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Stable RNA–DNA–RNA Polymerase Complexes Can Accompany Formation of a Single Phosphodiester Bond†

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ABSTRACT: Incubation of RNA polymerase with poly[d(A-T)$_n$] template results in a binary enzyme–DNA complex. Further addition of the dinucleotide UpA and [α-^{32}P]UTP results in catalytic formation of the labeled trinucleotide UpApU until substrate exhaustion. In contrast, incubation of binary enzyme–DNA complexes with ApU and [α-^{32}P]ATP results in labeled ApUpA formation to an extent that is stoichiometric with the amount of enzyme present despite an excess of substrates. The occurrence of ApUpA in a stable DNA–enzyme–RNA ternary complex is shown by gel exclusion chromatography, Millipore filtration, and the ability of ternary complexes to support subsequent RNA chain elongation. Radioactivity is not bound to Millipore filters when purified, labeled ApUpA is added to enzyme–DNA binary complexes. Hence, phosphodiester bond formation is required for stable ternary complex formation. The absence of the σ subunit of RNA polymerase or the addition of rifampicin to the reaction before ribonucleotide substrates results in catalytic ApUpA formation instead of stable ternary complexes.

DNA-dependent RNA polymerase is possibly one of the most extensively studied enzymes in *Escherichia coli*; for example, see Chamberlin (1976). Nevertheless, uncertainty remains as to how RNA chain initiation occurs and whether this step in DNA transcription can be regulated at a level beyond promoter recognition.

Kinetically the formation of the first phosphodiester bond is much faster than the formation of open promoter complexes by RNA polymerase (Rhodes & Chamberlin, 1975) even in instances in which there are relatively high rates of dissociation from open promoter complexes (Seeburg & Schaller, 1975). This difference in rates has been used to argue that RNA chain initiation is an almost certain event after proper promoter recognition and therefore unlikely to be a point at which regulation might occur (Chamberlin et al., 1976; Seeburg et al., 1979).

Recently, a phenomenon termed abortive inititation has been discovered in which RNA polymerase has been shown to form and release short oligonucleotides rather than to continue to elongate RNA chains (Johnston & McClure, 1976). These events occur when RNA polymerase is bound to natural promoters either in the presence or in the absence of the initiation inhibitor rifampicin. It is evident that the formation of a stable RNA–DNA–RNA polymerase ternary complex is not obligatory after promoter complex formation in the presence of ribonucleoside 5′-triphosphates even though such complexes can be formed after many rounds of phosphodiester bond formation (Rhodes & Chamberlin, 1974). The process of abortive inititation is of interest to us because of the potential regulatory implications accompanying formation of the first phosphodiester bond as well as the possible priming activities anticipated for the abortive oligoribonucleotides produced in this fashion.

It is experimentally difficult to dissect out determinants of the rate of formation of the first phosphodiester bond from those related to steps just before initiation and those that occur just after initiation (Chamberlin, 1974; Krakow et al., 1976). Early efforts to deduce properties of RNA chain initiation were based upon indirect assays which often used rifampicin as an indicator of the first phosphodiester bond formation since the drug could be shown to inhibit RNA chain initiation but not elongation. These studies led So & Downey (1970) to suggest that a stable ternary complex can accompany the formation of a single phosphodiester bond. Analogous rifampicin challenge experiments (Mangel & Chamberlin, 1974) have been complicated by the finding that rifampicin not only does not prevent phosphodiester bond formation but also stimulates the abortive variant of this reaction to the extent that such oligoribonucleotides are the major reaction product (Johnston & McClure, 1976; McClure & Cech, 1978). Evidently a direct assay is needed for the formation of the first phosphodiester bond in RNA chain initiation. Direct measurements

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of single additions to substrates that cannot elongate but can initiate have been recently reported such as dinucleoside monophosphate (BpB')[1] primers with alternating synthetic DNA templates (Oen & Wu, 1978) or ribonucleoside 5'-monophosphate (pB) primers (Shemyakin et al., 1978). Major differences in the rate of product formation are observed that are sequence specific, and these differences have been ascribed to either substrate affinity constant differences or deduced differences in ternary complex stabilities (Oen & Wu, 1978; Shemyakin et al., 1978). Abortive initiation has been suggested to be possibly a normal accompaniment of RNA chain initiation, but the stability of the ternary complexes has not been directly demonstrated (McClure et al., 1978).

We have been led to attempt to characterize factors affecting the formation of the first phosphodiester bond with synthetic, alternating DNA template systems. This is because of an earlier demonstration that ppGpp can inhibit GpC-primed synthesis of GpCpG with poly[d(I-C)$_n$] templates (Cashel et al., 1976). Here we wish to describe evidence of a stable ternary complex forming in a better characterized system such as the formation of ApU*pA from ApU primers and [α-^{32}P]ATP with poly[d(A-T)$_n$] templates. In contrast, the UpA*pU nucleotide triplet formed with UpA primer and [α-^{32}P]UTP is not associated with a stable ternary complex but is made in amounts that greatly exceed the enzyme present. We think that this is the first direct demonstration of the formation of a stable ternary complex accompanying formation of a single phosphodiester bond and that this phenomenon is an important one because it relates to the mechanism of abortion of RNA chain initiation.

Materials and Methods

Reaction Mixtures. Standard reaction mixtures (usually 25 μL) incubated at 25 °C contained 40 mM KCl, 50 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 1 mM β-mercaptoethanol, 1 A_{260}/mL synthetic, alternating DNA template specified, and 80–100 μg/mL RNA polymerase prepared according to Burgess & Jendrisak (1975). The enzyme was further purified free of trace nucleotidase contamination by a reverse ammonium sulfate fractionation. σ factor was removed by Bio-Rex 70 column chromatography to produce core enzyme preparations. Dinucleoside 3',5'-monophosphates present as primers were used at concentrations ranging from 0.1 to 1 mM together with 1–10 μM of the appropriate ribonucleoside 5'-[α-^{32}P]-triphosphate. The reactions requiring preincubation with an effector generally were for 5 min before the addition of nucleotide substrates to initiate the reaction. Variations in the order of addition of reactants revealed that the most rapid rates occurred when reactions were initiated by adding nucleotide substrates (data not shown). Purities of nucleotides were verified by thin-layer chromatography; nucleotides were purchased from ICN as high-performance LC grade compounds.

Product Verification. Reaction mixtures incubated at room temperature were usually terminated by mixing 5-μL aliquots with 10 μL of ice-cold 10% acetic acid or by spotting 5 μL directly on nucleotide-binding cellulose thin layers [either DEAE or poly(ethylenimine) (PEI)]. In some instances, analysis of reaction products was by the WASP solvent system of Johnston & McClure (1976) to confirm identities by published procedures. More often ascending thin-layer chromatography in 0.5 M LiCl on PEI-cellulose sheets was used. This system, developed by Randerath & Randerath (1967), quickly separates ribonucleoside triplets of the general structure BpB'pB from the labeled ribonucleoside 5'-triphosphate which remains origin bound. Oligonucleotide product identities were verified by the two-dimensional fingerprint procedures normally employed for RNA sequencing (Brownlee, 1972). In all cases the reaction mixtures were sampled directly. These products were eluted and subjected to secondary digestions with RNase A, a mixture of RNase A, RNase T_2, and RNase T_1, or *E. coli* phosphomonoesterase, and the products of these reactions were visualized after one-dimensional separation methods. For example, the triplet presumed to be UpA*pU from UpA and pp*pU was isolated from a fingerprint chromatogram (pH 3.4 electrophoresis in the first dimension and chromatography on DEAE-cellulose thin layers in the second dimension). The isolated UpA*pU was shown to be resistant to alkaline phosphatase, to form A*pU after RNase A digestion (shown on PEI-cellulose thin layers developed with 0.1 M acetic acid), and to form radioactive A*p after digestion with the RNase mixtures. These procedures have allowed unequivocal verification of ApU*pA and UpA*pU as well as GpC*pG and CpG*pC as reaction products which can be accurately measured from one-dimensional thin-layer chromatographic procedures.

In general, analysis of the formation of nucleotide triplets could be easily and quickly performed by the direct analysis on the thin-layer system. The resolved products were visualized by autoradiography and counted by Cerenkov emissions. The major advantages of this method are the ability to accommodate many samples quickly and the minimal streaking of products as compared to the WASP chromatographic system.

Gel Chromatography. Exclusion gel chromatography for microsamples was accomplished by dispensing a reaction mixture aliquot on a conical Eppendorf (yellow) pipet tip containing Sephadex G-50 equilibrated in reaction buffer. Two-drop fractions were collected and counted directly as well as subjected to thin-layer chromatographic resolution of nucleotide triplets from labeled substrates. In this manner, labeled nucleotide products that were present in the void volume as well as in the included volume could be visualized and quantitated relatively easily.

Millipore Filter Binding. A modification of the procedure of Jones & Berg (1966) was used. Essentially, 50–100-μL reactions were terminated by the addition of 8 mL of ice-cold Tris-HCl (pH 8) and 50 mM NaCl, and the mixtures were incubated for 30 min and then filtered slowly through previously soaked 4.5-cm Millipore filters. The filters were then washed with an additional 10 mL of the buffer. Where indicated (see, for example, Table IV) the wash buffer was altered to be identical with the treatment for decay studies. The filters were then counted for Cerenkov emissions.

Results

With synthetic poly[d(A-T)$_n$] templates, RNA polymerase catalyzes the condensation of [α-^{32}P]ATP to ApU to form ApU*pA. Visualization of reaction mixtures subjected to fingerprinting procedures (data not shown) verifies the absence of larger oligomers, indicating the absence of trace amounts of UTP in the system as well as the inability of ApU primers to participate in further RNA chain elongation of ApU*pA. Figure 1a shows that with holoenzyme (E^σ) an amount of ApU*pA is formed within 2 min that is roughly stoichiometric with the level of enzyme added and does not increase further at later times. Contrasting behavior is noted in Figure 1a with

[1] A phosphate to the left of a base indicates a 5' residue as in pB; a phosphate to the right of a base (Bp) indicates it to be a 3' residue. Thus, the dinucleoside monophosphate BpB', a 3',5'-diester, is indicated. The presence of [^{32}P]phosphate positioned in the nucleotidyl phosphate is indicated by an asterisk.

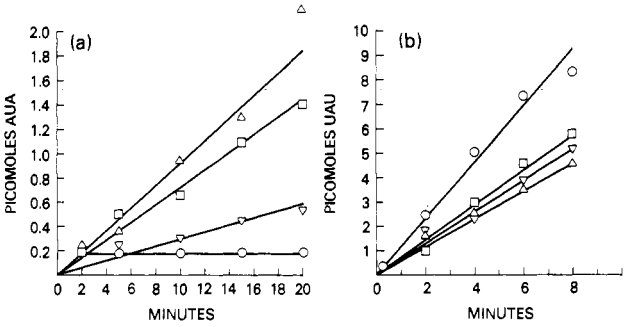

FIGURE 1: Rates of formation of ApU*pA and UpA*pA. ApU*pA (panel a): aliquots of 5 μL were removed from 40-μL reactions and chromatographed as described under Materials and Methods. The amounts of product plotted are for a 5-μL aliquot. Final concentrations were DNA = 12.5 μg/mL, polymerase = 75 μg/mL, ApU = 400 μM, [α-^{32}P]ATP = 5 μM (sp act. = 775 cpm/pmol), and rifamicin = 25 μg/mL. UpA*pU (panel b): same as above except UpA = 1 mM for core enzyme and 166 μM for holoenzyme reactions and [α-^{32}P]UTP = 5 μM (sp act. = 155 cpm/pmol). All reactions were incubated at 24 °C. (○) E^{σ}; (□) E^{σ} plus rifampicin; (▽) E; (△) E plus rifampicin.

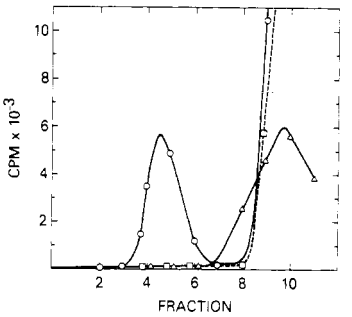

FIGURE 2: Exclusion column chromatography. ApU*pA: 50-μL reactions containing 6.6 μg of polymerase, 0.5 μg of DNA, 150 μM ApU, 4 μM [α-^{32}P]ATP, and 40 μg/mL rifampicin where indicated were incubated for 15 min at 25 °C prior to application to Sephadex G-50. Two-drop fractions were collected and counted; 10-μL portions of each fraction were applied to PEI-cellulose and chromatographed. UpA*pU: a reaction mixture of 20 μL containing 1.8 μg of polymerase, 0.2 μg of DNA, 250 μM UpA, and 4 μM [α-^{32}P]UTP was incubated for 6 min at 25 °C and treated as above. (○) ApU*pA minus rifampicin; (△) ApU*pA plus rifampicin; (□) UpA*pU minus rifampicin.

Table I

	K_m values (mM) associated with[a]			
	UpApU formation		ApUpA formation	
enzyme	UpA	UTP	ApU	ATP
E	1	0.066	1.8	
E^{σ}	0.15	0.005[c]		0.002[d]
E + Rif[b]	0.9		0.8	
E^{σ} + Rif	0.27	0.012	0.26	0.014

[a] Reactions were run like those in Figure 1 and as described under Materials and Methods. Dinucleotide concentrations varied from 0.05 to 2.5 mM and triphosphate concentrations from 0.5 to 20 μM. K_m values were determined from the resulting Lineweaver–Burk plots. [b] Rif = rifampicin. [c] V_{max} = 5.4 pmol of UpApU per min. [d] V_{max} = 0.06 pmol of ApUpA per min.

core enzyme (E) or in the presence of rifampicin (whether or not σ factor is present). ApU*pA formation under these conditions persists after 2 min at linear rates for at least 20 min, leading to product accumulations that are much greater than the amount of enzyme present, typically at least 10–20-fold. Why does the reaction stop in one instance and not in the others?

There is a determinant of this behavior specified by the sequence. Figure 1b shows that holoenzyme will continue to form large amounts of the triplet UpA*pU when UpA primers are present with [α-^{32}P]UTP even though the same enzyme will only make a limited amount of ApU*pA. Rates of UpA*pU synthesis are much faster than those of ApU*pA for both the core and holo forms of the enzyme, with or without rifampicin. Sustained accumulations of the UpA*pU are seen in Figure 1b that are linear with time and persist until the radioactive substrate is almost completely consumed. It is this difference in rates and extent of UpA*pU and ApU*pA formation that holds our interest as a model for abortive and nonabortive RNA chain initiation. We note that the differences presented here are in agreement with recent determinations using slightly different conditions (Oen & Wu, 1978; Shemyakin et al., 1978). We have also observed analogous behavior by using poly[d(I-C)$_n$] templates. With respect to stoichiometry, the formation of GpC*pG behaves much like that of ApU*pA just described and the formation of CpG*pC is like that of UpA*pU (data not shown).

Table I shows our estimates of the binding constants for the substrates participating in the formation of UpA*pU and ApU*pA under the conditions described in parts a and b of Figure 1. Although, in principle, differences in substrate binding constants could account for the nearly 10-fold difference in rates of UPA*pU formation as compared to ApU*pA, it seems that there are neither K_m nor V_{max} differences large enough to provide such an explanation. Interestingly, the presence of the σ factor is associated with moderate changes in the binding constants observed for UpA and UTP in the UpA*pU reaction.

Since rifampicin is known to stimulate abortive RNA chain initiation (Figure 1), rifampicin could stimulate ApU*pA formation by allowing release of ApU*pA from the enzyme–DNA complex, thereby allowing another round of product formation. This explanation assumes that a stable DNA–enzyme–RNA ternary complex exists after ApU and ATP condense.

Any excess of product formation over stoichiometry with polymerase concentrations can be ascribed either to the presence of some core enzyme which makes product catalytically (as shown in Figure 1a) or to something less than an absolutely stable DNA–enzyme–RNA ternary complex. In some experiments, particularly with prolonged incubations at temperatures above 25 °C, such increases do occur. Thus, these observations suggest that a fairly stable ternary complex might be formed in such reactions.

Identification of a Stable Ternary Complex. (a) Physical Evidence. Figure 2 shows that ApU*pA radioactivity can be detected in the exclusion (void) volume of a micro-Sephadex G-50 column when the triplet is formed in the presence of template by RNA polymerase. Thin-layer analysis of the column fractions shows that the radioactivity in the void volume in fact migrates with ApU*pA rather than the substrate or some other product oligonucleotide. The data indicate that ApU*pA is also present in the included column volume, as if partial dissociation from the ternary complex occurs. Figure 2 shows that UpA*pU formed in the analogous reaction is not found in the excluded volume and instead is found only in the included volume, as if it exists in dissociated form as free nucleotide. For both UpA*pU and ApU*pA reactions, single omission control experiments lacking template, enzyme, or primer show that the triplet does not form (data not shown). Figure 2 shows that the addition of rifampicin eliminates ApU*pA activity in the excluded volume even though it stimulates total ApU*pA synthesis 10 times (Figure 1).

Table II: Indication of Stable Complexes

expt	triplet formed	recovered radioact. (cpm) excluded vol	filter bound
1	ApUpA[a]	38 000	32 000
2	ApUpA[a]	14 100	12 400
3	UpApU[b]	0	180

[a] Reaction mixtures were prepared by using ApU (200 μM) as initiator and [α-^{32}P]ATP (2 μM). After ApU*pA formation was allowed for 15 min at 25 °C, aliquots of the reaction mixture were subjected to exclusion gel chromatography or Millipore filtration as described under Materials and Methods. [b] For UpA*pU formation, UpA was at 200 μM and [α-^{32}P]UTP was at 6 μM. Synthesis was allowed for 8 min at room temperature, forming 250 000 cpm of UpA*pU as judged by a thin-layer chromatographic assay.

Table III: A Stable Ternary Complex Requires Phosphodiester Formation[a]

expt	triplet formed	input act. (cpm)	other additions	% input retained by Millipore filters
1	ApU*pA	11 200		0
2	ApU*pA	11 200	DNA	0
3	ApU*pA	11 200	polymerase	0
4	ApU*pA	11 200	DNA, polymerase	0
5	ApU*pA reaction	3 300		32
6	UpA*pU	24 000		0
7	UpA*pU	24 000	DNA	0
8	UpA*pU	24 000	polymerase	0
9	UpA*pU	24 000	DNA, polymerase	0
10	UpA*pU reaction	250 000		0

[a] The UpA*pU and ApU*pA triplets were purified from reaction mixtures by preparative chromatography. Additions to the purified oligoribonucleotides were made as indicated in 100-μL reactions; DNA = 0.5 μg of poly[d(A-T)$_n$] and polymerase = 2.2 μg of holoenzyme. Incubations were for 15 min at 25 °C before Millipore filtration. In the case of experiment 5 listed as ApU*pA complex, a complete reaction mixture containing ApU*pA (or UpA*pU, experiment 10) was Millipore filtered for comparison.

Table II shows that the ApU*pA radioactivity recoverable in the column void is nearly equivalent to the activity bound to Millipore filters. This observation strongly suggests that the ApU*pA triplet can be rather stably bound to RNA polymerase. Table II further indicates that UpA*pU formed neither appears in exclusion column volumes nor binds to Millipore filters. Moreover, ApU*pA appearing in the included volume of Sephadex G-50 columns is also not bound to Millipore filters, suggesting that it is not enzyme bound. Neither purified ApU*pA nor UpA*pU radioactivity is Millipore bound (see Table III).

Does the complex containing ApU*pA radioactivity require phosphodiester formation in order to become stabilized? Table III shows that when previously purified ApU*pA or UpA*pU triplets are mixed together with holoenzyme in the absence of template, no radioactivity is Millipore bound (experiments 3 and 8). These triplets also do not form sufficiently stable binary complexes with template to allow their migration in the excluded column volumes (data not shown). In fact, admixture of either triplet with holoenzyme even in the presence of template does not lead to radioactivity retained on the filter (Table III, experiments 4 and 9). These binding studies suggest that a stable ternary complex is not formed unless condensation of a phosphodiester bond accompanies its formation. Simple admixture of the components present in the complex does not result in stable complex formation as judged by a comparison with ApUp*A radioactivity bound to Millipore filters when phosphodiester bond formation does accompany complex formation (Table III, experiment 5).

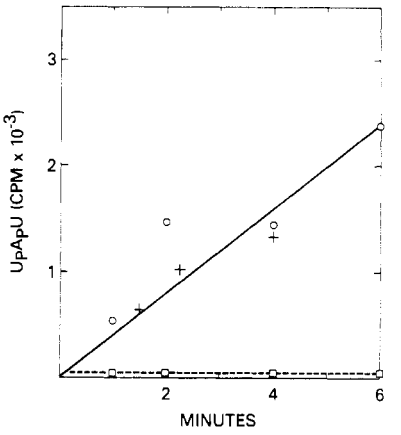

FIGURE 3: Heparin resistance. 25-μL reaction mixtures containing 0.25 μg of DNA, 2.2 μg of polymerase, 150 μM UpA, and 4 μM [α-^{32}P]UTP were sampled as described under Materials and Methods. (○) No heparin added; (+) 80 μg/mL heparin added at 1 min; (□) 80 μg/mL heparin added 5 min before poly[d(A-T)$_n$].

(*b*) *Functional Evidence.* The stable ternary complex seems functional. For example, the addition of unlabeled UTP and ATP (each 100 μM) to exclusion column isolates of radioactive ApU*pA complexes (1400 cpm) results in the conversion of 45% of the label to a trichloroacetic acid (Cl_3AcOH) precipitable form after a 30-min incubation at 25 °C. When an equivalent amount of purified radioactive ApU*pA or UpA*pU is mixed with fresh enzyme and template, less than 5% of the triplets are converted into a Cl_3AcOH-precipitable form. This indicates that a large portion of the stable ternary complex is capable of further elongation and therefore functional for further transcription.

Abortive Initiation from Binary Complexes. The formation of a stable ternary complex bearing the ApU*A oligoribonucleotide is capable of further elongation. Figure 1 shows that the formation of the other ribonucleotide triplet possible with poly[d(A-T)$_n$] occurs in a catalytic rather than a stoichiometric fashion. The amount of UpA*pU formed greatly exceeds the amount of enzyme present. Does RNA polymerase remain bound to the template while forming the UpA*pU triplet? Figure 3 shows that heparin addition prior to triplet formation can abolish UpA*pU formation. Addition of equivalent heparin concentrations after the UpA*pU reaction is in progress results in no inhibition (Figure 3), suggesting that the enzyme remains bound to the template. We have also noticed (Figure 1) that rifampicin addition prior to stable ternary (ApU*pA-bearing) complex formation results in catalytic formation of large amounts of ApU*pA in a manner at least formally analogous to UpA*pU formation. Rifampicin addition after stable complexes are formed does not increase the subsequent rate of ApU*pA formation (data not shown).

Generally, we strongly suspect that differences in the stability of the ApU*pA and UpA*pU ternary complexes can account for the differences in the extent of formation of the two triplets. Analogous but less complete experiments have verified similar properties for the poly[d(I-C)$_n$] template dependent formation of GpCpG and CpGpC triplets. Again the triplet containing two purines appears to form in amounts stoichiometric with the enzyme whereas the amounts of CpG*pC formed continue to accumulate until the substrate is consumed (data not shown).

Table IV: Conditions Influencing Stability of ApUpA-DNA-RNA Polymerase Complexes

expt	conditions	temp (°C)	% radioact. retained on filters
A[a]	50 mM NaCl	0	100
	50 mM NaCl	5	90
	50 mM NaCl	10	100
	50 mM NaCl	15	65
	50 mM NaCl	20	30
	50 mM NaCl	25	23
	50 mM NaCl	37	14
B[b]	0.25 M NaCl	0	120
	0.5 M NaCl	0	105
	1 M NaCl	0	70
C[b]	20% glycerol	0	87
	20% glycerol + 0.5 M NaCl	0	91
	0.1% $NaDodSO_4$[c]	0	11.4
	50 μM EDTA[c]	0	76
D[b]	20 μg/mL heparin	0	92
	20 μg/mL heparin + calf thymus DNA	0	103
	blank (no synthesis)	0	5-10

[a] 100% retention is 850 cpm. [b] 100% retention is 1350 cpm. [c] $NaDodSO_4$ = sodium dodecyl sulfate; EDTA = ethylenediaminetetraacetic acid.

Conditions Influencing Ternary Complex Stability. The ApU*pA-bearing ternary complex is sufficiently stable so that equivalent amounts of complex can be recovered by exclusion column chromatography or by Millipore filtration (Table II). Therefore, factors destabilizing ternary complexes should cause a loss of Millipore filter bound ApU*pA activity. Table IV shows that the ternary complexes are quite stable to changes in ionic strength as well as to polyanions. At 50 mM NaCl, complex degradation does not occur at 10 °C, but instability is noticeable at temperatures greater than or equal to 15 °C. Neither glycerol nor EDTA destabilizes the complex, but 0.1% sodium dodecyl sulfate virtually completely destroys it (Table IV).

Existence of σ Factor in Stable Ternary Complexes. It is known that σ factor has a catalytic function in promoter site selection (Travers & Burgess, 1969) and also that the association of σ factor with core enzyme is markedly diminished by the time the nascent RNA chain gets at least 10–12 residues long (Krakow & van der Helm, 1971). Nondenaturing gel electrophoresis analysis of σ factor release as described by Krakow (1971) indicates that the formation of ApUpA and UpApU as well as UpApU condensed with 3′-dATP does not cause σ release (data not shown).

Discussion

We have demonstrated that the formation of a single phosphodiester bond can result in a stable complex containing DNA template, RNA polymerase, and the product oligonucleotide functionally poised for further elongation. The formation of a stable ternary complex is not an inevitable consequence of the formation of the first phosphodiester in any RNA chain. With synthetic alternating templates, the stable complex is formed when a dinucleoside monophosphate primer begins with a purine (such as with ApU or with GpC) but not when the primer begins with a pyrmidine (as for UpA or CpG primers). Doubtless this specificity relates in part to the earlier observations that RNA chains initiated on such alternating templates almost inevitably start with a purine triphosphate (Maitra et al., 1967). Why are stable complexes formed in the presence of dinucleotides with a 5′-purine residue? Perhaps the enzyme has been fooled by the primer to make the first phosphodiester in a manner usually followed for the second one. This process presumably cannot occur with primers containing a pyrimidine on the 5′ terminus. Such a question might be explored by measuring stabilities of single-base adducts to appropriate ternary complexes. For example, we have found UpA + [α-^{32}P]UTP incubated with 3′-dATP does not give radioactivity appearing in the void volume of exclusion columns (data not shown), suggesting that the basis of stability is more complex.

As discussed at the beginning of this paper, kinetic considerations indicate that there is no a priori necessity that a stable ternary complex need exist because of the rapidity with which subsequent phosphodiester bonds can be formed. We are interested in the nature of formation of the stable ternary complex and in particular why phosphodiester bond formation is a requirement. Evidently it is not sufficient to admix the components of the complex and simply allow them to form a complex as judged from Millipore filter experiments (Table III). Evidently there are some configurational changes occurring that do not involve the release of σ factor (Stender, 1979).

Repetitive oligonucleotide formation is easier to study than initiating oligonucleotides that participate in stable complexes. However, the characterization of these abortive reactions does not seem to give a reliable measure of in vivo promoter strengths (Johnston & McClure, 1976; McClure & Cech, 1978; McClure et al., 1978). It remains to be seen whether the formation of stable ternary complexes is in fact related to in vivo promoter strength. The work of McClure et al. (1978) suggests that abortive RNA chain initiation may accompany normal RNA chain initiation at appreciable rates. If this is indeed the case in the cell, these oligonucleotides could possibly be used as primers to amplify gene expression of genes with complementary sequences. Our studies indicate that dinucleotide monophosphate and trinucleoside diphosphate primers can be readily out competed by equal concentrations of purine ribonucleoside 5′-triphosphates as initiators. We note that as the $r(A\text{-}U)_n$ chains become longer their effectiveness as primers at low concentrations dramatically increases (unpublished data). Clearly, suggestions of this sort need validation with naturally occurring sequences rather than precisely alternating templates and primers.

The existence of stable ternary complexes that are functionally active provides experimental possibilities for manipulations of elongation conditions such as the omission of single substrates for sequencing and addition of various concentrations of substrates for determinations of elongation kinetic constants and for attempts to allow the formation of a second phosphodiester bond to compare with the effects accompanying the first.

Acknowledgments

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Promoter Melting by T7 Ribonucleic Acid Polymerase As Detected by Single-Stranded Endonuclease Digestion†

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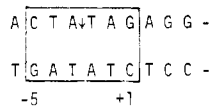
ABSTRACT: Highly purified T7 RNA polymerase is frequently contaminated with an endonuclease, probably the T7 gene 3 product, which is specific for single-stranded DNA. Since the double-stranded template is resistant, endonuclease activity can be used as an enzymatic probe for the regions of double-stranded DNA melted by the polymerase. In the absence of nucleoside triphosphates (NTPs) the T7 RNA polymerase melts the 10 base pair promoter sequence



as detected by the appearance of nuclease cleavages at all phosphodiester bonds of this region in the noncoding (upper) strand. In addition, a highly specific much more efficient cleavage is produced between the A and T in the noncoding strand (↓) which defines the center of twofold symmetry in the 6 base pair palindrome which includes the initiating nucleotide at the right end (position +1). All cleavages disappear when the temperature is lowered from 37 to 20 °C; the latter temperature was demonstrated by filter binding techniques to result in the loss of polymerase binding to the promoter. In the presence of NTPs, the cleavages in the initiation region disappear and the phosphodiester bonds in the noncoding strand most susceptible to nuclease attack are located downstream of the promoter in the direction of RNA synthesis. Some of the downstream phosphodiester bonds become more susceptibile than others, suggesting that the polymerase pauses at certain sequences. Alkylation of the single sulfhydryl group at the active site of the RNA polymerase alters the cleavage pattern seen in the promoter region, suggesting that the binding mode is altered, which may account for the inactivation which accompanies the alkylation. At high concentrations of T7 RNA polymerase all bonds of double-stranded DNA become mildly susceptible to attack by the accompanying endonuclease in the absence of NTPs, regardless of whether the fragment contains a promoter. This suggests that the RNA polymerase can bind to all regions of a double-stranded DNA regardless of sequence and in a process of one-dimensional diffusion increase the probability of melting of the double strand. When the polymerase encounters a promoter it "melts in" to form a binary complex of greatly increased lifetime in which the noncoding strand is relatively free in solution and susceptible to endonuclease attack, while the coding strand is protected in the active center groove.

We have recently found that preparations of the phage-specified T7 RNA polymerase are often contaminated by low levels of a single-stranded endonuclease which appears to be the product of phage gene 3. While the presence of this enzyme at low levels has no detectable effect on the doubled-stranded T7 DNA template, the enzyme does efficiently attack single-stranded regions of the template produced when the T7 RNA polymerase melts the template, particularly at the region of the well characterized T7 promoter sequence (Oakley & Coleman, 1977; Oakley et al., 1979). Only the noncoding strand is attacked specifically, suggesting that the coding strand is bound tightly and protected from endonuclease attack. This specific RNA polymerase induced nuclease

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