A Novel Form of RNA Polymerase from *Escherichia coli*

(M13/φX174/rifampicin)

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ABSTRACT   **A new form of RNA polymerase, termed RNA polymerase III, has been recognized as a large fraction of the rifampicin-sensitive enzyme in *E. coli*. It is physically separable from RNA polymerase (holoenzyme, RNA polymerase I) by gel filtration and is distinguished by its capacity to discriminate between M13 and φX174 viral DNA templates in priming DNA synthesis. This template specificity is manifested only with saturating levels of DNA unwinding protein and characterizes the priming of DNA synthesis on viral single strands in cell-free extracts and *in vivo*. RNA polymerase III has less than 5% of the specific activity of RNA polymerase I in transcribing duplex DNA of phages λ and T4, salmon sperm DNA, and the copolymer poly[d(A-T)]. Rifampicin inactivation of RNA polymerase III releases a factor, presumably a small subunit, which can be isolated and used to confer on RNA polymerase I the properties of III, namely, discrimination between M13 and φX174 templates in priming DNA synthesis, and a relative inability to transcribe duplex DNA.**

The observation that rifampicin, a specific inhibitor of the β-subunit of bacterial RNA polymerase, blocks conversion of M13 single-stranded circular viral DNA (SS) to the duplex replicative form (RF) *in vivo* first led us to postulate a role for RNA priming in DNA synthesis (1). Silverstein and Billen (2) found that conversion of φX174 SS to RF was not sensitive to rifampicin under similar conditions. Soluble extracts of gently lysed *Escherichia coli*, like the intact cells, were rifampicin-sensitive in the conversion of SS to RF of M13, but not of φX174 (3). Further studies (4, 5, 20) have shown that replication of φX174 SS is primed by a multienzyme RNA synthetic system using many of the same factors required for host chromosome replication. Recently, reconstitution of M13 SS to RF has been achieved (6) with purified DNA polymerase III holoenzyme (7), DNA unwinding protein (8, 9), and RNA polymerase I (holoenzyme). These enzymes resemble cells and crude extracts in producing M13 RF with a full-length linear complementary strand and a unique gap (3, 6), but they have the nonphysiological property of catalyzing rifampicin-sensitive conversion of φX SS to RF.

A search for the enzymatic basis of M13 DNA-specific priming has led us to purify a new form of RNA polymerase, termed RNA polymerase III, the subject of this communication. This enzyme is assayed by its ability, in the presence of DNA unwinding protein, to prime DNA polymerase III holoenzyme replication of M13, but not φX, DNA. RNA polymerase III, purified approximately 100-fold by ammonium sulfate fractionation and gel filtration, is estimated to be 50% pure by sodium dodecyl sulfate–acrylamide gel analysis.

Abbreviations: SS, single-stranded circular viral DNA; RF, double-stranded, circular viral DNA; φX, bacteriophage φX174.

It is separated from RNA polymerase I during gel filtration. In addition to its selectivity in priming DNA synthesis, it is distinguished from RNA polymerases I and II by the remarkable property of being inactive in transcription of duplex DNA under RNA polymerase I assay conditions. Upon exposure to rifampicin, a small factor is released from RNA polymerase III which renders RNA polymerase I (holoenzyme) both template-specific in priming viral DNA synthesis and inactive in transcribing duplex DNA. These studies raise questions about the physiological role of RNA polymerase III, its structure and interconversion with RNA polymerase I, and the role of each in the RNA synthetic events of the cell.

MATERIALS AND METHODS

Materials were from previously described sources (7). *E. coli* B, harvested ¹/₄ through the logarithmic phase of growth on rich medium, was purchased from Grain Processing Corp., Muscatine, Iowa.

Enzymes. DNA polymerase III holoenzyme was prepared as described (7). DNA unwinding protein was prepared according to Weiner *et al.* (9). We thank Dr. Michael Chamberlin for gifts of RNA polymerase I holoenzyme and core (10) as well as for rifampicin-resistant holoenzyme. Unless otherwise noted, RNA polymerase I was prepared by a minor modification of the method of Babinet (11).

Templates. M13 and φX174 SS were prepared as described previously (12). Salmon sperm DNA was purchased from Sigma. Phage T4 and λ DNAs were the generous gifts of Dr. J. Thorner and Dr. J. M. Syvanen of this department.

Buffers. Buffer I contained 20% glycerol, 0.05 M Tris·HCl (pH 7.5), 1 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM EDTA, and 0.15 M ammonium acetate. Buffer II contained 30% sucrose, 0.02 M Tris·HCl (pH 7.5), 1 mM dithiothreitol, 2 mM $MgCl_2$, 0.1 mM EDTA, and 0.25 M ammonium acetate. Buffer III was the same as buffer II but with only 0.04 M ammonium acetate.

Assays of RNA Polymerase. DNA synthesis on M13 or φX SS was measured in a final volume of 25 μl containing: 5 μl of assay buffer (10% sucrose, 0.05 M Tris·HCl (pH 7.5), 20 mM dithiothreitol, 0.05 M NaCl, 0.2 mg/ml of bovine serum albumin), 3 μl of deoxynucleoside triphosphate mixture [150 μM [α-³²P]dCTP (400 cpm/pmole); 400 μM each of dATP, dGTP, and dTTP; 40 mM $MgCl_2$], 1 μl of ribonucleoside triphosphates (25 mM ATP, 5 mM each of GTP, CTP, and UTP), 2.5 μl of DNA (500 pmoles of nucleotide), 3.5 μl of water, and 5 μl of DNA unwinding protein (1.8 μg). These components (20.0 μl) were mixed and incubated for 2 min at 30°. Water, RNA polymerase, and DNA polymerase

TABLE 1. *Purification of RNA polymerase III*

Fraction	Total units	Total protein, mg	Specific activity, units/mg of protein
I. Extract	90	11,000	0.008
II. Ammonium sulfate I	74	595	0.12
III. Ammonium sulfate II	30	90	0.33
IV. Gel filtration I	27	60	0.45
V. Gel filtration II	12	17	0.71

III holoenzyme (0.05 unit) were then added to chilled tubes to a final volume of 25 μl. After a 10-min incubation at 30°, acid-insoluble nucleotide was determined (13). One unit of RNA polymerase III primes 1 nmole of deoxynucleotide incorporation into M13 RF per min at 30°.

RNA synthesis was measured in a volume of 50 μl containing: 0.05 M Tris·HCl (pH 7.5), 0.4 mg/ml of bovine serum albumin, 4 mM 2-mercaptoethanol, 15 mM MgCl$_2$, 1 mM [^{3}H]ATP (10^3 cpm/pmole), 1 mM each of GTP, CTP, and UTP, and 25 μg/ml of DNA template. Acid-insoluble nucleotide was measured after 10 min at 30°.

RESULTS

Purification of RNA Polymerase III. All operations were at 0–4°. A summary of the purification is in Table 1.

Preparation of Extract. Two hundred grams of frozen *E. coli* B cell paste (see *Materials and Methods*) was broken into small pieces and placed in a 1-liter Waring Blendor. Lysis buffer [10% sucrose, 0.05 M Tris·HCl (pH 7.5), 0.2 M ammonium sulfate, and 10 mM spermidine·HCl] was added to the suspension to a volume of one liter and the cells were suspended with three full-power bursts. Lysozyme (100 mg) was added and the suspension was immediately transferred to five 250-ml plastic bottles placed on ice. After 30 min the bottles were transferred to a 37° bath for 5 min and centrifuged at 0° for 60 min at 12,000 rpm in the Sorvall GSA rotor. The clear amber supernatant is Fraction I (755 ml).

Ammonium Sulfate I. Solid ammonium sulfate (0.3 g/ml of Fraction I) was added with rapid stirring. After 20 min, the suspension was centrifuged for 10 min at 12,000 rpm in the GSA rotor. Pellets were successively suspended in one-fifth the volume of Fraction I in buffer I plus: (a) 0.30 g (b) 0.24 g (c) 0.22 g (d) 0.20 g (e) 0.18 g and (f) 0.16 g of ammonium sulfate per ml. Suspensions were performed with glass–Teflon homogenizers and were followed at once by centrifugation for 10 min at 12,000 rpm in the GSA rotor. The supernatant from each centrifugation was mixed with an equal volume of saturated, neutralized ammonium sulfate and, after 10 min, centrifuged as before. Each precipitate was dissolved in 10 ml of buffer II and fractions of high specific activity (usually d-f) were pooled: Fraction II (44 ml).

Ammonium Sulfate II. Fraction II was mixed with an equal volume of saturated ammonium sulfate and centrifuged after 10 min. The centrifugation here and subsequently was for 5 min at 16,000 rpm in the Sorvall SS34 rotor. Pellets were successively suspended in one-fifth the volume of Fraction I in buffer II plus: (a) 0.24 g (b) 0.22 g (c) 0.20 g (d) 0.18 g and (e) 0.16 g of ammonium sulfate per ml. After each centrifuga-
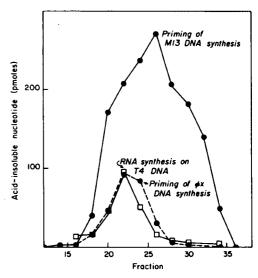

FIG. 1.   Gel filtration. Fraction III enzyme was applied to a Bio-Gel A-5m column (240 ml, see *Results*) and 6-ml fractions were collected. Fractions 25 to 33 were used for further purification of RNA polymerase III.

tion, the supernatants were precipitated with saturated ammonium sulfate as before. Each precipitate was dissolved in 5 ml of buffer III; fractions of high specific activity (usually c and d) were pooled: Fraction III (12 ml).

Gel Filtration I. Fraction III was applied to a Bio-Gel A-5m column (100–200 mesh, 240 ml) equilibrated with buffer II. Fractions that primed DNA synthesis on M13, but not ϕX-174, SS (Fig. 1) were pooled: Fraction IV (60 ml).

Gel Filtration II. Fraction IV was concentrated by addition of 0.3 g of solid ammonium sulfate per ml. After 30 min, the suspension was centrifuged and the precipitate was dissolved with 1.5 ml of buffer III (1.5 ml) and applied to a Bio-Gel A-5m column (100–200 mesh, 60 ml) equilibrated with buffer III. Fractions of peak RNA polymerase III specific activity were pooled: Fraction V (14 ml). This fraction was stable for at least 2 weeks at 0°.

Requirements for Specific Priming of M13 DNA Replication. Discrimination by RNA polymerase between M13 and ϕX DNA for priming DNA synthesis requires two factors: (i) the novel form of RNA polymerase, designated RNA polymerase III to distinguish it from the classic holoenzyme, called RNA polymerase I, and (ii) DNA unwinding protein in an amount sufficient to coat the single-stranded DNA (Table 2). The unwinding protein markedly stimulates the RNA polymerase I-primed reaction (6) but does so for both M13 and ϕX DNA replication; the effect on the RNA polymerase III-primed reaction is to suppress specifically the ϕX DNA replication.

RNA polymerase III has feeble transcriptional activity. RNA polymerase III was 15- to 70-fold less active than RNA polymerase I in transcription of standard templates, such as λ, T4, and salmon sperm DNA, and poly[d(A-T)] (Table 3). When the two polymerases were present in the same incubation, the RNA polymerase I remained active, thus indicating the absence of a diffusible inhibitor to account for the inactivity of RNA polymerase III.

TABLE 2. *Requirements for specific priming of M13*

Form of RNA polymerase	Unwinding protein	Template for DNA synthesis (pmoles)	
		M13	ϕX
I	−	13	15
III	−	225	107
I	+	232	122
III	+	188	6

DNA synthesis on M13 or ϕX SS was assayed with 0.01 unit of RNA polymerase III or RNA polymerase I (*Materials and Methods*). DNA unwinding protein was added, where indicated.

The properties of RNA polymerase I observed with the preparation obtained by the Babinet procedure were also observed with another preparation prepared by M. Chamberlin. RNA polymerase I holoenzyme primed M13 and ϕX replication equally well, while the core enzyme was totally inactive (Table 4), in contrast to the behavior of RNA polymerase III, which primed M13, but not ϕX, replication and was inactive in transcription of poly[d(A-T)]. Rifampicin at 5 μg/ml of completely inhibited RNA polymerase III, as it is known to do to RNA polymerase I.

Rifampicin Releases a Diffusible Factor from RNA Polymerase III. RNA polymerase III treated with rifampicin and then precipitated with ammonium sulfate to separate proteins from free, unbound rifampicin was inactive in priming DNA, but when mixed with RNA polymerase I prevented it from priming ϕX SS while still permitting it to prime M13. This result suggested that rifampicin released a factor from RNA polymerase III that could confer the M13-specificity property on RNA polymerase I. This diffusible factor, released by rifampicin, was isolated by Sephadex G-150 gel filtration (Fig. 2), using as an assay its inhibition of the priming of ϕX DNA synthesis by RNA polymerase I. Little or none of this factor activity was seen on gel filtration in a control experiment with RNA polymerase III that had not been exposed to rifampicin. As judged by gel filtration, the factor is smaller than hemoglobin, but its size and other properties remain to be determined.

The rifampicin-induced release of a diffusible factor was also studied with the aid of a rifampicin-resistant RNA polymerase I (I-rif^R) (Table 5). When I-rif^R was mixed with RNA polymerase III in the absence of rifampicin (Exps. 1, 3, and 5), RNA-primed DNA synthesis and transcription showed ap-

TABLE 3. *Transcription of duplex DNA*

Form of RNA polymerase	RNA synthesis (pmol) on:				DNA synthesis (pmol) on:	
	Salmon sperm	T4	λ	poly-[d(A-T)]	M13	ϕX
I	62	84	146	132	56	41
III	4	3	2	8	49	3
I + III	63	50		138		

As described in *Materials and Methods*, 0.005 unit (defined for priming of DNA synthesis) of RNA polymerase I (1 μg) or RNA polymerase III (2 μg) was added. In some assays, 0.005 unit of each polymerase was mixed and added together to the assay.

TABLE 4. *Comparison of forms of RNA polymerase I and III in priming of DNA synthesis and in transcription*

Form of RNA polymerase	DNA synthesis (pmol) on:		RNA synthesis (pmol) on poly[d(A-T)]
	M13	ϕX	
I-holoenzyme (Chamberlin*)	12	10	13
I-holoenzyme [Babinet (11)]	38	36	83
I-core (Chamberlin*)	0	0	10
III	66	5	2
III + rifampicin	0	0	0

Holoenzyme and core RNA polymerase I, RNA polymerase I prepared by the method of Babinet (11), and Fraction IV RNA polymerase III (0.007 unit) were assayed as described in *Materials and Methods*. Where indicated, the enzyme was incubated for 5 min at 0° with 5 μg/ml of rifampicin before addition to the assay.

* Holoenzyme RNA polymerase I has been purified by an unpublished procedure employing phosphocellulose chromatography (Prof. M. Chamberlin and Dr. N. Gonzalez, University of California, Berkeley).

proximately additive results. In contrast, when rifampicin-treated RNA polymerase III was mixed with RNA polymerase I (Exps. 2, 4, and 6), the priming of ϕX replication was depressed whereas that of M13 was stimulated; RNA
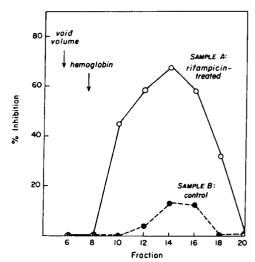

FIG. 2. Isolation of a factor released from RNA polymerase III by rifampicin. Samples of RNA polymerase III (100 μg in 0.1 ml buffer III) were incubated for 5 min at 0° (*A*) with rifampicin (2 μg) or (*B*) without the drug. Each sample was then mixed with 100 μl of saturated ammonium sulfate. After 10 min at 0°, the precipitates were collected by centrifugation (0°, 5 min, 16,000 rpm in the Sorvall SE12 rotor). Each precipitate was suspended twice in 3 ml of a mixture of equal volumes of buffer I and saturated ammonium sulfate and collected by centrifugation (as above). Rifampicin-treated (sample *A*) and untreated RNA polymerase III (sample *B*), dissolved in 100 μl buffer I were each filtered over a Sephadex G-150 column (0.6 × 8 cm, equilibrated with buffer I at 4°). Aliquots (3 μl) of the 100-μl fractions were assayed for their ability to inhibit RNA polymerase I-primed ϕX SS → RF. Incorporation of 20 pmoles of deoxynucleotide into the acid-insoluble fraction was observed in the uninhibited reaction.

TABLE 5.   *Rifampicin release of a diffusible factor from RNA polymerase III*

Experiment	Form of RNA polymerase	Rifampicin	DNA synthesis (pmol) on:		RNA synthesis (pmol) on T4
			M13	ϕX	
1	III	—	55	5	0
2	III	+	0	0	0
3	I-rif^R	—	11	8	11
4	I-rif^R	+	8	11	10
5	I-rif^R + III	—	96	17	9
6	I-rif^R + III	+	36	2	2

Rifampicin-resistant RNA polymerase I (I-rif^R, 1 μg) and RNA polymerase III (2 μg, rifampicin-sensitive) were assayed as described in *Materials and Methods*. Where indicated, the enzymes were incubated for 5 min at 0° with 2 μg of rifampicin per ml.

synthesis on a T4 duplex DNA template was likewise depressed upon mixing of the two rifampicin-treated enzymes. In sum, rifampicin, known to act on the β subunit, appears to release a small factor from RNA polymerase III, which can cause RNA polymerase I to discriminate between M13 and ϕX SS and to become relatively inert in transcription of duplex DNA.

DISCUSSION

The capacity of cell extracts to discriminate in the replication of M13 and ϕX single-stranded DNA in a rifampicin-sensitive reaction was not maintained in a purified RNA polymerase-multienzyme system. Our efforts to restore discriminatory ability to the purified system have led us to a new form of RNA polymerase, termed RNA polymerase III. This enzyme is physically separable from RNA polymerase I (Fig. 1) and is further recognized by (*i*) its selective ability to prime the replication of M13, but not ϕX DNA, and (*ii*) its inability to transcribe standard duplex DNA. The close relation of RNA polymerase III to RNA polymerase I is shown by its sensitivity to rifampicin. Upon exposure to rifampicin, RNA polymerase III releases a factor, presumably a low-molecular-weight subunit, that inhibits RNA polymerase I transcription and confers the ability to prime M13 but not ϕX replication. This factor may be the major difference between RNA polymerases I and III.

Modification of RNA polymerase functions by addition or substitution of subunits has been shown in phage T3 and T4 infections of *E. coli* (14, 15) and in SP82 infection of *B. subtilis* (16). Chao and Speyer (17) found that *E. coli* RNA polymerase is changed in chromatographic properties and template specificity when cells enter a stationary phase. However, this form of RNA polymerase is not separated from RNA polymerase I by gel filtration and, while inactive in transcription of natural duplex DNA, it is still quite active on poly [d(A-T)]. In contrast, RNA polymerase III has been obtained from cells growing exponentially, is separable from

RNA polymerase I by gel filtration, and is unable to transcribe poly [d(A-T)]. Ishihama and coworkers (18) identified yet another form of the enzyme, which they called RNA polymerase II. It contains a different σ subunit with a molecular weight of 56,000 rather than 90,000. Yet RNA polymerases I and II were not distinguishable in their capacities to transcribe duplex DNA.

The ability of RNA polymerase III to maintain the cellular discrimination between M13 and ϕX and its abundance in extracts of gently lysed cells suggest that RNA polymerase III may constitute a significant fraction of the cellular RNA polymerase. The complete inability of cell extracts to prime ϕX replication with rifampicin-sensitive enzymes points to a predominance of RNA polymerase III over RNA polymerase I or else to a specific repressor of the latter in priming the replication of ϕX single-stranded DNA (19). These studies raise questions that can be answered only by purification of RNA polymerase III to homogeneity so that the factor releasable by rifampicin can be characterized and its functions in regulating transcriptional activity properly assessed.

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