Escherichia coli Deoxyribonucleic Acid Dependent Ribonucleic Acid Polymerase Transcriptional Pause Sites on SV40 DNA F1†

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ABSTRACT: We have studied elongation on SV40 DNA F1 by *E. coli* RNA polymerase looking specifically at the length of the transcript as a function of time. By running the transcription reactions at 18 °C with limited enzyme and adding heparin or rifampicin after elongation has started, we have achieved almost exclusive initiation from the SV40 DNA preferred promoter site [Zain, B. S., Weissmann, S. M., Lebowitz, P., & Lewis, A. M., Jr. (1973) *J. Virol. 11*, 682–693]. In the region within 1500 nucleotides of the initiation we observe nine prominent sites and a number of minor sites where hesitation during elongation occurs. The positions of these hesitation points or pause sites are not effected by changes in the salt concentration, the simultaneous lowering of the concentrations of all the NTPs, or by increases in the RNA polymerase concentration, implying that the pause sites are a consequence of the RNA, DNA, and RNA polymerase ternary complex. The pause sites are not an artifact of the lowered temperature (18 °C) used in the experiments since they are also observed at 37 °C. The first four of these sites have been sequenced by using the 3′-*O*-methyl analogues of the ribonucleotide triphosphates. We have found no sequence homology between the pause sites. The kinetics of the pause reactions do not fit a first-order model but do correspond to a scheme where continuation through a pause site and termination at a pause site are both represented. For one of the pause sites, the relaxation time for continuation through the pause site was determined to be ~2.5 min and for termination ~50 min at 18 °C. If the concentration of one of the NTPs is lowered to 10 μM, the strength of a pause site can be increased if that NTP is contained in the pause. Also, minor pause sites are observed at regions in the RNA sequence which are rich in the NTP that has the lowered concentration. When GTP is replaced by ITP during transcription, a new set of pause sites quite different from the normal sites of hesitation are observed. The major new pause sites occur at or near sequences in the RNA which are rich in I-U residues preceded by a region rich in C residues. This indicates, as has been previously noted, that sequences where the DNA·RNA hybrid is quite stable followed by a region that is very unstable may cause termination. When BrUTP replaced UTP, very little effect was observed on the pause sites. The addition of ρ termination factor causes termination to increase in all the pause sites with a length greater than 300 nucleotides. In the type of experiments performed here, those pause sites had continuation relaxation times greater than 45 s at 37 °C. This implies that regardless of the nature of a pause, ρ will cause at least some termination at all hesitation sites with a relaxation time greater than 45 s. All the results are discussed in terms of a kinetic model for the termination of elongation.

An important element in prokaryotic gene expression is the termination of transcription (Roberts, 1976; Adhya & Gottesman, 1978; Rosenberg & Court, 1979). The extent to which termination occurs is tightly modulated depending on the environmental conditions encountered by the organism (Zurawski et al., 1978a,b). Proteins like ρ termination factor and bacteriophage λN protein are involved in termination such that ρ factor stimulates it at some specific sites (Roberts, 1969) and λN protein represses termination (Adhya & Gottesman, 1978).

The feature which governs termination at a specific site on a DNA template has not yet been implicitly determined, but three types of features stand out from the sequenced termination regions. These include (1) a dyad symmetry in the sequence of the RNA or DNA, approximately 20 ± 4 nucleotides behind the site of termination found in all the sequenced terminators (Rosenberg & Court, 1979), (2) a sequence rich in guanosine and cytosine residues followed by at least five successive uridine residues just prior to the site of termination found in most of the ρ-independent terminators (Gilbert, 1976), and (3) in some cases, a specific sequence conserved in a number of the terminator regions (Stauffer et al., 1978; Küpper et al., 1978; Rosenberg et al., 1978).

The termination reaction can be divided into three parts: cessation of elongation, release of the RNA, and release of RNA polymerase from the template (Neff & Chamberlin, 1978). The order of events for the last two parts is unknown, but all the evidence up to the present points to a mechanism where RNA polymerase hesitates prior to termination with or without added protein factors (Rosenberg & Court, 1979).

In this study, we have followed elongation of RNA by *Escherichia coli* RNA polymerase by looking at the length of the transcript as a function of time. Transcription using SV40 DNA F1 as a template is discontinuous, i.e., RNA polymerase hesitates at specific places (pause sites)[1] on the SV40 genome. The pausing of polymerase during transcription has been observed before on T_7 and λDNA (Maizels, 1973; Darlix & Horaist, 1975), but the use of modified conditions has led to questions about the natural occurrence of these hesitation sites (Adhya & Gottesman, 1978). Here we provide strong evidence that the pausing of polymerase on SV40 DNA F1 is not an artifact of the conditions employed.

To understand pausing during transcription further, we have developed conditions where only one specific initiation site is used on the SV40 genome. With this one initiation site and

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[1] For convenience we have termed the specific sized RNA transcripts, where RNA polymerase hesitates during elongation, pause sites.

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the sequence of SV40 DNA (Reddy et al., 1978), we have been able to determine the sequence of some of the pause sites. Also, we have looked at the kinetics of the pause reactions, and from these results we propose a kinetic model for the termination of elongation.

Materials and Methods

Materials. ATP, GTP, UTP, and CTP were high-performance liquid chromatography purified compounds and, with [^{3}H]UTP, were purchased from ICN Pharmaceuticals. ITP,[2] BrUTP, 3′-*O*-methyladenosine 5′-triphosphate, 3′-*O*-methylcytidine 5′-triphosphate, 3′-*O*-methyluridine 5′-triphosphate, and 3′-*O*-methylguanosine 5′-triphosphate were products of P-L Biochemicals. Rifampicin and heparin were obtained from Sigma Chemical Co. and ^{3}H-labeled 28S, 18S, and 5S RNAs were purchased from Miles Laboratories. [α-^{32}P]- and [γ-^{32}P]ATP were supplied by Amersham Corp., and the restriction endonuclease *Hin*fI was from Bethesda Research Laboratories. Ribonucleases T_1 and pancreatic RNase were products of Boehringer Mannheim and AMV reverse transcriptase was provided by Joseph Beard (Life Sciences, Inc.). All other reagents were the purest grades commercially available.

SV40 DNA F1 was isolated from infected TC-7 African green monkey cells by a modification of the Hirt extraction essentially as described by Hallick et al. (1978). The F1 DNA was more than 98% pure as judged by 1.4% agarose gels (Shen et al., 1976) and had A_{260}/A_{280} ratios greater than 2.0.

E. coli RNA polymerase holoenzyme (EC 2.7.7.6), a generous gift from Robert W. Woody, was prepared as described (Reisbig et al., 1979). Using the methods outlined by McConnell & Bonner (1972), we found no detectable polynucleotide phosphorylase or polyphosphate kinase activity in the preparation.

ρ protein was a gift from Malcolm Winkler of Charles Yanofsky's laboratory. It had been purified by the method of L. Finger and J. P. Richardson (unpublished results) and stored in 50 mM Tris (pH 7.9), 0.1 mM EDTA, 100 mM KCl, 100 mM $MgCl_2$, 0.1 mM DTT, and 50% glycerol at −20 °C.

DNA Markers. ^{32}P-Labeled DNA markers were obtained by cutting 1 μg of SV40 DNA with *Hin*fI restriction endonuclease and labeling the 3′ ends by using [α-^{32}P]dATP and AMV reverse transcriptase (Youvan & Hearst, 1979). After labeling, unincorporated [α-^{32}P]dATP was removed from the SV40 DNA fragments by gel filtration on a Sephadex G-100 column (1 × 10 cm). The buffer used in the separation was 200 mM NaCl, 20 mM Tris (pH 7.4), 10 mM EDTA, and 0.5% $NaDodSO_4$ (G-100 buffer). ^{32}P-Labeled *Hin*fI SV40 restriction fragments, which came off the column in the void volume, were precipitated by adding 5 μg of carrier tRNA (previously phenol extracted) and 2.5 volumes of ethanol, cooling to −70 °C for 1 h, and centrifuging for 5 min in an Eppendorf 3200/30 microcentrifuge. For high yields, the tubes were cooled to −70 °C a second time for 5 min and centrifuged again for 5 min. The supernatant fluid was discarded. The pellet, containing the ^{32}P-labeled fragments, was dried under vacuum, redissolved in 10 mM Tris (pH 7.4) and 1 mM EDTA, and stored at 4 °C.

RNA Synthesis. The standard assay for RNA synthesis on SV40 DNA F1 using *E. coli* RNA polymerase was divided into two steps: (1) initiation and (2) elongation. Initiation was accomplished by adding 0.72 μg of RNA polymerase to 100 μL of reaction buffer (180 mM KCl, 30 mM Tris, pH 7.9, 6 mM $MgCl_2$, and 6 mM β-ME) containing 10 μg of SV40 DNA F1, 10 μM [γ-^{32}P]ATP (50 Ci/mmol), 20 μM UTP, and 10 μM GTP at 18 °C. After incubation at 18 °C for 5 min, elongation was started by adding 100 μL of reaction buffer (preincubated at 18 °C) which contained 320 μM ATP, 310 μM UTP, 320 μM GTP, 330 μM CTP, and 200 μg/mL heparin to the initiation mixture. The final concentrations for the components during elongation were, thus, RNA polymerase 3.6 μg/mL, SV40 DNA F1 50 μg/mL, heparin 100 μg/mL, and each NTP 165 μM, in reaction buffer. The addition of all four NTPs with the concurrent start of elongation has been designated as time 0.

In some experiments, uniform labeling with [α-^{32}P]ATP or [^{3}H]UTP was substituted for the end labeling with [γ-^{32}P]-ATP. In these, [α-^{32}P]ATP (3.03 Ci/mmol) or [^{3}H]UTP (1.2 Ci/mmol) replaced cold ATP or UTP at the same concentration in both the initiation and elongation steps of the standard assay. Quantitative determination of the total amount of label incorporated into RNA showed that the use of [^{3}H]UTP and [α-^{32}P]ATP gave comparable results.

Reactions in which [α-^{32}P]ATP or [γ-^{32}P]ATP was used as the label were stopped by adding 0.1 volume of 2 M Tris–acetate and 0.4 M EDTA. The solution was immediately phenol extracted and the phenol removed. When [α-^{32}P]ATP was used as a continuous label, the RNA transcripts were purified as described below. When [γ-^{32}P]ATP was used as an end label, the RNA transcripts were precipitated and dried by the methods described under DNA Markers and used directly for gel electrophoresis.

Reactions in which [^{3}H]UTP was used as the label were stopped by removing an aliquot of the reaction mixture and placing it on a Whatman DE81 filter. The filters were washed and counted, and the radioactivity was corrected according to the method of Spindler et al. (1978). The sample size varied between experiments so all data have been normalized to the activity of 1 μg of RNA polymerase.

The initiation step of the reaction remained the same in all the experiments except when ApA was used. In these experiments ApA (165 μM) was added to the initiation system before addition of RNA polymerase. The final concentration of ApA during elongation was maintained at 165 μM.

When the reaction conditions were varied, the changes occurred in the elongation step. Reactions in which the temperature was increased to 37 °C were accomplished by incubating the initiation mixture at 37 °C for 2 min after the incubation for 5 min at 18 °C. The elongation mixture (preincubated at 37 °C) was then added. Reactions in which the concentration of the NTPs were varied, and where rifampicin replaced heparin, were accomplished by changing all the appropriate components in the elongation mixture. Reactions in which the salt concentrations were varied were accomplished by adding an appropriate volume of elongation mixture made up with no KCl to the initiation mixture to give the final concentration of KCl.

Purification of RNA Transcripts. Dry Bio-Gel P-6 resin was mixed with an excess amount of G-100 buffer and allowed to stand overnight. The resin was then washed three times with the same buffer and stored with excess buffer at 4 °C.

Holes were punched in the lid and the bottom of a 1.5-mL polypropylene snap-cap tube with an 18-gauge needle and the bottom of the tube was plugged with siliconized glass wool. Bio-Gel P-6 in G-100 buffer (at room temperature) was packed into the 1.5-mL tube until the tube was completely full.

[2] Abbreviations used: ITP, inosine 5′-triphosphate; BrUTP, 5-bromouridine 5′-triphosphate; RNAP, RNA polymerase; ApA, adenylyl-(3′–5′)-adenosine; β-ME, β-mercaptoethanol; AMV, avian myeloblastosis virus.

The lid was put into place and pressurized nitrogen gas was applied to the top of the tube to force most of the excess liquid out of the bottom of the tube. The 1.5-mL tube was then inserted into a 13 × 100 mm Falcon culture tube which had been previously punctured approximately 2.5 cm from the top with a heated 18-gauge needle.

The apparatus was centrifuged in a swinging bucket rotor for 3 min at 2000 rpm. This forces some excess buffer out of the beads. An aliquot of G-100 buffer equal in volume to the amount of sample that is going to be analyzed is added to the Bio-Gel P-6 resin in the 1.5-mL tube and the tube and culture tube apparatus undergo centrifugation again for 3 min at 2000 rpm. The contents of the culture tube are discarded, and the sample (the volume should not exceed 35 μL) is added to the Bio-Gel P-6 in the 1.5-mL tube. The 1.5-mL tube and culture tube are centrifuged for 3 min at 2000 rpm. The contents of the culture tube (RNA transcripts in G-100 buffer), which is the same volume as the sample which was put on the Bio-Gel P-6, is counted and precipitated as described above.

The type of swinging bucket rotor, the centrifuge time, and centrifuge speed are not important. Comparable results have been obtained by using at least three different rotors, at different centrifuge times and speeds. But the centrifuge time and speed should not be varied between the three centrifugations of the 1.5-mL tube-culture tube apparatus. Changes in the centrifuge time and speed between the three centrifugations will result in a change in the volume of liquid forced through the Bio-Gel P-6 resin.

We have found no detectable difference in the results obtained from samples handled in this way as compared with samples purified on Sephadex G-100 columns, even when samples contained up to 10 mM in NTPs.

Sequencing RNA Transcripts. Sequencing the RNA with 3′-*O*-methylnucleoside triphosphates and *E. coli* RNA polymerase essentially followed the methods of Axelrod et al. (1978). Initiation of transcription was similar to the standard assay except that [α-^{32}P]ATP (100 Ci/mmol) replaced [γ-^{32}P]ATP and ApA (165 μM) was added. During initiation the reaction mixture was divided into five 20-μL aliquots, one for analyzing the positions of each of the four NTPs and one for the control reaction. At the start of elongation, 10 μL of reaction buffer which contained the appropriate NTPs and heparin was added to give a final concentration of 10 μM ATP, 6.7 μM in the NTP being analyzed (except UTP which was 13.2 μM), 165 μM in the remaining two NTPs, and 100 μg/mL heparin. A solution containing the 3′-*O*-methyl analogue of the NTP being analyzed (5 μL) was added to the appropriate reaction mixture at various times after the start of elongation to give optimal termination in the region desired. The final concentration of the 3′-*O*-methyl analogues was ATP 1.0 mM, CTP 0.5 mM, GTP 0.5 mM, and UTP 1.0 mM. The elongation time used before addition of the 3′-*O*-methyl analogue to give termination in a particular region was 0 min for RNA transcripts 0–90 nucleotides[3] in length, 2.5 min for RNA transcripts 90–160 nucleotides in length, 3.5 min for RNA transcripts 160–240 nucleotides in length, and 4.5 min for RNA transcripts 200–300 nucleotides in length. The positions of the pause sites were determined by running samples of our standard reaction in lanes adjacent to the sequencing lanes and directly comparing the pause sites with the sequencing bands. Reactions were allowed to continue for another 10 min and then stopped and phenol extracted, and the RNA product was precipitated as described above.

In these experiments both ATP and the nucleoside triphosphate being analyzed are at fairly low concentrations (10 μM for ATP, 6.7 μM for the NTP). We have run control reactions where the ATP concentration was brought up to 165 μM and we found no changes in the autoradiographs of the sequencing gels.

T_1 and Pancreatic Ribonuclease Digestion. [γ-^{32}P]ATP-labeled 5-min RNA transcripts purified through Bio-Gel P-6 were digested with T_1 and/or pancreatic ribonuclease in 10 mM Tris (pH 7.4) and 1 mM EDTA. For complete digestions, reactions were done at 37 °C for 1 h with enough enzyme to give a 20:1 (w/w) ratio of carrier tRNA to enzyme. After 1 h of incubation, 2 volumes of a solution which was 8 M urea, 20 mM Tris (pH 7.4), 1 mM EDTA, 0.05% xylene cyanol, and 0.05% bromophenol blue was added. The mixture was then analyzed by gel electrophoresis.

Gel Electrophoresis. Gel electrophoresis followed the methods developed by Maxam & Gilbert (1977). Sequencing gels (20%, 12%, 8%, and 6% polyacrylamide) were slabs 0.8 mm thick and 40 cm long and typically were run at 1250 V for variable lengths of time depending on the region to be sequenced. Polyacrylamide (3%)–urea (7 M) gels were slabs 0.8 mm thick and 16 cm long and were run at 200 V until the bromophenol blue ran off the end of the gel.

Precipitated samples were taken up in 7 μL of 8 M urea, 20 mM Tris (pH 7.4), 1 mM EDTA, 0.05% xylene cyanol, and 0.05% bromophenol blue (loading buffer), denatured, and loaded on to the gels according to the methods of Peattie (1979). Samples which had been uniformly labeled with [α-^{32}P]ATP were adjusted with loading buffer so that the same amount of radioactivity was loaded on each sample well. All gels were run with at least two sample wells containing the SV40 DNA *Hin*fI restriction fragments, denatured prior to loading. The length of the ssDNA *Hin*fI restriction fragments was calibrated against RNA transcripts from pause site 1 (122 bases), pause site 2 (164 bases), pause site 3 (248 bases), pause site 4 (279 bases), 18S RNA (1980 bases), and 28S RNA (4590 bases). The RNA lengths for the pause sites were determined from sequencing analysis (see Results) and the 18S and 28S RNA lengths were determined from the molecular weight values given by Lehrach et al. (1977).

Autoradiography and densitometry techniques have been described by Huang & Hearst (1980).

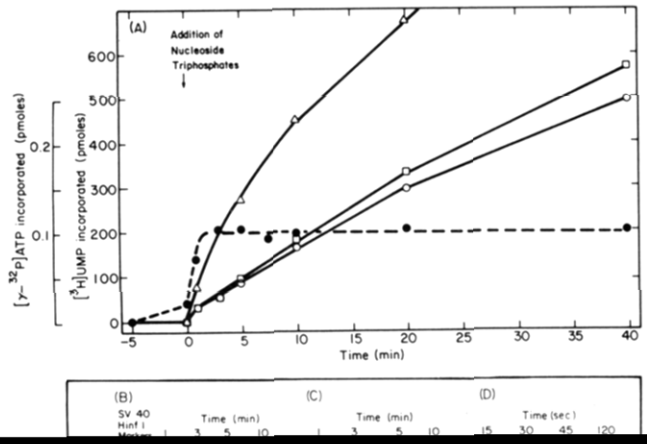
We have found that when RNA transcripts were directly precipitated from reaction buffer (as in the case of labeling with [γ-^{32}P]ATP) and loaded onto the 3% polyacrylamide–7 M urea gels, some radioactivity remained in the sample wells (see Figures 1, 7, and 9[4]). The amount of radioactivity did not depend on the size of the transcripts, and it did not affect the results. It seems to be precipitated radioactive ATP which for some unknown reason does not enter the gel.

Results

At reduced temperatures (18–24 °C) *E. coli* RNA polymerase has a preferred initiation site on SV40 DNA F1 (Zain et al., 1973). To decrease the initiation of RNA transcripts at places other than the preferred site, we have modified the previously determined conditions (Zain et al., 1973) by lowering the molar concentration of enzyme to half the molar concentration of supercoiled SV40 DNA, by making a two-step

[3] The numbering of the bases throughout this paper follows that of Reddy et al. (1978).

[4] Figure 9, discussed in this paper, is included in the supplementary material.



excluded from the Bio-Gel P-6 used in the RNA purification (data not shown).

To observe the actual process of elongation over time, we have labeled the RNA transcripts with ^{32}P and separated them on 3% polyacrylamide–7 M urea gels. Figure 1B shows a time course of the production of RNA transcripts under the standard assay conditions. From this figure, one can see that polymerase transcription is discontinuous. The intensity of bands marking places of discontinuous RNA synthesis first increases and then decreases with time. The bands in the autoradiograph must represent places where polymerase pauses during elongation. The major pause sites which are observed in at least two consecutive time points have been labeled 1 through 9, from the shortest to the longest discernible transcript. There are no pause sites prior to pause site 1 as do

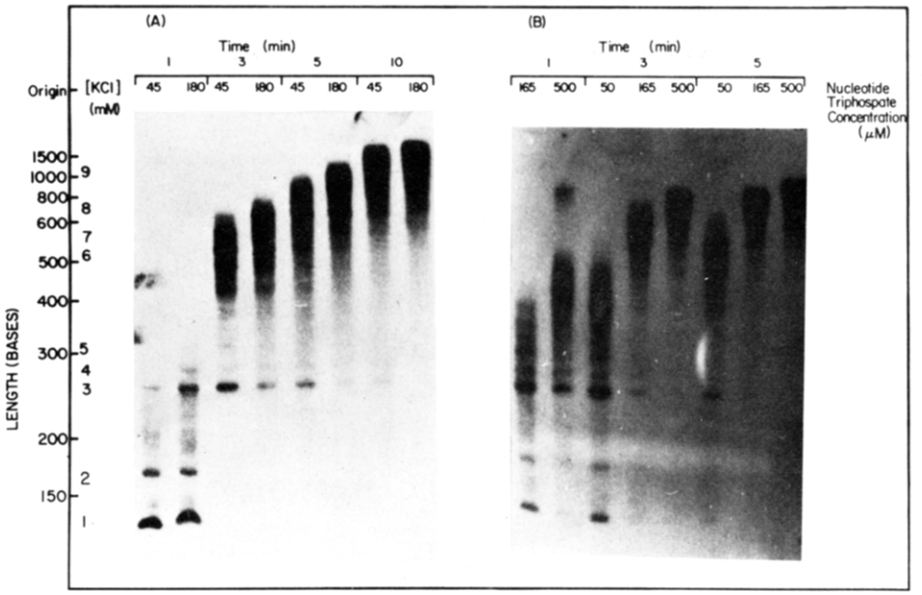


FIGURE 2: Autoradiographs of 3% polyacrylamide–7 M urea gels of the salt and total nucleotide concentration effects on RNA polymerase transcriptional pause sites with SV40 DNA F1 as a template. (A) Salt effects at 45 and 180 mM KCl are shown with the concentration designated at the top of each lane. (B) Nucleotide concentration at 50, 165, and 500 μM for each NTP is shown with the concentration designated at the top of each lane. The reactions were done under standard assay conditions with 165 μM ApA and continuously labeled with [α-^{32}P]ATP.

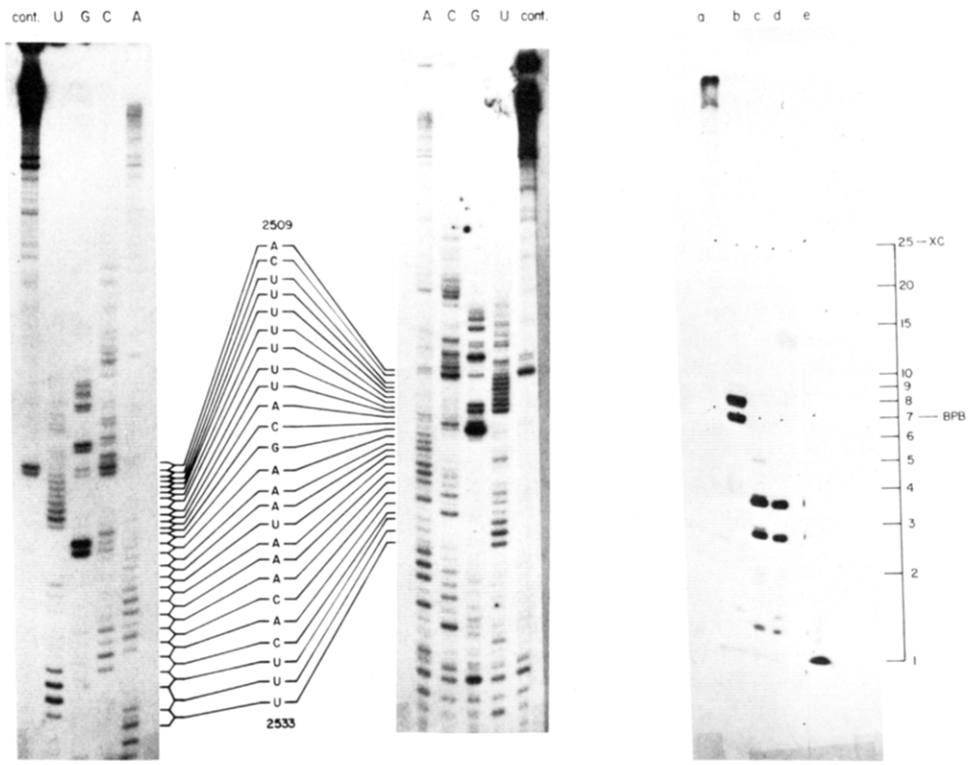


FIGURE 3: Sequencing and ribonuclease digestion of RNA polymerase transcripts of SV40 DNA F1. The left panel shows the autoradiograph of a 12% polyacrylamide–7 M urea gel of transcripts 23–47 nucleotides from the promoter site under standard assay conditions with [α-^{32}P]ATP, 20 μCi per lane. The center panel shows a similar gel over the same region under standard assay conditions with [α-^{32}P]ATP, 20 μCi per lane, and 165 μM ApA. The right panel is the autoradiograph of a 20% polyacrylamide–7 M urea sequencing gel of 5-min SV40 DNA F1 transcripts labeled with [γ-^{32}P]ATP, purified through Bio-Gel P-6 and digested with (a) no ribonuclease, (b) T_1 ribonuclease, (c) pancreatic ribonuclease, and (d) T_1 and pancreatic ribonuclease. Lane e is [γ-^{32}P]ATP. Numbers represent base length of oligonucleotides as determined by a semilogarithmic plot using ATP (1 base), bromophenol blue (~7 bases), and xylene cyanol (~25 bases) as markers. The mobilities of bromophenol blue and xylene cyanol are from experiments using partial digestion products of tRNA as standards (J.-P. Bachellerie, personal communication).

lated types of experiments. In the first experiment, we added ApA as an initiating dinucleotide which should base pair with the thymidines at 2555 and 2556 and give only single bands in the sequencing gel. This is observed in Figure 3, center panel. In the second type of experiment, we have done a complete ribonuclease T_1 digest of RNA transcripts produced under standard assay conditions. Two RNA oligomers seven and eight nucleotides in length (pppAAUAAAGp and pppAAAUAAAGp) are observed (Figure 3, right panel), as predicted from the promoter site (Zain et al., 1974). We have also done a complete digest on the RNA transcripts using pancreatic ribonuclease to show that oligomers three and four

FIGURE 4: Sequence of SV40 DNA in the region of the RNA polymerase transcriptional pause sites. This sequence and the numbering system come from the study of Reddy et al. (1978). Transcription initiates at residues 2556 and 2555 (as marked) and continues transcribing the early strand. The late strand is a representation of the RNA produced when T's are replaced by U's. The underlined sequences are the first four pause sites (see text for details). The sequence positions of the pause sites are as follows: pause site 1, 2434–2437; 2, 2386–2389; 3, 2306–2309; 4, 2280–2283.

nucleotides in length (pppAAUp and pppAAAUp) are produced as predicted from the preferred initiator sequence. Densitometer tracings of the autoradiographs of the digests are in agreement with the sequencing gel and show that the first band has 30% and the second band 70% of the combined radioactivity. We have found that 95% of all the radioactivity is localized in the two oligomers. From these results we believe that the 5′ end of essentially all the pause sites is from the preferred promoter site at 2556 and 2555 on the SV40 genome.

A simple kinetic scheme for RNA polymerase discontinuous elongation on SV40 DNA F1 can be represented as

$$I \xrightarrow{k_0} P_1 \xrightarrow{k_1} P_2 \xrightarrow{k_2} P_3 \xrightarrow{k_3} \text{etc.}$$

I, P_1, P_2, and P_3 represent the initiation complex and the major pause sites, and k_0, k_1, k_2, and k_3 are the kinetic constants for the release of a polymerase molecule from a pause site. In this scheme, pause sites can only be observed if their relaxation time $(1/k)$ is greater than the pause site previous to it. Thus $1/k_0 < 1/k_1 < 1/k_2$, etc., and $k_0 > k_1 > k_2$, etc. The determination of the kinetic constants for the pause sites other than P_1 are very complex, but the equation for any one pause site can be simplified to a first-order rate equation if at some time t' the RNA polymerase molecules have passed through all the previous pause sites (see supplementary material, part I). Figure 1B shows that after 3 min there are essentially no RNA transcripts paused at sites 1 and 2. We have also found that after 30 s there are no transcripts of length shorter than pause site 1 (data not shown). Thus the kinetic equation for pause site 1 is

$$[P_1] = [P_1]_{t'} e^{-k_1(t-t')} \quad (1)$$

where t' is the time when all the RNA transcripts must be of equal or larger size than the RNA transcript of P_1 (30 s) and $[P_1]$ and $[P_1]_{t'}$ are the concentrations of P_1 at time t and t', respectively. A similar equation can be written for pause site 3 (P_3) when t' is equal to 3 min.

By end labeling of the RNA transcripts with [γ-^{32}P]ATP (as in standard assay conditions) and loading of the same amount of radioactivity from each sample, the radioactivity in each band at a certain time point is directly proportional to the concentration of the pause site at that time. Thus the area under the peak of a densitometer tracing can be used to determine $[P_1]$ at any time t. A semilogarithmic plot of the normalized area from densitometer tracings of pause sites 1 and 3 vs. time is shown in Figure 5A. Equation 1 predicts that such a plot should give a straight line, but as observed in Figure 5A, this is not the case. It can be seen that a residual quantity of radioactivity remains even after long time periods. This indicates that while polymerase pauses at specific places, it also terminates to some degree at the same site. The kinetic scheme for such a termination component can be represented by eq 2, where T_1, T_2, and T_3 represent the termination of

$$\begin{array}{ccccccccc} I_0 & \xrightarrow{k_0} & P_1 & \xrightarrow{k_1} & P_2 & \xrightarrow{k_2} & P_3 & \xrightarrow{k_3} & \text{etc.} \\ & & \downarrow{\scriptstyle k_1'} & & \downarrow{\scriptstyle k_2'} & & \downarrow{\scriptstyle k_3'} & & \\ & & T_1 & & T_2 & & T_3 & & \end{array} \quad (2)$$

polymerase at a specific pause site.[5] From this scheme we have derived the linear equation (see supplementary material, part I)

$$\ln([P_1]_{obsd} - [T_1]_{t=\infty}) = -K_1(t - t') + \ln([P_1]_{obsd,t'} - [T_1]_{t=\infty}) \quad (3)$$

[5] We have defined termination as a complete stop or end of transcription where there is no continuation of elongation.

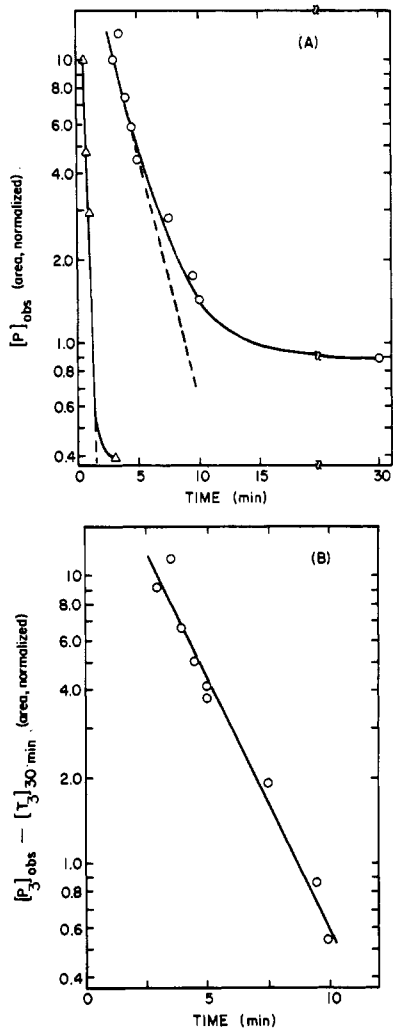


FIGURE 5:. First-order kinetics of pause sites 1 and 3 represented as semilogarithmic plots. (A) Semilogarithmic plot of the normalized area of pause site 1 (▲) and pause site 3 (○) as a function of time. (B) Semilogarithmic plot of the normalized area of pause site 3 minus the normalized area of pause site 3 at 30 min as a function of time (see text for details).

where $[P_1]_{obsd}$ and $[P_1]_{obsd,t'}$ are the concentrations of pause site 1 at times t and t', respectively, $[T_1]_{t=\infty}$ is the concentration of T_1 at infinite time, and $K_1 = k_1 + k_{1'}$.

A plot of eq 3 for pause site 3 (Figure 5B) shows that the data fit remarkably well to a straight line. The best fit of eq 3 to the data yields a slope where $K_1 = 0.40\ min^{-1}$.

While the values of k_1 and $k_{1'}$ cannot be determined with absolute precision, eq 4 can give an estimate of values for k_1

$$k_1 = K_1\left(1 - \frac{[T_1]_{t=\infty} - [T_1]_{t'}}{[P_1]_{obsd,t'} - [T_1]_{t=\infty}}\right) \quad (4)$$

and $k_{1'}$. When this equation is used, $k_3 = 0.38\ min^{-1}$ and $k_{3'} = 0.02\ min^{-1}$, giving a relaxation time for polymerase going through pause site 3 of ~2.5 min and a termination time of ~50 min. (A derivation of eq 4 and an explanation of the determination of k_3 and $k_{3'}$ are given in the supplementary material, part I.) An approximate value for k_1 (from the initial slope in Figure 5A) for pause site 1 is $2.4\ min^{-1}$, which gives a relaxation time of about 25 s.

The reasons for the pause sites must somehow be based on the sequence of the DNA at or near the pause site. We have sequenced the first four pause sites using 3′-*O*-methyl analogues of the NTPs (see supplementary material, part II), and these are presented in Figure 4.

The pause sites are shown as a group of four nucleotides. While the number of nucleotides in a pause site cannot be implicitly deduced from the sequencing gel, we have found that when the concentration of ATP is lowered to 10 μM, a strong pause site occurs on the cytidine residue at position 2509 with decreasing strength on residues 2508, 2507, and 2506 (data not shown). Because of this and the broadness of the bands as seen in the audioradiographs, we feel that the pause sites outlined in Figure 4 contain at least three or four nucleotides. This idea is further strengthened by the knowledge that natural terminator sites in vitro involve three to five nucleotides (Küpper et al., 1978; Rosenberg et al., 1978).

There seems to be a lack of sequence homology between the first four pause sites. All the pause sites which have been sequenced are preceded by a sequence of at least four pyrimidine residues, and the pyrimidine region is preceded by a region high in purine residues. But even this observation is made with reservations. Pause site 2 at 2385 is somewhat removed from the pyrimidine cluster.

It has been known for a number of years that if the concentration of one of the nucleotides is lowered to 15 μM, RNA polymerase can be induced to pause at specific sites during elongation (Darlix et al., 1968; Darlix, 1974; Darlix & Fromageot, 1974; Gilbert et al., 1974; Darlix & Horaist, 1975). The lowering of all the NTP concentrations to 50 μM had little effect on the positions of all the pause sites, but it did lower the elongation rate (see above). Lowering only one NTP to 10 μM and leaving the other three at 165 μM also dropped the total elongation rate; however, changes in the pause sites become dramatic (Figure 6). In general, there are very few new pause sites created by lowering the concentration of any one of the NTPs. But the intensity of a particular pause site was either increased or decreased depending on which NTP concentration had been placed at 10 μM. The results are summarized in Table I. In Table I we have approximated the positions of the pause sites beyond pause site 4 by comparing the electrophoretic mobilities of the RNAs to a set of standard RNAs and single-stranded DNAs.

We have compared the sequences of the first four pause sites with the intensities of the pause sites as a function of which NTP concentration was varied. From these comparisons it is apparent that if the NTP which has the lowered concentration is contained within the four nucleotides in which RNA polymerase pauses, the intensity of that pause site increases over that of the control reaction. In conjunction with this, if the lowered NTP is not contained in the actual pause site, then the intensity of the band at that pause site is decreased. For example, guanosine is only contained in pause sites 1 and 3, and both pause sites are very strong in relation to the other bands in the autoradiograph (Figure 6). Also, uridine is involved with pause sites 1 and 2, and they are very intense, while pause sites 3 and 4 are very weak where uridine residues are not needed for continued elongation.

An exception to this is adenine in pause site 2. One would predict that lowering the ATP concentration to 10 μM should give a very strong band at site 2. This is not the case. Our sequencing procedures are accurate in finding the initial residue of a pause site but are not accurate enough to determine the number of nucleotides at which RNA polymerase pauses (see above). If that size varies according to the site, the estimate of four nucleotides for all sites may be incorrect and possibly only the two NTPs, UTP and CTP, are important in pause site 2, as observed.

In all cases where the concentration of the NTP is lowered, weak pause sites become more intense (e.g., when CTP is made

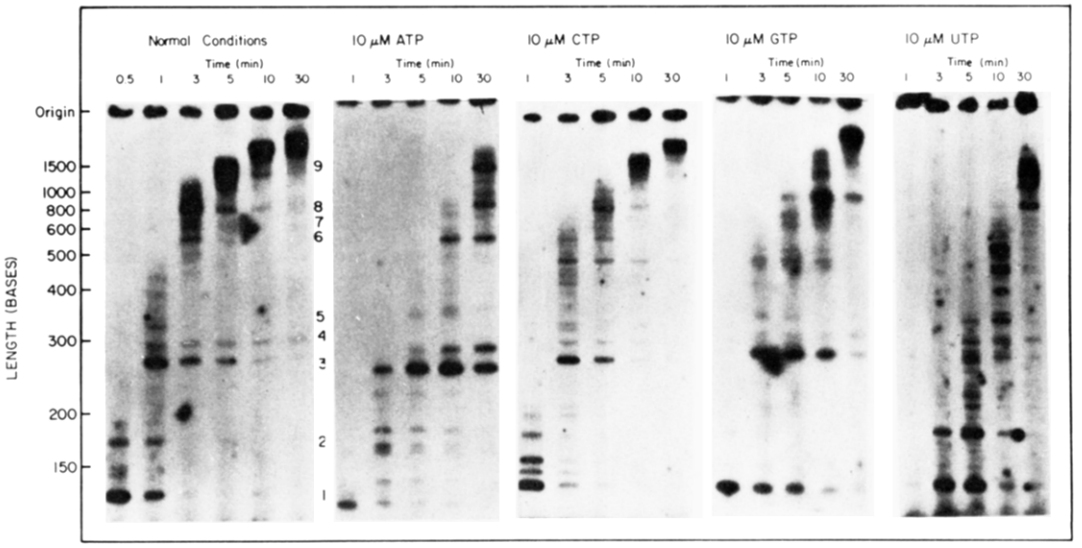


FIGURE 6: Autoradiographs of 3% polyacrylamide–7 M urea gels of the low nucleotide triphosphate concentration effects on RNA polymerase transcriptional pause sites. The nucleotide triphosphate specified has been lowered to 10 μM in concentration while the other three NTP's were kept at 165 μM. Reactions were done under standard conditions with [γ-^{32}P]ATP as the label.

10 μM the two weak bands at 2429 and 2416 are increased). When the analysis presented above is used, these more intense bands must represent weak sites where the lowered NTP is needed in the actual pause site. We also find new weak pause sites which do not occur under standard assay conditions. The sequence of the region of the RNA resolved on our 3% acrylamide–7 M urea gels (Figure 4) shows there are sequences where one nucleotide is dominant. As expected, when the concentration of an NTP is lowered, at regions where that NTP is needed many times in succession, new pause sites stand out (e.g., the weak site observed at 2088 when CTP is at 10 μM corresponds to a sequence of six out of seven residues, which are C at position 2100).

Structures such as DNA–RNA hybrids and/or RNA secondary structure have been implicated in the control of termination. The nucleotide 5′-triphosphate derivatives ITP and BrUTP, which can be incorporated into RNA by *E. coli* RNA polymerase, have been used to study such structures (Lee & Yanofsky, 1977; Neff & Chamberlin, 1978; Adhya et al., 1979). When ITP is incorporated into RNA instead of GTP, the stability of the RNA–DNA hybrid and/or RNA secondary structure should be greatly decreased (Chamberlin, 1965). The substitution of ITP for GTP has little effect on the elongation rate (Neff & Chamberlin, 1978) but has profound effects on the RNA transcripts of SV40 DNA F1 (Figure 7). We observe a whole new set of pause sites different from both the control reaction and the changes observed at low GTP concentrations (Figure 6). Lee & Yanofsky (1977) have pointed out that RNA transcripts where IMP has replaced GMP have a slightly higher electrophoretic mobility on 7 M urea gels than normal RNA transcripts. Even if this change in mobility of the RNA is taken into account in our system, there are a number of strong bands which cannot be correlated to any of the normal pause sites. When one looks at the sequence around the very strong band at 2114, observed when ITP replaces GTP, one sees a stretch of 10 guanosine and uridine residues, sequence 2091–2100, preceded by a region rich in cytidine. Similar types of sequences which are rich in guanosine and uridine are observed at or near the pause sites 2153, 2254, and 2368.

When BrUTP replaces UTP, the elongation rate drops by about a factor of 4 from that of the normal, but the pause sites are affected very little (Figure 7). Table I shows that all the

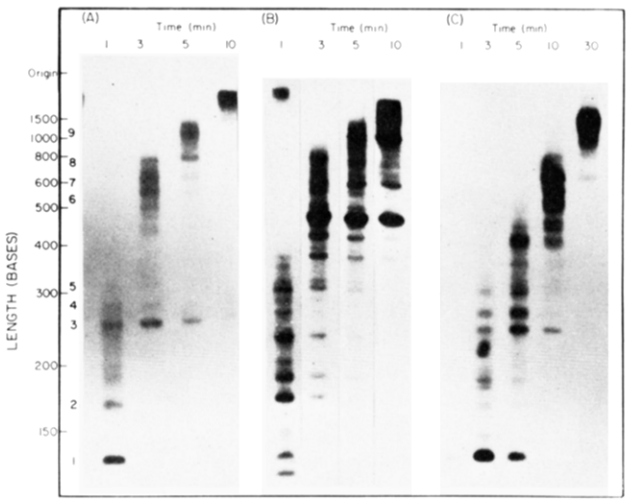


FIGURE 7: Autoradiographs of 3% polyacrylamide–7 M urea gels of ITP and BrUTP effects on RNA polymerase transcriptional pause sites. Under standard assay conditions GTP was replaced by ITP (B) at the same concentration (10 μM during initiation, 165 μM during elongation) and UTP was replaced by BrUTP (C) at the same concentration (20 μM during initiation, 165 μM during elongation). (A) is the control reaction. Reactions were done under standard assay conditions with 165 μM ApA and [α-^{32}P]ATP as a continuous label.

bands observed can be correlated directly to normal pause sites or ones produced from lowering the UTP concentration to 10 μM.

ρ interaction with paused RNA polymerase is thought to cause termination (Rosenberg & Court, 1979; Adhya & Gottesman, 1978). Under our standard assay conditions at 18 °C it takes approximately 10 ρ monomers per RNA polymerase to get optimal inhibition of total incorporation of [^{3}H]UMP (Figure 8A). This high ratio of ρ to RNA polymerase does not seem to be a result of the methods of preparation of ρ but is due to the low temperature used in the assay. At 37 °C, six ρ molecules give the highest inhibition, in agreement with the results of Finger & Richardson (1979).

After 20 min the incorporation of [^{3}H]UMP stopped completely when ρ was added at the beginning of the elongation step (Figure 8B). The inhibition is mainly due to termination of transcripts larger than 300 nucleotides in length (Figure 8D). Even pause sites which are hardly noticeable under

Table I: Location of Pause Sites under Various Conditions[a]

assigned no.	std assay conditions	ATP,[b] 10 μM	CTP, 10 μM	GTP, 10 μM	UTP, 10 μM	+ITP, −GTP	+BrUTP, −UTP	secondary structure[c] sequences	stem size (base pairs)	loop size (bases)	energy[d] (kcal/mol)	5 U's[e] (G,C)
		2510 (s)				2441 (w)						
1	2434 (s)	2432 (w)	2434 (s)	2433 (s)	2434 (s)		2432 (s)					
	2428 (w)		2429 (s)			2430 (w)						
	2415 (w)		2416 (s)					2402–2425	6	5		
2	2392 (w)	2388 (w)			2391 (s)	2386 (s)						
	2374 (s)	2372 (w)										
						2368 (s)						
								2325–2418	10	83	−11.7	
						2322 (s)						
3	2308 (s)	2305 (s)	2308 (s)	2308 (s)	2312 (w)		2305 (s)					2310
						2254 (s)						
4	2277 (w)	2271 (s)	2277 (w)		2277 (w)		2277 (s)					
					2271 (w)							
5	2243 (w)				2243 (w)		2236 (s)					
								2222–2312	14	76	−11.7	
						2197 (s)		2195–2233	10	28	−13.1	
						2153 (s)		2122–2192	10	60	−16.7	
				2121 (w)	2114 (w)	2114 (s)	2119 (w)					
			2099 (s)									
				2088 (w)								
					2061 (w)							
6	2022 (w)	2022 (w)	2022 (w)	2022 (w)			2019 (w)	2015–2037	8	14	−15.5	
						2006 (s)						
				1960 (w)								
7	1939 (w)						1946 (w)					
								1908–1946	9	29	−15.5	
8	1832 (s)	1848 (s)	1856 (s)	1866 (s)	1872 (w)		1856 (s)	1856–1904	9	39	−13.4	
9	1421 (s)	1472 (s)						1447–1464	8	9	−13.2	1433

[a] Location of the pause sites is given in the sequence numbering system of Reddy et al. (1978). The values were determined from plots of the mobility of each band in relation to *Hin*fI restriction fragments of SV40 DNA F1. The letter in parentheses beside each sequence position stands for whether the band was strong (s) or weak (w) with relation to the other bands on the autoradiograph. [b] All other conditions remained the same except for the change denoted. [c] These are the computer predicted double-helical structures found in the same region where pause sites occur (Tinoco and McMahon, personal communication). [d] The stability of the predicted double-helical structures is calculated by a set of rules developed by Tinoco and his co-workers (Tinoco et al., 1973; Borer et al., 1974). [e] RNA polymerase transcribed regions which would produce five or more continuous uridine residues and are preceded by ten bases where at least 50% of the residues are guanosine or cytidine.

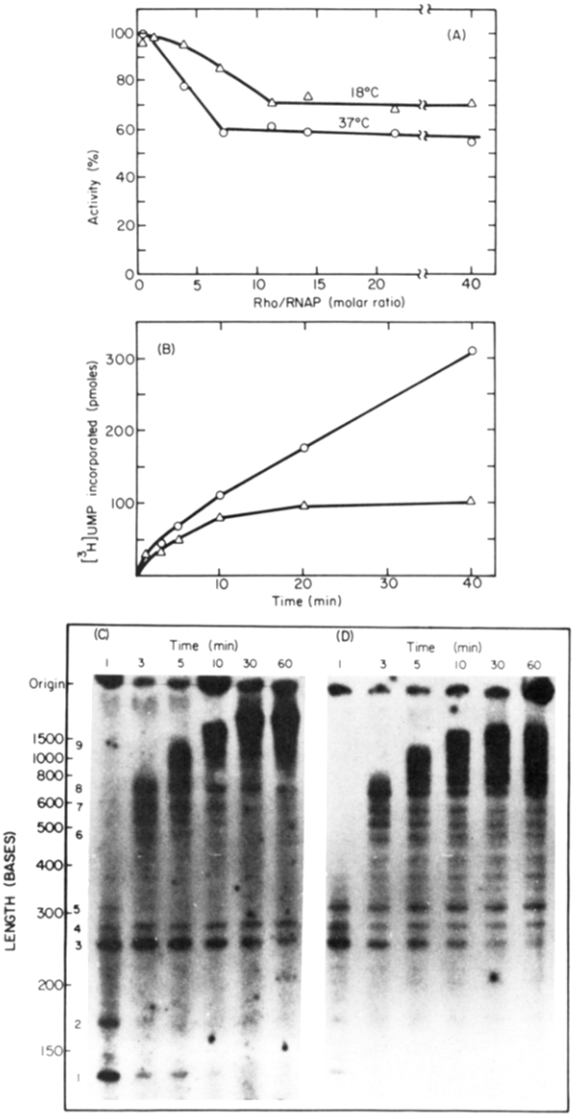


FIGURE 8: Effect of ρ factor on transcription of SV40 DNA F1. (A) The percent of the total incorporation of [^{3}H]UMP into RNA by RNA polymerase as a function of ρ concentration in a 10-min reaction at 18 °C (○) and in a 3-min reaction at 37 °C (△). The molecular weights of RNA polymerase and ρ used to determine the ρ/RNAP ratio are 490 000 (Burgess, 1976) and 50 000 (Roberts, 1969), respectively. (B) Total incorporation of [^{3}H]UMP as a function of time in a standard assay with ρ (△) and without ρ (○). The ratio of ρ to RNA polymerase was 15. Autoradiographs of 3% polyacrylamide–7 M urea gels of a standard reaction without ρ (C) and with ρ (D). All the reactions described used standard conditions except the [KCl] was changed to 45 mM, rifampicin (50 μg/mL) replaced heparin, and when ρ was added the ratio of ρ to RNA polymerase was 15.

normal conditions become terminators in the presence of ρ. In the assay presented here, the pause sites which are observed at longer lengths must have increasingly larger relaxation times and/or significant ability to cause termination (see above). The assay and the results with ρ (Figure 8D) imply that ρ can cause extensive termination at all pause sites which have a relaxation time equal to or greater than that of pause site 5. We can estimate that this pause site must have a relaxation time between 2.5 (the relaxation time of pause site 3) and 4 min at 18 °C.

Discussion

In this presentation, we have described the discontinuous transcription of *E. coli* RNA polymerase using SV40 DNA F1 as a template. The pausing of polymerase on various templates has been observed before (Darlix et al., 1968; Gilbert et al., 1974; Maizels, 1973; Darlix & Horaist, 1975), but in most of these studies the conditions for elongation have been modified by lowering one of the NTP's concentration. Figure 5 shows that the SV40 DNA F1 pause sites are altered when any one of the NTP's concentration is placed at 10 μM while the rest are left at 165 μM. This result makes it questionable whether pause sites induced by lowering the concentration of one of the NTPs are representations of in vitro or in vivo pause sites under normal conditions.

The *E. coli* RNA polymerase transcriptional pauses on SV40 DNA F1 are not artifacts of the assay system. Varying the salt concentration, the concentration of all the NTP's simultaneously, the temperature, and the RNA polymerase concentration or replacing heparin with rifampicin had essentially no effect on the location of the pause sites. These hesitation places must represent some feature in the transcriptional complex which signals a marked decrease in the rate of elongation at a specific position on the SV40 genome.

The sequenced elongation pause sites on SV40 DNA F1 have relaxation times in the range 0.5–3 min at 18 °C. When these values are extrapolated to 37 °C and one notes that the rate of elongation is dependent on those pause sites, the observed rates are less than 1 min ($\simeq$45 s) for the longest pause site which we have sequenced to base resolution (pause site 4). Rosenberg et al. (1978) have shown that RNA polymerase pauses at or near the t_{R1} terminator site of bacteriophage λ when ρ factor is not present in the assay. From their data we calculate the pause to have a relaxation time in the range 2–4 min at 37 °C. If we consider the ternary complex of RNA polymerase, RNA, and DNA found at the Trp attenuator (Stauffer et al., 1978) which is stable for at least 60 min, a pause, the relaxation rate may be hours. This difference in relaxation rates between our SV40 DNA pause sites and those of natural in vitro pause sites indicates that the factors governing hesitation at the pause sites of SV40 DNA F1 would only approximate those of the natural pause sites.

The first step in termination, whether it is ρ dependent or independent, seems to be cessation of elongation (Rosenberg & Court, 1979). The exact nature of the signal is unknown, but an abundance of evidence points to three types of features found at terminator sites. These include (1) a structural feature found in the RNA transcript or DNA near the termination site, (2) a sequence of bases recognized by RNA polymerase, and (3) a G-C rich region between 10 and 15 nucleotides behind the termination point followed by a region of five or more successive U's.

(1) The idea of secondary structure forming in the transcribed RNA just prior to the termination site in the Trp operon (Miozzari & Yanofsky, 1978; Stauffer et al., 1978), the Phe operon (Zurawski et al., 1978), and the Thr operon (Gardner, 1979) has been extensively documented. Rosenberg & Court (1979) have pointed out that in the region preceding the terminator site by approximately 20 nucleotides, some form of dyad symmetry occurs in all the sequenced terminators. Pause sites 2, 3, and 6 have the required dyad symmetry necessary to form the proposed secondary structure requirement (Table I). However, the loop sizes are much larger than those found at natural terminator sites, making this explanation for pausing unlikely.

(2) Of the known ρ-dependent and -independent terminator sites, many seem to have distinctive sequences. The ρ-dependent terminators on λt_{R1} (Rosenberg et al., 1978) and tyrosine tRNA SU_3 (Küpper et al., 1978) end in exactly the same sequence, 5′-CAAUCAA$(OH)_2$-3′. SV40 DNA F1

pause site 2 has a very similar sequence, 5′-CAAUCAU-$(OH)_2$-3′. Stauffer et al. (1978) have pointed out that most of the operon attenuators which have been sequenced show a conserved $GCCN_{12}GG/UGC\ N_{0-1}U_6$ sequence. None of the SV40 DNA F1 pause sites show any resemblance to this sequence.

(3) A feature which is common to all ρ-independent terminators is the existence of a G-C rich region followed by an A-T rich region at the termination site. Gilbert (1976) has suggested that possibly the RNA polymerase will pause in the region of high G-C and become suceptible to termination, since RNA–DNA hybrids with high G-C content are more stable than corresponding DNA–DNA duplexes. Table I shows that only one SV40 DNA F1 pause site (pause site 3) has this property, but it is the only place in which this property occurs in the region which has been studied. We also observe one very strong pause/terminator site when ITP replaces GTP near the RNA sequence of 5′-CCCCACCTGTGT-3′. When IMP replaces GMP in the RNA, this sequence would give a stable region followed by an unstable region in the RNA–DNA hybrid.

Thus, while some of the pause sites resemble the features found at some of the nautral terminator sites, no one feature can explain all of the pause sites. In view of this, and with the knowledge that pause sites have quite different relaxation times (consider pause site 1 and pause site 3), we believe that a number of specific features (possibly all mentioned above and perhaps others) make up the signal for hesitation of elongation.

Two factors, ρ and bacteriophage λN protein, are known to be involved in termination both in vitro and in vivo. ρ increases termination with a release of the RNA, DNA, and polymerase complex, while λN protein represses termination (Adhya & Gottesman, 1978). The results presented here indicate that ρ will cause at least some termination at all pause sites with a relaxation time greater than 2.5–3.0 min at 18 °C or greater than ≅45 s at 37 °C. When the kinetic scheme we have developed is inspected, one sees the action of ρ would be to greatly *increase* $k_{1'}$, the kinetic constant for termination, in relation to k_1, the constant for continued elongation (see eq 2). Extending the model to bacteriophage λN protein, we would expect the opposite to occur; namely, the termination constant ($k_{1'}$) would *decrease* as compared to the continuation constant (k_1).

The complexity of the SV40 *E. coli* RNA polymerase system has limited the extent to which we could characterize the kinetics of the pausing reaction. Simpler systems such as the bacteriophage λt_{R1} (Rosenberg et al., 1978) or the Trp operon (Stauffer et al., 1978) where only one well-characterized pause/terminator site is observed may be more useful in determining the validity of the kinetic model presented here.

Acknowledgments

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Supplementary Material Available

Kinetic details of the mechanistic scheme and a figure showing the sequence of RNA transcripts of RNA polymerase on SV40 DNA F1 (8 pages). Ordering information is given on any current masthead page.

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Kinetic Analysis of Guanosine 5′-Triphosphate Hydrolysis Associated with Tubulin Polymerization†

Marie-France Carlier* and Dominique Pantaloni

ABSTRACT: The correlation between the time courses of pure tubulin assembly and accompanying guanosine 5′-triphosphate (GTP) hydrolysis has been studied at different tubulin concentrations in the range where the rate of assembly varies with a strong cooperativity. One GTP molecule was found hydrolyzed per molecule of tubulin dimer incorporated in the microtubule. This hydrolysis was not strictly coupled to polymerization and occurred in a subsequent step. Consequently, in the first stages of assembly, tubulin–GTP complex is the transient major constituent of microtubules. Kinetic data of GTP hydrolysis have been treated within a model of two consecutive first-order reactions:

$$[\text{tubulin–GTP}]_{\text{free}} \xrightarrow{k_1} [\text{tubulin–GTP}]_{\text{MT}} \xrightarrow{k_2} [\text{tubulin–GDP}]_{\text{MT}} + [\text{P}_i]$$

GTP hydrolysis proceeded at an intrinsic rate $k_2 = 0.25\ \text{min}^{-1}$ independent of tubulin concentration. Simultaneous measurements of polymerization, GTPase activity, and incorporation of [^{3}H]GTP followed by unlabeled GTP chase indicated that before its hydrolysis GTP bound to microtubules was exchangeable while after hydrolysis GDP remained locked in the E site. The possibility is discussed that after assembly tubulin undergoes a conformation change which could trigger GTP hydrolysis and sequestration of GDP.

The complete understanding of the mechanism and regulation of microtubule assembly requires a thorough investigation of the relationship between tubulin polymerization and accompanying guanosine 5′-triphosphate (GTP) hydrolysis.

The question has been considered by many authors, and definite agreement has been reached on several points. It is now established that GTP is hydrolyzed only at the "exchangeable" (E) site of tubulin during polymerization, P_i is released in the medium, and GDP remains blocked in the microtubule (Jacobs et al., 1974; Kobayashi, 1975; Weisenberg et al., 1976; David-Pfeuty et al., 1977). In the absence of free GTP in the medium, polymerization of the preformed tubulin–GTP complex occurs concomitant with the hydrolysis of one molecule of GTP per molecule of tubulin in the microtubule (MacNeal & Purich, 1978a).

Hydrolysis of GTP is involved in two aspects of microtubule assembly, namely, the mechanism and energetics of tubulin–tubulin interaction and the regulation of the equilibrium or steady-state microtubules $\rightleftarrows$ tubulin. Indeed, the irreversible process of GTP hydrolysis at steady state has been the support for a "head-to-tail" polymerization mechanism proposed by Margolis & Wilson (1978) and first explicited by Wegner (1976) in the case of actin, another cytosqueleton contractile protein exhibiting the similar feature of nucleotide hydrolysis accompanying assembly. Puzzling findings raised the problem of the role of and requirement for GTP hydrolysis in microtubule assembly: microtubules can be obtained and stablized in the presence of large amounts of nonhydrolyzable analogues of GTP (Arai & Kaziro, 1976; Penningroth & Kishner, 1977; Weisenberg & Deery, 1976) and have almost the same equilibrium dissociation constant as microtubules formed in the presence of GTP (Karr et al., 1979). It was demonstrated in this laboratory (Carlier & Pantaloni, 1978) and confirmed by others (Karr et al., 1979; Zackroff et al., 1980) that GDP allowed elongation of microtubules if not nucleation and that the equilibrium dissociation constant of microtubules stabilized in the presence of GDP was only twice larger than the one obtained in the presence of GTP.

Use of 8-azido-GTP as a photoaffinity probe indicated that hydrolysis occurred on the β subunit of tubulin concurrently with polymerization (Geahlen & Haley, 1977, 1979). Although experiments performed with drugs inhibiting assembly (David-Pfeuty et al., 1979) show that the GTPase activity of tubulin can be induced in the absence of polymerization, it is nevertheless well established that during microtubule as-

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