JOURNAL OF VIROLOGY, Dec. 1973, p. 1384-1394

Vol. 12, No. 6
Printed in U.S.A.

Cold-Sensitive *Pseudomonas* RNA Polymerase

II. Cold-Promoted Restriction of Bacteriophage CB3 and the Lack of Host-Dependent Bacteriophage-Specific RNA Transcription

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Received for publication 12 April 1973

Cold-sensitive restriction of *Pseudomonas* phage CB3 by *Pseudomonas aeruginosa* strain PAT2 involves some aspect of CB3 specific RNA synthesis at 20 C. Experiments using chloramphenicol treatment and RNA-DNA hybridization establish that the amount of CB3 RNA present at 20 C is consistent with the known percentage of phage yielder cells at 20 C. Thus, it appears that nonyielder cells of PAT2 synthesize little or no phage-specific mRNA. Burgess technique extracted PAT2 RNA polymerase (RNAP) is cold sensitive when assayed in vitro with CB3 DNA at 20 C. However, it is not cold sensitive when either calf thymus or PAT2 DNA are the templates for transcription. Low ionic strength assay conditions eliminate the cold sensitivity of PAT2 RNAP. The effect of low ionic environments on transcription initiation along with the in vivo and in vitro suppression of cold sensitivity by host rifampin resistance suggests that the inability of CB3 to reproduce in PAT2 at 20 C is a cold-sensitive step in host RNAP initiation. Our modified RNAP extraction procedure for PAT2 and PAO1C also results in the recovery of cold-sensitive PAT2 RNAP with respect to CB3 DNA templates and points to basic enzymological differences between the two hosts. A model is presented for the unusual influence of temperature on the initiation process of both PAT2 and PAO1C on RNAP transcription.

The binding of phage DNA to host cell membrane after penetration has been observed for several unrelated phages. The relationship between DNA injection, attachment, and the transcription from this DNA of the first immediate early (IE) phage-specific mRNA molecules is not known. However, it has been shown for coliphage T4 (4, 12, 19, 35) that these first transcripts are synthesized by host DNA dependent RNA polymerase (EC 2.7.7.6). Other phages such as T3 (32), T7 (33), and ϕe of *Bacillus subtilis* (31) have a similar course of initial RNA synthesis.

In the accompanying report (28) we conclude that the inability of phage CB3 to develop in *Pseudomonas aeruginosa* PAT2 at 20 C is not due to the absence of host membrane binding of phage genomes at this temperature. On the other hand, functions expressed by the phage in a permissive infection, such as the immediate transient depression of the rates of RNA synthesis, the synthesis of phage DNA, and the inhibition of host DNA synthesis and its degradation, are not detected in the conditional host, PAT2, at nonpermissive temperature.

The cold-sensitive event can be bypassed by a 37 C temperature pulse applied early after infection (21). An exposure to the permissive temperature for only the first 5 min of the latent period followed by a shift down to 20 C promotes normal phage yields from PAT2. Thus, some aspect of phage development after DNA membrane binding but prior to phage DNA synthesis is facilitated by the increase in temperature. In this report we examine the production of viral specific mRNA by PAT2 infected with CB3. We consider that the presence of CB3-specific RNA at 20 C would implicate host determinants occurring after transcription of the IE phage mRNA species in the cold-sensitive response to infection, whereas their absence would implicate the PAT2 RNA polymerase (RNAP). We find that PAT2 RNAP fails to transcribe CB3 DNA at low temperatures in vitro. The apparent defect in the molecule resides in the initiation function of RNA transcription.

This investigation was taken in part from a

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thesis submitted by R. J. S. in partial fulfillment of the requirements for the Ph.D. degree in Microbiology at the University of Michigan. A preliminary communication was presented at the 1971 and 1972 meetings of the American Society for Microbiology.

MATERIALS AND METHODS

Organisms. *Pseudomonas aeruginosa* strains PAO1C, PAT2, and CB3 bacteriophage have been previously described (30). *Escherichia coli* D10 was obtained from Theophil Staehelin (Department of Zoology, University of Michigan). *Pseudomonas* phage, PX 4, is a psychrophilic phage able to infect the PAT2 strain at temperatures up to 32 C (22). The virulent *Pseudomonas* phage LP was isolated in this laboratory. This phage infects PAO1C (unpublished observation) and is serologically and morphologically unrelated to either PX 4 or CB3. Phage infections were routinely checked to estimate the number of infected bacteria as described previously (30).

Cultivation of microorganisms. Growth of *Pseudomonas* and *E. coli* D10 bacteria in medium consisting of tryptone, 0.5%; glucose, 0.1%; and yeast extract, 0.25% (TGE) has been described (30). When bacteria or phage were grown in volumes larger than 100 ml, KNO_3 was added to 0.4%. In some experiments, Vogel and Bonner (VBG) medium (36) supplemented with 1.8% glucose and 0.005 mg of any required growth nutrient per ml was used. Recovery medium (RM) which was used for plating survivors of mutagenesis consisted of 1% glucose, 0.5% tryptone, 1.0% yeast extract, and 1.0% Casamino Acids (pH 7.0). Large volumes of cells (50 to 100 liters) were grown in TGE broth in a New Brunswick Scientific Co. Fermacell (model F 250). For this the inoculum was 8 to 10 liters of early log-phase cells grown at 37 C in the same medium. After inoculation the fermentor was maintained at 37 C with vigorous aeration. At a cell density of 2×10^8 to 5×10^8 cells/ml, the culture was cooled to 14 to 16 C by circulation of cold water in the fermentor jacket and was harvested with the aid of a continuous flow Sharples centrifuge. The cell paste was immediately frozen at −20 C. Fermacell inocula and cell crops were routinely checked for the expected cultural characteristics with regard to bacterial strain nutritional requirements, colony morphology, phage resistance, and sensitivity markers.

Reagents. The sources of reagents used in this and the accompanying report (30) were the same. Lysozyme (3× crystallized), 3,5-dihydroxytoluene (orcinol), bovine serum albumin (BSA), yeast ribonucleic acid, L-valine, and L-isoleucine were purchased from the Nutritional Biochemical Co. Dithiothreitol, A grade (DTT), and rifampin, B grade, were obtained from Calbiochem. Uracil-2-^{14}C (55 mCi/mmol) and ammonium sulfate (enzyme grade) was purchased from Schwarz Mann. ^{14}C-adenosine 5′-triphosphate (462 mCi/mmol) was obtained from New England Nuclear Corp. The sodium salts of 5′ triphosphates of cytidine, uridine, and guanosine were purchased from P & L Biochemicals Inc. Whatman Cellulose-Phosphate, PC11 (7.4 meq/g) and Whatman DEAE-cellulose, DE 52 (1.0 meq/g dry weight) were obtained from Reeve Angel. Calf thymus (CT) DNA was purchased from Worthington Biochemical Corp.

Analytical methods. Concentration of the purified protein, RNAP, was calculated assuming an extinction coefficient of $E^{1\%}_{280} = 6.5$ (27). RNA determinations were performed either by the orcinol method (20) by using yeast RNA as a standard or spectrophotometrically assuming an extinction coefficient at 260 nm of $E^{1\%}_{260} = 250$ (12). DNA was determined spectrophotometrically assuming an extinction coefficient at 260 nm of $E^{1\%}_{260} = 200$ (19).

Mutagenesis. PAT2 cells were grown overnight in TGE broth at 37 C to early log phase and subcultured for 2 to 4 cell doublings. The cells were then washed once at ambient temperature in 0.9% sterile NaCl and the final pellet was suspended in 0.1 M sodium citrate (pH 5.4) to a cell density of 2×10^8/ml. Then, 1.0 ml of the cells was added to an equal volume of freshly prepared *N*-methyl-*N*′-nitro-*N*-nitroso-guanidine (NTG) at 1 mg/ml (Aldrich Chemical Co) in the same buffer. The cells were incubated for 30 min at 30 C, centrifuged, and suspended in RM. This suspension was incubated without aeration for 2 h at 37 C prior to selecting the PAT2 mutants on VBG-valine and isoleucine supplemented minimal medium agar plates containing 0.025 mg of rifampin per ml. Plates were incubated for at least 3 days at 37 C before colonies were picked and restreaked for isolation three times on the same agar medium.

Labeling RNA for hybridization. PAT2 or PAO1C was grown overnight in TGE medium containing uracil as described (30). After starvation (incubation in the absence of uracil) the cells were exposed to 0.003 mg of uracil per ml for 30 min and then were diluted into TGE or TGE-CB3 phage containing broth with only ^{14}C-uracil present at 1.25 μCi/ml. Labeling was permitted for 5 min at 37 C and for 17 min at 20 C before beginning the extraction of nucleic acid. Infected cells which were to be used for RNA hybridization competition experiments were manipulated in an identical fashion except that unlabeled uracil (0.01 mg/ml) was substituted for ^{14}C-uracil.

Nucleic acid isolation. (i) CB3 DNA. CB3 DNA was isolated as described previously (30) except that bacteriophage DNA was not radioactively labeled.

(ii) PAT2 DNA. PAT2 was grown overnight in TGE broth at 37 C to a cell density of between 5×10^7 and 10^8 cells/ml. The culture was washed in 0.15 M NaCl and the cell pellet was resuspended in 0.3 M NaCl to a cell density of between 2.5×10^9 and 5.0×10^9 cells/ml. These cells were then frozen and held for at least 18 h at −20 C. Volumes of 5.0 ml were thawed and extracted by the Marmur method (16). Resultant DNA suspensions had a 260/280 nm ratio of between 1.91 and 2.08. DNA was stored in 0.15 M NaCl, 0.02 M sodium-citrate, pH 7.0, (SSC) at 4 C at concentrations greater than 0.2 mg/ml.

(iii) RNA. RNA for hybridization experiments was extracted by using a modification of the technique of Taylor et al. (34). Labeled and unlabeled cells (5.0 ml) which were infected or uninfected were quickly cooled in an ice bath, treated with 0.5 ml 0.1 M

sodium azide, and then washed and resuspended at 4 C in 5 ml of Tris-hydrochloride, pH 7.4, KCl, and $MgCl_2$ (TKM) all at 10^{-3} M. These cells were then centrifuged and suspended in 4.0 ml of TKM and to this suspension was added 1.0 ml of TKM containing 1 mg of lysozyme, 0.2 mg of deoxyribonuclease, and 1 mg of BSA. The cells were then frozen and thawed three times with each melting occurring over a 10-min period. The 5.0 ml of frozen-thawed cells had 0.5 ml of 20% sodium lauryl sulfate (SLS) added; 5 min later an equal volume of redistilled phenol saturated with SSC was added and the RNA was extracted at 60 C for 15 min on a reciprocating shaker (300 cycles per min). The phenol phase was discarded after centrifugation and phenol extraction of the aqueous phase was performed twice more. Finally the aqueous phase was extracted six times with 1.5 to 2.0 volumes of TKM saturated ether. Samples (10 ml each) of RNA were dialyzed against two 1,500-ml changes of 2× SSC at 4 C for 18 h. RNA prepared in this manner had a 260/280 nm ratio of 1.86 to 1.94. Radioactive preparations had less than 0.096% DNA as determined by alkaline hydrolysis (30). When desired, 0.5- to 1.0-liter volumes of infected cells were concentrated by centrifugation and resuspended to 5.0 ml before beginning extraction. Other infected cells were treated with 0.2 mg of chloramphenicol (CM) per ml for 5 min prior to the addition of CB3. At this level of CM, protein synthesis as determined by the incorporation of a labeled amino acid was inhibited during pretreatment (unpublished observation).

DNA-RNA hybridization. (i) Template filters. The methods used for the preparation of filter templates, hybridization, and washing of filters were essentially those of Gillespie and Spiegelman (11). Schleicher and Schuell, Bac-T-Flex, B6 filters were prepared on the day of use. DNA, 0.5 ml, was denatured by boiling for 15 min followed by quick cooling and the addition of 2.0 ml of cold 7.2× SSC. DNA was fixed to filters by drying filters overnight under a vacuum and then by heating under a vacuum at 80 C for 2.5 h. As little as 5×10^{-7} mg of CB3 DNA per filter was saturating for the RNA produced in a permissive infection (data not presented). With host PAT2 RNA, saturation was not detected at DNA levels of 0.04 mg/filter. Routinely, CB3 DNA on filters was at least 4×10^4 in excess of added RNA. A level of 2.5×10^{-2} mg of PAT2 DNA was used per filter so that the percentage of counts hybridizable to both DNAs in permissive infection RNA preparations was approximately equal (see below). A retention of more than 95% of the denatured DNA on filters was confirmed by using the procedure of Brown and Weber (5).

(ii) Hybridization. DNA filters were incubated in scintillation vials (previously treated with Siliclad) for 22 to 23 h at 60 C in the presence of RNA. In the vial was the test RNA and enough phenol saturated with 2× SSC to give a final volume of 1.0 ml. The RNA for direct hybridization was ^{14}C-labeled. For competition experiments a mixture of ^{14}C-labeled and unlabeled RNA was used. After incubation, the filters were washed with 20 ml of 2× SSC, treated with RNase (2 ml SSC containing boiled RNase [0.02 mg/ml]) for 1 h at 25 C, and then washed again and dried before being counted (30).

(iii) Controls. Hybridization experiments included control filters which were processed as experimental filters through prewashing, curing, hybridization, washing, and counting. The controls included two filters lacking DNA, two filters with DNA for each type of DNA used which were not reacted with the test RNA, and two filters lacking DNA which had only test RNA added to them. In all cases these controls produced counts no greater than the samples from which filters were omitted.

DNA dependent RNA polymerase. (i) Buffers. Buffers described here and used in the isolation of RNAP were essentially those of Burgess (6). Double distilled water was used for all solutions. Stock solutions of the following were diluted prior to use: 1 M Tris-hydrochloride, pH 7.9; 1 M Tris-hydrochloride, pH 7.5; 0.1 M EDTA, pH 7.9; 1 M $MgCl_2$; 1 M KCl, and 0.1 M DTT. Buffer A was 10.0 mM Tris-hydrochloride, pH 7.9; 10.0 mM $MgCl_2$; 0.1 mM EDTA; 0.1 mM DTT, and 5% glycerol. Buffer C was $MgCl_2$-free buffer A. Buffer G was buffer A minus the pH 7.9 Tris-hydrochloride but with 50.0 mM Tris-hydrochloride, pH 7.5, and 200.0 mM KCl.

(ii) Enzyme isolation. RNAP was isolated from *E. coli* D10 and strains of *P. aeruginosa* by the technique of Burgess (6). This isolation procedure produces sonicated cell crude lysate (fraction 1), a ribosome-free high-speed supernatant fraction (Fraction 2), an $(NH_4)_2SO_4$ precipitated fractionation (fraction 3), a pooled DEAE-cellulose peak (fraction 4), a pooled phosphocellulose (PC) peak (fraction 5), and the flow-through material from PC (PCFM). Chromatography columns were prepared and equilibrated according to Burgess (6). When used, 10 to 30% glycerol gradients with ionic strengths of 0.04 and 1.0 (buffer A with KCl) were prepared by using a Beckman density gradient former and subsequently centrifuged in a Spinco SW 27 rotor. An LKB Ultrorac fraction collector coupled to an LKB Uvicord II with an E. H. Sargent SR recorder was used for the collection of material from glycerol gradients and chromatography columns.

(iii) Modified enzyme isolation. Our modification of the Burgess procedure consisted of lysing cells in KCl-free buffer G, eluting from DEAE with NaCl instead of KCl, and finally a stepwise elution from PC by using a procedure described by Whitely and Hemphill (37). For this stepwise elution from PC, we used fraction 4 (DEAE pool) desalted by dialysis against buffer C followed by the elution of RNAP from PC by 0.05 M NaCl increments of buffer C.

(iv) Enzyme assays. RNAP assays were performed using the conditions described by Burgess (6). Reaction tubes (11 by 72 mm) were Siliclad treated. For optimal results these assays did not require BSA, probably reflecting the use of Siliclad-coated tubes. All assays were performed in buffer B and were 40 mM Tris-hydrochloride, pH 7.9, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 0.15 M KCl, and 0.15 mM unlabeled UTP, GTP, and CTP. A 0.125 μCi amount of ^{14}C-ATP (462 mCi/mmol) was used per assay. Sufficient unlabeled ATP was added to each tube to bring

the total ATP concentration to 0.037 mM. Assays had between 0.0150 and 0.0175 mg of the PCFM. Components were added to the cold assay tubes in the following order: labeled and unlabeled triphosphates plus diluent, if any, then the proteins, and finally the DNA. The concentration of DNA was 0.035 mg/tube. Assays were incubated for 15 min at 37 C or 52 min at 20 C after which the tubes were chilled and the contents were processed on filter paper squares to determine total acid precipitable counts. All trichloroacetic acid contained 0.01 M sodium-pyrophosphate (6). As pointed out by both Chamberlin (8) and Burgess (7), specific activity determinations of RNAP obtained by using these methods are not satisfactory to measure the amount of active enzyme in a preparation. The rate-limiting step in transcription is seldom chain elongation (7, 8); therefore, the determination of nucleotide incorporation into acid insoluble material does not adequately reflect a true turnover number as it would with most enzymes. Consequently, assays to determine temperature effects on RNAP are reported in the Results section in terms of the radioactivity incorporated above controls in each assay. The above qualifications on specific activity and a rate limiting step will become more apparent in the studies presented below. Assays of RNAP used the fully permissive temperature, 37 C, as an estimate of 100% normal enzyme activity. Radioactivity incorporated at 20 C for each template was compared to incorporation at 37 C by using an identical template to indicate the influence of temperature on the efficiency of transcription. The variability between experiments for the enzyme efficiencies at 20 C was ± 2% of the reported efficiency. The variability between replicate assays did not exceed 3%. All assays were performed on at least three occasions and incorporation of label was linear with time, protein concentration, and dependent on the presence of all four nucleoside-triphosphates. Controls for the enzyme assays (which were subtracted from appropriate experimental filter papers) included: a reaction tube lacking template for each protein tested, a tube with template but no RNAP for each DNA species, and a blank filter processed with the experimental filters. Radioactivity incorporated in the presence of RNAP which was acid precipitable was both alkali labile and completely eliminated by treatment with boiled RNase before acid precipitation.

(v) Enzymatic impurities. Purified PAT2 RNAP and PCFM were tested for the presence of RNase and DNase activity. These fractions did not cause acid solubilization of radioactive RNA and DNA. The PC enzyme fractions were tested for 1 h at 37 C by incubating under assay conditions 0.015 mg of each protein with either ^{14}C-labeled purified PAT2 RNA (700 counts/min) or ^{14}C-CB3 DNA (1,300 counts/min) (30). By these criteria there was no RNase or DNase activity in the fractions.

RESULTS

Direct DNA-RNA hybridization. Direct hybridization was used to determine if CB3-specific transcripts are synthesized at cold temperatures. The results of one such experiment are shown in Table 1. Section A shows that uninfected PAT2 possesses no RNA capable of hybridizing to CB3 DNA but has RNA hybridizing to homologous host DNA, in this case 4.05%. This is an important control. Since there is no RNA in uninfected cells which is homologous to bacteriophage DNA, the RNA present in infected cells which does hybridize to phage DNA must be CB3 phage specific. Section B of Table 1 shows that the RNA labeled under permissive conditions in CB3 infected PAT2 during the first 5 min ("early") of the 37 C latent period has material homologous to both host and CB3 DNA (4.36 and 4.58%, respectively). The results of testing labeled RNA synthesized at 37 C by infected cells late in the latent period are also shown in section B. In the late preparation more RNA hybridizes to phage DNA than to host DNA (18.1 and 2.41%, respectively) in contrast to the early RNA preparations. Two other DNA-RNA hybridization experiments using RNA from cells infected with CB3 produced similar percentages of hybridization after 37 C infections.

The mRNA produced by infected cells under nonpermissive conditions presents a markedly different pattern of hybridization. In these studies, the first 17 min at 20 C is comparable to the early (5 min) interval at 37 C. The RNA produced during this early interval by CB3 infected PAT2 cells at 20 C is 2.33% hybridizable to PAT2 DNA but only 0.33% hybridizable to CB3 DNA. Results with other early RNA preparations from 20 C CB3 infected cells gave hybridization percentages of 0.14 and 0.13% with CB3 DNA. These direct annealing assays indicate that in a nonpermissive infection, PAT2 in-

TABLE 1. *Direct DNA-RNA hybridization of 20 and 37 C infected and uninfected PAT2*

DNA on filter	Temp/time of RNA sample	Counts per min added	Counts per min hybridized (%)
A. PAT2	(Control cells)	1,635	4.05
CB3	(Control cells)	1,635	0.00
B. PAT2	37 C, early[a]	1,089	4.36
CB3	37 C, early	1,089	4.58
PAT2	37 C, late[b]	507	2.41
CB3	37 C, late	507	18.10
PAT2	20 C, early	6,447	2.33
CB3	20 C, early	6,447	0.33

[a] Early RNA was isolated from PAT2 cells infected with CB3 and labeled for the first 5 min at 37 C or for 17 min at 20 C.

[b] Late RNA was isolated from PAT2 cells infected with CB3 and labeled for 25 to 30 min at 37 C.

fected with CB3 synthesizes very little phage-specific mRNA and normal levels of host RNA. In contrast, permissively infected cells synthesize both phage and host mRNA during the early fraction of the phage latent period.

Competition DNA-RNA hybridization. Competition hybridization was used to study the nature of the small amount of phage-specific RNA synthesized in PAT2 at 20 C. The ability of RNA isolated from PAO1C infected at 20 C to compete against early, ^{14}C-labeled RNA from both PAO1C and PAT2 at 37 C is shown in Fig. 1. This PAO1C early, 20 C RNA competes identically against both PAO1C and PAT2 37 C infected cell preparations. This establishes the similarity of the mRNA from both infected hosts at the permissive temperature to the RNA synthesized in the fully permissive PAO1C at 20 C. Figure 2 shows that CB3 infections at a permissive temperature produce at least two classes of phage specific RNA, early and late, which do not compete with one another (curve 1). In the critical combinations (curve 3), much more PAT2 20 C early RNA is needed to produce the same degree of competition as PAO1C 20 C early RNA (curve 2). The amount of PAT2 early RNA in line 3 is 6.6 times more than the amount necessary from PAO1C in line 2 to give similar (20%) competition. This value of 6.6 is called a competition ratio. The 6.6-fold increase in the amount of PAT2 RNA over PAO1C RNA means that at 20 C only 15.1% of the expected

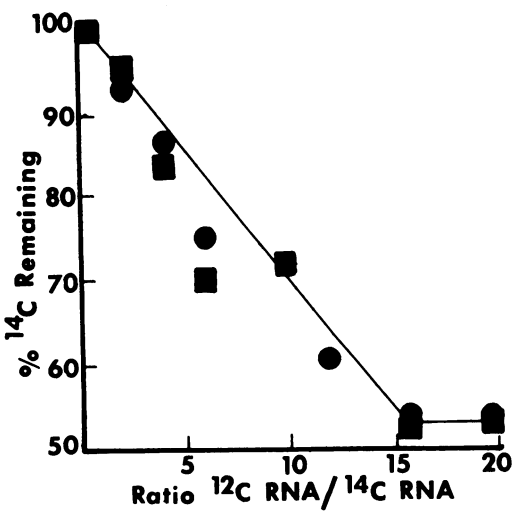

FIG. 1. *CB3 DNA-RNA competition hybridization between early RNA from 20 C PAO1C and 37 C PAO1 or PAT2. Symbols: ■, PAO1C 20 C, early RNA versus PAO1C 37 C, early ^{14}C-RNA; ●, PAO1C 20 C, early RNA versus PAT2 37 C, early ^{14}C-RNA. Experimental conditions are as described in Materials and Methods.*

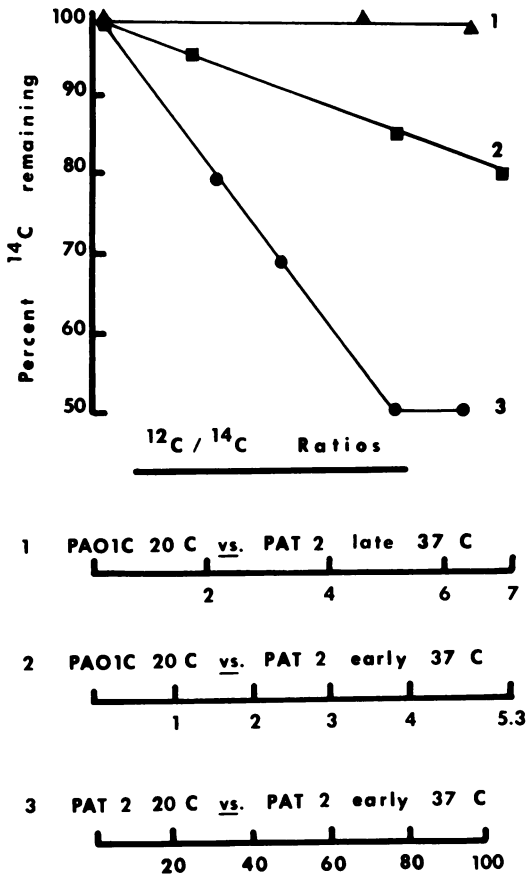

FIG. 2. *CB3 DNA-RNA competition hybridization between 20 C early RNA from infected PAT2 or PAO1C and 37 C early or late RNA. Symbols: 1. ▲, PAO1C 20 C, early RNA versus PAT2 37 C, late ^{14}C-RNA; 2. ■, PAO1C 20 C, early RNA versus PAT2 37 C, early ^{14}C-RNA; ●, PAT2 20 C, early RNA versus PAT2 37 C, early ^{14}C-RNA. Experimental conditions are as described in text.*

amount of CB3 RNA is produced in PAT2. Table 2 lists the results of five different experiments which produced an average competition ratio of 6.82 or an average RNA production in PAT2 at 20 C of 14.5%. Interestingly, this value is approximately the percent phage yielders obtained by single cell-burst experiments (20). In these experiments, between 13 and 15% of infected PAT2 cells were found to be phage yielders at 20 C. Therefore, these data raise the possibility that PAT2 nonyielders may not synthesize any phage mRNA at 20 C.

Early RNA prepared from PAO1C (Fig. 1) or from PAT2 (Fig. 2) 20 C infections is able to compete against only 50% of the early phage-specific RNA produced at 37 C in either host. This fact will be considered in the light of subsequent data and incorporated into a model

presented in the last section of this investigation.

CM was used to determine whether the synthesis of phage-specific mRNA in infected PAT2 cells at 20 C is dependent on protein synthesis. Figure 3 shows that early RNA synthesized by CB3 infected PAT2 cells at 20 C in the presence of CM competes equally effectively against RNA from 37 C CM-treated or untreated cells infected with CB3. In respect to the CM insensitivity, the synthesis of this CB3 RNA in PAT2 yielder cells is like that of the IE species of T4 mRNA transcribed under the direction of *E. coli* RNAP (4, 12, 19, 35). We assume that these IE host enzyme transcribed messages are probably absent in nonyielder cells of PAT2 at 20 C. Thus, we isolated RNAP from PAT2 to determine whether the restriction of phage CB3 is due to a cold sensitive property of this enzyme.

TABLE 2. *Summary of CB3-specific competition hybridization*

Expt[a]	Competition ratio	Percentage
1	6.16	16.2
2	6.60	15.1
3	8.96	11.3
4	7.34	13.6
5	5.85	17.1
	Average 6.82	14.5

[a] Each experiment used a different RNA preparation from PAT2 infected with CB3 at 20 C to compete against 37 C infected PAT2. The competition ratio is obtained from comparing the amounts of 20 C, PAT2 early RNA necessary to produce equivalent competition to PAO1C 20 C early RNA.

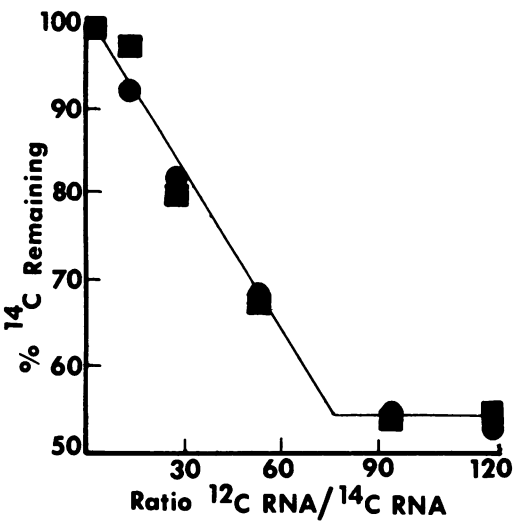

FIG. 3. *DNA-RNA hybridization competition between phage-specific RNA synthesized at 20 C in the presence of CM and CM-treated or untreated 37 C infected PAT2. Symbols: ■, early PAT2 20 C RNA synthesized in the presence of CM versus early ^{14}C-RNA synthesized in the presence of CM; ●, early PAT2 20 C RNA synthesized in the presence of CM versus early ^{14}C-PAT2 37 C RNA synthesized in the absence of CM. Experimental conditions are as described in Materials and Methods.*

RNA polymerase, notes on purification. The Burgess procedure for the isolation of the enzyme RNAP from *E. coli* D10 results in PC fractions that behave as predicted. The PC binding protein lacks sigma factor by SLS polyacrylamide gels (not shown) and hence is called the core enzyme. The enzymatic activity of *E. coli* D10 core is significantly stimulated by the PCFM fraction (presumed to contain sigma). *E. coli* D10 sigma stimulates core activity on PAT2 and CB3 DNA templates by 2.4- and 3.7-fold, respectively (Table 3). The sigma factor of RNAP is a positive control element which produces a specific asymmetric initiation of transcription (35).

Several unusual features of RNAP are evident with the strains of *P. aeruginosa* used in this investigation. PC chromatography does not separate the holoenzyme of our strains of *Pseudomonas* into core and sigma fractions as determined by SDS polyacrylamide gel electrophoresis (not shown). Whitely and Hemphill (37) have however, observed a core protein separation of *P. aeruginosa* RNAP much like that observed for *E. coli* D10. The subunit composition for *P. aeruginosa* strains used by us is otherwise identical to *E. coli* D10. PC chromatography of *P. putida* produces both core and sigma proteins (14). However, the isolation procedure was unlike that used here. The RNAP of our strains behaves as does *E. coli* D10 through all phases of purification prior to PC chromatography including the change in sedimentation behavior known to occur in low- (0.04) and high- (1.0) ionic strength 10 to 30% glycerol gradients (2, 6). After chromatography on PC, as mentioned above, holoenzyme is retained on the column; however, a heat-labile factor is found in the PCFM which when added to either homologous or heterologous *Pseudomonas* holoenzyme inhibits enzyme activity between 50 and 97%. The inhibition varies with the DNA template and the assay temperature. The identity of this factor is under study and it may be an ATPase which is released from the DEAE RNAP pools (fraction 4) in an activated form by PC chromatography. An ATPase of molecular weight 68,000 has recently been found associated with the RNAP of *E. coli* B (23) and it is also found in the PCFM.

PAT2 and PAO1C RNA polymerase holoenzymes. The in vitro transcription by Bur-

gess-purified RNAP holoenzymes from PAT2 and PAO1C are shown in Table 4. The enzymes isolated from either the conditionally permissive, PAT2, or the fully permissive, PAO1C, hosts synthesize equivalent amounts of RNA on either CT or PAT2 DNA templates after 15 min at 37 C or after 52 min at 20 C. When CB3 DNA is used as a template the enzyme is only 8% as efficient at this temperature as it is at 37 C (387 counts/min versus 4,830 counts/min). Thus, it is apparent that PAT2 RNAP in vitro shows cold sensitivity at 20 C. The transcription of CB3 DNA by PAO1C RNAP is also, but to a lesser degree, impaired at 20 C since it synthesized only 49.6% of the RNA that it transcribes at 37 C. Nevertheless, PAO1C is fully permissive at 20 C (21).

The effect of low ionic strength assay buffer (M buffer) on RNAP enzyme activity was tested. M buffer is similar to the assay buffer used in studies of eukaryotic RNAP (9) and to the buffer used by Johnson et al. (14) with *P. putida* RNAP. M buffer has the same concentration of DNA, enzyme, and triphosphates as the B assay but is 20 mM Tris-acetate (pH 7.9), 4 mM magnesium-acetate, 1 mM $MnCl_2$, and 60 mM ammonium-acetate. When PAT2 PC-purified holoenzyme is simultaneously assayed in both buffers, the specific activity of the enzyme in M buffer increases 3.2- to 125-fold (Table 5) over the activity of the same enzyme in B buffer (Table 4). In addition, the cold sensitivity of PAT2 RNAP toward CB3 DNA disappears. Interestingly, the greatest increase in activity occurs with CB3 DNA at 20 C where a 125-fold stimulation of enzyme synthesis is present (387 counts/min versus 48,500 counts/min). This dramatic influence at 20 C by the low ionic strength M buffer on CB3 DNA-PAT2 RNAP indicates that as yet unknown interactions occur between this template and PAT2 host enzyme. Assays of PAO1C holoenzyme (Table 5) are similar to those of PAT2 enzyme but the stimulation of specific activity is far lower for CB3 DNA at both temperatures. The milder effects of M buffer on PAO1C indicate intrinsic differences between the enzymes from the two hosts and suggest that the reversal of PAT2 cold sensitivity by M buffer results from a buffer effect on the cold-sensitive RNAP site.

TABLE 3. *Assay of E. coli D10 DNA polymerase using PAT2 and CB3 DNA*[a]

Phosphocellulose fraction	Counts/min with DNA	
	PAT2	CB3
Core	390	260
Sigma (PCFM)	124	86
Core and sigma	1,092	1,061

[a] Assay conditions as described in Materials and Methods with 15 min incubation at 37 C.

TABLE 4. *Effect of temperature on the RNA polymerases of PAT2 and PAO1C*[a]

Enzyme source	Assay temp (C)	Counts/min with DNA		
		CT	PAT2	CB3
PAT2	37	6,560	706	4,830
	20	6,979	681	387
PAO1C	37	3,567	402	1,860
	20	3,581	435	921

[a] Experimental conditions as described in Materials and Methods with assays of Burgess purified holoenzyme in B buffer for 15 min at 37 C and for 52 min at 20 C.

In view of the dramatic alteration in specificity using this low ionic strength buffer ($\Gamma/2$ = 0.035) over the B buffer ($\Gamma/2$ = 0.235), enzyme was isolated from PAO1C and PAT2 by using a modification of the Burgess enzyme extraction. When PAO1C RNAP is extracted in KCl-free buffers and eluted from DEAE and PC with NaCl, the procedure results in the recovery of PCFM fractions that inhibit the PC binding, sigma containing holoenzyme (data not shown). Table 6 gives the levels of transcription in M buffer of PAO1C RNAP purified by this modified Burgess method. The PAO1C enzyme produced by this procedure is more active at 20 C with all DNA templates.

Analogously, isolation of PAT2 RNAP by the modified Burgess technique also results in changed enzyme properties. With assay in the M buffer, this procedure produces enzyme fractions from PC that behave like those of *E. coli* D10 (see Table 3). PAT2 core enzyme has little activity alone (Table 7). Sigma (PCFM) has high activity even after having been chromatographed on PC three times in the hope of eliminating this background. However, when PAT2 core and PCFM (sigma) are both included in an assay at 37 C, there is a marked stimulation by sigma occurring with all three template DNAs. The magnitude of the stimulation at 37 C is similar to that seen in the *E. coli* D10 PC fractions. Moreover, this stimulation is cold sensitive with CB3 DNA at 20 C. Although the reconstituted holoenzyme is not as efficient at 20 C as at 37 C with either CT or PAT2 DNA, the greatest loss of stimulation occurs with CB3 DNA. PAT2 sigma does not stimulate PAT2 core enzyme with CB3 DNA at the low temperature in contrast to its behavior with CT and

TABLE 5. *Effect of M buffer on PAT2 and PAO1C RNA polymerase*

Enzyme source	Assay temp (C)	Counts/min with DNA and (stimulation by M buffer)[a]		
		CT	PAT2	CB3
PAT2	37	20,000 (3.2)	5,500 (7.8)	38,000 (7.9)
	20	33,600 (4.1)	4,630 (6.8)	48,500 (125)
PAO1C	37	3,020 (0.84)	4,570 (11.3)	4,390 (2.4)
	20	10,130 (2.8)	6,433 (14.7)	5,852 (6.4)

[a] Experimental conditions as described in the text and in Table 4. The stimulation of enzyme activity was computed by dividing the counts per minute incorporated by these same PC holoenzymes in a duplicate B buffer assay (Table 4) into the counts per minute incorporated by them in an M buffer assay.

TABLE 6. *Modified Burgess extracted PAO1C RNA polymerase assayed in M buffer at 37 and 20 C*[a]

Assay temp (C)	Counts/min with DNA		
	CT	PAT2	CB3
37	5,080	7,020	15,810
20	7,500	6,740	21,420

[a] Experimental conditions are as described in Materials and Methods.

TABLE 7. *Sigma (PCFM) stimulation of modified Burgess-extracted PAT2 RNA polymerase assayed at 37 C and 20 C in M buffer*[a]

Assay temp (C)	Phosphocellulose fraction	Counts/min with DNA		
		CT	PAT2	CB3
37	Core	144	109	124
	Sigma	937	670	1,070
	Core + sigma	2,857	3,014	3,948
	Sigma stimulation	2.64	3.75	3.28
20	Core	337	227	411
	Sigma	1,025	1,652	1,328
	Core + sigma	1,700	2,620	1,745
	Sigma stimulation	1.25	1.40	0.97

[a] Experimental conditions are as described in Table 6.

PAT2 DNAs. In contrast to the case with PAT2, both modified Burgess extraction of PAO1C and the usual Burgess extraction of PAO1C produce enzymes behaving similarly.

Rifampin and cold sensitivity. The B and M assay buffers differ in their ionic strengths. Therefore, in the M buffer, PAT2 RNAP may be aggregated, whereas in the B buffer it may be in a monomeric state (2) and the changes in specific activity may reflect these states. As well, changes in the ionic strength of the magnitude used in these assays are known to dissociate the initiation complexes between RNAP and DNA promoter sites (29). Furthermore, Bautz and Bautz have shown (1) that the formation of the initiation complex can also be altered by an ionic strength of 0.1 or higher. The beta subunit of the enzyme RNAP is known to be involved in the initiation process of RNA transcription (15, 24, 39) and in *E. coli*, a mutation affecting this subunit confers resistance to rifampin (13). Accordingly, we isolated mutants of PAT2 specifically associated with RNAP initiation by selecting rifampin resistant mutants and determining their effect on CB3 infection at 37 C and 20 C.

Rifampin-resistant mutants. Of 312 rifampin-resistant mutants of PAT2 studied, 44 were found permissive for CB3 at 37 C and 20 C by cross-streak testing. Controls establishing the parentage of one of the permissive PAT2 mutants, Rif 4, used both nutritional markers and phage sensitivity patterns (Table 8). At 37 and 20 C, PAO1C has an efficiency of plating CB3 of 1.0 (21). Rif 4 has an efficiency of plating of 0.91.

Rif 4 RNA polymerase. The Burgess isolation procedure produces Rif 4 holoenzyme eluting from PC (data not shown) and also shows the inhibition of its enzyme activity by its PCFM as seen with other *Pseudomonas* RNAPs isolated in this manner. The in vitro rifampin resistance of Rif 4 is shown in Table 9. Data in this table also shows that Rif 4 RNAP is not cold sensitive with CB3 DNA in vitro at 20 C. Rif 4 is much more active with all templates at 20 C than it is at 37 C (Table 10). The Rif 4 enzyme in B buffer behaves superficially as did both wild-type enzymes when they were assayed in M buffer. The identical enzyme isolation procedures and in vitro assay conditions show that the enzymes of PAO1C, PAT2, and Rif 4 react toward CB3 DNA as do intact cells to CB3 infection. This observation suggests that cold-promoted restriction of CB3 by PAT2 is related to the ability of PAT2 RNAP to form an initiation complex for the synthesis of IE phage mRNA.

TABLE 8. *Characterization of Rif 4*[a]

Test	*Pseudomonas* strain		
	PAO1C	Rif 4	PAT2
Isoleucine-valine auxotroph	−	+	+
CB3-sensitive at 37 C	+	+	+
CB3-sensitive at 20 C	+	+	−
PX 4-sensitive at 37 C	−	−	−
PX 4-sensitive at 20 C	−	+	+
LP-sensitive at 37 C	+	−	−

[a] Phages used to characterize these strains are described in Materials and Methods.

TABLE 9. *Effects of rifampin in vitro on PAT2 and Rif 4 RNA polymerase holoenzymes assayed on CB3 DNA at 37 and 20 C*

Assay conditions[a]	Counts/min with enzyme and temp			
	PAT2		Rif 4	
	37 C	20 C	37 C	20 C
Control	606	48	811	1,197
Rifampin	56	0	746	1,642
Inhibition (%)	92	100	9.4	none

[a] Enzymes were assayed in B buffer for 15 min at 37 C or for 52 min at 20 C. Rifampin was used at a final concentration of 0.0125 mg per ml.

TABLE 10. *Rif 4 Burgess extracted RNA polymerase holoenzyme assayed in B buffer at 37 and 20 C*[a]

Assay temp (C)	Counts/min with DNA		
	CT	PAT2	CB3
37	2,325	609	811
20	4,147	1,329	1,897

[a] Experimental conditions are as described in Table 4.

DISCUSSION

Phage CB3 and its hosts PAT2 and PAO1C are useful for studies on an unusual interaction between host RNAP and phage transcription. Hybridization competition experiments point out a peculiarity of these hosts when PAO1C and PAT2 are compared with respect to their transcription of CB3 DNA at the fully permissive temperature. Namely, the small amount of RNA synthesized at 20 C in PAT2 or the normal amounts in PAO1C at 20 C only compete against approximately 50% of the CB3 specific RNA transcribed at 37 C (Fig. 1 and 2). CM treatment during infection (Fig. 3) also results in the production of IE phage transcripts from 20 and 37 C infections giving similar competition percentages. This could be the consequence of transcription through the normal IE RNA termination signal, thus transcribing RNA at 37 C which cannot be competed by 20 C IE mRNA. Therefore, if the absence of termination at 37 C is responsible for the ineffectiveness of 20 C phage-specific RNA to compete with 37 C RNA, then this would be the first transcription termination system affected by temperature. This possibility will be excluded below.

An alternative view of the hybridization data may be formed by considering the influence by temperature on transcription initiation at the DNA promoter site of CB3 DNA. If we assume that at 37 C both PAO1C and PAT2 initiate and transcribe IE phage-specific DNA sequences, and furthermore that they initiate and transcribe the complementary strand of the phage genome, this would account for the observed hybridization competition and it would also explain the in vitro data showing that, in PAO1C at 20 C, the fully permissive RNAP holoenzyme synthesizes 49.6% of its 37 C levels of RNA (Table 3). An excess of CB3 RNA transcription at 37 C is implicit in our model, whereas at 20 C each host enzyme is influenced differently by temperature with respect to its ability to transcribe phage DNA. Thus, at 20 C, PAT2 is unable to initiate transcription (nonyielders) and PAO1C transcribes only the cistrons needed for a successful infection.

This model is consistent with the studies of Matsukage (18) which show that *E. coli* RNAP in vitro can be influenced to synthesize an excess of bacteriophage T7 RNA. Optimum conditions allow *E. coli* RNAP to transcribe only IE mRNA from the r-strand of T7 DNA, whereas a change in the assay conditions result in transcripts of IE, r-strand specific mRNA, and RNA thought to be complementary to the l-strand of T7 DNA.

The inability of RNAP from these pseudomonads to be separated by standard purification on PC into core and sigma is fortunate since it allows a purification of the complete enzyme. However, once this separation is accomplished with PAT2 by the modified Burgess enzyme isolation, the ability to reconstitute the enzyme to full efficiency is lost. The PC-purified holoenzymes from PAT2 and PAO1C are not cold sensitive with either PAT2 or CT DNA and in fact synthesize equivalent amounts of RNA at the two temperatures (Table 4). Nevertheless, the attempted in vitro reconstitution of PAT2 core with PAT2 sigma obtained by the modified Burgess technique (Table 7) does not give the combination full efficiency at 20 C. The in vitro reconstitution of core and sigma at 20 C shows that PAT2 sigma is less able to stimulate transcription on CT and PAT2 DNAs than for

reconstitution at 37 C. However, the greatest cold sensitivity of reconstituted PAT2 holoenzyme is with CB3 DNA since no stimulation is evident (Table 7). PAO1C RNAP purified by the modified Burgess procedure, however, produces holoenzyme from PC, thus pointing up a basic enzymological difference between these molecules.

The apparent cold sensitivity of PAO1C holoenzyme in B buffer at 20 C (i.e., 49.6% of its 37 C level of RNA) is interesting in light of our model. When in vitro RNA transcription between PAT2 and PAO1C are compared at 20 C, a ratio of 6.2 is obtained. This indicates that PAT2 produces 16.1% of the expected levels of CB3 RNA at 20 C when it is compared to PAO1C. Measurement by homology indicates that RNA which is made at 20 C in vivo by PAT2 is 14.5% of the level transcribed by PAO1C at 20 C (Table 2). These values (16.1 and 14.5%) are very near the percentages of PAT2 yielder cells (13 to 14%) seen at 20 C (21). Thus, both in vivo (single cell-burst experiments and nucleic acid homology studies) and in vitro (RNA transcription) experiments establish a similar value for the ratio of restricting host permissiveness to permissive host synthesis of phage or its gene transcripts. These conclusions are also implicit in our model, since the two host enzymes are predicted to respond differently to CB3 at low temperature.

Low ionic strengths influence the initiation of transcription (1, 17, 24, 39), and our experiments show temperature and initiation differences between PAT2 and PAO1C at different ionic strengths. The greatest stimulation by M buffer (125-fold) occurs at 20 C with PAT2 RNAP by using CB3 DNA as a template. Since PAO1C allows substantial transcription in buffer B at 20 C, one would not expect as great a low ionic strength stimulation for this enzyme as is detected in PAT2. The stimulation of synthesis at 20 C of PAT2 RNAP by low salt concentration suggests that end product inhibition of transcription may not be responsible for the cold sensitivity of PAT2 RNAP in high salt. Low ionic strengths have been reported to favor end-product inhibition (3, 10, 28, 38). This is obviously not the case with PAT2. High salt favors termination of the transcript (26, 38) and reinitiation. Thus the assays in high salt showing cold sensitivity and the elimination of cold sensitivity in low salt suggest that a temperature dependent read-through is not likely as an alternative explanation for excess 37 C transcription in these hosts.

The beta subunit of *E. coli* RNAP is involved with the initiation of transcription and with resistance to the antibiotic rifampin (24, 39). The effect of low ionic environments on initiation and the role of rifampin in inhibiting initiation support the contention that cold sensitivity in PAT2 reflects the inhibition of initiation of transcription with CB3 DNA at 20 C. A further indication of the involvement of initiation is that rifampin resistance in Rif 4 confers permissiveness in vivo and in vitro (Tables 8 and 10). The beta subunit of RNAP is also the polypeptide to which sigma binds in *E. coli* (2). Thus, pleiotropic effects on RNAP through the action of the beta subunit must also be considered. For example, the ability of sigma to recognize the promoter site (35) may be influenced by temperature through some conformation change of the beta subunit and account for the observations in our system. In either case, however, it is apparent from our studies that the cold sensitivity of CB3 growth in PAT2 reflects a unique interaction between host RNAP and CB3 DNA resulting in an alteration of some aspect of transcription, presumably from the evidence, initiation. Rifampin can cause pleiotropic effects in *E. coli* (13) and it has been shown that rifampin resistance may result in the acquisition of cold sensitivity in *E. coli* (25) as well as the loss of cold sensitivity we have reported here for *Pseudomonas*.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant AI-07533 from the National Institute of Allergy and Infectious Diseases.

R. J. S. was a predoctoral fellow of the National Institutes of Health under fellowship 5-FO1-GM-38971 from the National Institute of General Medical Sciences. The F. G. Novy fellowship from the Department of Microbiology to R. J. S. is also gratefully acknowledged.

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