Proc. Natl. Acad. Sci. USA
Vol. 74, No. 2, pp. 520–523, February 1977
Biochemistry

Virion-associated RNA polymerase required for bacteriophage N4 development*

(RNA synthesis/rifampicin-resistant RNA polymerase/temperature-sensitive mutant/hybridization competition)

S. C. FALCO, K. VANDER LAAN, AND L. B. ROTHMAN-DENES

Department of Biophysics and Theoretical Biology, University of Chicago, Chicago, Illinois 60637

Communicated by E. Peter Geiduschek, November 15, 1976

ABSTRACT ***Escherichia coli* phage N4 transcription is resistant to rifampicin, an inhibitor of the host RNA polymerase, even when the drug is added prior to infection. A rifampicin-resistant RNA polymerase has been detected in disrupted N4 virions. This enzyme shows a requirement for the four ribonucleoside 5′-triphosphates and exogenous denatured DNA. With denatured N4 DNA, the preferred template, transcription is asymmetric. The virion RNA polymerase apparently is necessary for phage development because a conditional lethal N4 mutant shows temperature-sensitive RNA synthesis *in vivo* as well as a temperature-sensitive RNA polymerase in disrupted virions.**

Bacteriophage N4 contains double-stranded DNA of molecular weight 40×10^6 and infects *Escherichia coli* K12 strains (1). N4 infection causes cessation of host DNA synthesis, but the host chromosome is not degraded (2). Host RNA and protein synthesis are not shut off except for functions dependent on adenosine 3′:5′-cyclic monophosphate (3).

N4 infection induces phage-specific transcription in cells pretreated with rifampicin which completely inhibits *E. coli* RNA polymerase (4). All N4 transcription is independent of the host RNA polymerase; therefore, N4 provides a new model of interaction between *E. coli* and its viruses (5).

Two classes of N4 RNA, which comprise all N4 transcripts, have been detected after infection of cells pretreated with rifampicin (5). Class I RNA synthesis does not require translation of the phage genome after infection, because it appears in cells treated with chloramphenicol prior to infection (4, 5). However, the expression of at least two N4 genes, cistrons 3 and 4 (6), is necessary for class II RNA synthesis (4, 5). These results suggest the existence of two distinct rifampicin-resistant RNA polymerase activities in N4 infected cells.

Because RNA polymerase activity was observed in the absence of postinfection protein synthesis, a virion-encapsulated RNA polymerase was postulated (4). This paper describes the detection of such an activity and its requirement for N4 development.

MATERIALS AND METHODS

Bacterial and Phage Strains. *E. coli* strain W3350 was used in all experiments. Temperature-sensitive conditional lethal mutants of N4 were isolated by hydroxylamine mutagenesis in this laboratory. N4*ts*150 was selected from mutants showing temperature-sensitive RNA synthesis *in vivo*. The genetic characterization and properties of N4*ts*150 will be reported elsewhere.

Preparation of N4 Virion-Associated RNA Polymerase. Phage were purified by CsCl buoyant density centrifugation. One volume of N4 virions at a concentration of 8×10^{13} plaque-forming units/ml ($A_{260} = 300$) in 15 mM Tris·HCl (pH 7.5)/10 mM $MgCl_2$ was added to four volumes of 4 M guanidine·HCl/10 mM Tris·HCl/10 mM EDTA/10 mM dithiothreitol brought to pH 8 by addition of NaOH. After two cycles of freezing and thawing, the viscosity of the solution was decreased by sonication (fraction Ia). Dialysis overnight at 4° against 10 mM Tris·HCl (pH 8)/1 mM EDTA/1 mM 2-mercaptoethanol yielded fraction Ib.

Viral DNA was removed by batch fractionation with hydroxylapatite (Bio-Gel HT, Bio-Rad Laboratories). Hydroxylapatite was equilibrated with a solution containing 4 M guanidine·HCl (brought to pH 7 with KOH), 0.24 M potassium phosphate (pH 7), and 10 mM 2-mercaptoethanol. One volume of fraction Ia was treated with three volumes of hydroxylapatite (75% vol/vol) in the above buffer for 1 hr at 4°. The hydroxylapatite was then removed by centrifugation. The supernatant was dialyzed as described above to yield fraction II. This procedure removes more than 97% of the viral DNA with a recovery of 60% of the activity present in fraction Ib. Fraction II, which has a protein concentration of 400–500 μg/ml, can be stored at −20° in the presence or absence of 50% glycerol without loss of activity for at least 1 month. However, repeated freezing and thawing results in a loss of activity.

Assay for RNA Synthesis. The standard reaction mixture (0.1 ml) contained 80 mM Tris·HCl (pH 8), 10 mM $MgCl_2$, 0.4 mM sodium phosphate (pH 7), 0.2 mM dithiothreitol, denatured N4 DNA at 60 μg/ml, fraction II protein at 60 μg/ml, 1 mM each of ATP, CTP, and UTP, and 0.1 mM GTP ([^{3}H]-GTP or [^{14}C]GTP, 7.8 cpm/pmol). Incubation for 15 min at 37° was terminated by addition of 1 ml of cold 10% trichloroacetic acid/0.1 M sodium pyrophosphate. Acid-insoluble radioactivity was collected on Whatman GF/A filters and assayed in toluene-based scintillation fluid. Under these conditions, 25 pmol of GMP was incorporated per microgram of protein.

Phage DNAs were prepared by phenol extraction (7), and *E. coli* DNA was obtained as previously described (8). DNAs were denatured by heating at 100° for 5 min followed by rapid cooling at 0°.

RESULTS

Properties of N4 virion-associated RNA polymerase

Disruption of N4 virions is essential for the detection of RNA polymerase activity. The reaction requirements are summarized in Table 1. Activity required the presence of four ribonucleoside triphosphates. Deoxyribonucleoside triphosphates were not incorporated. Furthermore, in the presence of CTP, GTP, and UTP, dATP did not substitute for ATP. Similar results were obtained with dCTP and dUTP (data not shown). The presence of Mg^{2+} was required, the optimal concentration being 10 mM. Substitution of Mn^{2+} was not efficient with either ribonucleoside or deoxyribonucleoside triphosphates as substrate. The enzyme was inhibited by NaCl, with 50% inhibition

* *Dedicated to Prof. Luis F. Leloir on the occasion of his 70th birthday.*

Downloaded from https://www.pnas.org by 44.211.24.175 on November 9, 2024 from IP address 44.211.24.175.

Table 1. Requirements for *in vitro* RNA synthesis by N4 virion-associated RNA polymerase

Deletions or additions	Activity (%)
None	100*
Omit ATP	3.9
Omit CTP	1.3
Omit UTP	3.9
Omit GTP†	2.4
Omit 4 rNTPs, add 4 dNTPs‡	0
Omit ATP, add dATP	5.8
Omit Mg^{2+}	0
Omit Mg^{2+}, add Mn^{2+} (5 mM)	9
Omit 4 rNTPs, omit Mg^{2+}, add 4 dNTPs‡ + Mn^{2+} (5 mM)	0
NaCl (0.1 M)	50
NaCl (0.25 M)	16
Rifampicin (5 μg/ml)	84
Rifampicin (50 μg/ml)	84
Streptolydigin (5 μg/ml)	101
Streptolydigin (50 μg/ml)	88

* Conditions of assay as described in *Materials and Methods*. 155 pmol GMP incorporated.

† [^{14}C]ATP (0.1 mM, 7.0 cpm/pmol) was the labeled nucleotide. UTP, GTP, and CTP were present at 1 mM.

‡ [^{3}H]dTTP (0.1 mM, 15 cpm/pmol) was the labeled nucleotide. dATP, dGTP, and dCTP were present at 1 mM. rNTP = ribonucleoside triphosphate; dNTP = deoxyribonucleoside triphosphate.

occurring at 0.1 M. Glycerol stabilized the activity (see *Materials and Methods*) and enhanced the rate of incorporation: with 25% glycerol there was a 50% increase in activity. Neither rifampicin nor streptolydigin had an appreciable effect on the N4 enzyme, even at 10-fold higher concentration (50 μg/ml) than is necessary to completely inhibit the *E. coli* polymerase. Synthesis of RNA was proportional to the amount of protein added to the reaction over a 4-fold range of protein concentration (Fig. 1A). After an initial rapid rate, the rate of synthesis of RNA was approximately linear for at least 1 hr (Fig. 1B).

In Table 2 several DNAs are compared with respect to their ability to serve as template for the N4 virion RNA polymerase. Exogenous DNA was required but, surprisingly, native DNA was not effective. There was a preference for denatured N4 DNA, but other denatured DNAs such as T7 DNA also served as template. The rate of synthesis depended on the concentration of denatured N4 DNA (Fig. 1C). T4 DNA is not effective either in its native or denatured state. The synthetic duplexes poly[d(A-T)] and poly(dG)·poly(dC) were unable to promote synthesis.

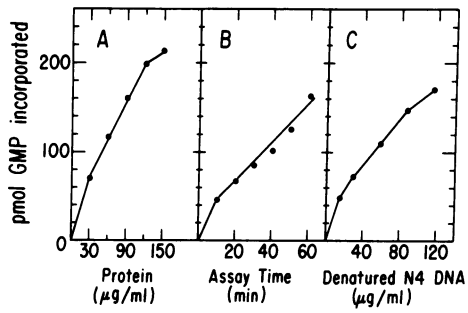

FIG. 1. Effect of protein concentration (A), assay time (B), and DNA concentration (C) on N4 virion-associated RNA polymerase activity. Conditions of assay were as described in *Materials and Methods*.

Table 2. Template specificity of N4 virion-associated RNA polymerase

Template DNA added	Concentration (μg/ml)	Incorporation of GMP (pmol/15 min)
None	—	1.5
N4 DNA	105	0.6
Denatured N4 DNA	105	191
T4 DNA	104	0
Denatured T4 DNA	104	1.1
T7 DNA	107	5
Denatured T7 DNA	107	73
poly[d(A-T)]*	108	0
poly(dG)·poly(dC)	95	5
poly(dG)·poly(dC)†	95	0

Conditions of assay as described in *Materials and Methods*, except that, where indicated, denatured N4 DNA was replaced with other DNAs.

* [^{14}C]ATP (0.1 mM, 7.0 cpm/pmol) was the labeled nucleotide. UTP, GTP, and CTP were present at 1 mM.

† [^{3}H]CTP (0.1 mM, 6.1 cpm/pmol) was the labeled nucleotide. ATP, GTP, and UTP were present at 1 mM.

Characterization of the product

The product of the reaction catalyzed by the N4 virion RNA polymerase was sensitive to alkali and RNase but insensitive to DNase (data not shown). Analysis of the RNA synthesized, by sedimentation in a 5–20% sucrose gradient, showed it to be heterogeneous in size, ranging from 4 S to 24 S with most of the product between 8 S and 14 S (data not shown).

Denatured N4 DNA directed the synthesis of RNA hybridizable to N4 DNA. To determine the relationship between this RNA and *in vivo* N4 RNA, two types of experiments were performed: RNA·RNA annealing and RNA·DNA hybridization competition.

N4 antimessenger RNA is not detectable in infected cells (5). The ability to form RNA·RNA hybrids between *in vitro* labeled RNA and excess unlabeled RNA obtained from cells at different times after infection was tested (9). In no case was more than 9% of the input RNA detected in ribonuclease-resistant duplexes (Table 3). These results indicate that the virion-associated RNA polymerase transcribes denatured N4 DNA asymmetrically.

In the absence of postinfection protein synthesis, only class I N4 transcripts are made (5). ^{3}H-Labeled RNA synthesized *in vitro* was allowed to compete with unlabeled *in vivo* RNA for hybridization to excess N4 DNA bound to nitrocellulose filters (Fig. 2). RNA obtained at 7 min after N4 infection from cells

Table 3. Asymmetry of N4 RNA synthesized by N4 virion-associated RNA polymerase

In vivo unlabeled RNA	% [^{3}H]RNA in duplex
None	9
Chloramphenicol, 6 min	2
7 min	4
33 min	5

[^{3}H]RNA was synthesized with [^{3}H]GTP (40 cpm/pmol), denatured N4 DNA (90 μg/ml), and fraction II (60 μg/ml). Incubation was for 1 hr at 33°. The RNA was phenol-extracted twice, heated to 90° and quick-cooled, and treated with pancreatic DNase (10 μg/ml) and then with proteinase K (10 μg/ml) followed by two more phenol extractions. Samples containing [^{3}H]RNA (0.2 μg/ml, 3300 cpm) were annealed, where indicated, with *in vivo* unlabeled RNA at 2 mg/ml (refer to legend of Fig. 2) as described (9).

Downloaded from https://www.pnas.org by 44.211.24.175 on November 9, 2024 from IP address 44.211.24.175.

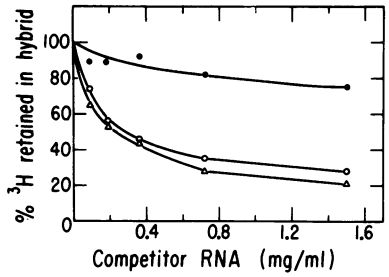

FIG. 2. Analysis of *in vitro* synthesized RNA by competitive RNA·DNA hybridization. RNA was synthesized and extracted as described in the legend of Table 3. RNA·DNA hybridization competition was performed as described (5), with 1 μg of N4 DNA per filter and 5250 cpm of ^{3}H-labeled RNA input. Efficiency of hybridization in the absence of competitor: 17.7% (931 cpm), mean of two determinations. Competitor RNAs: (●) from chloramphenicol-pretreated (100 μg/ml, 10 min before infection) *E. coli* cells 7 min after N4 infection; (○) from *E. coli* cells 7 min after N4 infection; (△) from *E. coli* cells 33 min after infection. Similar results were obtained when the efficiency of hybridization was 40%.

pretreated with chloramphenicol (class I RNA) decreased the label in the hybrid by only 25%. RNAs obtained from untreated cells at either 7 min or 33 min after N4 infection were better competitors. These RNAs, which contain both class I and class II transcripts (5), decreased the label by 70 to 80%. Because the competition curves for these RNAs had not leveled off in the experiment shown, an 80% level of competition may be a low estimate of their competing ability.

Involvement of N4 virion RNA polymerase in N4 development

Experiments with N4*ts*150, a mutant of N4 that is temperature-sensitive for growth, demonstrated that the N4 virion RNA polymerase is required for N4 development. When *E. coli* cells were treated with rifampicin and chloramphenicol and then were infected with wild-type N4, pulse-labeling with [^{3}H]-uridine yielded the characteristic pattern shown in Fig. 3A. At 33° the rate of RNA synthesis increased for the first 10 min of infection, leveled off until about 22 min, and then declined slowly. At 43° the rate increased rapidly, reached a maximum at 5 min after infection, and then gradually decreased to the 33° level. When cells infected at 33° were shifted to 43° at 7 min after infection, there was an initial rise in the rate of [^{3}H]uridine incorporation followed by a gradual decline to the same level as at 33° or 43°.

After infection by N4*ts*150, quite different results were obtained (Fig. 3B). At 33°, the permissive temperature, the pattern was similar to that observed after wild-type N4 infection.

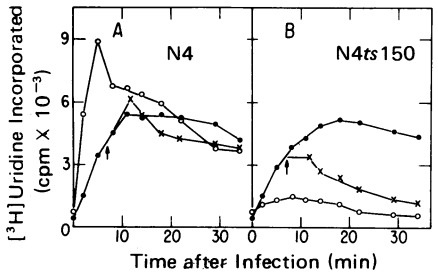

FIG. 3. Rate of N4 class I RNA synthesis *in vivo* in cells infected with N4 or N4*ts*150. Two-minute pulses of [^{3}H]uridine were performed as previously described (4). Chloramphenicol (200 μg/ml) and rifampicin (200 μg/ml) were added 15 min prior to infection. Values are plotted at the middle of the pulse period. Arrow at 7 min indicates time of shift to 43°. ●, 33°; ○, 43°; ×, shift to 43.

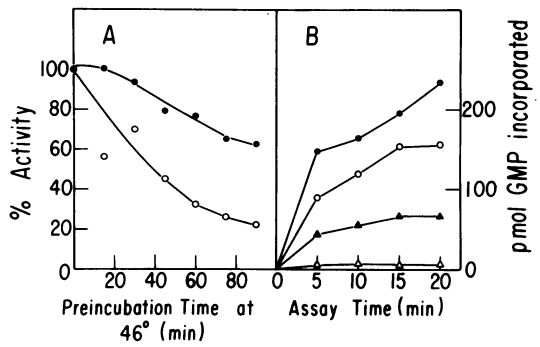

FIG. 4. Temperature sensitivity of wild-type N4 and N4*ts*150 virion-associated RNA polymerases. (A) Fraction II from N4 (200 μg/ml) (●) or N4*ts*150 (260 μg/ml) (○) virions were incubated in 50% glycerol at 46°. Enzyme activity was measured at 37° as described in *Materials and Methods* except that the assay mixture contained 25% glycerol and 100 μg of N4 fraction II or 130 μg of N4*ts*150 fraction II per milliliter. 100% activity: N4, 260 pmol; N4*ts*150, 208 pmol. (B) Fraction II samples from N4 (70 μg/ml) or N4*ts*150 (77 μg/ml) were preincubated for 10 min at the assay temperature (37° or 46°) in the standard assay mixture lacking N4 denatured DNA, which was added to start the assay. Acid-insoluble radioactivity was determined at the indicated times. N4, 37° (●); N4, 46° (▲); N4*ts*150, 37° (○), N4*ts*150, 46° (△).

In contrast, at 43°, the nonpermissive temperature, little incorporation of [^{3}H]uridine was observed above the background level of uninfected cells. Furthermore, when infection at 33° was followed by an increase to 43° at 7 min after infection, the rate of incorporation decreased, approaching the low 43° level.

These *in vivo* observations suggest that N4*ts*150 might possess a temperature-sensitive virion RNA polymerase. To test this possibility, disrupted N4*ts*150 virions were prepared and the temperature-sensitivity of their RNA polymerase activity was compared to that of RNA polymerase from N4 virions. Several experimental protocols were used, and in all cases the virion RNA polymerase activity from N4*ts*150 showed significantly greater temperature sensitivity than did the wild-type N4 activity. Fig. 4A shows that N4*ts*150 virion RNA polymerase activity decayed more rapidly at 46° than did the N4 activity. In Fig. 4B the RNA polymerase activities from wild-type N4 and N4*ts*150 disrupted virions are compared at two different reaction temperatures, 37° and 46°. When assayed at 37°, the activities were similar. At 46° the wild-type N4 activity was present, albeit reduced, but N4*ts*150 polymerase was completely inactive.

DISCUSSION

An RNA polymerase activity can be measured when the virions of *E. coli* bacteriophage N4 are disrupted. This activity requires the four ribonucleoside triphosphates, Mg^{2+}, and denatured DNA as template and is resistant to both rifampicin and streptolydigin. With denatured N4 DNA, the most effective template, the product synthesized is heterogeneous in size, ranging from 4 S to 24 S.

The experiments summarized in Table 3 demonstrate that the N4 virion-associated RNA polymerase yields asymmetric transcription on denatured N4 DNA. This transcriptional specificity is unexpected. T7 RNA polymerase transcribes only the *r* strand of native T7 DNA. However, when the separated strands of T7 DNA are used, the specificity for strand selection is lost (10). Similarly, *E. coli* RNA polymerase transcribes native T4 DNA asymmetrically but transcribes denatured T4 DNA symmetrically (11). However, in this case, some specificity is

Downloaded from https://www.pnas.org by 44.211.24.175 on November 9, 2024 from IP address 44.211.24.175.

retained because little late T4 mRNA is made with either native or denatured DNA (11). Whether the specificity observed in the N4 system resides in the enzyme or in the DNA template remains to be elucidated.

RNA·DNA hybridization competition experiments show that at least 80% of the RNA made *in vitro* is also made *in vivo* and that both *in vivo* classes of transcripts are made *in vitro*. In the infected cell, class II transcription requires N4 protein synthesis. This indicates a lack of transcriptional specificity in our preparation of virion-associated RNA polymerase. Because N4 DNA extracted from virions is not a template for this activity, we suggest that either the structure of N4 DNA in the infected cell allows the virion-associated polymerase to transcribe only N4 class I RNAs or a factor which provides transcriptional specificity is missing from our preparation. The inability of our polymerase preparation to use native N4 DNA as template does not appear to be due to the use of guanidine·HCl for the disruption of the virions. In our hands, disruption by freezing and thawing is a much less efficient method of exposing the virion-associated polymerase and, also, yields an activity that requires denatured DNA (but see ref. 12 and further comments below).

A mutant of N4, N4*ts*150, which is temperature-sensitive for growth, shows temperature-sensitive RNA synthesis *in vivo*. When N4*ts*150 virions are disrupted, the RNA polymerase activity observed *in vitro* is also temperature-sensitive. These results indicate that the virion-associated RNA polymerase is not a host cell contaminant and is necessary for N4 growth.

While this manuscript was in preparation, an N4 virion-associated RNA polymerase which transcribes native N4 DNA was reported (12). The RNA synthesized was N4 specific and, in competition experiments, was completely replaced by RNA from cells pretreated with chloramphenicol. We have not yet been able to obtain an activity with these characteristics.

Requirement of the host RNA polymerase for bacteriophage development has been well documented. Coliphages T4 and λ and *Bacillus subtilis* phages SP01, SP82, and ϕ29 require the host enzyme for transcription throughout their development (13–17). Coliphages T3 and T7 and *Pseudomonas putida* phage gh-1 require the host polymerase to transcribe their early genes, one of which codes for a new RNA polymerase necessary for late transcription (18–22). On the other hand, *B. subtilis* phage PBS2 shows transcriptional independence from its host, but the mechanism involved is not yet known (23, 24).

N4 transcription is independent of the host RNA polymerase (5). The results presented in this paper allow us to formulate a model for N4 development. We propose that the virion-associated RNA polymerase, which we have shown to be required for class I RNA synthesis *in vivo*, is injected into the host cell with the viral DNA. This polymerase is responsible for the expression of N4 genes, specifically cistrons 3 and 4, which are necessary for the synthesis of class II transcripts. A different RNA polymerase or a modified form of the virion-associated RNA polymerase transcribes class II genes, inasmuch as class II RNA synthesis is not temperature-sensitive in N4*ts*150 infected cells (after class I transcription has occurred) and the N4*ts*150 mutation is not in either cistron 3 or 4 (unpublished observations).

The existence of nucleic acid polymerizing activities in animal viruses is well established. N4 provides the first example of a bacteriophage in which such a virion-associated activity is required for its development.

We gratefully acknowledge the help of K. Agarwal, N. Cozzarelli, R. Haselkorn, and K. McEntee in the preparation of the manuscript. This research was supported by Research Grant AI 12575 and Research Career Development Award 1 K04-AI-00078 from the National Institute of Allergy and Infectious Diseases to L.B.R.-D. S.C.F. is a trainee on USPHS GM 780.

1. Schito, G. C., Rialdi, G. & Pesce, A. (1966) *Biochim. Biophys. Acta* **129**, 482–490.
2. Schito, G. C., Pesce, A. & Satta, G. (1969) *Gen. Microbiol.* **17**, 141–150.
3. Rothman-Denes, L. B., Haselkorn, R. & Schito, G. C. (1972) *Virology* **50**, 95–102.
4. Rothman-Denes, L. B. & Schito, G. C. (1974) *Virology* **60**, 65–72.
5. Vander Laan, K., Falco, S. C. & Rothman-Denes, L. B. (1976) *Virology*, in press.
6. Schito, G. C. (1973) *Virology* **55**, 254–265.
7. Bolle, A., Epstein, R., Salser, W. & Geiduschek, E. P. (1968) *J. Mol. Biol.* **31**, 325–348.
8. Marmur, J. (1961) *J. Mol. Biol.* **3**, 208–218.
9. Geiduschek, E. P. & Grau, O. (1970) in *First Lepetit Colloquium on Biology and Medicine: RNA Polymerase and Transcription*, ed. Silvestri, L. (North Holland, Amsterdam and London), pp. 190–203.
10. Chamberlin, M. & Ring, J. (1973) *J. Biol. Chem.* **248**, 2235–2244.
11. Brody, E. N. & Geiduschek, E. P. (1970) *Biochemistry* **9**, 1300–1309.
12. Pesce, A., Casoli, C. & Schito, G. C. (1976) *Nature* **262**, 412–414.
13. Haselkorn, R., Vogel, M. & Brown, R. D. (1969) *Nature* **221**, 836–838.
14. Takeda, Y., Oyama, Y., Nakajima, K. & Yura, T. (1969) *Biochem. Biophys. Res. Commun.* **36**, 533–538.
15. Geiduschek, E. P. & Sklar, J. (1969) *Nature* **221**, 833–836.
16. Spiegelman, G. B. & Whiteley, H. R. (1974) *J. Biol. Chem.* **249**, 1476–1482.
17. Holland, M. & Whiteley, H. R. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 2234–2237.
18. Summers, W. C. & Siegel, R. G. (1969) *Nature* **223**, 1111–1113.
19. Chamberlin, M., McGrath, J. & Waskell, H. (1970) *Nature* **228**, 227–231.
20. Maitra, U. (1971) *Biochim. Biophys. Acta* **43**, 443–450.
21. Dunn, J. J., Bautz, F. A. & Bautz, E. K. F. (1971) *Nature New Biol.* **230**, 94–96.
22. Towle, H. C., Jolly, J. F. & Boezi, J. A. (1975) *J. Biol. Chem.* **250**, 1723–1733.
23. Price, A. & Frabotta, M. (1972) *Biochem. Biophys. Res. Commun.* **48**, 1578–1585.
24. Clark, S., Losick, R. & Pero, J. (1974) *Nature* **252**, 21–24.

Downloaded from https://www.pnas.org by 44.211.24.175 on November 9, 2024 from IP address 44.211.24.175.