles form III of the sea urchin embryo in its chromatographic properties. We have yet to determine whether these two enzymes have similar catalytic properties. Certainly the minor component in rat liver is a smaller proportion of the total activity than that of form III in sea urchin embryos. Attempts to define the properties and possible function of the third RNA polymerase are in progress.

Polymerases I and II should not be simply equated with the Mg^{++}/low salt and Mn^{++}/high salt "activities" reported by others.[2-4] Jacob *et al.*[13] have reported essentially equivalent levels of both of these activities in isolated nucleoli. The results of the present experiments suggest their results are due to differential ion effects on a single polymerase species within the nucleolar complex.

The specific localization of the RNA polymerases within the nucleus raises several significant questions concerning the regulation of RNA synthesis in eukaryotic organisms. From their distribution it seems reasonable to postulate that polymerases I and II are involved in the synthesis of ribosomal RNA in the nucleolus[14] and the synthesis of DNA-like RNA in the nucleoplasm, respec-

tively. Preliminary experiments reported elsewhere,[1] however, give no evidence of inherent specificity of transcription of DNA by the isolated sea urchin polymerases I, II, and III. The apparent lack of specificity might be simply related to the assay conditions or to the presence of nonspecific initiation sites in the heterologous (nicked?) DNA employed. It is also possible that other factors are present *in vivo* which might confer specificity on, or increase the activity of, one or all polymerase activities. The maximal level of activity attainable *in vitro* with the solubilized polymerase is at least an order of magnitude less than that required to account for the apparent rate of RNA synthesis *in vivo* (Roeder and Rutter, manuscript in preparation).

If the RNA polymerases play a role in determining transcriptive specificity, then a change in the level or activity of the respective polymerases might occur, especially when the relative rates of synthesis of the major classes of RNA are substantially altered. Changes in the levels and ratios of the sea urchin polymerases are observed during early development and have been correlated with RNA synthesis in the intact embryos (Roeder and Rutter, manuscript in preparation). Selective changes in the activities of the RNA polymerases might also be elicited by hormones in specific target tissues. It has been reported that the Mg^{++}/low ionic strength RNA polymerase activity increases at an earlier time than does the Mn^{++}/high ionic strength activity in liver after the administration of certain hormones (see Tata, ref. 15). The possibility that these effects are mediated by changes in the polymerase deserves a careful experimental test.

Defining the changes in levels and/or activities of the RNA polymerase species may provide an insight into the mechanism of transcriptive regulation during various functional transitions, such as those involved in embryonic development, hormonal stimulation, and in various pathological states such as viral infections or carciogenesis. The methods developed here appear appropriate for such analyses, as well as for the initial steps in the isolation of the various polymerase species, so that structure-function and regulatory relationships can be effectively investigated at the molecular level.

* Supported by National Institutes of Health grant HD-02126 and National Science Foundation grant GB 6470.

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