Structure, Properties, and Recognition (Schimmel, P. R., Soll, D., & Abelson, J. N., Eds.) p 133, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY.
Schreier, A. A., & Schimmel, P. R. (1975) *J. Mol. Biol. 93*, 323–329.
Schwarz, U., Luhrmann, R., & Gassen, H. G. (1974) *Biochem. Biophys. Res. Commun. 56*, 807–814.
Sprinzl, M., Kramer, E., & Stehlik, D. (1974) *Eur. J. Biochem. 49*, 595.
Stout, C. D., Mizuno, H., Rao, S. T., Swaminathan, P., Rubin, J., Brennan, T., & Sundaralingam, M. (1978) *Acta Crystallogr., Sect. B 34*, 1529.
Sussman, J. L., Holbrook, S. R., Warrant, R. W., Church, G. M., & Kim, S. H. (1978) *J. Mol. Biol. 123*, 607.
Tabor, C. W., & Tabor, H. (1976) *Annu. Rev. Biochem. 45*, 285–306.
Takeda, Y., & Ohnishi, T. (1975) *Biochem. Biophys. Res. Commun. 63*, 611–617.
Urbanke, C., & Maass, G. (1978) *Nucleic Acids Res. 5*, 1551–1560.
Wagner, R., & Garrett, R. A. (1978) *FEBS Lett. 85*, 291–294.
Weiner, L. M., Backer, J. M., & Rezvukhin, A. I. (1974) *FEBS Lett. 41*, 40.
Woese, C. (1970) *Nature (London) 226*, 817–820.
Woo, N. H., Roe, B. A., & Rich, A. (1980) *Nature (London) 286*, 346–351.
Wright, H. T., Manor, P. C., Beurling, K., Karpel, R. L., & Fresco, J. R. (1979) *Transfer RNA: Structure, Properties, and Recognition* (Schimmel, P. R., Soll, D., & Abelson, I. N., Eds.) p 145, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY.

Function of Transcription Termination Factor ρ in a Model Transcription System Using Synthetic Deoxyribonucleic Acid as Template†

Katsuya Shigesada* and Mutsuo Imai

ABSTRACT: The function of a transcription termination factor, ρ, has been studied by using several synthetic DNAs with simple repetitive base sequences as templates for transcription. ρ actually exhibits various effects on transcription depending on the base sequence of the template: (1) ρ terminates poly(A) synthesis with poly(dA)·poly(dT), poly(dT), or oligo(dT), leading to release of RNA from RNA polymerase. ρ also inhibits the synthesis of other homoribopolymers such as poly(U) directed by poly(dA)·poly(dT) and poly(C) and poly(I) directed by poly(dG)·poly(dC), presumably by a similar mechanism. (2) ρ inhibits the synthesis of another homoribopolymer, poly(G), directed by poly(dG)·poly(dC) at the step of initiation rather than propagation of transcription. (3) ρ stimulates rather than inhibits the synthesis of poly(A-C) and poly(G-U) directed by poly[d(A-C)]·poly[d(G-T)], presumably by enhancing the dissociation of transcription complexes. (4) ρ has no influence on the synthesis of poly(A-U) and poly(G-C) directed by poly[d(A-T)] and poly[d(G-C)], respectively. In the first case, but not otherwise, the effect of ρ is coupled with its RNA-dependent nucleosidetriphosphate phosphohydrolase activity, as is ρ-mediated transcription termination on natural templates. The implication of these results is discussed in reference to the current view that ρ acts on transcription complexes that have ceased elongation and causes release of RNA in an energy-requiring reaction.

The protein factor ρ from *Escherichia coli* causes termination of RNA synthesis at specific sites on the DNA template, with eventual release of RNA from DNA and RNA polymerase (Roberts, 1969, 1976). ρ has also an RNA-dependent nucleosidetriphosphate hydrolase activity (ρ NTPase)[1] (Lowery-Goldhammer & Richardson, 1974), which is required for its termination function (Howard & de Crombrugghe, 1976; Galluppi et al., 1976). Accumulated evidence has indicated that RNA polymerase by itself can recognize a termination site, temporarily halting its propagation (pause) there prior to and independent of the action of ρ (Darlix & Horaist, 1975; Rosenberg et al., 1978). These findings have culminated in the current view that the primary function of ρ is to release RNA from paused transcription complexes through its interaction with nascent RNA by an energy-dependent mechanism (Roberts, 1976; Richardson & Conaway, 1980; Shigesada & Wu, 1980). However, the molecular bases for the ρ-catalyzed termination as well as for the pausing of RNA polymerase are still poorly understood. Recently, DNA and RNA sequences have been determined for a number of ρ-dependent termination sites. Some of them have in common a specific feature, dyad symmetry preceding the termination point, which has been implicated in the pausing of RNA polymerase (Rosenberg et al., 1978; Küpper et al., 1978). However, there also have been reported different types of terminators lacking this feature (Wu et al., 1981; Reisbig & Hearst, 1981). Thus, no general explanation can yet be offered about the exact nature of the signal for the pausing event. Moreover, despite the abundance of sequence data, no definite clue has been obtained regarding another important question of what region or structural feature of nascent RNA serves as the target for the action of ρ.

† From the Department of Biochemistry, Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606, Japan. *Received April 30, 1982.* This work has been supported by grants from the Ministry of Education, Science and Culture, Japan.

[1] Abbreviations: NTP, nucleoside 5′-triphosphate; NTPase, nucleosidetriphosphate phosphohydrolase; AMP-PNP, 5′-adenylyl imidodiphosphate; GMP-PNP, 5′-guanylyl imidodiphosphate; UMP-PCP, uridine 5′-(β,γ-methylenetriphosphate); $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

0006-2960/82/0421-5849$01.25/0

In an attempt to elucidate the minimal essential features of DNA or RNA structure that are required for ρ-mediated termination, we have studied the termination function of ρ by using synthetic DNA of simple and well-defined base sequences as a template for transcription. It has been generally conceived that ρ might not work on such templates since they would lack specific terminator sequences by nature (Goldberg & Hurwitz, 1972). However, Darlix & Fromageot (1974) have previously pointed out that ρ did inhibit transcription of poly(dA)·poly(dT) and poly(dG)·poly(dC). We have confirmed their observation and further found that ρ acts to terminate the synthesis of certain homoribopolymers directed by these templates through a mechanism dependent on ρ NTPase activity. This suggests that these templates have some structural elements which can evoke the termination reaction of ρ. This paper describes the basic characteristics of the function of ρ as manifested in the model transcription system by using the above two DNAs and a few other synthetic DNAs having simple alternating base sequences.

Materials and Methods

Enzymes and Chemicals. DNA-dependent RNA polymerase was purified from *Escherichia coli* A19 as described by Berg et al. (1971). The preparation was more than 95% pure, and its content of σ factor was about 60% as judged from its electrophoretic pattern on a $NaDodSO_4$–polyacrylamide gel. ρ factor was purified from the same strain by the procedure of Roberts (1969) with some modifications as described previously (Shigesada & Imai, 1978). Both the RNA polymerase and ρ preparations were essentially free of nucleases: they caused no detectable degradation of ^{3}H-labeled 16S and 23S rRNA from *E. coli*, and in their presence, discrete mRNA transcripts were produced from λ DNA. They contained no detectable template-independent RNA synthesizing activities such as polynucleotide phosphorylase or poly(A) polymerase (August et al., 1962; Sippel, 1973): the addition of inorganic phosphate (0.1 mM), which is known as a potent inhibitor of polynucleotide phosphorylase, to the transcription reaction mixture with or without ρ factor did not affect RNA synthesis. RNA synthesis was completely inhibited by the addition of rifampicin prior to the start of the reaction, whereas poly(A) polymerase is insensitive to this drug. The following synthetic DNAs were obtained from the indicated sources; the size of each DNA (weight average), where specified by the supplier, is indicated in parentheses: poly(dA)·poly(dT) (5.1×10^5 daltons), poly(dG)·poly(dC) (5.2×10^5 daltons), poly[d(A-T)]·poly[d(A-T)] (1.5×10^6 daltons), poly[d(G-C)]·poly[d-(G-C)] (6.3×10^4 daltons), poly(dT) (3.7×10^5 daltons), and oligo$(dT)_{10}$ were purchased from P-L Biochemicals, Inc. Poly[d(A-C)]·poly[d(G-T)] was purchased from Boehringer Mannheim GbmH. ^{3}H-Labeled nucleoside triphosphates including β,γ-imido analogues of ATP and GTP were supplied from The Radio Chemical Center, Amersham. Unlabeled nucleoside triphosphates, β,γ-imido-ATP and -GTP, and β,γ-methylene-UTP were obtained from P-L Biochemicals, Inc. [γ-^{32}P]ATP and [γ-^{32}P]GTP were prepared by the method of Glynn & Chappell (1964). All other chemicals were reagent grade.

Transcription of Synthetic DNA Templates. All DNA templates used here, either single stranded or double stranded, contained no more than two kinds of bases in each strand. In studies with double-stranded DNAs, transcription of each strand was investigated separately by supplying only one or two of the four ribonucleoside triphosphates at one time. A standard transcription reaction mixture, in a final volume of 50 μL, contained 40 mM Tris-HCl buffer (pH 8.0), 10 mM $MgCl_2$, 0.1 mM NTP as substrate for transcription, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.01–1.0 μg of RNA polymerase as indicated, and 0.2–0.6 μg of ρ where appropriate. Where indicated, 1 mM ATP or GTP was added as a supplementary substrate for ρ NTPase. Exceptionally, a lower concentration (2 mM) of $MgCl_2$ was used for transcription of poly(dA)·poly(dT) and poly(dG)·poly(dC) to form poly(U) and poly(C), respectively, because optimal effects of ρ on these particular transcription reactions were observed at this concentration. The reactions were performed for 30 min at 37 or 30 °C as indicated and stopped by adding 2.0 mL of ice-cold 3.5% perchloric acid containing 0.1 M $Na_4P_2O_7$ together with 200 μg of yeast RNA as carrier. The precipitated material was collected on a Whatman GF/C glass fiber filter, washed, and counted for radioactivity in a toluene-based scintillator.

Assay of RNA-Dependent NTPase Activity of ρ. Nascent RNA-dependent NTPase activity of ρ was measured by using the same transcription reaction mixtures described above supplemented with either [γ-^{32}P]ATP or [γ-^{32}P]GTP (0.2 mM, 0.2 μCi/nmol), whichever was not complementary to the template strand to be transcribed. After incubation for 30 min at 37 °C, the reaction was stopped by adding 500 μL of 5% (w/v) suspensions of activated charcoal in 10 mM $Na_4P_2O_7$ (neutralized to pH 6.0 with NaH_2PO_4), and radioactivity not adsorbable with charcoal was determined essentially as described (Lowery-Goldhammer & Richardson, 1974).

Results

Effects of ρ on Transcription of Various Synthetic Templates. In the present study, we first tested the following synthetic DNA duplexes with simple repetitive base sequences: poly(dA)·poly(dT), poly(dG)·poly(dC), poly[d(A-T)]·poly[d-(A-T)], poly[d(G-C)]·poly[d(G-C)], and poly[d(A-C)]·poly-[d(G-T)].

These templates were incubated with RNA polymerase in the presence of one or two appropriate NTPs at a time so as to allow selective transcription from each of their component strands. Under conditions of minimum salt concentrations as described under Materials and Methods, transcription occurred to measurable extents from all strands of the above templates (Table I). When ρ was added to the reaction, it affected total RNA synthesis differently, depending on the template (Table I and Figure 1).

First, ρ inhibited the synthesis of homoribopolymers directed by poly(dA)·poly(dT) and poly(dG)·poly(dC) with all possible combinations of ribonucleoside triphosphates as the substrate for transcription if suitable reaction conditions were provided as described below. Further, ρ also inhibited transcription of poly(dG)·poly(dC) when ITP was substituted for GTP. The inhibition with increasing concentrations of ρ showed saturation kinetics (Figure 1), thus resembling ρ-dependent transcription termination on natural templates (Roberts, 1969). The amount of ρ required for a half-maximal inhibition was around 1 μg/mL, being comparable to that reported for ρ-dependent termination on natural DNA (Roberts, 1969).

Second, ρ stimulated rather than inhibited the transcription from either strand of poly[d(A-C)]·poly[d(G-T)], which has non-self-complementary alternating base sequences.

Finally, ρ did not significantly affect the transcription of poly[d(A-T)]·poly[d(A-T)] and poly[d(G-C)]·poly[d(G-C)], both of which have self-complementary base sequences.

These effects of ρ as described were observed reproducibly in repeated experiments using several preparations of RNA polymerase either purified by us or kindly supplied from Dr. A. Ishihama and two preparations of ρ purified by us. As already noted under Materials and Methods, all preparations

Table I: Effect of ρ on Transcription of Various Synthetic Duplex DNAs[a]

template	substrate for transcription	supplementary substrate for ρ NTPase	RNA synthesis (pmol of [^{3}H]NMP incorporated) (A) −ρ	(B) +ρ	(B/A) X 100
poly(dA)·poly(dT)	[^{3}H]ATP		563	279	50
		GTP	434	156	36
	[^{3}H]UTP		37	38	103
		GTP	31	24	77
poly(dG)·poly(dC)	[^{3}H]GTP		95	41	43
	[^{3}H]ITP		267	128	48
		ATP	218	45	21
	[^{3}H]CTP		19	9	47
poly[d(A-C)]·poly[d(G-T)]	[^{3}H]ATP + CTP		17	30	176
	[^{3}H]GTP + UTP		51	109	214
poly[d(A-T)]	[^{3}H]ATP + UTP		184	179	97
poly[d(G-C)]	[^{3}H]GTP + CTP		327	321	98

[a] RNA synthesis was performed with the indicated combination of DNA templates and NTP substrates in the presence and absence of ρ as described under Materials and Methods. The amounts of reaction components used were the following: DNA, 0.01 A_{260} unit; ^{3}H-labeled or unlabeled NTP as substrate for transcription, 0.1 mM; supplementary NTP as substrate for ρ NTPase where indicated, 1 mM; ρ, 0.5 μg; RNA polymerase, 0.06 μg for poly(G) synthesis and 0.3 μg for all other cases. Note that a lesser amount of RNA polymerase was exceptionally used for the synthesis of poly(G), because the effect of ρ on this reaction became negligible if greater amounts were used, as explained in the text. The reaction was carried out at 37 °C for 30 min. The results were expressed as picomoles of [^{3}H]NMP incorporated into acid-precipitable materials.

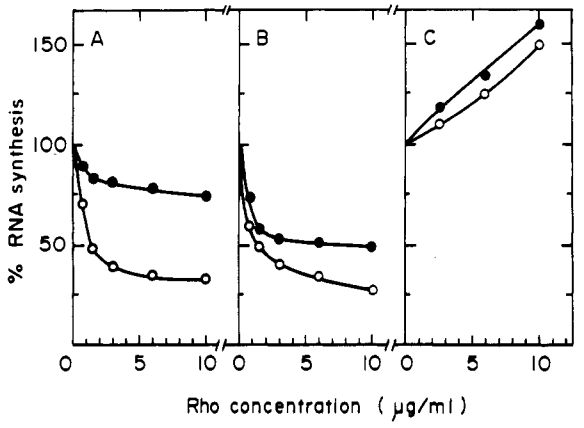

FIGURE 1: Effect of varying concentrations of ρ on transcription with synthetic DNA. RNA synthesis with the indicated templates was measured in the presence of varying concentration of ρ. The reaction systems synthesizing poly(A) and poly(U) were fortified with GTP (1 mM) as a supplementary substrate for ρ NTPase. Other reaction conditions are the same as in Table I. The results are expressed as percent of RNA synthesized in the absence of ρ. (A) Synthesis of poly(A) (○) and poly(U) (●) with poly(dA)·poly(dT); (B) synthesis of poly(G) (○) and poly(C) (●) with poly(dG)·poly(dC); (C) synthesis of poly(A-C) (○) and poly(G-U) (●) with poly[d(A-C)]·poly[d(G-T)].

of RNA polymerase and ρ were virtually free from RNase and miscellaneous RNA-synthesizing activities. Therefore, it is unlikely that our results are artifacts due to contaminating enzymes.

The apparent extent of the ρ effect in some of the above transcription systems was found to be critically affected by reaction conditions. The effect of ρ on the synthesis of poly(A), poly(U), and poly(I), but not of the other products, was markedly enhanced when the reaction was supplemented with a high concentration (1 mM) of nucleoside triphosphate which is not complementary to the template (Table I); data obtained under such conditions were presented in Figure 1. Since AMP-PNP and GMP-PNP, nonhydrolyzable analogues of ATP and GTP, respectively, had no such influence, the effect of supplementary nucleotides may be associated with the requirement of NTPase activity for the action of ρ as investigated in detail below. The inhibition of poly(G) synthesis was detectable only when the amount of RNA polymerase was limiting relative to that of the template. Note that in the experiment shown in Table I a 5-fold lesser amount of RNA polymerase was used for poly(G) synthesis than for the other transcription. The inhibitory effect became almost negligible if RNA polymerase concentration was increased several times over that level or the amount of poly(dG)·poly(dC) was decreased conversely, even when ρ was present at a saturating concentration. No such change was observed in the effect of ρ on the synthesis of the other RNAs including poly(I). Finally, the effect of ρ with either template tested was highly sensitive to ionic strength: it was decreased significantly in the presence of added KCl at 0.05 M and was almost abolished at 0.1 M or above.

Overall, the pattern of the effect of ρ described above is consistent with the previous report of Darlix & Fromageot (1974) but contradicts the report of Goldberg & Hurwitz (1972), who found that ρ did not affect the transcription of various synthetic DNA templates including those studied here. Neither of these reports, however, presented any experimental detail as to what exact combinations of template and substrate were tested and what reaction conditions were used. Therefore, the reason for the above discrepancy is difficult to assess, but one could easily miss the effect of ρ on such templates if one or other critical reaction condition as described above is not adequately fixed.

Relationship of the ρ Effect on RNA-Dependent NTPase Activity. To understand the mechanism of the action of ρ in the present transcription systems, we examined whether the observed effects of ρ require RNA-dependent NTPase activity as does ρ-mediated transcription termination on natural templates. To this end, measurements were first made of ρ-catalyzed hydrolysis of NTP as activated by nascent RNA produced in the transcription reaction mixture. As seen in Table II, this activity was detectable in all transcription systems that were affected by ρ, except for that synthesizing poly(G). No significant activity was found in the systems synthesizing poly(A-U) and poly(G-C). The lack of ρ NTPase activity in the latter three systems is consistent with a previous report that RNA molecules having highly ordered secondary structures do not activate the NTPase (Lowery & Richardson, 1977).

So that more direct evidence on the involvement of ρ NTPase activity could be obtained, experiments were done in which the transcription was allowed to proceed in the presence

Table II: Nascent RNA-Dependent NTPase Activity of ρ[a]

template	product RNA	^{32}P-labeled substrate for ρ	$^{32}P_i$ released (nmol)
poly(dA)· poly(dT)	poly(A)	GTP	0.06
	poly(U)	GTP	0.03
poly(dG)· poly(dC)	poly(G)	ATP	0
	poly(C)	ATP	0.29
poly[d(A-C)]· poly[d(G-T)]	poly(A-C)	GTP	0.76
	poly(G-U)	ATP	0.03
poly[d(A-T)]	poly(A-U)	GTP	0
poly[d(G-C)]	poly(G-C)	ATP	0

[a] Transcription of the indicated template was performed in the presence of ρ (0.5 μg) by using the same conditions as in Table I except that unlabeled NTPs were used as substrate for transcription and either γ-^{32}P-labeled ATP or γ-^{32}P-labeled GTP (0.2 mM) was added as a substrate for ρ. ρ NTPase activity was measured by the release of $^{32}P_i$ as described under Materials and Methods. The results were presented after correction for $^{32}P_i$ released in control reactions without ρ (about 0.01 nmol). Where the $^{32}P_i$ release did not significantly increase over the control value, the result was expressed as zero.

Table III: Effect of ρ on Transcription with β,γ Analogues of Ribonucleoside Triphosphates[a]

template	substrates for transcription	supplementary NTP	[^{3}H]NMP incorporated (pmol) (A) $-\rho$	(B) $+\rho$	(B/A) × 100
poly(dA)· poly(dT)	[^{3}H]AMP-PNP	none	81	81	100
		GTP	73	17	23
		CTP	68	52	76
		GMP-PNP	72	73	101
poly(dG)· poly(dC)	[^{3}H]GMP-PNP	none	79	45	57
poly[d(A-C)]· poly[d(G-T)]	[^{3}H]GMP-PNP + UMP-PCP	none	31	45	145

[a] RNA synthesis with the indicated templates was performed by using β,γ-imido or -methylene analogues of ribonucleoside triphosphates (0.1 mM) as substrates for transcription. The amount of RNA polymerase used was 0.06 μg for the transcription of poly(dA)·poly(dT) and poly(dG)·poly(dC) and 0.6 μg for that of poly[d(A-C)]·poly[d(G-T)]. Where indicated, a 1 mM sample of the appropriate NTP was added as substrate for ρ NTPase. Other conditions were the same as in Table I.

of β,γ-imido or -methylene analogues of ribonucleoside triphosphate. These analogues can serve as substrate for RNA polymerase but not for ρ NTPase (Howard & de Combrugghe, 1976). So far, we have examined three transcription systems which synthesize poly(A), poly(G), and poly(G-U). As shown in Table III, ρ failed to inhibit poly(A) synthesis with AMP-PNP directed by poly(dA)·poly(dT). However, the inhibitory effect of ρ was restored if any hydrolyzable nucleoside triphosphate such as GTP, CTP, or dGTP was added to the reaction mixture as a substrate for ρ NTPase. GMP-PNP, a nonhydrolyzable analogue of GTP, again showed no such effect. These results indicate that ρ NTPase activity is required for the action of ρ in this transcription system. In contrast, ρ affected poly(G) and poly(G-U) synthesis with analogues (GMP-PNP and UMP-PCP) essentially in the same way as with normal substrates. Thus, the effects of ρ on these transcription systems are independent of ρ NTPase activity.

Step of Transcription Affected by ρ. The preceding studies have suggested that the effect of ρ on poly(A) synthesis with poly(dA)·poly(dT) template is closely related to its termination function. To further characterize the mode of action of ρ on this template, we investigated the influence of ρ on the kinetics of poly(A) synthesis. For comparison, similar analyses were made in parallel on the synthesis of poly(G) and poly(G-U).

Table IV shows that ρ progressively inhibited the total synthesis of poly(A) with the time of reaction, whereas it scarcely affected the initiation of poly(A) chains as measured by incorporation of [γ-^{32}P]ATP. Consequently, the average size of poly(A) products was presumed to be greatly decreased in the presence of ρ as compared with the control obtained in its absence. This was confirmed by the sedimentation analysis of poly(A) chains in a glycerol gradient as described in detail below (Figure 3). Note that the length of poly(A) chains produced in the absence of ρ (3800 nucleotides at 30 min) is much greater than that of the template used (ca. 5×10^5 daltons, 700 base pairs in weight average). This implies that a reiterative copying of the template occurs on poly(dA)·poly(dT), as has been previously postulated for poly(A) synthesis on single-stranded DNA containing clustered thymidylate residues (Chamberlin & Berg, 1964; Falaschi et al., 1963). According to Chamberlin & Berg (1964), a reiterative transcription is presumed to occur when transcription has reached the 3′ end of a homopolymer template, where RNA polymerase may repeatedly slide back and forth along the template to continue elongation of preformed RNA chains. If this model is true, RNA polymerase involved in reiterative transcription is somewhat similar to that pausing at a terminator in that it temporarily stops its movement without losing its capacity for elongation. This raises the possibility of the effect of ρ on homoribopolymer synthesis being associated with the reiterative mode of transcription. This was supported by studies of the effect of ρ on poly(A) synthesis

Table IV: Effect of ρ on the Size of RNA Transcripts[a]

expt	template	product RNA	reaction time (min)	[^{3}H]HMP incorporated (pmol) $-\rho$	$+\rho$	[γ-^{32}P]NTP incorporated (pmol) $-\rho$	$+\rho$	av length of RNA (nucleotide residues) $-\rho$	$+\rho$
A	poly(dA)·poly(dT)	poly(A)	5	273	175	0.34	0.28	803	625
			10	863	638	0.55	0.53	1570	1200
			20	2590	1240	0.84	0.87	3080	1430
			30	3920	2030	1.03	1.30	3810	1560
B	poly(dG)·poly(dC)	poly(G)	30	777	330	2.18	0.82	356	402
C	poly[d(A-C)]·poly[d(G-T)]	poly(A-C)	30	642	1030	1.82	3.03	705	680
		poly(G-U)	30	1042	1310	3.40	4.30	613	609

[a] Each reaction system contained the following in a final volume of 1 mL: ^{3}H- and γ-^{32}P-labeled ATP or GTP (^{3}H, 0.01 μCi/nmol; ^{32}P, 0.5 μCi/nmol), 0.035 mM; CTP or UTP, 0.1 mM where appropriate; DNA, 0.2 A_{260} unit; RNA polymerase, 30 μg for experiments A and C and 2 μg for experiment B; ρ, 10 μg where indicated. GTP (1 mM) was supplemented as a substrate for ρ NTPase in experiment A. Other conditions were as described under Materials and Methods. The reaction was carried out at 37 °C. At the indicated times, 200-μL aliquots were removed, and the amounts of [^{3}H]NTP and [^{32}P]NTP incorporated into RNA products were measured. The average length of RNA chains was calculated from the ratio of incorporated ^{3}H and ^{32}P.

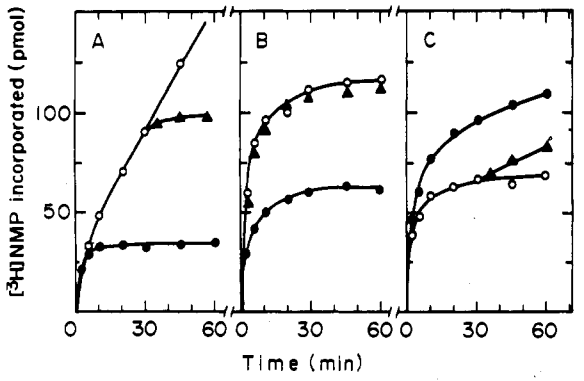

FIGURE 2: Time course of transcription in the presence of ρ and rifampicin. The transcription system containing each template with or without ρ (10 μg/mL) as described in Table I was scaled up to 0.5 mL for poly(A) and poly(G-U) synthesis or 2.5 mL for poly(G) synthesis. RNA synthesis was started by adding nucleoside triphosphate(s) to the reaction mixture which contained all other components and was preincubated at 37 °C for 5 min. Rifampicin (final concentration 10 μg/mL) was added at zero time to all reaction systems for poly(A) and poly(G) synthesis and at 30 min to the poly(G-U) synthesizing system where indicated. At appropriate time points, aliquots [50 μL for poly(A) and poly(G-U) synthesis and 250 μL for poly(G) synthesis] were withdrawn and determined for the amount of RNA synthesized. (A) Poly(A) synthesis with poly(dA)·poly(dT): (○) without ρ; (●) with ρ added during the preincubation; (▲) with ρ added at 30 min. (B) Poly(G) synthesis with poly(dG)·poly(dC): (○) without ρ; (●) with ρ added during the preincubation; (▲) with ρ added at 1 min. (C) Poly(G-U) synthesis with poly[d(A-C)]·poly[d(G-T)]: (○) without ρ; (●) with ρ added during the preincubation; (▲) with ρ added at 30 min.

when reinitiation of transcription was blocked by the presence of rifampicin. When rifampicin was added immediately after the start of RNA polymerase reaction, poly(A) synthesis proceeded in two distinct phases, a rapid rise in the initial several-minute period followed by a steady increase lasting for at least 1 h (Figure 2A). Conceivably, the first phase corresponds to the propagation of transcription from one end of the template to the other end and the second phase to the reiterative transcription. As seen in Figure 2A, ρ had virtually no influence on the first phase but caused an almost complete inhibition of the synthesis in the second phase. Further, we have found that ρ can also terminate poly(A) synthesis on single-stranded templates, such as poly(dT), oligo(dT), and denatured calf thymus DNA, all well-known for their capacity to direct the reiterative transcription (Table V). With any of these templates, the action of ρ required its RNA-dependent NTPase activity and resulted in a decreased size of poly(A) products, just as with poly(dA)·poly(dT) as described above. It was interesting to note that the transcription of calf thymus DNA became almost insensitive to ρ in the presence of all four NTPs, conditions which totally suppressed the reiterative synthesis of poly(A) (Chamberlin & Berg, 1964). This is consistent with and strengthens the suggested connection between the reiterative mode of transcription and the termination activity of ρ. A reiterative transcription and its termination by ρ also seems to occur in the synthesis of poly(U) directed by poly(dA)·poly(dT), and that of poly(C) and poly(I) directed by poly(dG)·poly(dC). In these transcription systems, persistent RNA synthesis was observed to greater or lesser extents in the presence of rifampicin, and such synthesis was effectively inhibited by ρ as described for poly(A) synthesis on poly(dA)·poly(dT) (data not presented).

In contrast to the case of poly(A) synthesis, ρ apparently inhibited the initiation of poly(G) and conversely enhanced that of poly(G-U) or poly(A-C) (Table IV). In these transcription systems, the average size of RNA products was

Table V: Effect of ρ on Poly(A) Synthesis with Single-Stranded Templates[a]

template	substrate	[3H]AMP incorporated (pmol) (A) −ρ	(B) +ρ	(B/A) × 100
poly(dT)	[3H]ATP	1200	517	43
oligo(dT)$_{10}$	[3H]ATP	25.3	14.5	57
denatured calf thymus DNA	[3H]ATP	237	160	68
	[3H]ATP + 3NTPs	74	69	93

[a] RNA synthesis with the indicated templates was performed in essentially the same way as in Table I. The following amounts of templates were used: poly(dT), 0.005 A_{260} unit; oligo(dT)$_{10}$, 0.025 A_{260} unit; heat-denatured calf thymus DNA, 1.0 μg. The concentration of each nucleoside triphosphate was 0.1 mM. Other conditions were as described under Materials and Methods. The reaction was carried out for 30 min at 37 °C with poly(dT) and calf thymus DNA or at 30 °C with oligo(dT)$_{10}$.

scarcely affected by ρ, being near or smaller than that of the respective template used. The synthesis of poly(G) continued for a long time like poly(A) synthesis in the absence of rifampicin, but it was completely inhibited upon addition of this reagent with a certain time lag (Figure 2B). A similar observation has been previously reported (Neff & Chamberlin, 1978). The residual synthesis of poly(G) after the addition of rifampicin was still partly inhibited by ρ if this was added before or at the same time as rifampicin, but not at all if added afterward. These results suggest that ρ interferes with the initiation of poly(G) synthesis at or prior to the step which is sensitive to rifampicin. On the other hand, the synthesis of poly(G-U) or poly(A-C) in the absence of ρ tended to level off spontaneously after an initial rapid increase (Figure 2C) as previously reported (Morgan, 1970). In the presence of ρ, however, the synthesis came to continue at a steady rate after the initial rise. A similar resumption of synthesis also occurred when ρ was added after the synthesis had reached the plateau phase. No such effect appeared in the presence of rifampicin regardless of whether ρ was added before or after the reagent. It thus seems that transcription on this template reaches a dead-end state after completion of RNA chain growth and that ρ relieves this block presumably by enhancing release of RNA from RNA polymerase.

Release of RNA from Transcription Complexes by ρ. The primary function of ρ is understood to be the release of RNA from transcription complexes (Richardson & Conaway, 1980; Shigesada & Wu, 1980). This activity was measured in the poly(A) synthesizing system by means of centrifugal analysis (Roberts, 1969) (Figure 3). When the transcription reaction mixture was subjected to centrifugation in a glycerol gradient under nondenaturing conditions, poly(A) chains produced in the absence of ρ sedimented in broad heterogeneous bands with rates ranging from 10 S to 30 S. In a parallel gradient run under denaturing conditions, components with larger *s* values decreased, while those with smaller *s* values increased in a reciprocal manner. The results suggest that at least a part of poly(A) products had existed as ternary complexes with RNA polymerase and the templates. In contrast, poly(A) chains produced in the presence of ρ sedimented in a single narrow band essentially at the same rate, regardless of whether they were centrifuged under denaturing or nondenaturing conditions. Note that their sedimentation rate (7–9 S) is much smaller than that of RNA polymerase (14–15 S) (Berg et al., 1971). This implies that those poly(A) products had been almost totally dissociated from RNA polymerase due to the action of ρ.

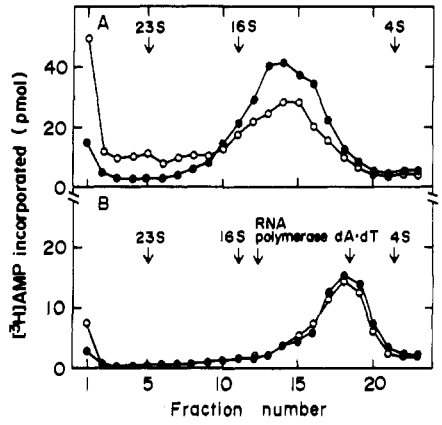

FIGURE 3: Sedimentation profiles of poly(A) products in a glycerol gradient. The conditions of poly(A) synthesis were as in Table I, except that 0.1 mM [^{3}H]ATP, 1.0 mM GTP, 1.2 μg of RNA polymerase, 0.025 A_{260} unit of poly(dA)·poly(dT), and 2.5 μg of ρ, where indicated, were used per 500 μL of reaction mixture. After incubation for 20 min at 37 °C, the synthesis was stopped by chilling and addition of EDTA to 20 mM, and each reaction mixture was divided into two equal parts, to one of which 10 μL of 5% sodium dodecyl sulfate ($NaDoSO_4$) was added. Each portion was layered on a 5-mL glycerol gradient (10–30%) containing 50 mM Tris-HCl (pH 7.4), 0.1 M KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM dithiothreitol. The gradients were centrifuged for 4.5 h at 50000 rpm at 4 °C in the Spinco SW 50.1 rotor, and 23 fractions were collected. [^{3}H]Poly(A) in each fraction was precipitated with perchloric acid and counted as before. The sedimentation rates of free poly(dA)·poly(dT), free RNA polymerase and ^{3}H-labeled stable RNAs from *Escherichia coli* were determined in parallel gradients, and their positions were indicated by arrows. (A) Poly(A) produced in the absence of ρ; (B) poly(A) produced in the presence of ρ: (○) untreated sample; (●) sample treated with $NaDodSO_4$.

ρ-induced release of nascent poly(A) chains was also demonstrated by a filtration assay using a glass fiber filter paper (Whatman GF/C), which is capable of binding DNA or RNA molecules complexed with proteins but not those staying in free forms (Strniste et al., 1973). When the transcription mixture after the reaction without ρ was passed through a glass fiber filter, about 60–80% of the radioactivity incorporated into poly(A) was retained on the filter. In contrast, this value decreased to 20–30% for poly(A) products formed in the presence of ρ. It should be stressed that the RNA-releasing activity of ρ as detected in either of the above methods completely depends on the presence of a hydrolyzable nucleoside triphosphate as does its inhibitory effect on poly(A) synthesis described before (data not presented). This precludes the possibility that the observed release of poly(A) products was an artifact due to a contaminating RNase activity.

Discussion

In this paper, we have characterized the effect of ρ on transcription by *E. coli* RNA polymerase of several synthetic DNAs with simple repetitive base sequences. ρ elicited various responses, depending on the base sequence of RNA transcripts. They can be classified into four cases in terms of their relationship with the RNA-dependent NTPase activity of ρ.

(1) ρ inhibits the synthesis of poly(A) on poly(dA)·poly(dT) and single-stranded templates such as poly(dT), oligo(dT), and denatured calf thymus DNA by a mechanism involving the NTPase activity. This effect is accompanied by a marked reduction in the size of product RNA as well as the release of RNA chains from RNA polymerase. The action of ρ on the above templates closely mimics ρ-mediated transcription termination as observed on natural templates (Roberts, 1969, 1976). ρ also inhibits the synthesis of other homoribopolymers such as poly(U) directed by poly(dA)·poly(dT), and poly(C) and poly(I) directed by poly(dG)·poly(dC), presumably by a similar mechanism.

(2) ρ inhibits the synthesis of another homoribopolymer, poly(G) directed by poly(dG)·poly(dC), at the step of initiation rather than propagation of transcription. The NTPase activity neither is detectable nor is necessary for this effect.

(3) ρ stimulates rather than inhibits the synthesis of poly(A-C) and poly(G-U) directed by poly[d(A-C)]·poly[d(G-T)], presumably by enhancing the dissociation of transcription complexes that have reached a dead-end state. Since the stimulatory effect still appears in the presence of nonhydrolyzable analogues of NTPs (Table III), it does not require the NTPase activity, although this activity is detectable.

(4) ρ has no influence on the synthesis of poly(A-U) and poly(G-C) directed by poly[d(A-T)] and poly[d(G-C)], respectively. The NTPase activity is not detectable in these transcription systems.

There are a number of difficulties in using synthetic templates as model systems for transcription. Since they naturally lack specific promoter and terminator sequences, their transcription often proceeds in anomalous manners as has been amply documented (Neff & Chamberlin, 1978; Morgan, 1970) and confirmed here as well. Nevertheless, the resemblance between the effect of ρ seen in the first case and ρ-catalyzed termination under normal conditions is so striking that the structure of those homopolymer templates or their transcription may have some crucial features which are needed for the termination activity of ρ.

Previous work has indicated that pausing of RNA polymerase at termination sites is prerequisite to ρ-catalyzed termination (Rosenberg et al., 1978) and that the primary function of ρ is to release RNA from paused RNA polymerase in an energy-requiring reaction (Richardson & Conaway, 1980; Shigesada & Wu, 1980). Recent sequencing studies on a number of ρ-dependent and independent termination sites have revealed some common features in their primary structure. In all ρ-independent sites so far examined, the transcripts end with a cluster of U residues, which is preceded by a GC-rich region of hyphenated dyad symmetry (Rosenberg & Court, 1979). A similar dyad symmetry, but not a U cluster, has been also found in a few typical ρ-dependent terminators such as t_{R1} in bacteriophage λ (Rosenberg et al., 1978) and the terminators at the end of the $tRNA_{Tyr}$ gene (Küpper et al., 1978). Accumulated evidence has suggested that the dyad symmetry as represented by a "stem and loop" structure in the RNA transcript may be responsible for the pausing event (Rosenberg et al., 1978; Farnham & Platt, 1981). More recently, however, other types of ρ-dependent termination sites have been described which lack this feature (Wu et al., 1981; Reisbig & Hearst, 1981), suggesting that the pause signals for ρ-dependent sites may be more complex or diverse. The kinetic analysis of poly(A) synthesis with poly(dA)·poly(dT) has indicated that a reiterative transcription occurs on this template and that ρ specifically affects this unusual mode of transcription but not the normal chain elongation along the template. According to the "slippage" model proposed by Chamberlin & Berg (1964), a reiterative transcription is presumed to occur when RNA polymerase has reached the 3′ end of a coding strand. At this point, RNA polymerase together with the growing end of the nascent RNA may slip back along the template by at least one base and then proceed again to copy the transcribable sequence thus newly created. If this model is true, RNA polymerase being engaged in the reiterative transcription approximates that undergoing a pause at natural termination sites in that it temporarily stops its

movement, while maintaining its capacity to resume chain elongation. Then the termination of reiterative transcription by ρ suggests that ρ has a versatile capacity to act on pausing transcription complexes, regardless of whether the pause is primarily elicited by a natural termination signal or by a physical barrier such as the 3′ end of a template strand. However, ρ does not act at all pause sites (Shigesada & Wu, 1980; Kassavetis & Chamberlin, 1981). This implies that some stringent conditions more than mere cessation of RNA chain elongation are required for the action of ρ.

Apparent cessation of RNA polymerase propagation at the end of the template and subsequent release of RNA by ρ also seem to occur on poly[d(A-C)]·poly[d(G-T)]. In this case, however, RNA polymerase is unable to continue chain elongation, and the action of ρ thereon does not require the NTPase activity. Accordingly, the conformation of RNA polymerase in this state may be different from that of one which has paused as discussed above. Under such circumstances, RNA products can apparently be dissociated from the polymerase by an interaction between ρ and the transcripts without the expenditure of free energy derived from hydrolysis of NTP. Consistent with this view is our observation that the stimulatory effect of ρ on the synthesis of both poly(A-C) and poly(G-U) was abolished when nascent RNA chains were destroyed by the action of RNase while it was being made (K. Shigesada, unpublished results).

The inhibitory effect of ρ on poly(G) synthesis (case 2) seems to be little associated with the termination function. Since ρ can bind to poly(dG)·poly(dC) (Goldberg & Hurwitz, 1972) and also to RNA polymerase (Darlix et al., 1971), the inhibition of RNA chain initiation could result either from competition between ρ and RNA polymerase for the site of binding on the template or from alteration in the affinity of RNA polymerase for this template upon its association with ρ. More exactly, the present results (Figure 2B) only indicate that ρ can inhibit poly(G) synthesis at or prior to the step sensitive to rifampicin. It has been shown that short oligonucleotides can be formed even in the presence of rifampicin (abortive initiation; Johnston & McClure, 1976). Since such products would be undetectable by the analytical method used here, the possibility is not excluded that ρ has actually worked after formation of short oligo(G) chains. Further studies are needed to discriminate between these possibilities and also to understand why the synthesis of poly(G) on poly(dG)·poly(dC) in particular is affected by ρ at early stage(s) of transcription. It is of interest in this connection that ρ comes to act at the termination step when poly(dG)·poly(dC) is transcribed with ITP as a substrate instead of GTP. It has been shown previously that the synthesis of poly(I) on this template proceeds continuously without reinitiation probably due to the reiterative transcription, whereas that of poly(G) undergoes frequent termination and reinitiation (Neff & Chamberlin, 1978). Similar to the case of poly(A) synthesis, ρ can effectively inhibit poly(I) synthesis continuing in the presence of rifampicin, and this inhibition is enhanced by the addition of a supplementary substrate for ρ NTPase (Table I). Thus, the poly(I) synthesizing system seems to satisfy the requirements of the ρ termination function both for the pausing of transcription as discussed above and for the RNA-dependent NTPase activity. Apparent enhancement of ρ-induced termination by substitution of ITP for GTP has also been observed in the transcription of T3 phage DNA as template (Adhya et al., 1979).

The present studies also focus on the nature of the RNA requirement for the termination activity of ρ. Previously, the specificity of ρ NTPase for RNA base composition has been extensively studied by using free RNA molecules as activators (Lowery & Richardson, 1977). Under such conditions, the NTPase is most strongly activated by ribocopolymers containing cytidine and having little secondary structures. In contrast, poly(U) can stimulate the NTPase to about 10% of the activity obtainable with poly(C), and poly(A) and poly(I) do so to only a few percent or less, with poly(G) being totally ineffective (Lowery & Richardson, 1977; Shigesada & Imai, 1978). It has been an intriguing problem whether the presence of cytidine in RNA transcripts is specifically required for the termination activity of ρ as well. The current finding that the synthesis of poly(A), and probably also of other homoribopolymers except poly(G), can be terminated by ρ has indicated that ρ exhibits little selectivity for RNA base sequence during the termination reaction. This implies that the interaction of ρ with nascent RNA in the process of termination may be somewhat different from that with free RNA molecules. This notion is also supported by the observation that ρ NTPase activity shows different sensitivity to magnesium concentration, depending on whether it is coupled or uncoupled with termination. With free poly(A) or poly(I) molecules, the NTPase activity is detectable only when Mg concentration is kept low (1–5 mM) and the concentration of activator is at least 10 μg/mL or higher. In the transcription system to form these homoribopolymers, however, ρ can cause termination optimally in the presence of much higher concentrations of magnesium (10–20 mM). Besides, this termination is observable even under such conditions where the total concentration of product RNA reaches no more than 0.1 μg/mL. We infer that nascent RNA molecules may have a more extended configuration than free molecules so as to facilitate their interaction with ρ, or the possible interaction between ρ and RNA polymerase during the termination may modify the functional characteristics of ρ NTPase, or both. Furthermore, RNA species terminated at several ρ-dependent sites so far analyzed have widely variable base sequences in their 3′-terminal regions and are not necessarily rich in cytidine (Rosenberg et al., 1978; Küpper et al., 1978; Wu et al., 1981). The possible importance of the interaction between ρ and noncytidine bases for efficient termination has been also suggested from the previous observation that a mutant ρ protein (*nit A* 702), which is severely defective in termination activity, has decreased ability to utilize ribocopolymers other than poly(C) as activator for the NTPase (Shigesada & Imai, 1978).

Finally, the observed efficacy of ρ in terminating poly(A) synthesis on single-stranded templates (Table V) has provided some clues to the question of whether ρ interacts with the template in addition to the nascent RNA during the termination reaction. Since those templates only consist of coding strands, it is evident that ρ does not require the presence of noncoding strands, at least in the homopolymer template systems. Further, the oligo(dT) template used here (10 nucleotides long) is so short that it may be wholly covered up by the RNA polymerase molecule and most of its nucleotide residues must be involved in RNA–DNA base pairing with the 3′ end of the growing poly(A) chain in the process of transcription. Then it follows that either ρ may not need any interaction with the template for its termination function or else it may somehow manage to contact with the particular DNA–RNA hybrid region.

In summary, the current studies suggest that ρ can manifest its termination activity on various synthetic templates when the transcription complex undergoes a pauselike process and provided that RNA transcripts are available for activation of

ρ NTPase. Due to their simplified primary structures, such artificial templates should prove useful for the study of the molecular details of the postulated interaction between ρ and paused transcription complexes. The results of experiments using poly(dG)·poly(dC) and poly[d(A-C)]·poly[d(G-T)] have also revealed that ρ can affect transcription in still different ways. Further investigation of such template systems will help broaden our understanding about the potential molecular activities of ρ and their relationships with its termination function.

Acknowledgments

We thank Dr. A. Ishihama for a kind gift of RNA polymerase and Dr. M. J. Champerlin and M. Brennan for critical reading of the manuscript.

References

Adhya, S., Sarkar, P., Valenzuela, D., & Maitra, U. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1613–1617.

August, J. T., Ortiz, P. J., & Hurwitz, J. (1962) *J. Biol. Chem. 237*, 3786–3793.

Berg, D., Barrett, K., & Chamberlin, M. (1971) *Methods Enzymol. 21*, 506–519.

Chamberlin, M., & Berg, P. (1964) *J. Mol. Biol. 8*, 708–726.

Darlix, J. L., & Fromageot, P. (1974) *Biochimie 56*, 703–710.

Darlix, J. L., & Horaist, M. (1975) *Nature (London) 256*, 288–292.

Darlix, J. L., Sentenac, A., & Fromageot, P. (1971) *FEBS Lett. 13*, 165–168.

Falaschi, A., Adler, J., & Khorana, H. G. (1963) *J. Biol. Chem. 238*, 3080–3085.

Farnham, P. J., & Platt, T. (1981) *Nucleic Acids Res. 9*, 563–577.

Galluppi, G., Lowery, C., & Richardson, J. P. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M. J., Eds.) pp 657–665, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Glynn, I. M., & Chappell, H. B. (1964) *Biochem. J. 90*, 147–149.

Goldberg, A. R., & Hurwitz, J. (1972) *J. Biol. Chem. 247*, 5637–5645.

Howard, B. H., & de Crombrugghe, B. (1976) *J. Biol. Chem. 251*, 2520–2524.

Johnston, D. E., & McClure, W. R. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M. J., Eds.) pp 413–428, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Kassavetis, G. A., & Chamberlin, M. J. (1981) *J. Biol. Chem. 256*, 2777–2786.

Küpper, H., Sekiya, T., Rosenberg, M., Egan, J., & Landy, A. (1978) *Nature (London) 272*, 423–428.

Lowery, C., & Richardson, J. P. (1977) *J. Biol. Chem. 252*, 1381–1385.

Lowery-Goldhammer, C., & Richardson, J. P. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2003–2007.

Morgan, A. R. (1970) *J. Mol. Biol. 52*, 441–466.

Neff, N. F., & Chamberlin, M. J. (1978) *J. Biol. Chem. 253*, 2455–2460.

Reisbig, R. R., & Hearst, J. E. (1981) *Biochemistry 20*, 1907–1918.

Richardson, J. P., & Conaway, R. (1980) *Biochemistry 19*, 4293–4299.

Roberts, J. W. (1969) *Nature (London) 224*, 1168–1174.

Roberts, J. W. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M. J., Eds.) pp 247–271, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Rosenberg, M., & Court, D. (1979) *Annu. Rev. Genet. 13*, 319–353.

Rosenberg, M., Court, D., Shimatake, H., Brady, C., & Wulff, D. L. (1978) *Nature (London) 272*, 414–423.

Shigesada, K., & Imai, M. (1978) *J. Mol. Biol. 120*, 467–486.

Shigesada, K., & Wu, C. W. (1980) *Nucleic Acids Res. 8*, 3355–3369.

Sippel, A. E. (1973) *Eur. J. Biochem. 37*, 31–40.

Strniste, G. F., Smith, D. A., & Hayes, F. N. (1973) *Biochemistry 12*, 596–608.

Wu, A. M., Christie, G. E., & Platt, T. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2913–2917.