

# Molecular structure of yeast RNA polymerase III: Demonstration of the tripartite transcriptive system in lower eukaryotes

(RNA nucleotidyltransferase III/subunits)

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**ABSTRACT** Homogeneous RNA polymerase III (RNA nucleotidyltransferase III) has been obtained from yeast. The subunit composition of the enzyme was examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The enzyme is composed of 12 putative subunits with molecular weights 160,000, 128,000, 82,000, 41,000, 40,500, 37,000, 34,000, 28,000, 24,000, 20,000, 14,500, and 11,000. The high-molecular-weight subunits and several of the smaller subunits of yeast RNA polymerase III are clearly different from those of enzymes I and II, indicating a distinct molecular structure. However, the molecular weights of some of the small subunits (41,000, 28,000, 24,000, and 14,500) appear to be identical to those of polymerases I and II. Thus, it is possible that the three classes of enzymes in yeast have some common subunits.

As in other eukaryotes, yeast polymerase II is inhibited by relatively low concentrations of alpha-amanitin; however, contrary to what has been found in higher eukaryotes, yeast polymerase III is resistant (up to 2 mg/ml) to alpha-amanitin, while yeast polymerase I is sensitive to high concentrations of the drug (50% inhibition at 0.3 mg/ml).

These results establish the existence of RNA polymerase III in yeast and provide a structural basis for the discrimination of the three functional polymerases in eukaryotes.

Since the original report that eukaryotic cells contained three major classes of DNA-dependent RNA polymerases (1), there have been many investigations of RNA polymerase (RNA nucleotidyltransferase) multiplicities in many species (2, 3). The general findings have been confirmed for polymerases I (A) or polymerase II (B) in every system examined. In contrast, until recently, polymerase III (C) has been absent in most comprehensive analyses of RNA polymerase activities. This fact raised serious questions concerning the existence of polymerase III as a major functional class of RNA polymerases and further implied that the earlier results might have been artifactual (2). The work of Roeder and his colleagues has substantially clarified this matter by demonstrating that in two higher eukaryotes (*Xenopus laevis* and mouse plasmacytoma MOPC 315) polymerase III was inhibited by very high levels of alpha-amanitin (4, 5). This characteristic was diagnostic in most higher organisms and allowed the discernment of polymerase III activity even when this enzyme was not clearly resolved chromatographically from the other polymerases. Furthermore, it was shown that polymerase III specifically transcribes 5S and 4S RNA genes in mouse plasmacytoma cells (6) and several small RNA species from adenovirus-infected cells (7). Finally, a subunit structure of polymerase III distinct from that of polymerases

I and II was found (8). Thus, a unique structural and functional role for this enzyme seems apparent in higher organisms.

In lower eukaryotes, however, the situation has remained unclear. Although the original studies of Roeder and of Rutter and coworkers (9) and later of others (10-12) reported the chromatographic evidence for the presence of polymerase III in yeast, there has never been a confirmation by isolation and characterization of the structure of this enzyme in any lower eukaryotes. In contrast, polymerases I and II have been isolated and their subunit structure defined for yeast (13, 14, †), *Dictyostelium* (15), and *Physarum* (16).

In this paper we report the characterization of yeast polymerase III which can be isolated by two different procedures. The homogeneous preparation contains two large polypeptides (160,000 and 128,000 daltons), one polypeptide of intermediate size (82,000 daltons), and 9 to 10 smaller peptides with stoichiometries varying from 1 to 2 moles per mole of enzyme. This structure is clearly different from yeast polymerases I and II and bears a general resemblance to polymerases III from *Xenopus* and mouse plasmacytoma. These results provide a convincing argument for the existence of class III polymerases in all eukaryotes and establish a structural basis for the discrimination of the three enzymes.

## MATERIALS AND METHODS

**Materials.** Materials and yeast cells were obtained as described previously (13). DNA-cellulose was prepared by the method of Alberts and Herrick (17), using denatured calf thymus DNA.

**RNA Polymerase Assay.** Enzymes were assayed as described previously (13). One unit corresponds to the incorporation of 1 nmol of UMP into RNA in 10 min at 30°.

**Enzyme Purification.** Yeast RNA polymerase III was purified to homogeneity by two methods. *Method A:* Polymerase III was obtained as a by-product of a large-scale purification of yeast polymerase I. The first three steps are batchwise treatment with phosphocellulose, batchwise treatment with DEAE-cellulose, and ion-filtration chromatography on DEAE-Sephadex. The details of these procedures, which are common to the purification of polymerase I, will be published elsewhere (13). Enzymes I and III are then separated by DNA-cellulose chromatography (Fig. 1). Whereas, enzyme I is eluted at 0.45 M KCl, polymerase III binds more strongly and is eluted in a highly purified form at 0.7 M KCl. Concentration of the enzyme and further purification, mainly the removal of a small amount of contamination by polymerase I, is achieved by DEAE-Sephadex chromogra-

Abbreviations: TGEM buffer, 0.02 M Tris · HCl (pH 8.0), 25% glycerol, 0.1 mM EDTA, 0.02 M 2-mercaptoethanol; NaDodSO<sub>4</sub>, sodium dodecyl sulfate.

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† G. Hager, M. Holland, and W. J. Rutter, submitted to *Biochemistry*.

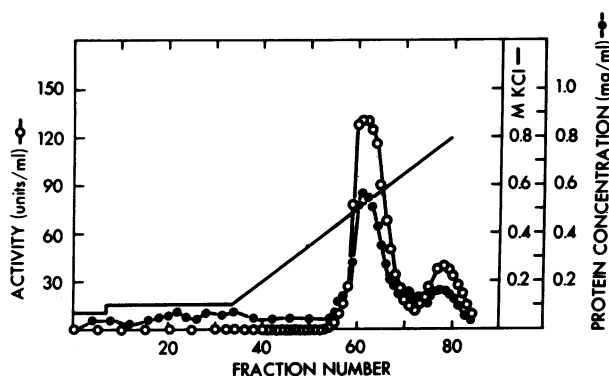


FIG. 1. DNA-cellulose chromatography of yeast RNA polymerases I and III. About 18 mg of RNA polymerases from the ion-filtration step from 600 g of cells (12) are loaded in a 25-ml column of calf thymus denatured DNA-cellulose. After the column was washed with 80 ml of buffer TGEM (0.02 M Tris · HCl, pH 8.0, 25% glycerol, 0.1 mM EDTA, and 0.02 M 2-mercaptoethanol) containing 0.1 M KCl, the enzymes were eluted with a linear gradient of 0.1–0.8 M of KCl in TGEM. Enzyme I elutes at 0.45 M KCl and enzyme III at 0.7 M KCl. Fractions of 2.4 ml were collected.

phy (Fig. 2). The pooled active fractions have a constant specific activity of 250 units/mg of protein. Between 3 and 4 mg of enzyme are obtained from 1200 g of cells. This represents an overall purification of about 1000-fold, with a yield of about 15–20%.

**Method B:** RNA polymerase III is purified by protamine sulfate fractionation, gel filtration on agarose A-5M, ion-filtration on DEAE-Sephadex, and DNA-cellulose chromatography. Enzyme (10–12 mg) with a specific activity of 250 units/mg of protein is obtained from 1200 g of yeast cells. The overall yield is 40–60%. Details of this procedure will be published elsewhere<sup>†</sup>.

Yeast RNA polymerase I and II were purified to homogeneity as described elsewhere (13, <sup>†</sup>).

**Polyacrylamide Gel Electrophoresis.** Nine percent and 12% acrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate (NaDodSO<sub>4</sub>) was carried out in 12 × 15 cm × 1.5 mm slab gels (Hoefer Scientific Instruments, San Francisco, Calif.). Buffers and solutions were prepared according to Laemmli (18). The gels were fixed by shaking them in 50% isopropanol, 10% trichloroacetic acid for 30 min at 30° and stained 12 hr in 25% isopropanol, 10% trichloroacetic acid, and 0.1% Coomassie blue at 23°. Gels were destained at 23° with 10% acetic acid. The slab was cut into 0.8 × 10 cm strips and scanned at 650 nm with a Gilford linear transport device attached to a Gilford spectrophotometer. The relative amount of each component present was determined from the areas under the peaks. To calibrate the relationships between mobility and molecular weight, a number of proteins of known molecular weight were subjected to electrophoresis under the same conditions. The following proteins and molecular weight values were used: *E. coli* RNA polymerase subunit β', 165,000 (19); *E. coli* RNA polymerase β, 155,000 (19); β-galactosidase, 130,000 (20); phosphorylase A, 94,000 (21); bovine serum albumen 63,000 (22); *E. coli* RNA polymerase α, 39,000 (19); carbonic anhydrase, 29,000 (23); and lysozyme, 14,300 (24).

Gel electrophoresis in two dimensions was carried out in slab gels. Native enzyme was used in the first dimension in 5% acrylamide gels under non-denaturing conditions (25). NaDodSO<sub>4</sub> gel electrophoresis was performed in the second dimension. The corresponding strip was cut, equilibrated 30 min at 23° and 5 min at 80° in Laemmli's sample buffer

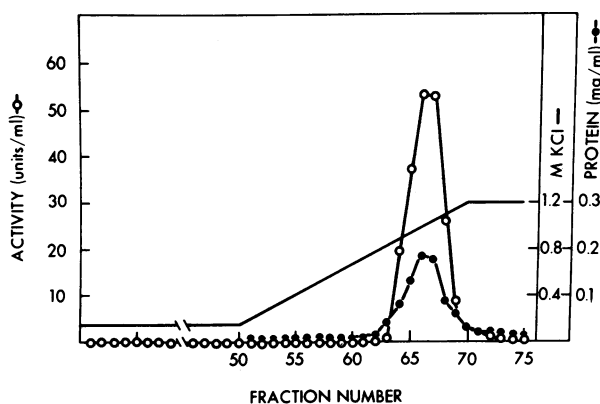


FIG. 2. DEAE-Sephadex chromatography of yeast RNA polymerase III. About 4 mg of enzyme from the DNA-cellulose step were diluted in small aliquots to 0.05 M KCl with TGEM and loaded on a 5-ml column of DEAE-Sephadex A-25. The column was washed with 25 ml of TGEM containing 0.12 M KCl and eluted with a linear gradient of 0.12–1.2 M KCl in TGEM. Fractions of 1.2 ml were collected.

without glycerol (18), and layered on top of a polymerized 3% acrylamide–0.1% NaDodSO<sub>4</sub> stacking gel and 9% or 12% acrylamide–0.1% NaDodSO<sub>4</sub> resolving gels.

### RESULTS

The peak fractions from the DEAE-Sephadex chromatography containing essentially pure polymerase III (method A) were subjected to electrophoresis in the presence of sodium dodecyl sulfate. Fig. 3 shows the staining pattern of peak fractions from DEAE-Sephadex chromatography (Fig. 2) in 9% acrylamide gels. The densitometer patterns of enzyme III subjected to 9% and 12% acrylamide gel electrophoresis are presented in Fig. 4. Twelve polypeptides, distinguished by their different migration in polyacrylamide gels, consistently copurify with the enzyme activity. Molecular weights were estimated from a standard curve prepared from the migration of known proteins. In 9% acrylamide gels, protein

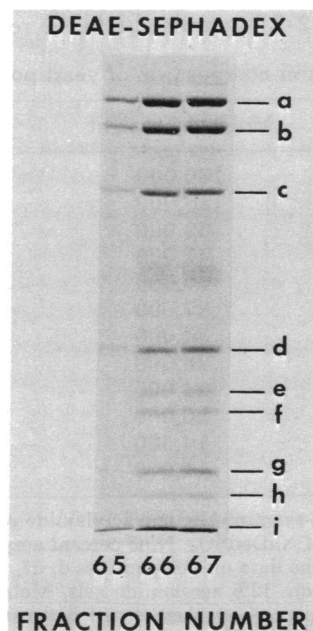


FIG. 3. Polyacrylamide-NaDodSO<sub>4</sub> gel electrophoresis of yeast RNA polymerase III. Enzyme (2–6 μg) from active fraction of DEAE-Sephadex chromatography. Gels contain 9% acrylamide.

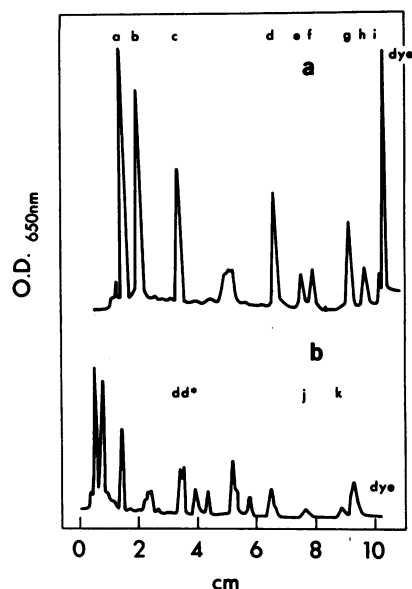


FIG. 4. Densitometer tracings of yeast RNA polymerase III subjected to polyacrylamide gel electrophoresis. (a) 9% Acrylamide; (b) 12% acrylamide.

bands appear at 160,000 (III<sub>a</sub>), 128,000 (III<sub>b</sub>), 82,000 (III<sub>c</sub>), 41,000 (III<sub>d</sub>), 37,000 (III<sub>e</sub>), 34,000 (III<sub>f</sub>), 28,000 (III<sub>g</sub>), 24,000 (III<sub>h</sub>), and 20,000 (III<sub>i</sub>). The same protein bands were also observed when 12% acrylamide gels (Fig. 4) were used except for the following: (i) polypeptides of 14,500 (III<sub>j</sub>), and 11,000 (III<sub>k</sub>) daltons, which migrate with the dye in 9% acrylamide gels, are now clearly evident; (ii) heterogeneity is found in the polypeptide of 41,000 (III<sub>d</sub>) daltons, which resolves into two polypeptides of 41,000 (III<sub>d</sub>) and 40,500 (III<sub>d</sub>\*) daltons; both are present in equimolar amounts; and (iii) polypeptides III<sub>g</sub> and III<sub>i</sub> show evidence of heterogeneity; a shoulder is discernible at the right side of each band. A summary of these results, including the estimated molar ratios, is shown in Table 1.

A group of three to four protein bands with individual molar ratios of 0.2–0.3 appears also in the region of 53,000

Table 1. Subunit composition of yeast polymerase III<sup>a</sup>

Polypeptide	Molecular weight	Molar ratio
a	160,000	1.0
b	128,000	1.0
c	82,000	1.0
d	41,000	2.0
d*	40,000	—
e	37,000	0.8
f	34,000	0.9
g	28,000	2.0
h	24,000	1.5
i	20,000	1.2
j	14,500	1.4
k	11,000	—

<sup>a</sup> Composition was examined by polyacrylamide gel electrophoresis in the presence of NaDodSO<sub>4</sub>. Nine percent acrylamide gels were used except for the data on polypeptides d, d\*, i, j, and k, which were obtained from 12% acrylamide gels. Molar ratios are normalized to polypeptide a: they varied between 10 and 20% in different preparations. They are estimated from the amount of dye adsorbed, a method which may not be accurate. The values shown correspond to those obtained from the data of Figs. 4 and 5. These data were obtained with enzyme purified by method A.

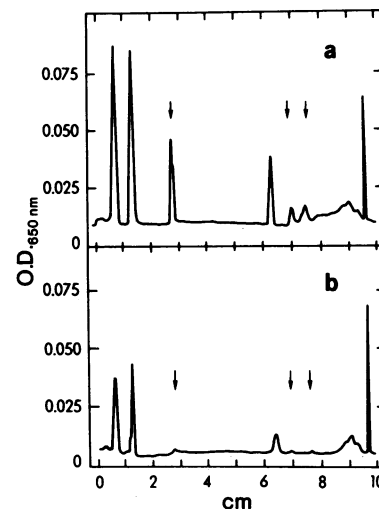


FIG. 5. Second dimension polyacrylamide gel electrophoresis of yeast RNA polymerase III. This figure shows the subunit composition of two components separated in the first dimension (5% acrylamide gels run under non-denaturing conditions, not shown). (a) Slowly migrating component; (b) fast migrating component. Arrows indicate the position of polypeptides III<sub>c</sub>, III<sub>e</sub>, and III<sub>f</sub>. The gels contain 9% acrylamide and 0.1% NaDodSO<sub>4</sub>.

daltons in enzyme purified by method A. However, in enzyme purified by method B, a single polypeptide with a molecular weight of 53,000 is observed in this region. The molar ratio of this polypeptide varied in three preparations between 0.46 and 0.86 and was constant across fractions containing activity from a sucrose gradient of purified enzymes. Thus, it is possible that this polypeptide is also a component of yeast polymerase III.

Analysis of the purified RNA polymerase III by polyacrylamide gel electrophoresis under non-denaturing conditions revealed the presence of two main protein bands. The subunit composition of each component was determined by performing electrophoresis under denaturing conditions in a second dimension (Fig. 5). The slower band contained all the subunits found in the purified enzyme. The faster band was identical except that subunits III<sub>c</sub>, III<sub>e</sub>, and III<sub>f</sub> were absent.

The general properties of the isolated yeast RNA polymerase III are summarized and compared to those of yeast polymerases I and II in Table 2. Yeast polymerase III shows many properties similar to those of class III polymerases from *Xenopus* and mouse plasmacytoma (4, 5). Polymerase III is readily distinguished from I and II by (i) its differential elution from DEAE-cellulose compared with DEAE-Sephadex; (ii) differential sensitivity to alpha-amanitin; and (iii) its biphasic salt activation profile.

The RNA polymerase from higher eukaryotic cells are readily discriminated by their differential sensitivity to the mushroom toxin, alpha-amanitin (6, 7, 26). Polymerase II from these cells is highly sensitive to the drug, while polymerase III is sensitive only at much higher levels of the toxin. Contrary to these sensitivities reported for the enzymes from higher cells, Schultz and Hall (27) have found anomalous sensitivity to alpha-amanitin for crude enzyme fractions from yeast. Putative polymerase III, identified by classical chromatographic and template activity criteria, is resistant to alpha-amanitin, while the putative polymerase I is sensitive to high concentrations of the drug (50% inhibition at 600 μg/ml). Huet *et al.* (28) have also reported the partial sensitivity of yeast polymerase A (I), and the structurally modified variant A\*, to this toxin.

Table 2. General properties of homogeneous yeast RNA polymerases I, II, and III

	I	II	III
Ammonium sulfate concentration (M) for elution from DEAE-cellulose (pH 7.9)	0.12	—	0.12
Ammonium sulfate concentration (M) for elution from DEAE-Sephadex (pH 7.9)	0.13	0.24	0.30
Alpha-amanitin concentration for 50% inhibition ( $\mu\text{g/ml}$ )	300	0.8	—
Salt concentration for optimal activity with native calf thymus DNA			
KCl (M)	0.11	0.25	0.13; 0.43
$(\text{NH}_4)_2\text{SO}_4$ (M)	0.03	0.10	0.04; 0.18

We have carried out a complete titration of the sensitivities of homogeneous RNA polymerases to alpha-amanitin (Fig. 6). We find that polymerase II is inhibited by low concentrations of the drug (50% inhibition at 0.8  $\mu\text{g/ml}$ ), polymerase I is sensitive to high concentrations (50% inhibition at 300  $\mu\text{g/ml}$ ), and polymerase III is resistant to concentrations of alpha-amanitin up to 2 mg/ml.

### DISCUSSION

The present results demonstrate conclusively the presence of polymerase III in yeast and document its peptide components. The enzyme contains two large subunits of 160,000 and 128,000 daltons, one intermediate size subunit of 82,000 daltons, and several smaller subunits. A comparison of the structures of yeast RNA polymerases I, II, and III is presented in Table 3. The molecular weight of the large subunits of yeast polymerase III (160,000 and 128,000) differ from those of yeast polymerase I (185,000 and 137,000) and II (170,000 and 145,000). In addition, the 82,000 dalton and possibly 53,000 dalton subunits are present in III, but absent in I and II. The molecular weights of certain of the smaller subunits seem to be identical for the three polymerases, particularly those of molecular weights 41,000, 28,000, 24,000, and 14,500. In addition, I and II have a 12,000 dalton subunit not present in III, and I and III have one 20,000 dalton subunit not present in II. This analysis demonstrates that the three

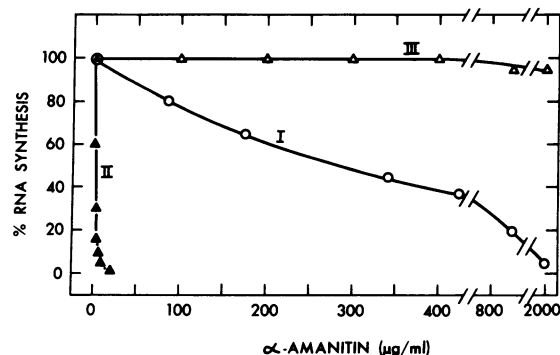


FIG. 6. Effect of alpha-amanitin upon the activity of yeast RNA polymerases. Enzymes I, II, and III were assayed in 0.03 M, 0.1 M, and 0.03 M  $(\text{NH}_4)_2\text{SO}_4$ , respectively.

Table 3. Subunits of yeast RNA polymerases I, II, and III

I*	II†	III‡
185	170	160
137	145	128
		82
		53
48		
44		
41	41	41
		40.5
36		37
	33	34
28	28	28
24	24	24
20		20
	18	
14.5	14.5	14.5
12	12	11

Numbers indicate subunit molecular weight  $\times 10^{-3}$ .

\* Data from Valenzuela *et al.* (13).

† Data from G. Hager, M. Holland, and W. J. Rutter, submitted to *Biochemistry*.

‡ Data from this paper.

RNA polymerases of yeast have a distinct molecular structure defined mainly by the unique large-molecular-weight subunits. Some of the smaller subunits may be common to two or to the three forms of yeast RNA polymerases. However, this possibility should be examined carefully, since these subunits could be identical in size, but have a unique primary sequence.

Although different when examined in detail, the overall subunit structure of RNA polymerase III from yeast clearly resembles the polymerase III from *X. laevis* and mouse plasmacytoma. The class III enzymes are distinguished particularly by the presence of subunits of intermediate size [82,000 and 53,000 in yeast; 89,000 and 70,000 in mouse plasmacytoma (8); 92,000 and 68,000 in *X. laevis* (8)]. Thus, the general structural homology in the proteins is evident.

Purified RNA polymerase III from yeast can be resolved by electrophoresis into the components. One component contains all the subunits. The other contains all the subunits except for polypeptides III<sub>c</sub> (82,000 daltons), III<sub>e</sub> (37,000 daltons), and III<sub>f</sub> (34,000 daltons). It is not yet clear whether this represents an equilibrium between two functional forms of the enzyme or represents simple dissociation of subunits during electrophoresis. But it seems likely that polypeptides III<sub>c</sub>, III<sub>e</sub>, and III<sub>f</sub> are more loosely bound to the protein, have distinct charges, and therefore separate during electrophoresis. This observation emphasizes the strong possibility that some of the multiple forms of RNA polymerases reported in the literature may be due to the elimination of more dissociable subunits during extensive fractionation procedures.

RNA polymerases I, II, and III from yeast, like enzymes I, II, and III from mouse plasmacytoma (8), can be distinguished on the basis of their subunit structure. This suggests that the three molecules are composed largely of products from different genes and are not artifacts of isolation and interconvertible assemblies of subunits representing variations of a common molecule.

The results of our studies provide an unequivocal interpretation of the anomalous alpha-amanitin inhibition pattern of the yeast enzymes (27, 28). Yeast RNA polymerase I,

identified structurally, is sensitive to high concentrations of alpha-amanitin whereas polymerase III is insensitive to all levels of this toxin tested. This contrasts with the pattern of alpha-amanitin inhibition in *Xenopus* (5), plasmacytoma MOPC<sub>315</sub> (4), and presumably other higher eukaryotes (8). In these instances, RNA polymerase III is sensitive to high levels of alpha-amanitin and polymerase I is insensitive to this compound. In all systems RNA polymerase II is sensitive to relatively low levels of the drug. The sensitivity to alpha-amanitin, therefore, cannot be used as the prime diagnostic test for the RNA polymerase in previously uncharacterized systems. Once the identity of the enzymes has been established on structural or functional grounds, however, alpha-amanitin remains a useful probe for distinguishing polymerase activities in complex systems, such as nuclei or chromatin.

The functional studies and structural analysis of Weinmann and Roeder and Sklar *et al.* with mouse plasmacytoma polymerase III (6–8) and the present work with the yeast enzymes now provide a basis for the distinction and nomenclature of all eukaryotic RNA polymerases based on the criteria of structure and function. Other criteria that have been used in the past, such as alpha-amanitin inhibition, chromatographic elution behavior, metal requirements, and salt optima, are inadequate because prominent exceptions exist. These criteria should be considered only presumptive evidence until the structure and functional role of the enzymes are defined.

The finding of polymerase III as a distinctive enzyme corroborates the tripartite apparatus for transcription eukaryotes. We predict that all eukaryotes, except perhaps evolutionarily transitional forms, will have the three major polymerases (I, II, and III), each with a unique transcriptional function. It has been demonstrated that yeast polymerase I as isolated has inherent specificity for transcription of ribosomal genes from naked yeast DNA (M. Holland, G. Hager, and W. J. Rutter, submitted to *Biochemistry*). We expect that polymerase III will also display such specificity. Of course, ancillary factors which enhance or modify the transcriptional specificity may exist in the chromatin or in the cytosol. In contrast, the transcriptive activity of polymerase II which transcribes many genes may be directed by other factors not resident in the isolated molecule. The range of transcriptional activity of the eukaryotic cell nucleus can now be understood in terms of the three enzymes. The availability of large quantities of enzymes and specifically defined templates makes feasible an experimental onslaught on the regulation of transcription of particular genes.

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