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Abortive Products as Initiating Nucleotides during Transcription by T7 RNA Polymerase†

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ABSTRACT: The kinetics of formation of abortive initiation products during transcription of a synthetic template (encoding the transcript GAUGGC) by T7 RNA polymerase have been determined. This study revealed that while total RNA was formed in the reaction as expected, the levels of the dinucleoside tetraphosphate guanylyl-3′,5′-adenosine-5′-triphosphate (pppGpA) and trinucleoside pentaphosphate guanylyl-3′,5′-adenosine-3′,5′-uridine-5′-triphosphate (pppGpApU) formed by premature termination of transcription reached a maximum after 10 min, and then decreased. Transcription of the same template, in the presence of either [γ-^{32}P]GTP and ATP, or GTP and [α-^{32}P]ATP, gave the ^{32}P-labeled dinucleotides *pppGpA and pppG*pA. Incorporation of each of these substrates into longer RNA transcripts in the same enzyme–template system was demonstrated. The incorporation was shown to require the presence of template in the reaction mixture. The requirement for base complementarity restricts the position of incorporation to that of initiating (5′) nucleotide. Transcription of a second template, which encodes an RNA transcript having the partial sequence GpA at two internal positions, in the presence of each of the labeled dinucleoside tetraphosphates, failed to bring about the synthesis of significant yields of any longer radiolabeled transcripts. It is concluded that dinucleoside tetraphosphate (and perhaps trinucleoside pentaphosphate) can function as initiating nucleotides when complementary to the nucleotide sequence at promoter regions. However, a dinucleotide is not used as substrate for subsequent chain elongation in T7 RNA polymerase catalyzed transcription reactions.

T7 RNA polymerase is now the enzyme of choice for the in vitro synthesis of RNA (Milligan et al., 1987), and it provides a convenient system for studying the individual steps in transcription (Martin & Coleman, 1987). As with other RNA polymerases, that encoded by the phage T7 utilizes a DNA template, variation of which allows the synthesis of virtually any sequence of RNA. The interactions between enzyme, template, and the nucleoside triphosphates have been the subject of footprinting (Ikeda & Richardson, 1986; Basu & Maitra, 1986; Muller et al., 1989) and kinetic studies (Martin

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& Coleman, 1987). More recently, an investigation of the processivity of T7 RNA polymerase has revealed that abortive initiation results in the production of oligonucleotides that are shorter than the full-length transcript due to premature release of the growing oligonucleotide before the full-length product has been made (Martin & Coleman, 1989).

Our interest in T7 RNA polymerase stems from a requirement for introducing unnatural bases, having unique hydrogen-bonding patterns, into oligoribonucleotides (Switzer et al., 1989; Piccirilli et al., 1990). We will report elsewhere the results of a study of the ability of T7 RNA polymerase to utilize templates containing N- and C-nucleotides[1] to direct the incorporation into RNA of N- and C-nucleoside triphosphates, in which kinetic analysis of abortive initiation products was used to compare the relative efficiencies of template–substrate combinations during transcription (Piccirilli et al., 1991). In the course of this work, we discovered that dinucleoside tetraphosphate produced by abortive initiation under normal transcription conditions was, when present at sufficiently high concentration, consumed in the reaction. We report here the results of a more detailed investigation of this phenomenon, which reveal that a dinucleoside triphosphate can be utilized as a substrate for T7 RNA polymerase and incorporated as the initial nucleotide in a template-dependent transcription reaction.

Materials and Methods

T7 RNA polymerase was obtained from Pharmacia as a solution of 70000 units/mL, having specific activity 175000 units/mg. RNAsin (10000 units/mL) and BSA[2] (molecular biology grade) were from Boehringer-Mannheim. Acrylamide and bisacrylamide were from Fluka and were of gel electrophoresis grade. Radioactive nucleotides were from New England Nuclear ([γ-^{32}P]GTP) and Amersham International ([α-^{32}P]ATP and [α-^{32}P]CTP).

Oligonucleotides were synthesized on an Applied Biosystems Model 380B DNA synthesizer, with use of cyanoethyl phosphoramidite chemistry, and purified by high-performance liquid chromatography on a Vydac C4 column. The dimethoxytritylated oligonucleotides were eluted using 20-min gradients of 20–30% or 25–35% acetonitrile in 0.1 M triethylammonium acetate, pH 7.0. The dimethoxytrityl group was cleaved by treatment with 80% acetic acid (room temperature, 20 min) and removed by extraction with diethyl ether. Templates were prepared by heating a 1:1 mixture (concentrations calculated by use of an average molar extinction coefficient of 8.4×10^3 M^{-1} per base) of the component strands in water to 70 °C for 5 min and then allowing them to cool slowly to room temperature.

Polyacrylamide gel electrophoresis was carried out by use of slab gels (48 × 16 cm, 0.06 cm thick for analytical, 0.15 cm thick for preparative gels) of 20% polyacrylamide/7 M urea according to the published procedure (Maniatis & Efstratiadis, 1980). Gels were prerun at 500 V 2–3 h before use and then run at 400–600 V for 8–12 h after application of the samples. On completion of the run, reference spots were placed on the gel with radioactive ink (0.4 μL of approximately 7 μCi/mL) to allow alignment of gel and autoradiogram for excision of bands. Bands were visualized by autoradiography with Kodak X-Omat film. For quantification, bands were excised, and the RNA was liberated for scintillation counting by treatment with Protosol (Du Pont; 0.3 mL, 35 °C, 4–6 h). Scintillation fluid (Kontron Kontrogel, 10 mL) and acetic acid (2 drops) were then added to the Protosol solution, and counts per minute of ^{32}P was determined by use of a Kontron Betamatic II liquid scintillation counter. Under these conditions, extraction of RNA from the gel was found to be quantitative, and scintillation counting efficiency was between 75 and 80%.

Transcription reactions were carried out at 38 °C in 20 μL of 40 mM Tris-HCl, pH 8.1, and contained $MgCl_2$ (20 mM), spermidine (1 mM), DTT (5 mM), RNAsin (50 units), nucleoside triphosphates as noted in the text (2.5 mM each), Triton X-100 (0.01%), template Tp6 or Tp19 (4 μg/mL), BSA (50 μg/mL), either [γ-^{32}P]GTP, [α-^{32}P]ATP, or [α-^{32}P]CTP (5–10 μCi), and T7 RNA polymerase (70 or 140 units as noted for individual experiments). For kinetic work, the reaction was initiated by the addition of enzyme, preequilibrated at 38 °C, to a mixture of the other components at the same temperature. Aliquots (4 μL) were removed from the reaction after 5, 10, 15, and 60 min and quenched by addition of an equal volume of sample buffer (7 M urea, 89 mM Tris–borate, 2 mM EDTA, 0.02% xylene cyanol, 0.02% bromophenol blue) and heating at 70 °C for 5 min. The quenched aliquots were then stored on ice prior to polyacrylamide gel electrophoresis.

*Synthesis of Guanylyl-3′,5′-adenosine [γ-^{32}P]Triphosphate (*pppGpA) and Guanylyl-3′,5′-[α-^{32}P]adenosine Triphosphate (pppG*pA).* The labeled dinucleoside triphosphates required for the incorporation experiment were synthesized by a scaled-up transcription reaction with T7 RNA polymerase using the template Tp6 and the nucleotides [γ-^{32}P]GTP (specific activity 220 mCi/mmol) and ATP (for the synthesis of *pppGpA) or [α-^{32}P]ATP (specific activity 210 mCi/mmol) and GTP (for the synthesis of pppG*pA). Each reaction was carried out for 1 h on a 200-μL scale under the conditions listed above. Reactions were quenched by addition of sample buffer and heating to 70 °C for 5 min. Each reaction mixture was then separated on a 20% polyacrylamide/7 M urea gel. The products were isolated from the gel as follows. After location of the products by autoradiography, the bands corresponding to dinucleotide were excised, and the gel slices were extracted with 0.5 M NaCl/0.1 M Tris-HCl, pH 8.0 (5 mL of each, 30 °C, 10 h). The supernatants were then diluted to 50 mL each, and the dinucleotides were adsorbed onto DEAE-cellulose (1.5 mL). The DEAE-cellulose was then washed with 20 mM triethylammonium bicarbonate, pH 8.0 (TEAB) (20 mL each), and each dinucleotide was eluted with 2 M TEAB (8 mL each). The solutions were evaporated, and traces of buffer were removed by three successive evaporations from water. The purity of the two dinucleotides was assessed by thin-layer chromatography on polyethyleneimine–cellulose plates (Merck) with co-spotted ATP and GTP, using the conditions of Randerath and Randerath (1965), and by gel electrophoresis (see text for details). The yields of purified dinucleotides were 170 nmol, 30%, and 120 nmol, 20%, for *pppGpA and pppG*pA, respectively.

*Incorporation of *pppGpA and pppG*pA into RNA.* Transcription reactions were run as described above except that either *pppGpA or pppG*pA (2.5 mM) replaced ATP in the reaction mixture, and a higher level of T7 RNA polymerase (140 units) was used than for the kinetic studies. Reactions were run for 1 h and analyzed by polyacrylamide gel electrophoresis in the usual way.

[1] N-nucleotides and C-nucleotides are nucleotides that have the heterocyclic base joined to C-1′ of ribose via a carbon–nitrogen bond or a carbon–carbon bond, respectively.

[2] Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane, TEAB, triethylammonium bicarbonate.

Tp6 TAATACGACTCACTATAG
ATTATGCTGAGTGATATCTACCG

RNA Transcript pppGAUGGC

Tp19 TAATACGACTCACTATAG
ATTATGCTGAGTGATATCCCTAGGGGGGGCCTAGGT

RNA Transcript pppGGGAUCCCCCCCGGAUCCA

FIGURE 1: Structures of the templates Tp6 and Tp19 and the RNA molecules that they encode.

RESULTS

The two templates used in this study, Tp6 and Tp19, were designed to produce RNA molecules 6 and 19 nucleotides long, respectively. Both templates were of the "overlapping" type (Milligan et al., 1987) consisting of a double-stranded portion comprising the consensus promoter for T7 RNA polymerase, followed by a single-stranded coding sequence. The structures of the two templates, and the RNA molecules that they encode, are shown in Figure 1.

As expected, transcription from template Tp6 with T7 RNA polymerase in the presence of the four nucleoside triphosphates, ATP, GTP, UTP, and CTP, gave hexamer as the major product, together with lesser amounts of abortive initiation products (see Figure 2, lane 1). The products of abortive initiation of transcription by T7 RNA polymerase have been analyzed in a very similar system and shown to have the sequences expected from premature termination of RNA synthesis (Martin & Coleman, 1987). In the present case, the short RNAs formed during transcription with the template Tp6 were therefore assigned to be pppGpA, pppGpApU, pppGpApUpG, and pppGpApUpGpG. Support for this assignment was obtained from the results of parallel transcription reactions using [γ-^{32}P]GTP and [α-^{32}P]CTP as the radioactive labels. With [γ-^{32}P]GTP as the labeling nucleotide, all products (dimer to hexamer) could be observed by gel electrophoretic analysis of the reaction mixture (see Figure 2). In contrast, transcription with [α-^{32}P]CTP revealed only full-length product, as expected from the sequence of the template (results not shown).[3]

Further support for the assignment of the bands came from parallel transcription reactions with template Tp6, in which selected nucleoside triphosphates were omitted, resulting in the exclusive synthesis of the shorter products (Figure 2). In the presence of only [γ-^{32}P]GTP, a small amount of dinucleotide, presumably *pppGpG, was formed. This is consistent with the ability of T7 RNA polymerase, in the absence of all the nucleotides required for full-length RNA synthesis, to insert an "incorrect" base at a low level (S. E. Moroney and S. A. Benner, unpublished results). Inclusion of ATP in the reaction mixture resulted in the production of dinucleotide tetraphosphate as the major product, the assignment of which as *pppGpA is consistent with its slightly increased mobility compared with that of *pppGpG (Frank & Köster, 1979). In the absence of ATP, transcription past the first position is no longer possible, and no oligoribonucleotides were formed (Figure 2, lane 2). Finally, transcription in the presence of [γ-^{32}P]GTP, ATP, and UTP gave pentanucleotide (*pppGpApUpGpG) as the major product (Figure 2, lane 3), consistent with the requirement for CTP to extend the product to full length.[4]

[3] At longer times (greater than 1 h), transcription with [α-^{32}P]CTP revealed additional radiolabeled products that migrated either faster or slower than the full-length transcript (hexamer). The sequence identities of these products are unknown.

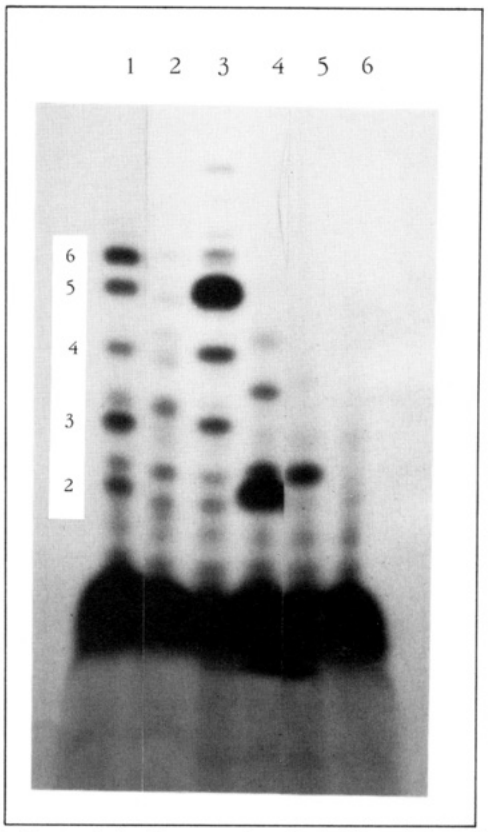

FIGURE 2: Transcription from template Tp6 with omission of selected nucleotides. All reactions were run for 30 min with 70 units of T7 RNA polymerase. Otherwise, conditions were as given under Materials and Methods. Reaction mixtures contained the following combinations of nucleoside triphosphates: (lane 1) [γ-^{32}P]GTP, ATP, UTP, CTP; (lane 2) [γ-^{32}P]GTP, UTP, CTP; (lane 3) [γ-^{32}P]GTP, ATP, UTP; (lane 4) [γ-^{32}P]GTP, ATP; (lane 5) [γ-^{32}P]GTP only; (lane 6) [γ-^{32}P]GTP, ATP, UTP, CTP, with no template. The assignment of abortive transcripts as discussed in the text is given along the left side.

For our investigation of the ability of T7 RNA polymerase to accept C-nucleotides in a transcription complex, we developed an assay for the formation of abortive initiation products (Piccirilli et al., 1991). By using [γ-^{32}P]GTP as radioactive label, we were able to observe all prematurely terminated transcripts. The same procedure was used in the present study to monitor the fate of each of the products formed during transcription of the template Tp6. Reactions were initiated by addition of enzyme to a mixture of the other components that had been preequilibrated at 38 °C. Aliquots were removed from transcription reactions at time points chosen so as to reveal the fate of the absortive initiation products, rather than to allow accurate determination of initial rates. The aliquots were quenched in 7 M urea solution, and the mixtures were separated into their components by polyacrylamide gel electrophoresis. Following electrophoresis, bands were visualized by autoradiography, excised from the gel, and quantified by scintillation counting. In this way, the amount of each product, and of total RNA, was determined.

When the reaction was allowed to proceed for 60 min, the product–time profile for total RNA synthesis (including all

[4] Added in Proof: To confirm the sequence assignments of the abortive products, transcription reactions using various α-labeled nucleoside triphosphates were performed, and each abortive product was subjected to enzymatic hydrolysis with RNase T1. The digestion products were separated and identified by two-dimensional thin-layer chromatography on cellulose (Nishimura, 1979). In each case, greater than 95% of the radioactivity appeared with the expected nucleotide.

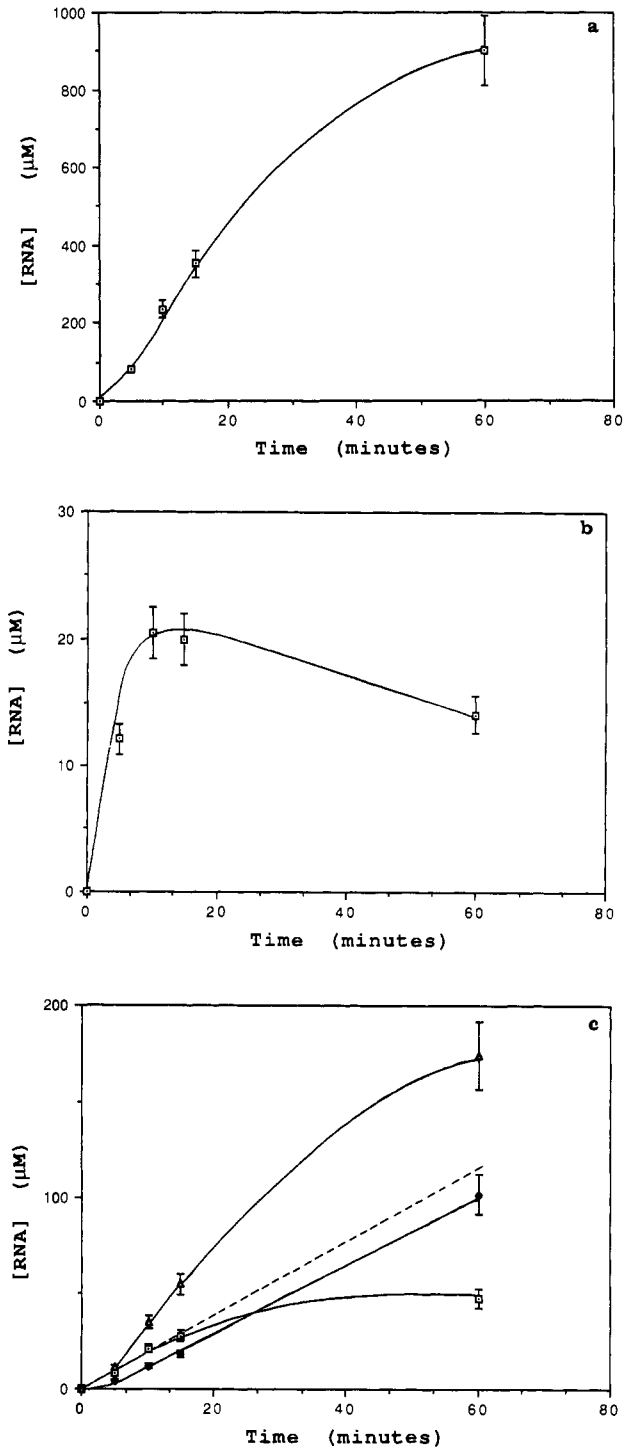

FIGURE 3: Time course of transcription of template Tp6 by T7 RNA polymerase: (a) total RNA synthesis; (b) synthesis of the abortive initiation product pppGpA; (c) synthesis of the abortive initiation products pppGpApU (□), pppGpApUpG (◆), pppGpAUpGpG (△). The dotted line represents the initial rate of pppGpApU synthesis.

abortive initiation products) shown in Figure 3a was obtained. This plot is similar to product–time profiles reported previously for T7 RNA polymerase (Chamberlin & Ring, 1973). It includes an initial lag phase that has been ascribed to rate-limiting preassociation of enzyme and template (Chamberlin & Ring, 1973). Figure 3b shows the corresponding data obtained by measuring the formation of prematurely terminated dinucleotide (*pppGpA) in the same reaction. In this case, the level of dinucleotide in the reaction mixture, after reaching a maximum of 20 μM at approximately 10 min, actually decreased. Similar behavior was apparently displayed by the abortive trinucleotide (*pppGpApU), which at the end of the transcription was present at a concentration (20 μM) considerably lower than that predicted from its initial rate of formation, as can be seen in Figure 3c.

There are three possible interpretations of this observation. First, it may be that the dimer and trimer are broken down during the reaction. This seems unlikely, given that the levels of the longer products (greater than three nucleotides in length) do not decrease. Alternatively, it may be that dinucleoside tetraphosphate and trinucleoside pentaphosphate, after being released from the enzyme–template complex, are subsequently consumed in the reaction, being incorporated into product by T7 RNA polymerase. Assuming that the requirement of complementarity between template and incoming substrate is maintained, when present in the reaction mixture at a sufficiently high concentration (about 20 μM), pppGpA or pppGpApU could bind to the enzyme–Tp6 complex as the initial nucleotide. Once bound, the oligonucleotide would be extended in the normal way by T7 RNA polymerase, accounting for its disappearance from the reaction mixture. This process presumably cannot occur for the longer abortive initiation products (greater than three nucleotides in length), which explains why their levels do not decrease as the reaction proceeds. The data do not rule out a third possibility of the ternary complex (enzyme–DNA–transcript) resuming transcription after having paused for a long time without the transcript ever dissociating from the enzyme–template complex. However, the steady-state concentration of dinucleotide (20 μM) cannot entirely be paused complexes since this concentration is greater than the template concentration (~90 nM). Thus, at least some of the dinucleotide must reversibly dissociate from the enzyme–DNA complex. The experiment below demonstrates directly that the dinucleotide can reassociate into a productive complex with enzyme and DNA template.

The concentration of nucleoside triphosphate utilized in the reaction (2.5 mM) is much higher than the K_m [0.16 mM] (Martin & Coleman, 1989). In view of the high concentration of normal substrates, it is surprising that the concentration of dinucleotide reaches a steady-state level of 20 μM, some 125-fold lower in concentration than the NTP's. This factor of 125 is roughly that expected for an additional Watson–Crick base pair and may reflect tighter binding of the dinucleotide for the enzyme–DNA complex as compared to GTP, assuming $K_m = K_d$. However, in the absence of a more detailed kinetic description, it is difficult to make inferences about the relative utilization of pppGpA as the initiating nucleotide in the presence of excess GTP.

The possibility that a dinucleoside tetraphosphate could be a substrate for T7 RNA polymerase catalyzed transcription was directly tested by synthesizing two samples of radioactively labeled pppGpA and determining their incorporation into RNA in reactions with the template Tp6. The result of transcription of Tp6 by T7 RNA polymerase with GTP and ATP as the only nucleotides present in the reaction mixture (vide supra) showed that the required dinucleoside tetraphosphate could be obtained in this way. By scaling up the reaction, the dinucleotide was conveniently prepared in two labeled forms. Synthesis of the dinucleotide with [γ-^{32}P]GTP as label afforded *pppGpA. Similarly, pppG*pA was prepared by using [α-^{32}P]ATP as label. In each case, the labeled dinucleoside tetraphosphate was separated from unreacted starting material by polyacrylamide gel electrophoresis and purified by elution from the gel, followed by anion-exchange chromatography. That both products were free of radioactive

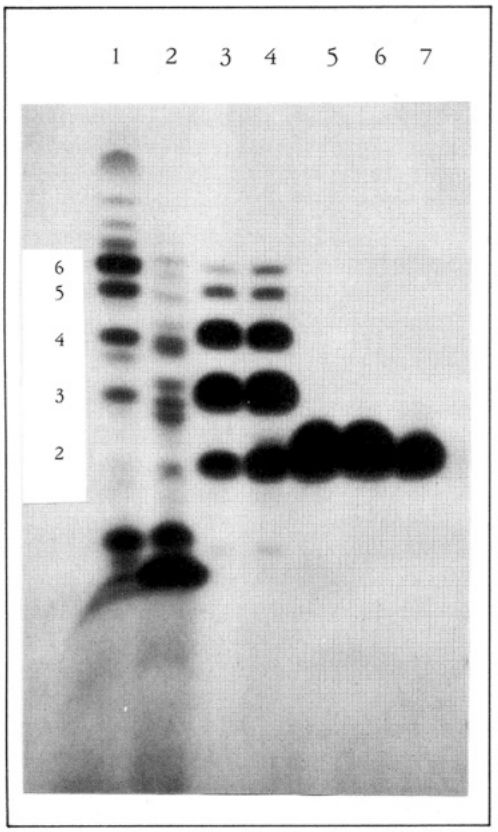

FIGURE 4: Incorporation of *pppGpA and pppG*pA into RNA by transcription from the template Tp6 with T7 RNA polymerase. Transcription reactions were carried out for 1 h with 140 units of enzyme. Otherwise, conditions were as described under Materials and Methods. (Lane 1) [γ-^{32}P]GTP, ATP, UTP, CTP; (lane 2) [γ-^{32}P]GTP, UTP, CTP; (lane 3) pppG*pA, GTP, UTP, CTP; (lane 4) *pppGpA, GTP, UTP, CTP; (lane 5) *pppGpA, GTP, UTP, CTP, no template; (lane 6) pppG*pA; (lane 7) *pppGpA. The assignment of absortive transcripts as discussed in the text is given along the left side.

contaminants was demonstrated by PEI–cellulose chromatography under conditions that resolved mono- and dinucleoside triphosphates (Randerath & Randerath, 1965) and also by polyacrylamide gel electrophoresis (Figure 4, lanes 6 and 7). The gel used to produce Figure 4 was run so that all radioactivity was retained, and by overexposure of the autoradiogram, it was shown that the levels of radioactive contaminants must be less than approximately 1%.

The radiochemical yield of the γ-^{32}P-labeled dinucleotide was slightly higher than that of its α-labeled congener, despite the use of radiolabel of approximately the same specific activity in each case. This is consistent with the presence of pppGpG as a minor impurity in the preparations of pppGpA, the contaminant being present as the γ-^{32}P-labeled dinucleotide in the preparation of *pppGpA and being unlabeled in the preparation of pppG*pA. The presence of such an impurity should have no effect on the outcome of the experiment, although, as discussed below, it may account for some artifacts observed in the incorporation experiments.

Although the requirement for base complementarity restricts the position in which pppGpA could be incorporated with use of template Tp6, we considered that, given an appropriate template sequence, incorporation of a dinucleoside tetraphosphate within the growing RNA chain may be possible. For this reason, we wished to test the incorporation of dinucleotide at both the initial and subsequent positions of an RNA transcript. To distinguish these two possibilities, we utilized two different templates in the incorporation experiments: Tp6, which encodes GpA as the initial dinucleotide, and Tp19, in which the partial sequence GpA occurs twice within the product RNA structure.

In the first experiment designed to test the incorporation of dinucleotide into RNA, transcription of template Tp6 was carried out with either *pppGpA or pppG*pA, in the presence of UTP, CTP, and GTP with use of twice the normal level of T7 RNA polymerase. In each case, the dinucleotide was incorporated into RNA, giving products up to six nucleotides in length (see Figure 4, lanes 3 and 4). The abortive initiation products had the same mobilities as those formed in the presence of the four natural nucleoside triphosphates. The level of product formation in the absence of the dinucleotide was determined by transcription of Tp6 in the presence of CTP and UTP, using [γ-^{32}P]GTP of the same specific activity as *pppGpA and pppG*pA as radioactive label. In this case (see Figure 4, lane 2) the pattern (relative mobilities of the products) of RNA formed was substantially different, and the level of incorporation of radioactivity into each transcript was much lower. Lane 1 of Figure 4 shows the control, in which equimolar amounts of all four nucleoside triphosphates were used in the reaction. The longer products formed in this case were always observed at high enzyme concentrations and long reaction times. Also apparent from the control reaction (lane 1) is the absence of dinucleotide, although it can be clearly seen in the corresponding reaction performed with less enzyme and for a shorter time (Figure 2, lane 1). Clearly, dinucleoside tetraphosphate is a substrate for the reaction and is extended by T7 RNA polymerase giving oligomers between three and six nucleotides in length.

Comparison of lanes 1, 3, and 4 (Figure 4) shows that the relative yields of abortive initiation products are quite different when pppGpA replaces ATP in the reaction. With dinucleoside tetraphosphate as initiating nucleotide, the yield of RNA drops off as the chain length increases (lanes 3 and 4). This was unexpected, as when bound to the enzyme–template complex the dinucleoside tetraphosphate should be extended in the usual way and should therefore give the same pattern of abortive initiation products as is seen in the control reaction. One possible rationale for this observation is that the preparation of dinucleotide contains an impurity that is an inhibitor of subsequent nucleoside triphosphate incorporation steps and therefore reduces the rate of formation of successively longer products.

That the T7 RNA polymerase catalyzed incorporation of dinucleotide is template-dependent can be concluded from two lines of evidence. First, the mobilities of products formed by transcription of template Tp6 with pppGpA (Figure 4, lanes 3 and 4) are identical with those of the major transcripts formed with the same template in the presence of the four nucleoside triphosphates (lane 1). This is evidence that the products seen in lanes 3 and 4 are the same as those formed in the control reaction (lane 1), since the mobility of short oligonucleotides is composition-dependent (Frank & Köster, 1979) and is a useful guide in structure determination. In the absence of both dinucleotide and ATP, some "read-through" is observed with template Tp6 (Figure 4, lane 2), but the resulting pattern of products is quite distinct from that seen in the presence of GTP, CTP, UTP, and either *pppGpA, pppG*pA, or ATP.

Second, omission of template Tp6 from a transcription reaction carried out with *pppGpA, GTP, CTP, and UTP under identical conditions gave no extension of the dinucleotide to longer products (Figure 4, lane 5). Finally, this experiment demonstrates that no degradation of the dinucleotide occurred

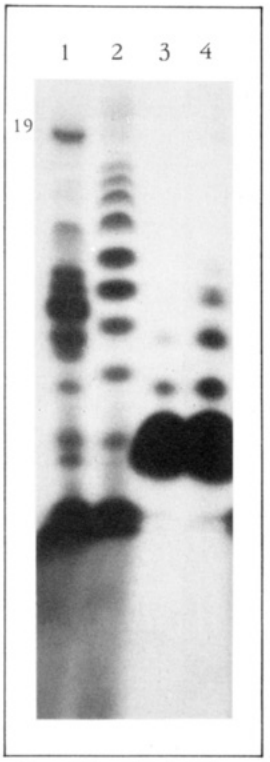

FIGURE 5: Incorporation of *pppGpA and pppG*pA into RNA by transcription from the template Tp19 with T7 RNA polymerase. Transcription reactions were carried out for 1 h with 140 units of enzyme. Otherwise, conditions were as described under Materials and Methods. (Lane 1) [γ-^{32}P]GTP, ATP, UTP, CTP; (lane 2) [γ-^{32}P]GTP, UTP, CTP; (lane 3) pppG*pA, GTP, UTP, CTP; (lane 4) *pppGpA, GTP, UTP, CTP.

under the reaction conditions (at least in the absence of template), since the autoradiogram shows all radioactive components formed in the reaction.

In similar experiments, T7 RNA polymerase catalyzed transcription of the template Tp19 was carried out, with and without added dinucleoside tetraphosphate. This template directs the synthesis of a 19-mer (see Figure 1 and lane 1 of Figure 5),[5] and the requirement for complementarity between bases in the coding sequence and substrate suggests that pppGpA could potentially be incorporated twice, although in neither case as the initiating nucleotide. In the absence of ATP, incorporation of pppGpA should lead to the synthesis of an 18-mer, transcription stopping prematurely due to the absence of the terminal nucleotide from the reaction mixture. Successful incorporation of *pppGpA would not lead to radiolabeled products if the dinucleotide were indeed a substrate, since the ^{32}P would be lost as inorganic pyrophosphate during phosphodiester bond formation. "Internal" incorporation could be observed in this case with the α-labeled dimer pppG*pA.

As can be seen from Figure 5 (lane 3), only traces of oligomers longer than trinucleotide were formed when the radioactive label was provided by pppG*pA, none of which were longer than four nucleotides in length. Also shown in Figure 5 is the result of the analogous experiment with *pppGpA as the radioactively labeled species (lane 4). As can be seen, the same products are formed in this case, up to approximately five bases in length. These transcripts are present at a low level and are most likely to be products initiated incorrectly with *pppGpA and pppG*pA. With *pppGpA as the radioactive substrate, the greater intensity of the products may reflect some correct initiation with *pppGpG, which is a possible contaminant in the preparation of *pppGpA (vide supra). Since the products are seen with both labeled dinucleotides, and longer products are absent from the reaction with pppG*pA, it can be concluded that the dinucleotide is not incorporated during extension of the RNA chain. Transcription of Tp19 with T7 RNA polymerase in the presence of only [γ-^{32}P]GTP, CTP, and UTP results in the production of a ladder of transcripts (lane 2). These are most likely to be oligomers of G, formation of which from templates having the partial sequence CpCpC as initial coding bases has been reported by others (Martin et al., 1988).

[5] Independent experiments that included sizing markers have established the length of the Tp19 transcript as 19 nucleotides.

Discussion

The property of abortive initiation of RNA polymerization reactions provides a useful means of analyzing the interaction between enzyme, template, and incoming nucleoside triphosphate. For example, it has been suggested that the tendency to release short transcripts prematurely is increased following incorporation of uridine residues (Martin et al., 1988). Similarly, we have used an assay for abortive initiation by T7 RNA polymerase to compare the abilities of thymidine and the isosteric C-nucleoside *N*-methylpseudouridine in template coding sequences to direct the incorporation into RNA of the triphosphates of adenosine and its C-nucleoside analogue formycin (Piccirilli et al., 1991). It was in the course of this study that we first observed disappearance from a reaction mixture of dinucleotide formed by premature termination of RNA synthesis.

By following the formation of each of the short oligonucleotides formed during transcription of the template Tp6, we have shown that the shortest abortive transcript, dinucleoside tetraphosphate, is consumed in the reaction. The most likely explanation for the disappearance of an oligonucleotide from a mixture of several is its utilization as a substrate during subsequent transcription cycles. If degradation were occurring (such as pyrophosphorolysis catalyzed by the enzyme), this would almost certainly affect the levels of each of the other transcripts as well. The lower level of trinucleotide, as compared to the level expected from the initial rate of formation (Figure 3c, dotted line), suggests that this abortive transcript is also utilized in subsequent transcription cycles. The abortive products of lengths four and five, however, do not show this behavior (Figure 3c, diamonds and triangles). Thus, it appears that the incorporation of di- and trinucleotides into the product represents the limit of tolerance for the polymerase, and longer oligonucleotides, although complementary to the template strand, cannot be incorporated as initiating nucleotides. This limit of tolerance suggests that the interactions of the enzyme with the DNA–RNA hybrid begin to change after incorporation of the fourth nucleotide in the growing RNA chain. Perhaps the first translocation event occurs at this stage and the interactions required for initiation of the enzyme are no longer present. This hypothesis is consistent with footprinting studies in which the Fe–EDTA protection pattern of the polymerase–promoter complex appears to change only slightly during formation of the first phosphodiester bond (Muller et al., 1989). Our results suggest that a similar protection pattern would be observed during formation of the second phosphodiester bond as well. However, once the third phosphodiester bond is formed, a conformational change may occur (such as the polymerase moving along the DNA template) in which the interactions necessary for enzyme initiation are no longer present.

The possibility of direct incorporation of the dinucleotide into RNA by T7 RNA polymerase was tested by inclusion in

a transcription reaction mixture of radioactively labeled substrate. We found that nanomole amounts of this material could be conveniently prepared enzymatically. The synthesis made use of a property of T7 RNA polymerase demonstrated by Ikeda and Richardson (1986), that transcription continues as far as possible with the available nucleotides, terminating when the requirement for complementarity with a template base is not satisfied by the available nucleoside triphosphates. Having two labeled forms of the dinucleotide pppGpA allowed us to answer several questions regarding its incorporation. The γ-labeled form *pppGpA and its α-labeled congener pppG*pA were readily prepared by transcribing the template Tp6 with [γ-^{32}P]GTP and [α-^{32}P]ATP as the respective sources of radioactive label.

That the dinucleotide could be used as a substrate by T7 RNA polymerase in transcription from template Tp6 was demonstrated by carrying out reactions with either *pppGpA or pppG*pA as the only radioactive species. Under the assumption that Watson–Crick base pairing between coding sequence and incoming triphosphate base(s) is maintained, we conclude that pppGpA is incorporated as the initial dinucleotide during transcription of template TP6. The incorporation was shown to be template-dependent, producing products with the same mobilities as transcription in the presence of the four mononucleoside triphosphates. However, the transcripts were produced in different relative amounts depending on whether pppGpA or γ-^{32}P supplied the radioactive label. Although each preparation of pppGpA was demonstrated to be free of radioactive contaminants, the presence of an impurity that inhibits nucleotide incorporation during chain elongation cannot be excluded. The reduction in yield of successively longer products is consistent with this explanation.

By using another template, Tp19, incorporation of pppGpA during chain elongation was tested. This template encodes an oligoribonucleotide of 19 bases (Figure 1), in which the partial sequence GpA occurs not at the 5′-end of the transcript but at 2 internal positions. With use of this template, it was established that the dinucleoside tetraphosphate could not serve as a substrate for incorporation by T7 RNA polymerase at an "internal" position in the nascent RNA chain.

The results of these experiments are consistent with an emerging picture of the active site of T7 RNA polymerase. It has been shown (Martin & Coleman, 1989) that the enzyme will accept not only GTP but also guanosine, GMP, and GDP as initiating nucleotides. Furthermore, in the same study, the K_m for GTP incorporation into transcripts as initial (5′) nucleotide was found to be 4-fold higher than the value for its subsequent participation during chain elongation. Both of these observations suggest that the active site binds the initial and subsequent nucleoside triphosphates differently. The results presented here reinforce this notion in that dinucleoside tetraphosphate can prime RNA synthesis but is ineffective as an elongation triphosphate. As a rationale for the formation of homopolymers of G with some templates, the concept of "slippage" of the nascent RNA transcript with respect to the coding DNA strand has been introduced (Martin & Coleman, 1987). The implication of this is that the active site of T7 RNA polymerase must be able to accommodate structures larger than GTP when commencing synthesis at the 5′-end of the transcript. The ability of T7 RNA polymerase to catalyze the incorporation of a dinucleoside tetraphosphate as initiating nucleotide reported here is consistent with this conclusion. Furthermore, our finding that the same dimeric substrate is not incorporated intact at an "internal" position of the RNA product suggests that the active site in the processive T7 RNA polymerase–template complex differs from that in the initiating event. Such a suggestion is also consistent with the observed differences in K_m for the two separate incorporation events (Martin & Coleman, 1989).

However, as our results were obtained with a single template–promoter sequence, it remains to be determined whether "abortive oligonucleotide initiation" is a general property of T7 RNA polymerase or whether such behavior is template- and/or sequence-specific. Finally, we note that initiation of RNA polymerization by di- or trinucleotides has been observed for *Escherichia coli* RNA polymerase (Downey et al., 1971) provided sequence complementarity to promotor regions is maintained. It appears that this same sequence restriction is required for short oligonucleotides to initiate T7 RNA polymerase as well. This property of initiation, along with abortive cycling (Martin & Coleman, 1988), may be general to all RNA polymerases and is likely to originate from similar mechanistic constraints imposed by the complex process of biological polymerization of RNA.

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