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UTP Allosterically Regulates Transcription by *Escherichia coli* RNA Polymerase from the Bacteriophage T7 A1 Promoter

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In the case of *Escherichia coli* RNA polymerase, UTP at elevated concentrations suppresses terminated transcript accumulation during multiple-round transcription from a DNA construct containing the T7 A1 promoter and T_e terminator. The step that is affected by UTP at elevated concentrations is promoter clearance. In an attempt to understand better the mechanism by which UTP regulates this step, we analyzed the effect of UTP on the formation of pppApU in the presence of only UTP and ATP. At elevated concentrations, UTP is a non-competitive inhibitor with respect to ATP in the formation of pppApU. This indicates that the effect of UTP on the formation of pppApU is mediated through an allosteric site. Moreover, the magnitude of the inhibition of pppApU formation is sufficient to account for the decrease in terminated transcript accumulation at elevated UTP concentrations. Thus, it appears that UTP modulates terminated transcript accumulation during multiple-round transcription from this DNA construct by allosteric regulation of promoter clearance at the point of transcription initiation.

Keywords: Escherichia coli RNA polymerase; promoter clearance; transcription initiation; allosteric regulation; transcriptional pausing

Introduction

RNA synthesis as catalyzed by *Escherichia coli* RNA polymerase is a complex multistep process. The first step is promoter search and open complex formation. This is followed by promoter clearance which involves all of the steps up to and including the formation of a stable ternary elongation complex consisting of the enzyme, the DNA template and the nascent RNA transcript. The process of elongation continues until a termination site is reached. At this point, the full-length RNA transcript is released along with the enzyme. Regulation may occur at any step along this pathway and may involve accessory proteins.[1–7]

In most cases, the role of nucleoside triphosphates in regulating the synthesis of full-length transcripts has been postulated to be mediated in a passive way by variations in their affinities for the active site at different points along the template.[4,8–12] To gain further insight into the role of nucleoside triphosphates in regulating the synthesis of full-length transcripts, we used several DNA constructs containing either just the T7 A1 promoter or the T7 A1 promoter along with the T7 T_e terminator. We investigated the effects of ATP and UTP on the production of full-length transcripts. These two nucleotides are involved in transcription initiation at the T7 A1 promoter. At elevated concentrations, UTP but not ATP suppressed the formation of full-length transcripts during multiple-round transcription from the DNA construct that contained the A1 promoter and the T_e terminator. The data obtained here are consistent with a model in which UTP regulates transcription through an allosteric site.

Abbreviations used: CIP, calf intestinal alkaline phosphatase.

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Results

The accumulation of transcripts from the A1 promoter is modulated by UTP levels during multiple-round transcription

The *Rsa*I–*Sal*I fragment from plasmid pARl707 contains the A1 promoter along with the T_e terminator from bacteriophage T7. The 5′ terminal

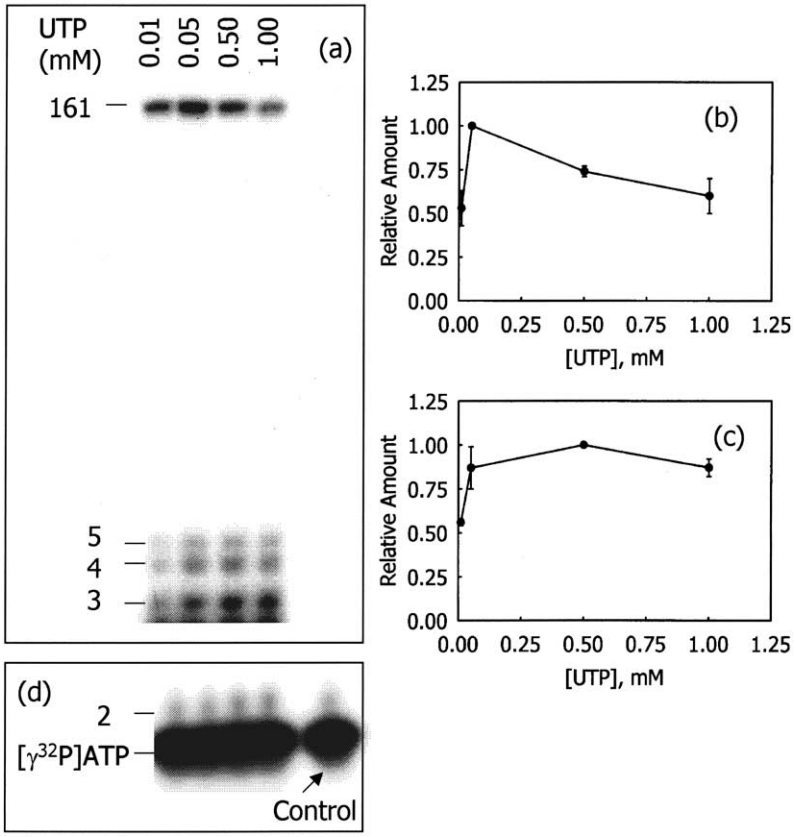

Figure 1. (a) Gel electrophoretic analysis (20% (w/v) polyacrylamide) of products formed during multiple-round transcription from the *Rsa*I–*Sal*I DNA fragment of pARl707 over a time span of 30 minutes as a function of UTP concentration at 37 °C. (All electrophoretic patterns presented here correspond to digital representations. In each case, we have optimized visualization of the bands by varying brightness and contrast settings.) Each reaction mixture contained 12.8 nM active enzyme molecules and 16 nM *Rsa*I–*Sal*I DNA fragments. The concentrations of CTP, GTP and [γ-^{32}P]ATP (1500–3000 cpm/pmol) were each 50 μM. UTP concentrations were varied as indicated in the Figure. Variations in the amounts of the terminated transcript (b) and the 4-mer product (c) formed as a function of UTP concentration. Each point is normalized to the value at a UTP concentration of 0.05 mM in the case of the terminated transcript, whereas they are normalized to the value at a UTP concentration of 0.5 mM in the case of the 4-mer product. Also, each point corresponds to the average of three experiments. (d) Short time exposure of X-ray film showing the variation of the 2-mer abortive product as a function of UTP concentration. The control contains [γ-^{32}P]ATP at the same concentration as that used in the reaction.

sequence of the transcript produced from the A1 promoter is pppApUpCpG.[13] During multiple-round transcription from the A1 promoter, the accumulation of product corresponding to the terminated transcript (161 nucleotides) varied as a function of UTP concentration (Figure l(a)). Maximum accumulation of terminated transcripts occurred at a UTP concentration of 0.05 mM. This accumulation was reduced by 40(±11)% at a UTP concentration of 1.0 mM (Figure l(b)).

In addition to the terminated transcript, abortive products were generated during this reaction time (Figure l(a)). Maximum accumulation of the 4-mer occurred at a UTP concentration of 0.5 mM and the accumulation was reduced by 16(±4)% at a UTP concentration of 1.0 mM (Figure l(c)). A similar UTP concentration dependency was observed for the 5-mer abortive product (plot not shown). The accumulation of the 3-mer reached a maximum at a UTP concentration of 0.5 mM and remained constant (plot not shown). At an exposure time of the X-ray film appropriate for analyses of the terminated transcript as well as the 5, 4 and 3-mers, it was not possible to analyze the 2-mer. In an attempt to determine the variation of the 2-mer as a function of UTP concentration, we decreased the exposure time of the X-ray film. The accumulation of the 2-mer could still not be analyzed under these conditions due to the presence of a contaminant in the [γ-^{32}P]ATP that appears to comigrate with the 2-mer product (Figure 1(d)). The intensity of the contaminant band is comparable to or less than the intensities of the bands corresponding to the reaction mixtures. Although this prevented a quantitative analysis of the 2-mer product, qualitatively it appears that the accumulation of this product reached a maximum at a UTP concentration of 0.5 mM and then decreased slightly at a UTP concentration of 1 mM. Based on these data, a mechanism involving increased abortive cycling cannot account for the decrease in the accumulation of terminated transcripts at elevated UTP concentrations. Because there are no bands between those corresponding to the terminated and abortive products (Figure 1(a)), arrested elongation complexes can also be eliminated as the cause of the suppression of terminated transcript accumulation at elevated UTP concentrations.

Electrophoretic analyses of terminated transcript accumulation as a function of time are given in Figure 2(a) (0.05 mM UTP) and (b) (1.0 mM UTP). Plots of the amount of terminated transcripts formed as a function of time are linear over the time span of 5–30 minutes at UTP concentrations of 0.05 and 1.0 mM (Figure 2(c)) as well as at UTP

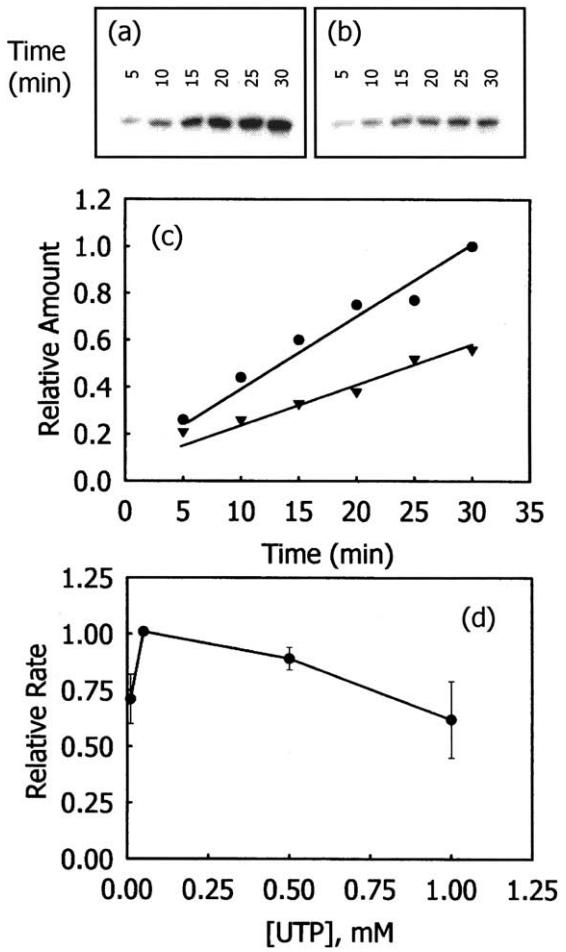

Figure 2. Gel electrophoretic pattern (20% (w/v) polyacrylamide) of terminated transcript formation as a function of time during multiple-round transcription from the *Rsa*I–*Sal*I DNA fragment of pAR1707 in the presence of (a) 0.05 mM UTP and (b) 1.0 mM UTP at 37 °C. The concentrations of enzyme and DNA fragment were the same as those given in the legend to Figure 1. The concentrations of [α-^{32}P]CTP (750–1000 cpm/pmol), GTP and ATP were each 50 μM. (c) Variations in the formation of terminated transcripts during multiple-round transcription in the presence of 0.05 mM (●) and 1.0 mM (▼) UTP as a function time. The data are normalized to the value at 30 minutes in the presence of 0.05 mM UTP. (d) Variation in the relative overall rates for terminated transcript accumulation as a function of UTP concentration. The values at 0.01 and 0.5 mM UTP correspond to the average of two determinations and the values at 0.05 and 1.0 mM UTP correspond to the average of six determinations. Each point is normalized to the value at 0.05 mM UTP.

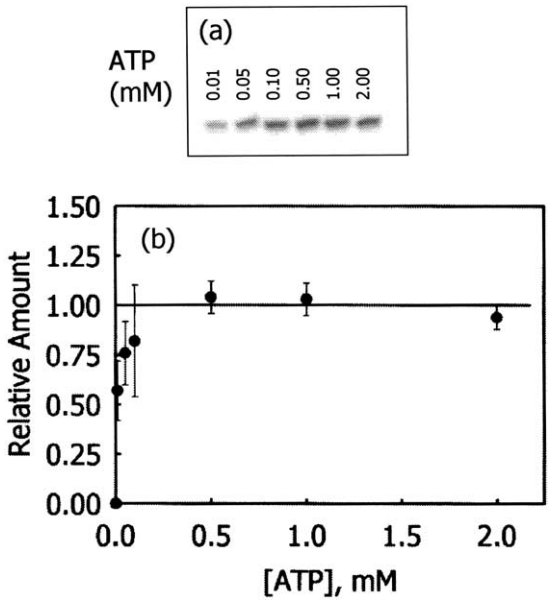

Figure 3. (a) Gel electrophoretic analysis (20% (w/v) polyacrylamide) of products from the *Rsa*I–*Sal*I DNA fragment of pAR1707 over a time span of 30 minutes during multiple-round transcription as a function of ATP concentration at 37 °C. The concentrations of enzyme and DNA fragment were the same as those given in the legend to Figure 1. The concentrations of [α-^{32}P]CTP (750–1000 cpm/pmol), GTP and UTP were each 50 μM. ATP concentrations are indicated in the Figure. (b) Variation in terminated transcript formation as a function of ATP concentration. Each point corresponds to the average of six independent experiments and the data are normalized by the average of the values over the ATP concentration range of 0.5–2.0 mM.

concentrations of 0.01 and 0.5 mM (data not shown). Based on promoter clearance studies that are presented later, most of the RNA polymerase molecules are in the recycling phase after approximately five minutes. Linear plots over the time range of 5–30 minutes indicate that most of the RNA polymerase molecules are able to recycle after termination, that the nucleoside triphosphates are not depleted during the course of the reaction and that there is no product inhibition. The dependency of the relative rate of terminated transcript accumulation on UTP concentration is illustrated in Figure 2(d). The maximum relative rate for terminated transcript accumulation occurred at a UTP concentration of 0.05 mM and that rate was reduced by approximately 38% at a UTP concentration of 1 mM. This is consistent with the data in Figure 1(b).

The ATP concentration in the studies reported in Figures 1 and 2 was 50 μM, whereas it was 2 mM in studies that will be discussed later on the effect of UTP concentration on the formation of pppApU from ATP and UTP. This concentration of ATP allowed us to simplify the analysis of the UTP concentration dependency of pppApU formation. To ascertain whether the UTP concentration dependency of terminated transcript accumulation is independent of ATP concentration, we analyzed the system at an ATP concentration of 2 mM on a 20% polyacrylamide gel. For these experiments, we used [α-^{32}P]CTP at a concentration of 50 μM to monitor the formation of terminated transcripts and the GTP concentration was 50 μM. Under these conditions, maximum accumulation of terminated transcripts occurred at a UTP concentration of 0.05 mM and this accumulation was reduced by 23(±6)% at a UTP concentration of 1.0 mM during

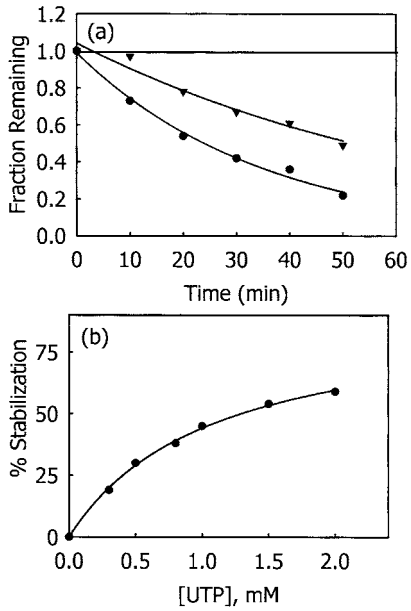

Figure 4. (a) Heparin displacement of the T7 A1 promoter (3.2 nM ^{32}P-end labeled *Rsa*I-*Sal*I fragment of pAR1707 at a specific radioactivity of approximately 18×10^6 cpm/nmol) from RNA polymerase (2.5 nM active enzyme molecules) in the absence (●) and presence (▼) of UTP (2 mM) at 37 °C. The curves through the data correspond to exponential fits. (b) Concentration dependency of UTP stabilization of the T7 A1 promoter–RNA polymerase complex (5 nM active enzyme molecules and 6.4 nM A1 fragment) against heparin displacement. Each point corresponds to the percentage increase in the amount of complex retained on the filter relative to that retained in the absence of UTP after a heparin challenge of 15 minutes. The curve through the data corresponds to a hyperbolic fit.

a reaction time of 30 minutes (average of five independent determinations; data not shown). Although the overall trends of UTP concentration dependencies for terminated transcript accumulation are the same at 0.05 and 2 mM ATP, the extent of inhibition in the presence of 2 mM ATP is less. We will address this point further in the Discussion.

In contrast to the results obtained with UTP, ATP at elevated concentrations did not suppress the accumulation of terminated transcripts during multiple-round transcription from the A1 promoter (Figure 3). These data provide additional evidence that the suppression of terminated transcript accumulation at elevated UTP concentrations is not due to product inhibition by either the terminated or abortive products. If product inhibition was the source of the variation in terminated transcript accumulation as a function of UTP concentration, then elevated concentrations of ATP should have produced the same results. Thus, although it has been reported that elevated concentrations of RNA inhibit transcription,[14] apparently insufficient terminated transcripts were produced to have any effect on RNA synthesis in this case.

In all of the studies discussed above, the reactions were initiated by mixing solutions containing the A1 promoter and RNA polymerase with solutions containing the nucleotides. Thus, the open complex was preformed for the first round of transcription. During the recycling phase, the holoenzyme has to reassociate with the A1 promoter and form an open complex. To ascertain whether the results are the same when the open complex is not preformed for the first transcriptional cycle, we initiated the reactions by mixing solutions containing the A1 promoter and nucleotides with solutions containing the protein. For these studies, we used [α-^{32}P]CTP at a concentration of 50 μM to monitor the formation of the terminated transcript. The concentration of ATP was 2 mM, the concentration of GTP was 50 μM and the concentration of UTP was varied. Under these conditions, maximum accumulation of terminated transcripts occurred once again at a UTP concentration of 0.05 mM and this accumulation was reduced by 25(±3)% at a UTP concentration of 1.0 mM during a reaction time of 30 minutes (average of three independent determinations; data not shown). This value is comparable to the value observed when the reaction was initiated by adding nucleotides to a solution containing the preformed open complex. Thus, starting with a preformed open complex does not alter the UTP concentration dependency of terminated transcript accumulation.

Interaction of UTP with the RNA polymerase–A1 promoter complex as monitored in filter binding assays

In previous filter binding studies, it was demonstrated that the A1 promoter is directly displaced from RNA polymerase by heparin.[15,16] We extended these studies in order to investigate the interaction of UTP with the RNA polymerase–A1 promoter complex. At elevated concentrations, UTP stabilized the RNA polymerase–A1 promoter complex against heparin disruption (Figure 4(a)). The concentration dependency for the UTP stabilization of the RNA polymerase–A1 promoter complex is illustrated in Figure 4(b). Analyses of several binding isotherms yielded a value of 1.3(±0.4) mM for the apparent K_d in the binding of UTP to the RNA polymerase–A1 promoter complex at 37 °C. These results indicate that the decrease in terminated transcript accumulation is not due to a UTP-mediated decrease in the stability of the RNA polymersae–A1 promoter complex at elevated UTP concentrations.

Interaction of UTP with RNA polymerase as monitored by fluorescence spectroscopy

In the presence of UTP (4 mM) but the absence of DNA, there was approximately a 62% decrease in the intensity of the fluorescence spectrum of RNA polymerase (44 nM) at a wavelength of 340 nm; however, there was no discernible shift in

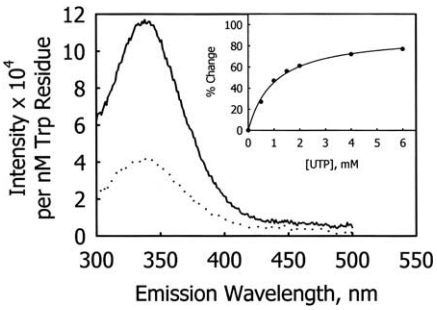

Figure 5. Emission fluorescence spectra of RNA polymerase (44 nM total enzyme concentration) in the absence (continuous line) and presence (dotted line) of UTP (4 mM). Each spectrum represents the average of five scans and has been normalized to the concentration of tryptophan residues present. The inset shows the fluorometric titration of RNA polymerase by UTP.

the wavelength maximum at 340 nm (Figure 5). In the absence of detailed information on the location of the tryptophan residues relative to the UTP binding site, it is not possible to state whether this perturbation is due entirely to a UTP-induced conformational change in RNA polymerase or if there is a contribution due to direct interaction of UTP with tryptophan residues resulting in quenching. The binding isotherm obtained in the fluorometric titration of RNA polymerase with UTP is hyperbolic (Figure 5, inset). Analyses of several titrations yielded a value of 0.8(±0.3) mM for the K_d of UTP binding to RNA polymerase at 25 °C. In studies that were limited to UTP concentrations of less than 0.2 mM, Wu and Goldthwait[17] observed no effect of UTP on the fluorescence spectrum of RNA polymerase. (No DNA was present.) Inspection of the data in the inset of Figure 5 indicates, in agreement with the data of Wu and Goldthwait,[17] that there is little if any effect of UTP on the fluorescence spectrum of RNA polymerase at UTP concentrations less than 0.2 mM.

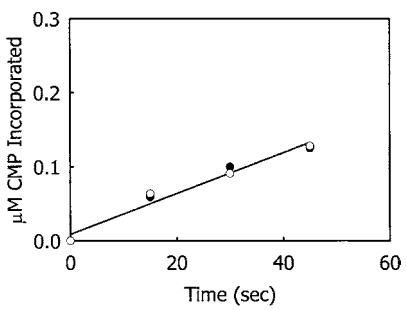

Figure 6. Incorporation of [^{32}P]CMP into RNA during elongation in the presence of 0.05 mM (●) and 1.0 mM (○) UTP, respectively, at 37 °C. The zero time point corresponds to the time of mixing the preformed A20 complex with the nucleotide cocktail containing [α-^{32}P]CTP. The concentrations of GTP, ATP and [α-^{32}P]CTP (300–500 cpm/pmol) in the reaction mixtures were all 50 μM.

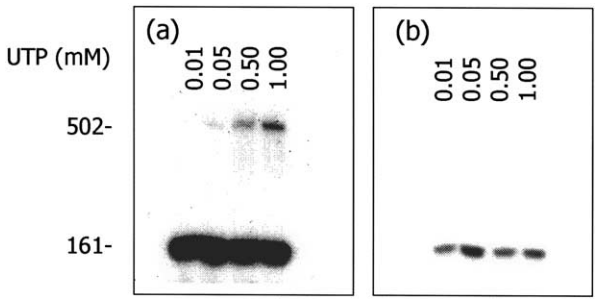

Figure 7. Gel electrophoretic analysis (8% (w/v) polyacrylamide) of termination efficiency as a function of UTP concentration during multiple-round transcription from the *Rsa*I–*Sal*I DNA fragment at 37 °C. The concentrations of enzyme and DNA fragment were the same as those given in the legend to Figure 1. The concentration of ATP was 2 mM and the concentrations of GTP and [α-^{32}P]CTP (750–1000 cpm/pmol) were each 50 μM. (a) and (b) correspond to the same autoradiogram at different brightness and contrast settings.

Effect of UTP on transcription elongation

In order to monitor the elongation process separate from promoter clearance, we used an elongation complex (A20) that was paused at position +20 of the transcript. Synchronously paused ternary complexes prepared by exclusion of one or more nucleotides from the reaction mixture have been used previously to study the elongation process.[9,18–21] For this purpose, we used the *Rsa*I–*Pvu*II DNA fragment isolated from plasmid pAR1435. This DNA fragment contains the A1 promoter but not the T_e terminator and is capable of producing a 1750 nucleotide runoff transcript. The incorporation of [^{32}P]CMP into RNA occurred at identical rates at UTP concentrations of 0.05 and 1.0 mM in the case of this DNA fragment (Figure 6). Similar results were obtained at a UTP concentration of 1.5 mM (data not shown). Because Rhodes and Chamberlin[22] reported that UTP at concentrations of either 5 or 10 mM competitively inhibited transcription elongation in the case of poly[r(A–U)] synthesis, we investigated the effect of UTP at a concentration of 5 mM in the case of the *Rsa*I–*Pvu*II DNA fragment. At this concentration of UTP, there was a 29(±4)% decrease in the rate of [^{32}P]CMP incorporation (data not shown). Values of 0.5 mM,[23] 0.7 mM[24] and 1.4 mM[25] have been reported for the total UTP concentration in *E. coli*. Thus, although UTP at concentrations equal to or greater than 5 mM inhibits transcription elongation, these UTP concentrations are far greater than the reported values in *E. coli* cells and this inhibition probably plays no role in modulating the overall process of transcription within the cell. Up to a concentration of at least 1.5 mM, it appears that UTP has little if any effect on transcription elongation in this system.

Table 1. Estimates of the termination efficiency at the T7 T_e terminator during multiple-round transcription as a function of UTP concentration

[UTP] (mM)	Termination efficiency (%T)	
	2 mM ATP[a]	0.05 mM ATP[b]
0.01	>99	99(±0.8)
0.05	99	97(±0.5)
0.50	98(±1)	96(±1.4)
1.00	97(±2)	94(±0.5)

Transcript formation was monitored by the incorporation of [^{32}P]CMP. Therefore, in the calculation of the termination efficiencies, the intensities of the bands were corrected for CMP content. The termination efficiencies were determined by using the equation, $\%T = (100)(\text{read through})/(\text{terminated} + \text{read through})$. The concentrations of UTP, GTP and [^{32}P]CTP were all 0.05 mM.

[a] Each value corresponds to the average of three independent determinations.

[b] Each value corresponds to the average of four independent determinations.

Effect of UTP on transcription termination

During multiple-round transcription from the *Rsa*I–*Sal*I fragment of pAR1707, read through occurred at the T_e terminator (Figure 7). There are no bands between the terminated (161 nucleotides) and the read through (502 nucleotides) transcripts that would be indicative of arrested elongation transcripts. As is apparent from the data in Table 1, there was a slight decrease in termination efficiency at the T_e terminator as a function of increasing UTP concentration during multiple-round transcription. The trends are similar at ATP concentrations of 0.05 and 2 mM. The values of the termination efficiency at a UTP concentration of 0.05 mM are comparable to the value obtained by Reynolds *et al.*[26] during single-round transcription at a nucleotide concentration of 0.04 mM. The data for multiple-round transcription indicate that the decrease in the amount of terminated transcript accumulation at elevated UTP concentrations is not due to enhanced read through at the T_e termination site.

Promoter clearance is modulated by UTP levels

Promoter clearance can be monitored by the appearance of short terminated transcripts as a function of time during single-round transcription. Such studies are illustrated in Figure 8 for the DNA construct containing the T7 A1 promoter and T_e terminator. At a UTP concentration of 0.05 (Figure 8(a)) but not 1.0 mM (Figure 8(b)), there is a prominent band that appears to correspond to paused transcripts. The length of the paused transcripts is between 70 and 80 nucleotides based on DNA markers (data not shown). The size of these paused transcripts corresponds to the location of the linker DNA that Studier used to construct plasmid pARl707 (personal communications). The linker DNA yields nucleotides 47–68 of the

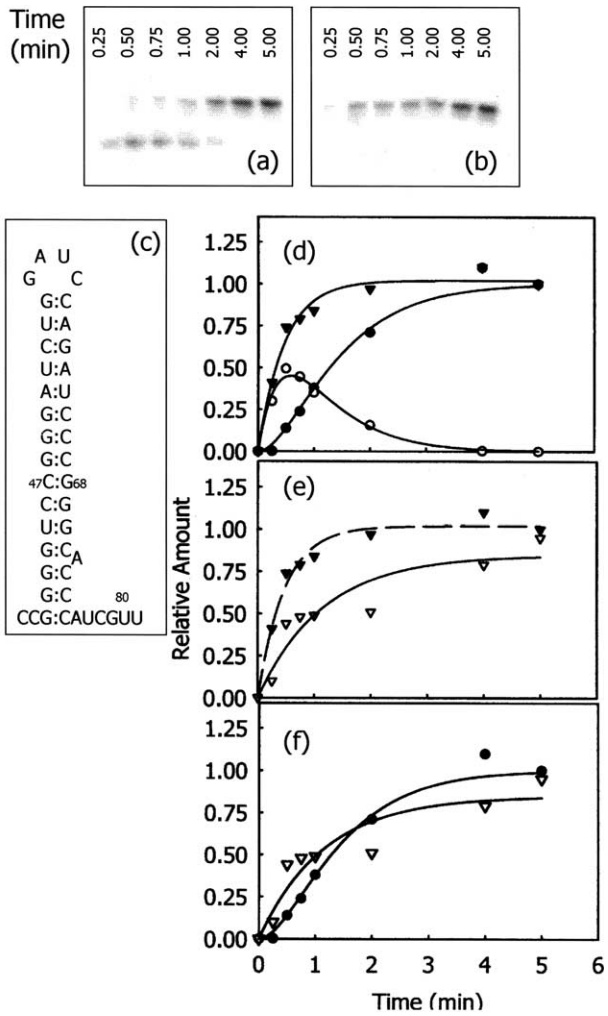

Figure 8. Gel electrophoretic analysis (20% (w/v) polyacrylamide) of products formed during single-round transcription from the *Rsa*I–*Sal*I DNA fragment of pAR1707 over a time span of five minutes in the presence of 0.05 (a) and 1.0 (b) mM UTP at 37 °C. Each reaction mixture contained 25.6 nM active enzyme molecules and 32 nM DNA fragment. The concentrations of CTP, GTP and [γ-^{32}P]ATP (1500–3000 cpm/pmol) were each 50 μm. (c) Sequence and structure of predicted hairpin. This structure was determined by using version 3.6 of RNAstructure (1996–2001) for Windows NT (D. H. Mathews, M. Zucker, & D. H. Turner). (d) Variations of terminated transcripts (●), paused transcripts (○) and the sum of terminated and paused transcripts (▼) as a function of time in the presence of 0.05 mM UTP. All values in this Figure are normalized to the value of the terminated transcript at five minutes in the presence of 0.05 mM UTP. The theoretical curves through the data in this Figure were obtained as indicated in Materials and Methods. (e) Variations of the sum of terminated and paused transcripts in the presence of 0.05 mM UTP (▼) and of terminated transcripts in the presence of 1.0 mM UTP (▽). (f) A direct comparison of the variations of terminated transcripts in the presence of 0.05 mM (●) and 1.0 mM (▽) UTP.

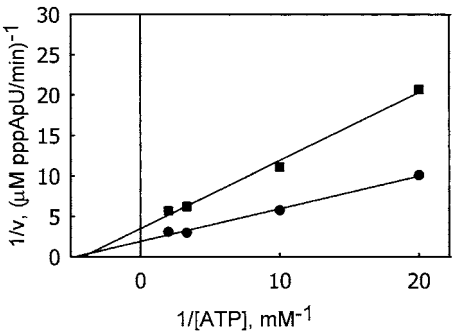

Figure 9. Double reciprocal plots of $1/v$ *versus* $1/[ATP]$ at [α-^{32}P]UTP concentrations of 0.05 mM (●) and 1.0 mM (■) mM. The reaction mixtures contained 2.5 nM active RNA polymerase molecules, 3.2 nM A1 promoter and the indicated amounts of ATP in the presence of either 0.05 or 1.0 mM [α-^{32}P]UTP (200–300 cpm/pmol). Each point corresponds to the average of single time point assays done in duplicate.

transcript that are capable of forming a hairpin (Figure 8(c)). Therefore, this site corresponds from a structural standpoint to a class I pause site.[27,28] The variations in the amounts of paused and terminated transcripts, respectively, as a function of time are shown in Figure 8(d) in the case of a UTP concentration of 0.05 mM. There is a lag in the appearance of terminated transcripts and the variation in the amount of paused transcripts goes through a maximum. We fitted the data by using a model for terminated transcript formation involving sequential first-order reactions for promoter clearance and pause site escape:

RNA polymerase-T7 A1 promoter + NTPs

$\downarrow k_1$

Paused transcripts + NTPs

$\downarrow k_2$

Terminated transcripts

The theoretical fit of the variation of paused transcripts as a function of time yielded unique values for the rate constants with small associated errors. Analyses of three separate data sets yielded a value of $1.9(\pm 0.1)\ min^{-1}$ for the rate constant for promoter clearance and a value of $1.2(\pm 0.2)\ min^{-1}$ for the rate constant for pause site escape. As is apparent from the theoretical curve through the data points in Figure 8(d), the model for promoter clearance and pause site escape involving sequential first-order processes provides a good fit to the experimental data for the variation of paused transcripts as a function of time. Back extrapolation of the experimental data corresponding to pause site escape indicates that this pause site is 90(±20)% efficient (based on three determinations); i.e. most of the RNA polymerase molecules pause at this site. In the case of the variation of terminated transcripts as a function of time, the theoretical fit did not yield unique values for the rate constants for promoter clearance and pause site escape. Moreover, the associated errors were very high. As is apparent in Figure 8(d), there are two well-resolved portions of the curve for the variation of the paused transcripts, whereas there is simply a gradual increase in the curve for the variation of terminated transcripts. Thus, it is easier to obtain reliable and unique estimates of the respective rate constants in the case of the paused transcripts. This is especially true if the values of the rate constants vary only slightly. Also shown in Figure 8(d) is the variation of the sum of paused and terminated transcripts as a function of time. These data obey first-order kinetics, and the value of the rate constant based on the analyses of three separate data sets is $1.5(\pm 0.2)\ min^{-1}$.

The variation of terminated transcripts as a function of time at a UTP concentration of 1.0 mM obey first-order kinetics (Figure 8(e)). Also shown in Figure 8(e) is the variation of the sum of the paused and terminated transcripts at a UTP concentration of 0.05 mM as a function of time (broken line) from Figure 8(d). This clearly illustrates that the clearance time at a UTP concentration of 0.05 mM is faster than that at a UTP concentration of 1.0 mM. The value of the rate constant for promoter clearance at a UTP concentration of 1.0 mM obtained from the analyses of three separate experiments is $0.9(\pm 0.2)\ min^{-1}$. This represents approximately a 53% decrease in the value of the rate constant for promoter clearance at a UTP concentration of 1.0 mM relative to the value at 0.05 mM UTP.

Although pausing occurred at a UTP concentration of 0.05 mM but not 1 mM, more terminated transcripts accumulated during multiple-round transcription at a UTP concentration of 0.05 mM over a time span of 30 minutes. This is due to the fact that promoter clearance and pause site escape at a UTP concentration of 0.05 mM are both faster processes than promoter clearance at a UTP concentration of 1.0 mM. Inspection of the data and the theoretical fits in Figure 8(f) indicates that accumulation of terminated transcripts at a UTP concentration of 0.05 mM does not exceed accumulation of terminated transcripts at a UTP concentration of 1.0 mM until about two minutes after initiation of the reactions for this data set. The range for all data sets is between two and four minutes.

The above experiments were conducted at an ATP concentration of 0.05 mM. Under conditions where the concentration of ATP was 2 mM and that of UTP was 0.05 mM, promoter clearance was too rapid to allow us to obtain a value of the rate constant for promoter clearance by using this approach. Therefore, we could not ascertain whether the magnitude of the UTP effect on promoter clearance displayed the same dependency on ATP concentration as that observed for terminated transcript accumulation.

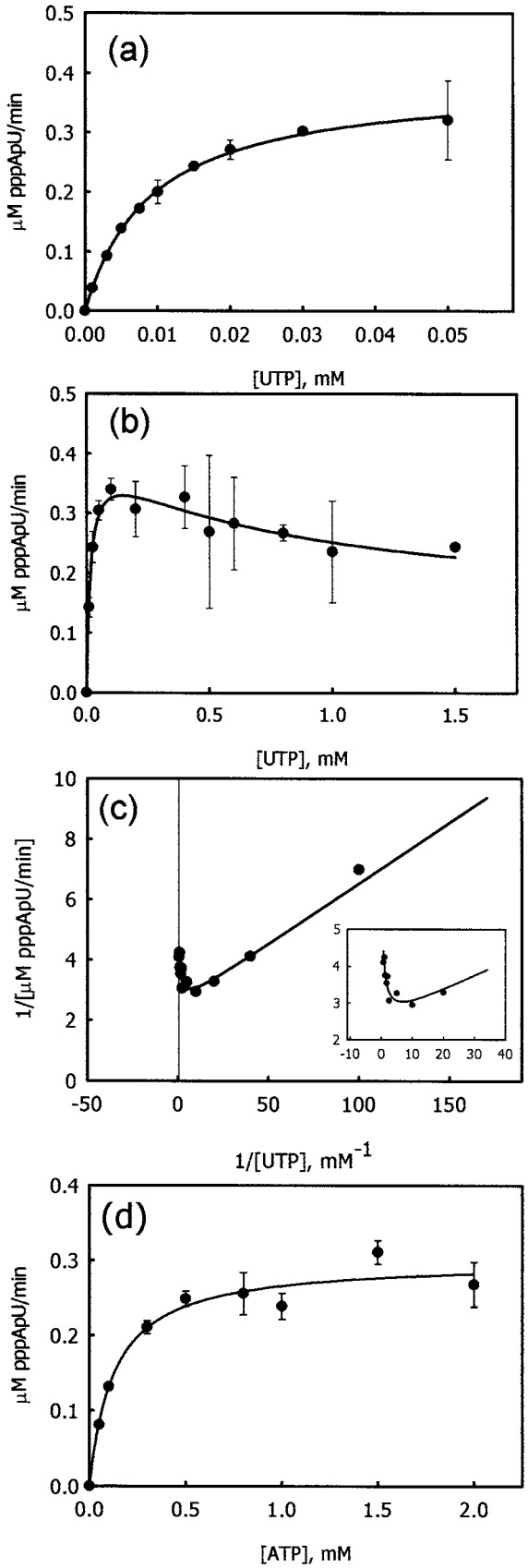

Figure 10. The reaction mixtures contained 2.5 nM active RNA polymerase molecules, 3.2 nM A1 promoter (*Rsa*I–*Sma*I fragment from pAR1707), 2 mM ATP and the indicated amounts of [α-^{32}P]UTP (200–300 cpm/pmol). Each point corresponds to the average of single time point assays done in duplicate. Concentration dependencies for the rate of pppApU formation over the [α-^{32}P]UTP concentration ranges of 0–0.05 mM (a) and 0–1.5 mM (b). (c) Double reciprocal plot of $1/v$ *versus* 1/[UTP] of the experimental data given in (b). The inset more clearly illustrates the fit at higher UTP concentrations. (d) Concentration dependency for the rate of pppApU formation over the ATP concentration range of 0–2 mM.

Effect of UTP on formation of first phosphodiester bond

In the reaction between ATP and UTP as catalyzed by RNA polymerase from the A1 promoter, elevated concentrations of UTP inhibited the formation of pppApU. On the paper chromatogram, there was no radioactivity above background in regions where longer products should migrate. Therefore, the decrease in radioactivity corresponding to the pppApU product at elevated UTP concentrations cannot be attributed to the conversion of pppApU to longer transcripts. To determine the mechanism of UTP inhibition of pppApU formation, we analyzed the reaction as a function of ATP concentration in the presence of 0.05 and 1.0 mM UTP, respectively. Double reciprocal plots of the experimental data illustrating the effect of UTP on this reaction are given in Figure 9. The two lines intersect on the abscissa. Thus, UTP at a concentration of 1.0 mM is a non-competitive inhibitor with respect to ATP. These results are consistent with an allosteric mechanism for UTP suppression of pppApU synthesis. Further analysis of the data by using the equation $V_{max,i} = V_{max}/(1 + [UTP]/K_d)$ where $V_{max,i}$ is the velocity of the reaction in the presence of 1 mM UTP yielded a value of 1.6($\pm$0.5) mM for the apparent K_d for UTP binding at the putative allosteric site. This value is nearly identical to that obtained in the experiments on the UTP-stabilization of the open complex against heparin disruption (i.e. $K_d = 1.3(\pm 0.4)$ mM).

The equilibria corresponding to an allosteric model for the mechanism of action of UTP at elevated concentrations under the condition that [ATP]$\gg$[EP$_o$] are:

$$\begin{array}{ccc} & \overset{K_{UTP,L}}{} & \\ EP_o\text{-}ATP_A + UTP & \rightleftharpoons & UTP_L\text{-}EP_o\text{-}ATP_A \\ + & & + \\ UTP & & UTP \\ \Updownarrow K_{UTP,A} & & \Updownarrow K_{UTP,A} \\ & \underset{K_{UTP,L}}{} & \\ EP_o\text{-}ATP_AUTP_A + UTP & \rightleftharpoons & UTP_L\text{-}EP_o\text{-}ATP_AUTP_A \\ \downarrow k_{cat} & & \downarrow \beta k_{cat} \\ EP_o + pppApU + PP_i & & UTP_L\text{-}EP_o + pppApU + PP_i \end{array}$$

In this formulation, EP_o–ATP_A corresponds to the open complex (EP_o) in which ATP is bound at the active site; $K_{UTP,A}$ and $K_{UTP,L}$ correspond to the apparent dissociation constants for the binding of UTP to the active (UTP_A) and allosteric (UTP_L) sites, respectively; and β corresponds to the fractional decrease in k_{cat} in the case of the ATP–RNA polymerase–A1 promoter open complex that has UTP bound at the allosteric site. The velocity equation for this scheme is:

$$V = \frac{(V_{max}\{[UTP]/K_{UTP,A} + \beta[UTP]^2/(K_{UTP,A})(K_{UTP,L})\}}{1 + [UTP]/K_{UTP,A} + [UTP]/K_{UTP,L} + [UTP]^2/(K_{UTP,A})(K_{UTP,L})} \quad (1)$$

In Figure 10(a) is a plot of the variation in the rate of pppApU formation as a function of UTP concentration over the range of 0–0.05 mM. Analyses of several data sets yielded a value of 9.3(±0.2) μM for the apparent $K_{UTP,A}$ for UTP binding at the active site and a value of 0.39(±0.01) μM pppApU/minute for V_{max}. If the concentration range for UTP is extended up to 1.5 mM, it is apparent that UTP at elevated concentrations suppresses the synthesis of pppApU (Figure 10(b)). In fitting the experimental data in Figure 10(b) to equation (1), the value of $K_{UTP,L}$ was kept constant at 1.3 mM and the other parameters were allowed to vary. This value for $K_{UTP,L}$ was obtained from the filter binding studies on the effect of UTP on the stability of the RNA polymerase–T7 A1 promoter complex. The estimates for the various parameters as determined in theoretical fits of four data sets are 0.28(±0.14) μM pppApU/minute for V_{max}, 8.2(±5.9) μM for $K_{UTP,A}$ and 0.5(±0.1) for β. As is apparent from the theoretical curve through the data points shown in Figure 10(b), the scheme given above involving two binding sites for UTP gives a good fit to the experimental data. Relative to the velocity of the reaction at a UTP concentration of 0.05 mM, there is a 27(±5)% decrease in the velocity at a UTP concentration of 1.0 mM. These studies were conducted at an ATP concentration of 2.0 mM. In comparable studies conducted at an ATP concentration of 0.05 mM, the velocity of the reaction at a UTP concentration of 1.0 mM was 50(±20)% less than that at a UTP concentration of 0.05 mM (based on six determinations; data not shown).

In Figure 10(c) is a double reciprocal plot of the data given in Figure 10(b). The theoretical curve through the data points is based on the estimates of the various parameters (V_{max}, $K_{UTP,A}$, $K_{UTP,L}$ and β) as determined above for this data set. At low UTP concentrations (i.e. at high 1/[UTP] values), the double reciprocal plot is linear and it appears that the reaction obeys classic Michaelis–Menten kinetics. However, as the concentration of UTP increases, the value of 1/velocity goes through a minimum and then increases again. This increase in 1/velocity indicates substrate inhibition at elevated UTP concentrations.

To test the hypothesis that the observed variation in pppApU formation as a function of UTP concentration is due to product inhibition, we investigated the variation in pppApU formation as a function of ATP concentration. If product inhibition is the source of the variation in pppApU production as a function of UTP concentration, then similar results should be obtained whether we vary the concentration of UTP or ATP. As is illustrated in Figure 10(d), ATP does not inhibit pppApU formation at concentrations as high as 2 mM. Thus, the suppression of pppApU formation at elevated UTP concentrations is not due to product inhibition. Analyses of several data sets yielded a value of 0.33(±0.01) μM pppApU/minute for V_{max}, and a value of 0.12(±0.01) mM for the apparent K_m, for the interaction of ATP with the open complex.

Discussion

Suppression of terminated transcript accumulation from the T7 A1 promoter at elevated UTP concentrations is due to inhibition of promoter clearance

In the presence of 0.05 mM ATP, the value of the rate constant for promoter clearance at a UTP concentration of 1.0 mM is approximately 53% less than that observed at a UTP concentration of 0.05 mM. The magnitude of the UTP effect on promoter clearance is sufficient to account for the suppression of terminated transcript accumulation at elevated UTP concentrations (i.e. approximately 40% suppression at 1 mM UTP relative to the value at 0.05 mM UTP). The difference in these two values appears to be due to the pausing of RNA polymerase molecules at a UTP concentration of 0.05 mM. Jin and Turnbough[29] observed a decrease in terminated transcript accumulation at elevated UTP concentrations in the case of a DNA fragment containing the *pyr* BI promoter-regulatory region. They attributed this phenomenon to increased stuttering from a homopolymeric region in the initially transcribed region of the DNA template. The DNA construct containing the T7 A1 promoter and T_e terminator does not contain a homopolymeric region in the initially transcribed region that can lead to stuttering synthesis of a string of uridine residues. Therefore, such a mechanism cannot account for the decrease in the accumulation of terminated transcripts from the A1 promoter at elevated UTP concentrations.

The magnitude of the UTP suppression of terminated transcript accumulation in the presence of 2 mM ATP is less than that in the presence of 0.05 mM ATP. The analysis of the mechanism of inhibition of pppApU synthesis by UTP appears to rule out competitive binding between ATP and UTP as a mechanism for this difference. Nierman and Chamberlin[8] obtained data that suggested that there is an allosteric nucleotide-binding site

that may be crucial for the process of productive initiation. If there is indeed an ATP allosteric binding site, then ATP may partially counteract the effect of UTP suppression of terminated transcript accumulation through this site. Although this provides a mechanism for the effect of ATP at higher concentrations, additional studies must be conducted to confirm it.

The pattern of abortive product formation as a function of UTP concentration in the case of the A1 promoter varies from those reported in other systems.[16,18,29,30] Moreover, there is a large variation in the reported patterns of abortive product formation as a function of nucleotide concentration.[16,18,29,30] The K_ms of the respective nucleoside triphosphates, the rates of extension and release of each oligonucleotide in the initial transcribed complexes[18,31,32] as well as any putative allosteric effects probably determine each specific abortive product pattern as a function of nucleotide concentration. Although elevated concentrations of UTP suppress the synthesis of both the abortive and terminated products from the A1 promoter, the overall UTP concentration dependencies are different. Heisler *et al.*[16] reported that there is a subpopulation of RNA polymerase molecules that are slow to clear the A1 promoter sites. Sen *et al.*[33] reported that few moribund (arrested) complexes appear to accumulate at A1 promoters. If there are trace amounts of arrested complexes that form at A1 promoters, then the arrested complexes that produce the abortive products may have a different UTP concentration dependency than the productive complexes that yield full-length terminated transcripts.

The rate constants for promoter clearance as well as for pause site escape are macroscopic constants that describe all of the events that occur in each process. Although promoter clearance from the A1 promoter has been investigated previously,[16,34] estimates of rate constants were not determined. Therefore, a direct comparison cannot be made with the current study. The value of the rate constant for escape from the pause site generated by the linker DNA (i.e. $1.2(\pm 0.2)\ \mathrm{min}^{-1}$) is comparable to those for escape from naturally occurring pause sites (i.e. $0.3–4.3\ \mathrm{min}^{-1}$).[27,28,35–38] The kinetics of pause site escape are first order in all cases. This suggests, as postulated previously, that the rate-determining step in pause site escape is a unimolecular (conformational) event.[4]

The formation of pppApU is inhibited at elevated UTP concentrations

The reaction in which only the first two nucleotides in the 5′ terminal sequence of the transcript are added and the formation of the dinucleotide product is monitored has served as a model for transcription initiation.[39] This reaction, however, also mimics the formation of dinucleotide product during abortive initiation. Moreover, Nierman and Chamberlin[8] reported abortive product formation in the absence of productive initiation at nucleotide concentrations below 3–4 μM in the case of the A1 promoter. However, as shown here, as well as other studies,[40] synthesis of abortive products still occurs in the case of the A1 promoter at nucleotide concentrations greater than or equal to 10 μM. This may be due to trace amounts of arrested complexes that occur at A1 promoters.[33] In spite of the problems associated with studies on the formation of pppApU from ATP and UTP, such investigations can still provide insight into the mechanism of action of UTP on modulating the accumulation of terminated transcripts from the A1 promoter.

At elevated concentrations, UTP suppresses the synthesis of pppApU. The pattern of inhibition of pppApU formation as a function of UTP concentration (in the presence of only UTP and ATP) is similar to that seen for the suppression of terminated transcript accumulation as a function of UTP concentration rather than that seen for suppression of abortive transcript synthesis (in the presence of all four nucleotides) as monitored by gel electrophoresis. This suggests that the results obtained on the formation of pppApU from ATP and UTP more closely reflect events that occur during productive initiation. In the presence of 2 mM ATP, the velocity of pppApU formation at a UTP concentration of 1.0 mM is $27(\pm 5)\%$ less than that at a UTP concentration of 0.05 mM. The magnitude of this change is sufficient to account for the suppression of terminated transcript accumulation during multiple-round transcription at elevated UTP concentrations (i.e. $23(\pm 6)\%$ suppression at 1 mM UTP concentration relative to the value at 0.05 mM UTP) in the presence of 2 mM ATP. The difference in these values is most likely due to pausing of RNA polymerase molecules at a UTP concentration of 0.05 mM. These results are consistent with UTP suppressing promoter clearance at the point of transcription initiation. Mechanistic studies on the inhibition of pppApU synthesis by UTP indicate the presence of a low affinity allosteric binding site for UTP which, when occupied, decreases V_{max} but does not alter the K_m for ATP.

In previous studies on the effect of UTP concentration (0–80 μM) on RNA chain initiation from the A1 promoter, Nierman and Chamberlin[41] observed no inhibition by UTP. Also, in previous studies on the effect of UTP concentration (0–150 μM) on pppApU formation from the A1 promoter, McClure *et al.*[39] observed no inhibition by UTP. Qualitatively these results agree with the results reported here over the same concentration ranges. The lack of inhibition of pppApU synthesis as a function of ATP concentration (0–2.0 mM) reported by McClure *et al.*[39] is consistent with the current study.

Chamberlin & Berg[42] reported both high and low efficiency inhibition of RNA synthesis by nucleoside triphosphates. They monitored reiterated synthesis of poly[r(A)] from homopolymeric regions of denatured calf thymus DNA (single-stranded

DNA). This system and the patterns of inhibition are significantly different from those in the case of the DNA construct containing the T7 A1 promoter and T_e terminator. Based on the many differences in the systems, it seems unlikely that inhibition of RNA synthesis by UTP in the case of the DNA construct used here corresponds to either the high or low efficiency inhibition described by Chamberlin & Berg.[42]

Shimamoto & Wu[43,44] studied the effect of UTP concentration on transcription initiation from a poly(dA-dT) template in which the initiating nucleotide was UpA. The results from these studies led Shimamoto & Wu[43,44] to propose that two UTP molecules bind to the enzyme–DNA complex during the formation of UpApU. Moreover, the second UTP acts as a positive effector in the process of initiation. In subsequent studies, Shimamoto *et al.*[45] obtained data indicating that UTP is not a positive effector in the initiation of transcription at the T7 A1 promoter site. This is consistent with the results obtained here over the UTP concentration range of 0–50 μM. Shimamoto *et al.*[45] did not investigate transcription initiation at millimolar concentrations of UTP, and therefore did not report UTP inhibition of transcription initiation. However, Shimamoto *et al.*[45] found that the third nucleotide (CTP) and the fourth nucleotide (GTP) were potent activators of productive initiation at the A1 promoter and postulated that this activation was mediated through an allosteric site. The step that appeared to be activated by these nucleoside triphosphates occurs after the formation of the first phosphodiester bond.

Foster *et al.*[46] reported the allosteric regulation of transcription elongation by nucleoside triphosphates. In quench flow kinetic studies, they monitored the incorporation of CMP into a stalled elongation complex as a function of CTP concentration. Their kinetic data were consistent with the binding of two CTP molecules to the RNA polymerase molecule and a mechanism involving non-essential activation by CTP. They reported values of 8.4 and 20 μM for the apparent dissociation constants for the binding of CTP to the putative allosteric binding site at approximately 23 °C under conditions where the active site was either empty or contained CTP. The relationship between the CTP-activation of elongation[46] and the UTP-suppression of terminated transcript accumulation reported here is unclear.

The interaction of UTP with RNA polymerase

The nucleotide-binding scheme in RNA polymerase is fairly complex. In the case of a promoter in which the transcript is initiated by a purine nucleotide, the presence of two nucleotide-binding sites is well established.[47] The *i* (initiation) purine site is template and Mg^{2+}-independent but is purine nucleotide-specific. The $i+1$ (polymerization) site is template and Mg^{2+}-dependent but displays no nucleotide preference. In steady-state kinetic studies, Anthony *et al.*[48] found that the apparent K_m for the *i* purine nucleotide site is approximately 0.15 mM, whereas the apparent K_m for the $i+1$ site is approximately 0.015 mM at 28 °C. In equilibrium dialysis binding studies conducted in the absence of a template but in the presence of Mg^{2+}, Wu and Goldthwait[49] found two binding sites for the purine nucleotides with respective K_ds of 0.15 and 0.015 mM at 25 °C.

The results presented here add to the complexity of the nucleotide-binding scheme in RNA polymerase. The kinetic studies establish the presence of a low affinity UTP binding site that is distinct from the active site (apparent $K_d = 1.6(\pm 0.5)$ mM at 37 °C). Studies on the stabilization of the open complex against heparin disruption support the presence of a low affinity UTP binding site (apparent $K_d = 1.3(\pm 0.4)$ mM at 37 °C). The fluorescence studies on the binding of UTP to RNA polymerase in the absence of the A1 promoter also indicate the presence of a low affinity UTP binding site ($0.8(\pm 0.3)$ mM at 25 °C). Wu and Goldthwait[49] reported a low affinity UTP binding site in equilibrium dialysis binding studies in the absence of DNA but the presence of Mg^{2+} ($K_d = 0.5$ mM at 25 °C). They saw no evidence of UTP binding to RNA polymerase with an affinity corresponding to a K_m (15 μM) for the binding of UTP to the polymerization site. Moreover, they could not induce the formation of a high affinity UTP binding site by the addition of a purine nucleotide (GTP, GMP, dATP or dAMP). Differences in the values of K_d for the low affinity UTP binding site may reflect differences in experimental conditions (temperature, absence/presence of a template, presence of other nucleotides and salt concentration) or differences inherent in the techniques that were used to obtain estimates of this parameter. It should be noted that the equilibrium dialysis binding studies of Wu & Goldthwait[49] also indicate the presence of a low affinity CTP binding site in the absence of DNA but the presence of Mg^{2+}. It is not clear whether CTP and UTP bind to the same low affinity site. Also, the relationship between the allosteric binding sites proposed in other studies[42–46] and the low affinity CTP and UTP binding sites is unclear.

The stabilization of the RNA polymerase–A1 promoter complex by the binding of UTP to the putative allosteric site suggests that there is a UTP-mediated conformational change in this complex. Apparently, this conformational change leads to an RNA polymerase–T7 A1 promoter complex that is transcriptionally less active. This conformational change must be different from the alteration that various RNA polymerase–promoter complexes are thought to undergo upon the binding of the appropriate initiating nucleoside triphosphate at the active site.

Conclusions

The data that we obtained here indicate that UTP modulation of the accumulation of terminated

transcripts during multiple-round transcription from a DNA construct containing the T7 A1 promoter and T_e terminator is mediated at the point of transcription initiation through an allosteric mechanism. What possible role could such a mechanism serve within the cell? Under nucleoside triphosphate concentrations within the cell, the active site of RNA polymerase is saturated and RNA synthesis is driven as a result. Therefore, in the case of a so-called constitutive gene containing a strong promoter, one would expect to have the continuous unregulated synthesis of transcripts regardless of the amount of mRNA required to maintain the steady-state level of a given protein. Excess transcripts would then have to be degraded. This would represent a waste of energy in the consumption of nucleoside triphosphates to produce mRNA transcripts that are not needed by the cell. A mechanism whereby RNA polymerase activity is suppressed or modulated at elevated nucleoside triphosphate levels would tend to ameliorate this effect and conserve energy. Our initial studies on the suppression of RNA synthesis from the T7 A1 promoter at elevated UTP concentrations support such a model for modulating RNA polymerase activity. It should be noted that CTP at elevated concentrations suppresses terminated transcript accumulation in the case of the A1 promoter whereas GTP does not (our unpublished data). Thus, it appears that the pyrimidine nucleoside triphosphates play a role in regulating transcription from the A1 promoter. Additional studies are required to further delineate the mechanism of action of the pyrimidine nucleotides and to obtain a more unified picture of the putative role of allosteric mechanisms in regulating transcription.

Experimental Procedures

Reagents and materials

[α-^{32}P]UTP, [α-^{32}P]CTP and [γ-^{32}P]ATP were obtained from ICN. *E. coli* K12 cell paste (3/4 log phase, enriched medium) was purchased from the University of Wisconsin. Restriction enzymes were obtained from New England Biolabs. T4 polynucleotide kinase and calf intestinal alkaline phosphatase (CIP) were purchased from Promega. All other reagents were of the highest purity available from commercial sources. Whatman cellulose nitrate filters (25 mm and 0.45 μm pore size) and Whatman 3 MM chromatography paper were purchased from Fisher.

Purification of RNA polymerase and isolation of holoenzyme

The method of Burgess & Jendrisak[50] as modified by Lowder & Johnson[51] to incorporate chromatography with a red agarose column instead of a DNA cellulose column was used for the purification of RNA polymerase. Separation of core polymerase and holoenzyme was carried out by chromatography on a Bio-Rex-70 (Bio-Rad) column.[52] In the final step in the purification of the holoenzyme, a modification of the method of Hager *et al.*[53] was used involving chromatography with Q Sepharose FF (Pharmacia) as outlined by Johnson & Chester.[52]

Characterization of holoenzyme

As judged from analyses of Coomassie Blue stained gels (sodium dodecyl sulfate–8.75% (w/v) polyacrylamide[54]) by using ImageQuant, the holoenzyme was at least 90% saturated with sigma. The number of active enzyme molecules present in the samples was estimated by using the procedure of Oen *et al.*[55] as modified by Solaiman & Wu.[56] The protocol is presented in detail by Johnson & Chester.[52] In the studies conducted herein, the percentage of active enzyme molecules was approximately 40%.

Isolation of plasmids and DNA fragments

Dr W. Studier (Biology Department, Brookhaven National Laboratory, Upton, NY) generously provided plasmids pAR1707 and pAR1435. Plasmid pAR1707 contains the A1 promoter site as well as the early termination site T_e from bacteriophage T7, whereas plasmid pAR1435 contains just the T7 A1 promoter cloned into the *Bam*H1 site of pBR322. Bacteria (DH5α competent cells containing either pAR1707 or pAR1435) were grown in LB medium (1% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto yeast extract, 1% (w/v) NaCl) containing 40 μg/ml ampicillin according to standard protocol.[57] Plasmids were isolated by using Qiagen columns as described in the protocol provided by the manufacturer. After digestion of the plasmids with the appropriate restriction enzymes, the fragments were separated on native 5% polyacrylamide gels. The electrophoresis buffer was TBE (90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, pH 8.3). The DNA fragment of interest was then isolated by electroelution from the gel. This was followed by volume reduction with *sec*-butanol, phenol extraction and then precipitation with ethanol. The precipitated fragment was then resuspended in buffer (10 mM Hepes, 50 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 10% (v/v) glycerol, 0.1 mM DTT at pH 8.0). Treatment of pAR1707 with *Rsa*I and *Sma*I yielded a 529 base-pair fragment containing the A1 promoter; treatment of pAR1707 with *Rsa*I and *Sal*I yielded a 963 base-pair fragment containing the A1 promoter along with the T_e terminator; and treatment of pAR1435 with *Rsa*I and *Pvu*II yielded a 2030 base-pair fragment containing the A1 promoter.

Radioactive labeling of the DNA fragment for heparin displacement studies

The *Sma*I–*Rsa*I fragment from pARl707 was dephosphorylated by using CIP according to standard protocol.[57] Labeling with ^{32}P was performed by mixing the dephosphorylated DNA fragment with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase according to standard protocol.[57] Unreacted [γ-^{32}P]ATP was removed by electrophoresis on a native 5% polyacrylamide gel. The protocol for electrophoresis and isolation of the labeled DNA fragment was the same as that given above for the isolation of DNA fragments from restriction digests.

Transcript analyses

All reactions throughout this study were conducted in Hepes buffer (10 mM Hepes, 50 mM KCl, 10 mM $MgCl_2$, 0.1 mM DTT at pH 8.0) at 37 °C. The purchased nucleotide samples were in the salt form. Therefore, when necessary, the salt concentration of each reaction mixture was adjusted to account for the salt added due to the respective nucleotides. All solutions were preincubated at 37 °C for ten minutes prior to initiation of the reaction. In the case of multiple-round transcription, the reaction volume was 20 μl and each reaction was terminated by the addition of 10 μl of stop reaction mixture (0.2 M EDTA, 0.01% (w/v) bromophenol blue, 50% glycerol). For monitoring the recycling phase as a function of time during multiple-round transcription, 10 μl aliquots were removed from the reaction mixture (80 μl) at the appropriate times and the reaction was stopped by the addition of 10 μl of stop reaction mixture. In the case of promoter clearance, the reaction was limited to a single round by the addition of heparin (final concentration of 40 mg/ml) along with the nucleotides. Aliquots of 10 μl were removed from the reaction mixture (80 μ1) at the appropriate times and mixed with 10 μl of stop reaction mixture. After stopping each reaction, the samples were heated in boiling water for one minute and then cooled on ice. The samples (10 μl) were applied to either an 8 M urea 20% or 8% (w/v) polyacrylamide gel and subjected to electrophoresis in TBE buffer. The gels were dried and then exposed to X-ray film (Fuji RX) at either room temperature or at −70 °C for varying lengths of time. The autoradiograms were analyzed by using ImageQuant (Molecular Dynamics).

Heparin displacement assay

The stability of the open complex between RNA polymerase and the A1 promoter (^{32}P-end labeled *Rsa*I–*Sma*I fragment of pARI707) in the absence and presence of UTP was determined by measuring retention of complexes on nitrocellulose filters after challenge with heparin (50 μg/ml) in Hepes buffer at 37 °C. After applying the respective samples to the filters, they were immediately filtered under vacuum. Each filter was then washed five times with 1 ml of Hepes buffer. The filters were dried in an oven at 50 °C for one hour and then subjected to Cerenkov counting. Background retention of the end-labeled DNA fragment was determined by filtering a reaction mixture containing everything except for RNA polymerase. In all cases, the background retention was less than 3%. For determination of the disruption of the open complex as a function of time, 50 μl aliquots were removed at appropriate times from the reaction mixture (350 μl) after the addition of heparin. For determination of the UTP concentration dependency for stabilization of the RNA polymerase–T7 A1 promoter complex against heparin disruption, 45 μl of each reaction mixture (50 μl) was removed after 15 minutes and analyzed as outlined above.

Transcription elongation assay

The A20 elongation complex was generated in the absence of a labeled radioactive nucleotide by incubating ApU, GTP, ATP and CTP (all at 0.05 mM) with a solution containing RNA polymerase (18.5 nM active enzyme molecules) and T7 A1 promoter (24 nM *Rsa*I–*Pvu*II DNA fragment from pAR1435) complexes in Hepes buffer. The nucleotide reaction mixture also contained heparin (60 mg/ml) to ensure that no initiation occurred after formation of the A20 complexes. After a five minute incubation, nucleotide solutions containing ATP (0.05 mM), GTP (0.05 mM), [α-^{32}P]CTP (0.05 mM at a specific activity of 700–1000 cpm/pmol) and the appropriate amounts of UTP were added and 25 μl aliquots were removed from the reaction mixture (350 μl) at appropriate times for analyses. RNA polymerase final concentration was 12 nM in active enzyme molecules, the T7 A1 promoter final concentration was 16 nM and heparin final concentration was 40 mg/ml. The aliquots were treated as described by Kingston *et al.*[58]

Fluorescence studies

All fluorescence studies were conducted by using a Spex Model 1681 spectrofluorometer that was interfaced to a Spex DM3000 computer. Measurements were made in the ratio mode by using front-face illumination.[59] Excitation and emission bandwidths were 1.8 and 4.5 nm, respectively. With front-face illumination, a linear dependence of fluorescence intensity on protein concentration was observed up to at least 2000 nM. Therefore, no corrections were required for inner filter effects. For fluorescence emission spectra, data were recorded every 1 nm with an integration time of one second. The excitation wavelength was 280 nM. Each spectrum was corrected for blank background emission and wavelength-dependent effects. All studies were conducted at 25 °C.

In the fluorometric titration of RNA polymerase by UTP, 11 measurements were made at 340 and 500 nm, respectively, at each UTP concentration. An integration time of two seconds was used in recording each data point. These measurements were then averaged and the differences between the measurements at 340 and 500 nm were determined and corrected for dilution.

Analysis of promoter clearance data

The model that we used to analyze the data obtained at a UTP concentration of 0.05 mM corresponds to sequential pseudo first-order processes for promoter clearance and pause site escape. Harcourt and Esson[60] were the first to report the integration of the differential equations for this type of series first-order reactions. By using a variation of these equations as presented by Frost and Pearson,[61] we fitted the experimental data in order to obtain estimates for k_1 and k_2. In the case of the variation of the paused transcript as a function of time, the data were fitted to the equation:

$$\gamma = 1 + [1/(1 - k_2/k_1)][(k_2/k_1)\exp(-k_1 t) - \exp(-k_2 t)]$$

where γ is the normalized value for the amount of paused transcripts present and t corresponds to time. In the case of the variation of terminated transcripts as a function of time, the data were fitted to the equation:

$$\beta = [1/(k_2/k_1) - 1][\exp(-k_1 t) - \exp(-k_2 t)]$$

where β is the normalized value for the amount of the terminated transcripts. In the analysis of the data for the sum of the terminated and paused transcripts at 0.05 mM UTP or the data for the terminated transcripts at 1.0 mM UTP, the equation:

$$\alpha = 1 - \exp(-kt)$$

was used where α is the corresponding normalized value. All fits of experimental data were done by using

Sigmaplot 4.0 for Windows and the appropriate equation.

Steady state kinetic analysis of transcription initiation

The steady state kinetics of product formation (pppApU) between UTP and ATP were performed by using the procedure of McClure.[62] Reaction mixtures (30 μl) contained in Hepes buffer RNA polymerase (2.5 nM active enzyme molecules), A1 promoter (3.2 nM), and the appropriate amounts of ATP and [α-^{32}P]UTP at a specific radioactivity of 360 cpm/pmol. Prior to the initiation of the reaction, the solutions were preincubated for ten minutes at 37 °C. Each reaction was terminated at the appropriate time by the addition of 10 μl of 0.2 M EDTA and then placed on ice. The samples were spotted on Whatman 3 MM paper in 5–6 μl aliquots with interim drying and resolved with ascending chromatography in WASP (water/saturated $(NH_4)SO_4$ (pH 8.0)/2-propanol, 18:80:2 (by volume) containing 5 mM EDTA). The chromatograms were dried at room temperature, cut into strips and analyzed for radioactivity by Cerenkov counting. The R_f value of pppApU was approximately 0.44. In studies in which the concentration of [α-^{32}P]UTP was varied over the concentration range of 0–1.5 mM, a standard curve based on the background radioactivity due to unincorporated [α-^{32}P]UTP that migrated to the area corresponding to a R_f value of 0.44 was used to obtain the corrected value for pppApU formation.

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