Transcription from Bacteriophage T7 and SP6 RNA Polymerase Promoters in the Presence of 3'-Deoxyribonucleoside 5'-Triphosphate Chain Terminators†

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ABSTRACT: RNA synthesis by T7 RNA polymerase or SP6 RNA polymerase is 100–1000 times more sensitive to the presence of the 3'-deoxyribonucleoside 5'-triphosphate chain terminators than is RNA synthesis by *Escherichia coli* RNA polymerase or Qβ replicase. These ribonucleotide analogues do not alter the specificity of each polymerase for its own promoters nor do they alter the site at which synthesis is initiated. Transcription by T7 RNA polymerase or SP6 RNA polymerase in the presence of relatively low concentrations of these chain terminators offers a useful route for determining the nucleotide sequence of any DNA segment that is inserted immediately downstream from a homologous bacteriophage promoter. This sequencing procedure was used to explore the effects that different dinucleotides have on the specificity of initiation at two different T7 RNA polymerase promoters.

Bacteriophage RNA polymerases are highly specific for their own promoters. For example, bacteriophage T7 RNA polymerase (EC 2.7.7.7.6) (Chamberlain et al., 1970) ignores almost all promoters, except those found in T7 DNA (Chamberlin & Ring, 1973). Similarly, SP6 RNA polymerase is highly specific for its own promoters (Butler & Chamberlin, 1982). Since recombinant DNA techniques can be used to introduce segments of DNA that contain these bacteriophage promoters into specific locations in other genomes, the homologous bacteriophage RNA polymerase can then be used for the exclusive transcription of the nucleotide sequence immediately downstream from the inserted promoter (McAllister et al., 1981; Melton et al., 1984). We conducted experiments to see whether chain-terminating nucleotide analogues can be used to determine the nucleotide sequence of the RNA synthesized from these bacteriophage promoters.

The addition of chain terminators to in vitro nucleic acid syntheses results in the production of a series of partially synthesized strands, whose different electrophoretic mobilities on a polyacrylamide gel identify the sequence of the nucleic acid. For example, the 2',3'-dideoxyribonucleoside 5'-triphosphates have been used with *Escherichia coli* DNA polymerase and single-stranded DNA templates (Sanger et al., 1977), the 3'-*O*-methylribonucleoside 5'-triphosphates have been used with *E. coli* RNA polymerase and double-stranded DNA templates (Axelrod et al., 1978), and the 3'-deoxyribonucleoside 5'-triphosphates have been used with Qβ replicase and single-stranded RNA templates (Kramer & Mills, 1978).

We constructed a number of plasmids containing bacteriophage promoters, including one that contained both a bacteriophage T7 promoter and an SP6 promoter directed toward each other from opposite ends of a polylinker. These plasmids were then used to study the effect that various chain-terminating ribonucleotides have on transcription by T7 and SP6 RNA polymerases. Sequencing gels were used to determine the effect of the chain terminators on the specificity and site of initiation of RNA synthesis. Sequencing gels were also used to determine the effect of different dinucleotides on the initiation of transcription. The results indicate that useful sequencing systems can be prepared that incorporate bacteriophage promoters and utilize the chain-terminating ribonucleotides in reactions containing bacteriophage RNA polymerases.

Experimental Procedures

Materials. 3'-Deoxyadenosine 5'-triphosphate (3'-dATP) was purchased from Miles Laboratories. 3'-Deoxycytidine, 3'-deoxyguanosine, and 3'-deoxyuridine were synthesized from methyl 3'-deoxy-β-D-furanoside (ICN) (Walton et al., 1965, 1966) and converted to the 3'-deoxyribonucleoside 5'-triphosphates (3'-dCTP, 3'-dGTP, and 3'-dUTP), as has been described for the 3'-*O*-methylribonucleoside 5'-triphosphate analogues (Axelrod et al., 1978). 3'-*O*-Methyladenosine 5'-triphosphate (3'-OmeATP) was obtained from P-L Biochemicals. [α-^{32}P]ATP, [^{3}H]ATP, and [α-^{32}P]-3'-dATP were purchased from Amersham. Restriction endonucleases, T4 DNA ligase, and calf intestine alkaline phosphatase were obtained from Boehringer Mannheim, and bacterial alkaline phosphatase was purchased from Bethesda Research Laboratories. SP6 RNA polymerase (Butler & Chamberlin, 1982) and pSP64 DNA were obtained from Promega Biotec. pT7-1 DNA, containing the class III T7 promoter, φ10, was purchased from United States Biochemical Corp. Qβ replicase was isolated from Qβ bacteriophage-infected *E. coli* Q13 by the procedure of Eoyang & August (1971) with the hydroxylapatite step omitted. *E. coli* RNA polymerase was purchased from Sigma Chemical Co. MDV-1 RNA was synthesized by Qβ replicase (Kramer et al., 1973). pBR322 DNA was a gift from Lisa Brunet of Columbia University. pAR864 DNA, containing the class III T7 promoter, φ10, and all strains of *E. coli* and bacteriophage T7 used in the preparation of T7 DNA and T7 RNA polymerase were generously provided by Dr. F. W. Studier of Brookhaven National Laboratory. The techniques for working with T7 bacteriophage are described in detail elsewhere (McDonell et al., 1977; Studier, 1969, 1975, 1979). Bacteriophage T7 DNA was isolated from *E. coli* B11' infected with T7 strain D111 (McDonell et al., 1977; Studier, 1969).

Isolation of T7 RNA Polymerase. A culture of *E. coli* B11' was lysed by infection with bacteriophage T7 *am* 29, 28 (gene

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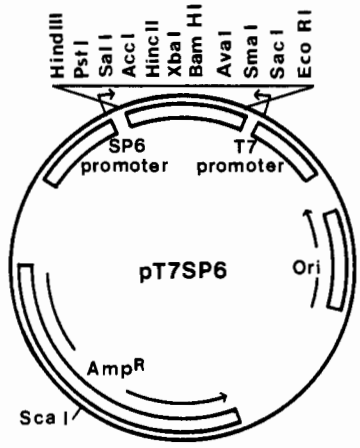

FIGURE 1: Structure of pT7SP6 DNA. The two bacteriophage promoters are directed toward the same polylinker, but from opposite directions.

3⁻, 5⁻) to obtain phage particles (McAllister & McCarron, 1977). *E. coli* BL15 [RNase I⁻, *rel*, (λ)] was then infected with these phage particles, and T7 RNA polymerase was isolated from the infected cells by the procedure of Chamberlin and his collegues (Chamberlin et al., 1970). Nuclease-free bovine serum albumin was added to the polymerase to a final concentration of 1 mg/mL, then diluted to half this concentration (v/v) with glycerol, and stored at −20 °C. This enzyme preparation is stable during storage for at least 1 year. Later work was carried out with T7 RNA polymerase that was isolated from *E. coli* BL15/pAR1219 (containing the T7 RNA polymerase gene) by the method of Davanaloo and her colleagues (Davanloo et al., 1984), with the Affi-Gel blue and GTP–agarose steps omitted.

Construction of a Recombinant Plasmid Containing the φ1.1A T7 Promoter. T7 DNA was digested with restriction endonuclease *Hpa*II, and the 138 base pair fragment containing the class II promoter φ1.1A was isolated by chromatography on a Sepharose 4B (Pharmacia) column and by polyacrylamide gel electrophoresis (Oakley & Coleman, 1977). This fragment was then inserted into the *Cla*I site of pBR322 DNA, and the resulting recombinant DNA was used to transform *E. coli* HB101. A tetracycline-sensitive clone was selected, and plasmid DNA was isolated from it (Holmes & Quigley, 1981) and assayed for its ability to serve as a template for T7 RNA polymerase. Clones containing active DNA were regrown in the presence of chloramphenicol, and plasmid DNA was isolated and then purified by chromatography on a Sephacryl 1000 (Pharmacia) column. The isolated plasmid was named pBR1.1A.

Construction of a Recombinant Plasmid Containing both a T7 Promoter and an SP6 Promoter (Refer to Figure 1). pSP64 DNA and pT7-1 DNA were cleaved with restriction endonucleases *Eco*RI and *Sca*I. Each plasmid was cleaved into two fragments, which were separated from each other by low melting point agarose gel electrophoresis and isolated from the agarose by phenol extraction and ethanol precipitation (Wieslander, 1979). The smaller fragment from pSP64 DNA, containing the SP6 promoter, a polylinker, and part of the ampicillin-resistance gene, was ligated to the larger fragment from pT7-1 DNA, containing the φ10 T7 promoter, an origin of replication, and the other part of the ampicillin-resistance gene. The resulting recombinant DNA was used to transform *E. coli* Jm105. Ampicillin-resistant clones were selected. DNA was isolated from 12 clones and analyzed by restriction mapping with endonucleases *Eco*RI and *Sca*I. Each of the isolated DNAs contained both promoters and served as a template for transcription by both SP6 RNA polymerase and T7 RNA polymerase. One clone, pT7SP6, was selected for further use.

Transcription with T7 RNA Polymerase. Reactions were performed according to the protocol of Chamberlin and his colleagues (Chamberlin et al., 1970). A total of 1–2 μg of DNA and 1–2 units (Chamberlin & Ring, 1973) of T7 RNA polymerase were incubated for 45–60 min at 37 °C in 50-μL reaction mixtures containing 50 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl) (pH 8.0), 12 mM $MgCl_2$, 5 mM dithiothreitol (freshly prepared), and 1 mg/mL bovine serum albumin or 4 mM spermidine, 100 μM ATP, 100 μM CTP, 100 μM GTP, 100 μM UTP, and 50–100 μCi of [α-^{32}P]ATP. For nucleotide sequence analysis, the reaction mixtures were supplemented with 50–100 μM 3′-dATP, 3′-dCTP, or 3′-dUTP, or with 25–50 μM 3′-dGTP, prior to the addition of the polymerase. Reactions were stopped by the addition of 10 μL of 200 μM ethylenediaminetetraacetic acid (EDTA) containing 1 mg/mL *E. coli* tRNA (Sigma). The transcripts were precipitated from the reaction mixtures by addition of 200 μL of ice-cold 4 M acetic acid, incubation at 0 °C for 10 min, and centrifugation in an Eppendorf centrifuge for 15 min at 4 °C. The pellets were washed with 500 μL of ice-cold 2 M acetic acid, spun 3 min, washed with 500 μL of 70% ethanol, spun 3 min, and dried in a vacuum.

RNA Syntheses with SP6 RNA Polymerase, E. coli RNA Polymerase, and Qβ Replicase. All reactions were carried out in a 50-μL volume. SP6 RNA polymerase reactions were carried out under conditions described by Butler & Chamberlin (1982). For sequence analysis, reactions containing 1 μg of pT7SP6 DNA, 2 units of SP6 RNA polymerase, 100 μM ATP, 100 μM CTP, 100 μM GTP, 100 μM UTP, 50 μM of one of the 3′-deoxyribonucleoside 5′-triphosphates, 40 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, 10 mM NaCl, 2 mM spermidine, and 10 mM dithiothreitol were incubated at 37 °C for 45 min. *E. coli* RNA polymerase reactions were carried out under conditions described by Stahl & Chamberlin (1977), and Qβ replicase reactions were carried out under conditions described previously (Kramer et al., 1973).

Removal of 5′-Terminal Phosphate Groups from Transcripts. When bacterial alkaline phosphatase was used, the transcripts were precipitated from the reaction mixture and then dissolved in 10 μL of 20 mM Tris-HCl (pH 8.0). A total of 100 units of bacterial alkaline phosphatase were added, and the RNA was incubated at 60 °C for 1 h. The transcripts were then reprecipitated. When calf intestine alkaline phosphatase was used, the reaction was stopped by the addition of EDTA to a final concentration of 10 mM. Two units of calf intestine alkaline phosphatase were then added per 50 μL of reaction mixture, and the RNA was incubated at 50 °C for 1 h. The transcripts were then precipitated.

Electrophoretic Analysis of Transcripts. The RNA was dissolved in 6 μL of 7 M urea containing 50 mM Tris–borate (pH 8.3), 100 μM EDTA, 1 mg/mL bromophenol blue, and 1 mg/mL xylene cyanol. This solution was heated for 2 min at 100 °C, chilled for 10 min at 0 °C, then applied to an 8% polyacrylamide gel [acrylamide:bis(acrylamide) ratio of 29:1], cast in 7 M urea, 50 mM Tris–borate (pH 8.3), and 100 μM EDTA, and run in 50 mM Tris–borate (pH 8.3) and 100 μM EDTA. The RNA was then analyzed by electrophoresis at 4000 V on a gel that was 0.4 mm thick and 90 cm long. Gels were thermostatically regulated at 65 °C.

RESULTS

Inhibition by Chain-Terminating Analogues. To study the inhibitory effect of nucleotide analogues on transcription with

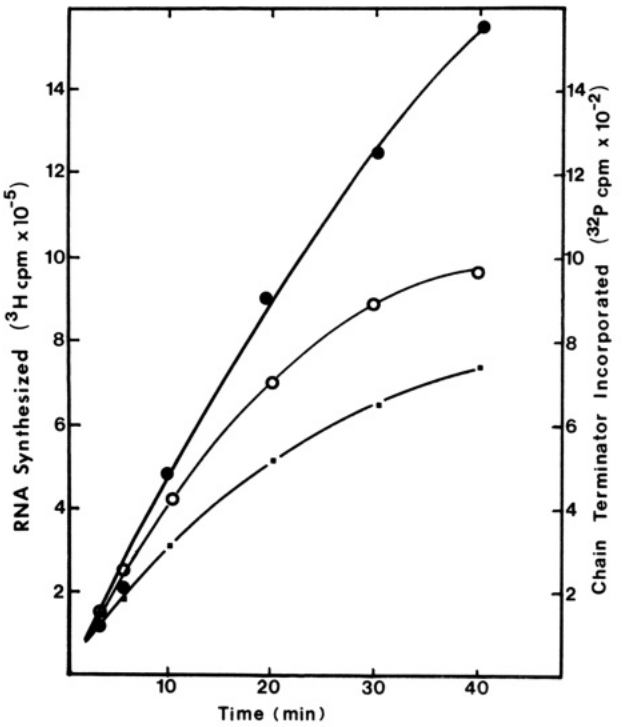

FIGURE 2: Effect of 3′-dATP on the synthesis of RNA by T7 RNA polymerase from a bacteriophage T7 DNA template. The synthesis of RNA in a control reaction containing 100 μM of each natural ribonucleoside 5′-triphosphate and [^{3}H]ATP as a label (●) was compared with the synthesis of RNA in a similar reaction containing both [^{3}H]ATP (■) and 40 nM [α-^{32}P]-3′-dATP (○). Aliquots were absorbed onto 25-mm Whatman 3MM filter paper disks and were then immersed in ice-cold 5% trichloroacetic acid containing 10 mM sodium pyrophosphate. The disks were washed 5 times with the same solution and once with ice-cold ethanol. They were then air-dried, and their radioactivity was determined in a scintillation counter. The results demonstrate that RNA synthesis is inhibited by the incorporation of 3′-deoxyribonucleoside analogues into the transcripts.

different RNA polymerases, the kinetics of the reactions were measured (an example is shown in Figure 2). The amount of radioactive precursor incorporated into the transcripts after 25 min in the presence of these analogues was compared with control reactions that did not contain the analogue. Table I shows the results of this comparison. These data indicate that T7 RNA polymerase and SP6 RNA polymerase are remarkably sensitive to the presence of the 3′-deoxyribonucleoside 5′-triphosphates. For example, only 80 nM 3′-dCTP in a reaction containing 100 μM CTP will inhibit T7 RNA polymerase activity by 50%. In general, RNA synthesis by T7 RNA polymerase or SP6 RNA polymerase is 100–1000 times more sensitive to the presence of the 3′-deoxyribonucleoside 5′-triphosphate chain terminators than is RNA synthesis by *E. coli* RNA polymerase or Qβ replicase.

Nucleotide Sequencing by Transcription. The high sensitivity of T7 RNA polymerase and SP6 RNA polymerase to the 3′-deoxyribonucleoside 5′-triphosphates suggests that DNA sequences can be determined by transcription in the presence of these chain terminators. To demonstrate the feasibility of this sequencing procedure, a plasmid, pT7SP6, was constructed which contained both an SP6 promoter and the class III T7 promoter, ϕ10, on opposite sides of the same polylinker (see Figure 1). In separate reactions containing the different 3′-deoxyribonucleoside analogues, both SP6 RNA polymerase and T7 RNA polymerase were used to transcribe the polylinker region downstream from their respective promoters. The resulting collections of terminated transcripts were analyzed by polyacrylamide gel electrophoresis (Figure 3). The homogeneity of the "sequence ladders" in each lane demonstrates that transcription with either RNA polymerase was initiated at a single nucleotide in the sequence. The nucleotide sequences that were determined with each RNA polymerase were, as expected, complements of each other. This plasmid will be useful as a general vector for the determination of the nucleotide sequence of a DNA segment inserted within its polylinker.

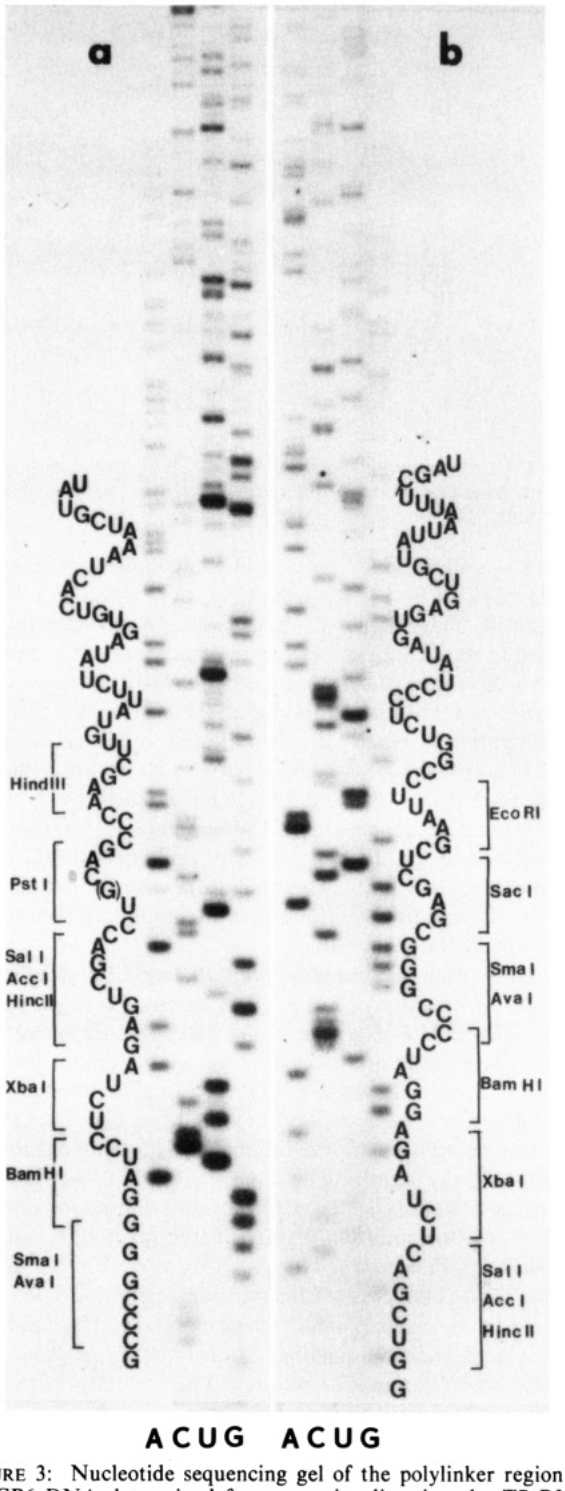

FIGURE 3: Nucleotide sequencing gel of the polylinker region of pT7SP6 DNA determined from opposite directions by T7 RNA polymerase (a) and SP6 RNA polymerase (b).

To see the effects of linearization of a plasmid template on transcription and termination, T7 RNA polymerase was used to determine the nucleotide sequence immediately downstream from the class II T7 promoter, ϕ1.1A, in plasmid pBR1.1A.

Table I: Concentration of Chain Terminator (μM) Required To Cause 50% Inhibition of RNA Synthesis in Reactions Containing 100 μM of Each Natural Ribonucleoside Triphosphate[a]

enzyme/ template	3′-dATP	3′-dCTP	3′-dGTP	3′-dUTP	3′-OMe-ATP
T7 RNA polymerase/ T7 DNA	0.050	0.080	0.090	0.025	10
SP6 RNA polymerase/ pSP64 DNA	0.180	0.330	0.190	0.150	20
E. coli RNA polymerase/ T7 DNA	50	90	100	40	120
Qβ replicase/ MDV-1 RNA	15	18	20	10	1000

[a] Increasing the concentration of each natural ribonucleoside triphosphate to 300 μM did not significantly alter the ratio of chain terminator to natural ribonucleotides that would cause 50% inhibition.

The results, shown in Figure 4, gave the expected nucleotide sequence (Sutcliffe, 1978; Oakley et al., 1979; Dunn & Studier, 1983, 1984) and demonstrated that the specificity of initiation is not affected by the linearization of the plasmid, though RNA polymerase activity was higher with supercoiled plasmid.

Relative Acceptability of the Chain Terminators by T7 RNA Polymerase. During product chain elongation in the presence of a chain-terminating analogue, there is a finite probability that an RNA chain will terminate at each position at which the natural ribonucleotide can be incorporated. Experimentally, this results in there being a smaller number of longer terminated strands than shorter terminated strands. For example, Figure 4c shows an electrophoretic separation of the products of a T7 RNA polymerase reaction carried out in the presence of 3′-dGTP. Since the sequence of each of the partially synthesized fragments was known, the number of moles of RNA in each band could be determined by measuring its radioactivity and dividing by the number of radioactive nucleotides in the fragment. A plot of the number of moles of RNA terminated at each site as a function of how many potential termination sites had been encountered by the polymerase is shown in Figure 5a. The number of strands terminated at each site decreased with increasing chain length. A semilogarithmic plot of the same data gave a straight line (Figure 5b), indicating that the number of moles of RNA terminated at each site form a decreasing geometric progression.

This relationship can be expressed by the exponential equation

$$T_n \sim (1 - F)^n$$

where T_n is the number of RNA strands terminated at the nth potential termination site, n is the number of potential termination sites encountered by the RNA polymerase, and F is the fraction of strands that terminate at each site. The coefficient of geometric progression, $1 - F$, represents the fraction of strands that are not terminated at each site. This equation is algebraically equivalent to

$$\log T_n \sim n \log (1 - F)$$

Therefore, a plot of $\log T_n$ vs. n gives a straight line with a slope equal to $\log (1 - F)$, from which F can be calculated.

When the concentration of the chain terminator is the same as the concentration of the natural ribonucleotide, as was the case for the experiment shown in Figure 5, then the fraction of strands that terminate at each site depends only on the acceptability of the analogue by the RNA polymerase compared to the acceptability of the natural nucleotide. The fraction of strands that terminate at each site is related to these acceptabilities by

$$F = A_{3'\text{-dNTP}}/(A_{\text{NTP}} + A_{3'\text{-dNTP}})$$

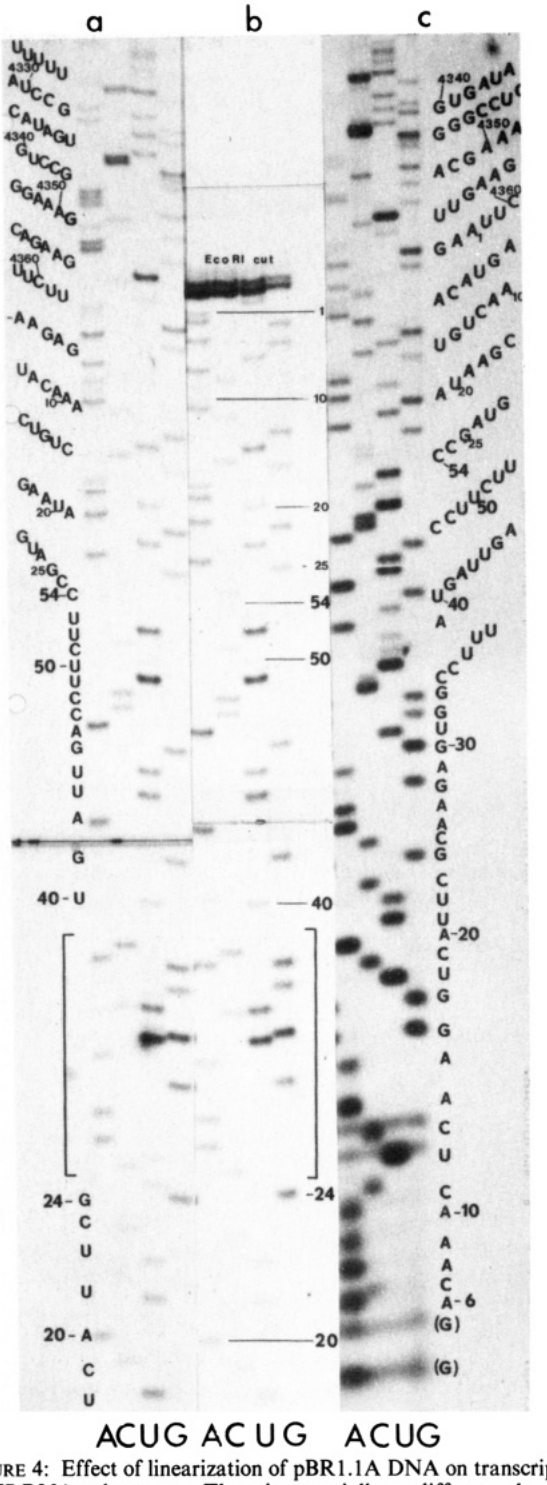

FIGURE 4: Effect of linearization of pBR1.1A DNA on transcription by T7 RNA polymerase. There is essentially no difference between the sequence ladder obtained from supercoiled template DNA (a) and the sequence ladder obtained from the same DNA after it was linearized by digestion with endonuclease *Eco*RI (b). A region of band compression (indicated by brackets) that occurred when the electrophoresis apparatus was not thermostatically regulated (running temperature between 40 and 50 °C) was eliminated when the terminated transcripts were separated on an electrophoresis apparatus that was thermostatically regulated at 65 °C (c). Transcription began with the T7 sequence downstream from the promoter (large numbers) and continued into the pBR322 sequence (small numbers).

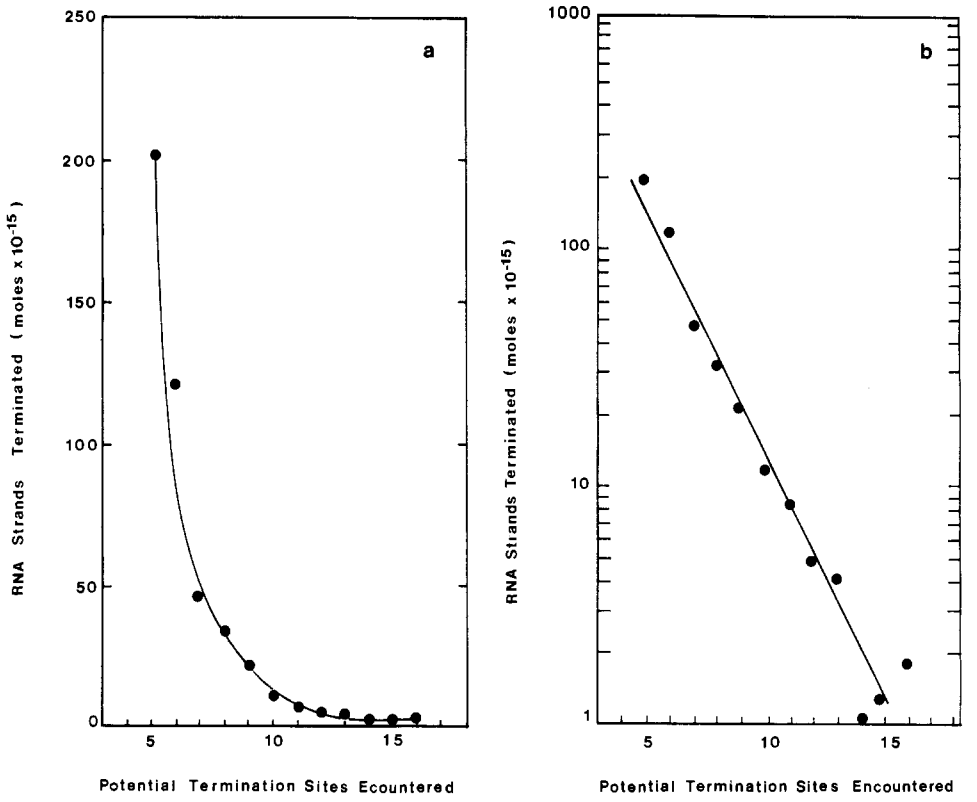

FIGURE 5: Termination of transcription by T7 RNA polymerase in the presence of 100 μM 3′-deoxyguanosine 5′-triphosphate and 100 μM each of each natural ribonucleotide. The number of RNA strands terminated at each termination site forms a decreasing geometric progression (a). When the results are replotted semilogarithmically, the data form a straight line with a slope equal to log $(1 - F)$, where F is the fraction of strands that terminate at each site (b).

Table II: Acceptability of Each 3′-Deoxyribonucleoside 5′-Triphosphate by T7 RNA Polymerase Compared with the Acceptability of Its Corresponding Natural Ribonucleotide[a]

3′-dATP	3′-dCTP	3′-dGTP	3′-dUTP
0.23	0.31	0.59	0.30

[a] The acceptability of the natural ribonucleoside 5′-triphosphate is assumed to be 1.

where $A_{3'\text{-dNTP}}$ is the acceptability of the analogue and A_{NTP} is the acceptability of the natural ribonucleotide. This equation is algebraically equivalent to

$$A_{3'\text{-dNTP}}/A_{\text{NTP}} = F/(1 - F)$$

Therefore, the relative acceptability of the analogue compared to the natural ribonucleotide can be determined once F has been calculated (from the slope of a linear regression on a plot of log T_n vs. n).

The experiment shown in Figure 4c provided data for each of the other 3′-deoxyribonucleoside 5′-triphosphates, and the results were plotted in the same manner as in Figure 5b. The relative acceptability values determined from each plot are shown in Table II. The results indicate that all four 3′-deoxyribonucleotides are accepted well by T7 RNA polymerase. 3′-dATP, 3′-dCTP, and 3′-dUTP have approximately 28% of the activity of their corresponding natural ribonucleotides, and 3′-dGTP is twice as acceptable, having 59% of the activity of GTP. In essence, T7 RNA polymerase barely discriminates against the 3′-deoxyribonucleotides, and this accounts for the great sensitivity of T7 RNA polymerase to these chain terminators.

Initiation of Transcription with Dinucleotides. Dinucleotides are frequently added to in vitro RNA polymerase reactions to serve as initiator sequences, yet little is known of their actual specificity. A comparison of the sequence ladders in gel autoradiographs of the transcripts produced when chain terminators are present can provide information about the effect that different dinucleotides have on the site where initiation occurs and on the homogeneity of initiation. For example, it is known that transcripts initiated under control of the class II T7 promoter ϕ1.1A in the absence of dinucleotides begin homogeneously with the sequence pppGpApGpGpGp... (Oakley et al., 1979). Figure 6 compares sequence ladders obtained by transcription under control of this promoter. When no dinucleotides were present in the reaction, each nucleotide in the sequence was represented by a single band. Similarly, a reaction carried out in the presence of the dinucleotide GpA gave single bands for each nucleotide. The electrophoretic mobility of each transcript initiated with GpA was less than the mobility of the corresponding transcript initiated with guanosine triphosphate, because the 5′-terminal triphosphate group provided additional charges. However, when the transcripts initiated with guanosine triphosphate were incubated with bacterial alkaline phosphatase to remove their 5′-terminal triphosphate groups and then mixed with the transcripts initiated with GpA (which have 5′-terminal hydroxyl groups) and analyzed electrophoretically, each nucleotide in the sequence was represented by only one band, indicating that transcription begins at the same position when GpA is used as an initiator sequence as it does when there are no dinucleotides present. On the other hand, when ApG was present in a reaction, the mobility of each transcript was decreased in comparison with the mobility of the corresponding transcript initiated with GpA. From the relative mobility of the bands, it can be inferred that transcripts initiated with ApG are one nucleotide longer than those initiated with GpA.

A more extensive survey of the effects of different dinucleotides on the specificity of initiation at the ϕ1.1A promoter is shown in Figure 7. Each reaction contained 3′-

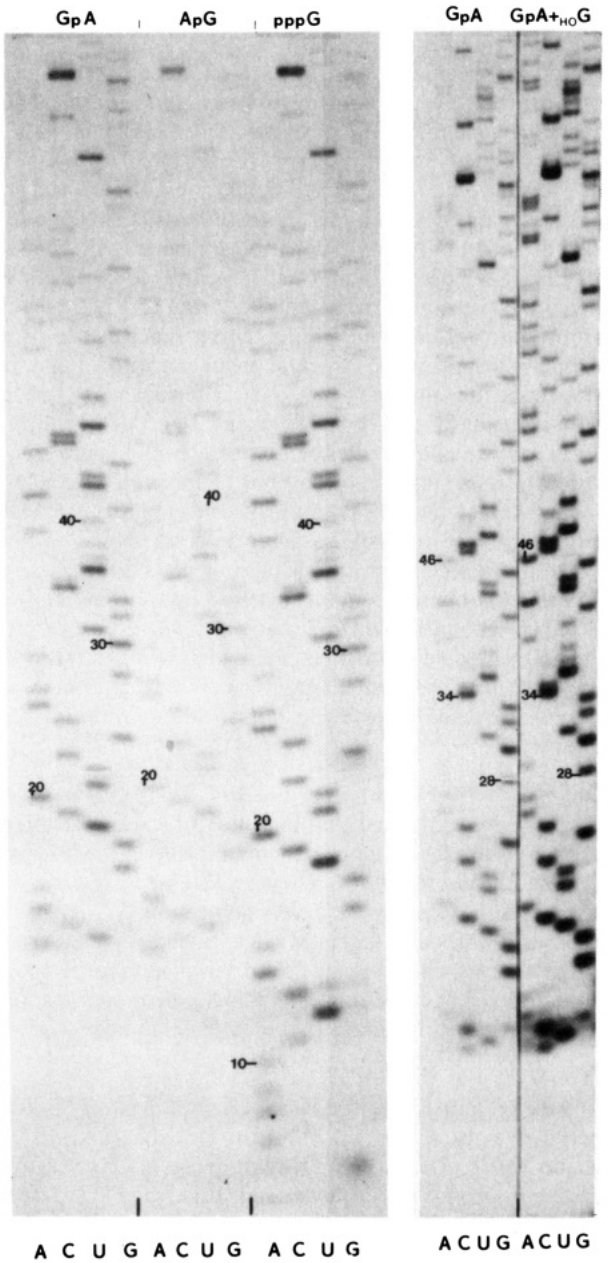

FIGURE 6: Electrophoretic separations of terminated transcripts obtained from T7 RNA polymerase reactions carried out in the presence of different dinucleotides. All transcripts were synthesized from pBR1.1A DNA, which contains the class II T7 promoter ϕ1.1A. Each reaction contained 200 μM dinucleotide and 100 μM each of ATP, CTP, GTP, and UTP. Each reaction also contained either 100 μM 3′-dATP, 3′-dCTP, 3′-dUTP, or 50 μM 3′-dGTP (indicated by the letter at the bottom of each lane). Reactions were initiated with GpA, ApG, and without any added dinucleotide (pppG). Numbers identify bands that correspond to nucleotides in the natural transcript (i.e., 20 is the band that results from the adenosine that normally occurs at position +20). A mixture of transcripts initiated with GpA and transcripts that were synthesized in the absence of dinucleotides and subsequently incubated with bacterial alkaline phosphatase to remove their 5′-triphosphate groups was analyzed (GpA + $_{HO}$G) in parallel with a control that did not contain phosphatase-treated RNA (GpA).

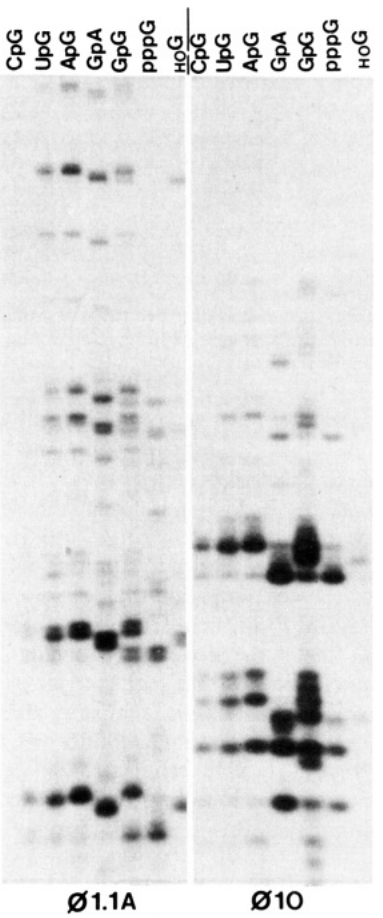

FIGURE 7: Electrophoretic separation of transcripts that indicate the effects of various dinucleotides on the initiation of transcription from both the class II T7 promoter, ϕ1.1A, and the class III T7 promoter, ϕ10. The reactions were carried out as described in the legend to Figure 6. pBR1.1A DNA was used as the template for transcription under control of the ϕ1.1A promoter, and pAR864 DNA was used as the template for transcription under control of the ϕ10 promoter. 50 μM 3′-dCTP was present in each reaction as a chain terminator. Control reactions were carried out in the absence of dinucleotides (pppG), and another set of controls was prepared by carrying out reactions in the absence of dinucleotides and then treating the transcripts with calf intestine alkaline phosphatase to remove their 5′-triphosphate groups ($_{HO}$G).

deoxycytidine 5′-triphosphate as the chain terminator. The results demonstrate that transcripts initiated with CpG or UpG, like those initiated with ApG, are one nucleotide longer than transcripts initiated with GpA. In addition, transcripts initiated with GpG gave double bands. One member of each doublet comigrated with the corresponding transcript obtained from a reaction intiated with ApG, CpG, or UpG, and the other member of each doublet comigrated with the corresponding transcript obtained from a reaction in which no dinucleotides were present. This result indicates that GpG is a relatively weak initiator sequence and the transcripts obtained in its presence consist of a mixture of strands initiated with GpG and strands initiated with guanosine triphosphate.

A similar survey was made of the effects of different dinucleotides on the specificity of initiation under the control of the class III T7 promoter ϕ10. These results are also shown in Figure 7. It is known that transcripts initiated under the control of this promoter, in the absence of dinucleotides, begin homogeneously with the sequence pppGpGpGp... (Rosa, 1979; Dunn & Studier, 1983, 1984). The results indicate that GpA did not serve as an initiator sequence with this promoter, since the transcripts comigrated with transcripts obtained from a reaction in which no dinucleotides were present. However, ApG, CpG, and UpG did serve as initiators, extending the length of the transcripts by one nucleotide, as shown by their decreased mobility in comparison with the mobility of dephosphorylated transcripts obtained from a reaction in which no dinuceotides were present. Transcripts initiated with GpG gave triple bands. An examination of the autoradiograph indicates that one member of each triplet resulted from initiation with GpG at the normal (+1) position, the second

Table III: Sequence at the 5′ End of Transcripts Synthesized in the Presence of Different Dinucleotides

dinucleotide	ϕ1.1A promoter	ϕ10 promoter
none	pppGAGGG...	pppGGG...
GpA[a]	HOGAGGG...	pppGGG...
ApG	HOAGAGGG...	HOAGGG...
CpG	HOCGAGGG...	HOCGGG...
UpG	HOUGAGGG...	HOUGGG...
GpG	HOGGAGGG... pppGAGGG...	HOGGGG... pppGGG... HOGGG...

[a] GpA did not serve as an initiator with the ϕ10 promoter. Similarly, CpA, ApC, and ApA were unable to serve as initiators with either the ϕ1.1A promoter or the ϕ10 promoter.

member of each triplet resulted from initiation with GpG at the abnormal (−1) position, and the third member of each triplet resulted from initiation with guanosine triphosphate at the normal position. Thus, GpG is a relatively weak initiator with the ϕ10 promoter, as well as with the ϕ1.1A promoter, and is unable to prevent normal initiation with guanosine triphosphate at the concentration that was used.

Table III lists the 5′-terminal sequences of the transcripts that were synthesized in the presence of different dinucleotides. In general, dinucleotides with a sequence identical with the first two nucleotides of the natural template initiate synthesis at the normal (+1) position, dinucleotides with a sequence unrelated to either of the first two nucleotides of the natural transcript do not serve as initiators, and dinucleotides of the form NpG initiate synthesis at the abnormal (−1) position.

Discussion

The results indicate that transcription by either T7 RNA polymerase or SP6 RNA polymerase is inhibited by relatively low concentrations of the 3′-deoxyribonucleoside 5′-triphosphate chain terminators. Apparently, these polymerases barely discriminate between the analogues and their corresponding natural ribonucleotides. The results also indicate that the chain-terminating ribonucleotides do not alter the specificity of each polymerase for its own promoters nor do they alter the site at which transcription is initiated. Consequently, these bacteriophage RNA polymerases, in conjunction with the chain-terminating ribonucleotide analogues, can be used to determine the nucleotide sequence of any DNA segment that is inserted immediately downstream from a homologous bacteriophage promoter.

The results also suggest some useful modifications that can be made to the sequencing procedure. If α-^{32}P-labeled chain terminators are present in the reaction, then only terminated strands will be radioactively labeled, eliminating the confusion that can be caused by the presence of aborted or paused transcripts in the reaction products. Labeled chain terminators are convenient because much lower concentrations of the analogue are required to terminate synthesis with these polymerases. Another modification to the basic procedure would eliminate band compression regions in sequencing gels that are due to the persistence of RNA secondary structures during electrophoresis. Often, a gel is run at higher temperatures to denature these structures (Sanger & Coulson, 1978), as in our experiments. However, it should be possible for the guanosine triphosphate in a bacteriophage RNA polymerase reaction to be replaced by inosine triphosphate, so that the transcripts will contain inosine in place of guanosine and be unable to form strong secondary structures (Mills & Kramer, 1979). Often, inosine triphosphate will not replace guanosine triphosphate for the initiation of nucleic acid syntheses. However, the results indicate that the inclusion of an appropriate dinucleotide initiator in a reaction would suppress natural initiation by guanosine triphosphate.

The strict specificity of these bacteriophage RNA polymerases for their own promoters suggests a number of useful sequencing strategies. For example, a heterologous DNA segment can be cloned into the polylinker of pT7SP6 DNA. Since this plasmid has a T7 promoter on one side of the polylinker and an SP6 promoter on the other side of the polylinker, plasmid DNA from a recombinant clone could be used as template for determining the nucleotide sequence of the inserted DNA segment from opposite ends. T7 RNA polymerase would be used to determine the sequence of one complementary strand, and SP6 RNA polymerase would be used to determine the sequence of the other complementary strand. The orientation of the cloned segment would be irrelevant.

Even very small quantities of comparatively impure DNA isolated from a single clone by the "mini-prep" procedure (Holmes & Quigley, 1981) can be used as templates for nucleotide sequence analysis, because the bacteriophage RNA polymerases ignore heterologous promoters. Furthermore, if a T7 or SP6 promoter could be inserted by transfection at a selected position in the genome of a cell, then the transformed cellular DNA could be isolated and used as template with the homologous bacteriophage RNA polymerase to determine the nucleotide sequence immediately downstream from the inserted promoter.

Added in Proof

We recently confirmed that inosine 5′-triphosphate effectively substitutes for guanosine 5′-triphosphate in the presence of a dinucleotide initiator. We also obtained excellent sequencing results using [α-^{32}P]-3′-dATP. Dr. W. T. McAllister and his colleagues at Rutgers Medical School have observed that the 3′-deoxyribonucleoside 5′-triphosphates are effective terminators for bacteriophage T3 RNA polymerase, as well as for T7 RNA polymerase (personal communication).

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Registry No. 3′-dATP, 73-04-1; 3′-dCTP, 69383-05-7; 3′-dGTP, 55968-37-1; 3′-dUTP, 69199-40-2; 3′-OMeATP, 30993-48-7; GpA, 6554-00-3; ApG, 3352-23-6; CpG, 2382-65-2; UpG, 3474-04-2; GpG, 3353-33-1; RNA polymerase, 9014-24-8.

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Cyclobutane Pyrimidine Dimers and (6-4) Photoproducts Block Polymerization by DNA Polymerase I†

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ABSTRACT: Bipyrimidine cyclobutane dimers and 6-4′-(pyrimidin-2′-one)-pyrimidine photoproducts are the major adducts formed in DNA following exposure to ultraviolet light. The relationship between the type and frequency of UV-induced DNA damage and the effects of such damage on DNA replication were investigated. UV-irradiated M13 phage DNA was employed in polymerization reactions with the Kenow fragment of *Escherichia coli* DNA polymerase I. The locations and frequencies of polymerase termination events occurring within a defined sequence of M13 DNA were compared with measurements of the locations and frequencies of UV-induced DNA damage of the same DNA sequence by using UV-specific enzymatic and chemical methods. The results indicate that both cyclobutane dimers and (6-4) photoproducts quantitatively block polymerization by DNA polymerase I.

Damage to DNA may have lethal, mutagenic, and carcinogenic consequences (Hanawalt et al., 1978; Friedberg & Bridges, 1983). One event central to such biological effects is DNA replication on a damaged template. Failure to replicate past a site of DNA damage will lead to cell death. Advances in nucleic acid technology make it possible to analyze, at the DNA sequence level, events that occur when DNA polymerases encounter sites of DNA damage (Moore & Strauss, 1979; Moore et al., 1981).

We have used ultraviolet light induced DNA damage as a model for such studies. At low UV doses (10-500 J/m^2) the major UV-induced DNA photoproducts are cyclobutane pyrimidine dimers (Lippke et al., 1981). If unrepaired, a single cyclobutane dimer constitutes a lethal event in bacterial or phage genomes (Howard-Flanders & Boyce, 1966; Radman et al., 1978; Benbow et al., 1974). These observations suggest that cyclobutane dimers, under some circumstances, may constitute quantitative blocks to DNA replication. Although studies have suggested that cyclobutane pyrimidine dimers inhibit polymerization by several different DNA polymerases (Moore & Strauss, 1979; Yoshida et al., 1981; Miyaki et al., 1983), precise quantitative measures of termination events at individual dimer sites have not yet been made.

Another class of UV-induced DNA lesions, (6-4) photoproducts,[1] have been described (Lippke et al., 1981; Wang, 1976; Franklin et al., 1982). These photoproducts also form between adjacent pyrimidines, but the structure of these lesions

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[1] Abbreviations: (6-4) photoproducts, 6-4′-(pyrimidin-2′-one)-pyrimidine class of ultraviolet light induced photoproducts; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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