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RNA polymerase: linear competitive inhibition by bis-(3'→5')-cyclic dinucleotides, ⌜NpNp⌝

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ABSTRACT

We have investigated the possible role of the bis-(3'→5')-cyclic dinucleotides ⌜UpUp⌝ and ⌜ApUp⌝ as kinetic inhibitors of the DNA dependent RNA polymerase enzyme of E. coli, using $T_7\Delta D_{111}$ deletion mutant DNA and several synthetic DNA polymers as templates. We have established that ⌜UpUp⌝ is a linear competitive inhibitor of the initiation phase of the polymerization (K_I = 28 μM using $T_7\Delta D_{111}$ DNA as a template), but that it has no effect when added during the elongation phase. The compound ⌜ApUp⌝ is an inhibitor of the reaction only when poly(dA-T)·poly(dA-T) is used as a template, and ⌜UpUp⌝ is an inhibitor of the reaction when poly(dA)·poly(dT) was employed as the DNA template.

INTRODUCTION

We recently reported a model for rotational translocation for the DNA dependent RNA polymerase (1) in which the active site surface topography of the enzyme specifically accommodates bis-(3'→5')-cyclic di(ribosemonophosphate); see Figure 1. One prediction of this model is that a bis-(3'→5')-cyclic dinucleotide should be an effective inhibitor of the enzyme at the initiation phase of the reaction when both symmetric binding loci are unoccupied. In contrast, during the elongation phase of the reaction when one or both of the symmetric binding loci are occupied, these cyclic compounds should neither bind to the enzyme nor act as inhibitors of the polymerization.

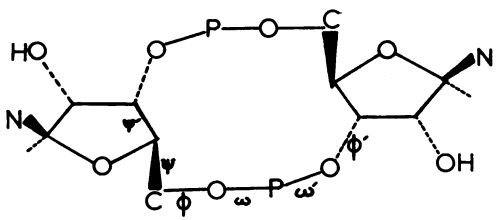

Figure-1 Symmetric representation of bis-(3'→5') di(ribose monophosphate).

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We have now devised a test system which allows one to clearly discriminate between the initiation phase and the elongation phase. This test system involves the formation of a precision stable ternary complex, [E•$T_7\Delta D_{111}$ DNA• pppApUpCpG], which is resistant to challenge by a salt jump or rifampicin as described by Kinsella (2). The cyclic dinucleotide inhibitors were added to a) the binary complex prior to the addition of any substrate, b) simultaneously with those substrates required to form a stable ternary complex, i.e., pppApU,CTP,GTP, or c) subsequent to the formation of the stable ternary complex and the salt jump challenge, when the remaining required substrates ATP and UTP were added to complete the reaction.

Several different bis-(3'→5')-cyclic dinucleotides have been synthesized by Hsu, et al., (3) using conventional phosphotriester condensation methods with recently reported selective blocking reagents. The compounds have been characterized by UV, CD, TLC, HPLC, as well as by ^{1}H-NMR and ^{31}P-NMR. The conformations of the rigid ribose rings have also been determined to be 3'-endo.

We have investigated the kinetic inhibition by bis-(3'→5')-cyclic di(uridine monophosphate) ⌜UpUp⌝ and bis-(3'→5')-cyclic (uridilyl-adenylyl) ⌜ApUp⌝ using $T_7\Delta D_{111}$ DNA and several synthetic DNAs as templates for the DNA dependent RNA polymerase of E. coli K_{12} [E.C. #2.7.76]. The inhibition observed is consistent with our model for rotational translocation and our suggestion for the topography of the active site surface.

MATERIALS AND METHODS

HPLC purified nucleoside triphosphates were purchased from ICN. ^{3}H-UTP was purchased from New England Nuclear and poly(dA)•poly(dT) and poly(dA-dT)• poly(dA-dT) from P. L. Biochemicals. The bis-(3'→5')-cyclic dinucleotides ⌜ApUp⌝ and ⌜UpUp⌝ were synthesized by a phosphotriester method (3). In each case, the final product was purified by HPLC and collected as a single peak. The purified product showed a single spot in cellulose TLC. Treatment of ⌜ApUp⌝ with Ribonuclease M (provided by Dr. M. Irie; Hoshi Pharm. College; Tokyo, Japan 142) and product analysis by cellulose TLC showed two spots, one corresponding to 3'-AMP and the other to 3'-UMP. Note that the notation ⌜ApUp⌝ is equivalent to ⌜UpAp⌝ since the cyclic dinucleotide is rotationally symmetric.

The DNA dependent RNA polymerase holoenzyme was isolated and purified from E. coli K_{12} by the method of Zillig (4); the purified holoenzyme had a σ/E ratio of 0.8 as judged by SDS gel electrophoresis. The T_7 phage ΔD_{111}

deletion mutant (containing only the A_1 major promoter) was obtained from W. Studier. The DNA was obtained by SDS-KCl cell rupture followed by two or more phenol extractions (5).

The dinucleotide tetraphosphate pppApU was prepared enzymatically by incubating RNA polymerase, $T_7\Delta D_{111}$ DNA, ATP, UTP, and rifampicin according to a variation of the method of Smagowich and Scheit (6). The product was purified by DEAE column chromatography and characterized by ascending cellulose TLC using the WASP (water:saturated $(NH_4)_2SO_4$:isopropanol = 18:80:2) or 1BAW (isobutyric acid:ammonium hydroxide:water = 66:1:33) solvent systems. The concentration of poly(dA-dT)·poly(dA-dT) and poly(dA)·poly(dT) was determined spectroscopically using the extinction coefficient 6.65 mM^{-1} cm^{-1} at 262 nm and 6.0 mM^{-1} cm^{-1} at 260 nm, respectively.

REACTION PROTOCOLS FOR KINETIC INHIBITION STUDIES USING $T_7\Delta D_{111}$ TEMPLATE

The reaction mixture (final concentration) was 40 mM Tris·HCl, pH 7.9; 0.1 mM EDTA; 80 mM KCl; 10 mM $MgCl_2$; 1 mM DTT; 1.66 nM $T_7\Delta D_{111}$ DNA; 20 nM RNA polymerase holoenzyme; 0.2 mM pppApU; 0.2 mM each of ATP, CTP, GTP; and 0.08 mM ^{3}H-UTP (containing 200 CPM/pmole). In experiments where a salt jump challenge was employed, the final concentration of KCl was 0.38 M. The concentration of the cyclic dinucleotide ($\lceil$ApUp$\rceil$ or $\lceil$UpUp$\rceil$) was 0.25 mM where used. The total reaction volume was either 0.1 or 0.05 ml. Protocols for assembly of the reaction components are presented below where S = components included in the preincubation mixture with the binary complex; N' = components added to the preincubated mixture prior to the salt jump challenge, where the mixture was made 0.38 M with KCl; and N = addition of remaining components subsequent to the salt jump challenge. The subscript denotes the time of incubation in minutes at 37°C for each successive segment of the reaction.

(a) $\{E\sigma + T_7\Delta D_{111}\ DNA + S\}_{10}\{KCl\}_{2.5}\{N\}_{10}$ (as in Table Ia)

(b) $\{E\sigma + T_7\Delta D_{111}\ DNA + S\}_{10}\{N'\}_1\{KCl\}_2\{N\}_{10}$ (as in Table Ib)

Reactions were terminated by the addition of 1.0 ml 5% TCA, and incubation in an ice bath for 20 minutes. The ^{3}H labeled RNA product was collected by filtration through a Whatman G-F/C glass filter disc which was prerinsed with cold 5% TCA. The discs were then rinsed three times with cold 5% TCA, once with cold ethanol, dried at 100°C and placed in a scintillation vial containing a toluene based cocktail. Radioactivity was determined using a Beckman L-100.

REACTION PROTOCOLS FOR KINETIC INHIBITION STUDIES USING A SYNTHETIC DNA TEMPLATE

The reaction mixture (final concentration) was 40 mM Tris·HCl, pH 7.9; 0.1 mM EDTA; 80 mM KCl; 10 mM $MgCl_2$; 1.0 mM DTT; 20 nM RNA polymerase holoenzyme; 50 µM poly(dA-dT)·poly(dA-dT); 0.2 mM ⌜ApUp⌝; and 0.1 mM ^{3}H-UTP (200 CPM/pmole) and 0.2 mM ATP. The total reaction volume was 0.1 ml.

When poly(dA)·poly(dT) was employed as the DNA template at a concentration of 50 µM, the RNA polymerase holoenzyme was 60 nM and only ^{3}H-UTP (200 CPM/pmole, final concentration of 0.28 mM) was added as a substrate. The cyclic dinucleotide (⌜UpUp⌝) concentration was 0.2 mM, and the final concentration of KCl was 0.38 M after the salt jump challenge.

These protocols are presented below where S, N', and N have the same meanings as before.

(c) $\{E\sigma + \text{poly(dA-dT)}\cdot\text{poly(dA-dT)} + S\}_{10}\{N\}_5$ (as in Table II)

(d) $\{E\sigma + \text{poly(dA)}\cdot\text{poly(dT)} + S\}_{10}\{KCl\}_2\{N\}_{10}$ (as in Table III)

The reactions were terminated and assayed for radioactivity as previously described.

DETERMINATION OF THE KINETIC CONSTANT K_m FOR pppApU

The reaction mixture (final concentration) was 40 mM Tris·HCl, pH 7.9; 0.1 mM EDTA; 80 mM KCl; 10 mM $MgCl_2$; 1.66 nM $T_7\Delta D_{111}$ DNA; and 20 nM RNA polymerase holoenzyme. Various concentrations of pppApU were included in a preincubation mixture for 10 minutes prior to the addition of CTP and GTP, to a concentration of 20 µM. After the salt jump challenge, when KCl was increased to 0.38 M and incubated for 1 minute, the substrates ATP, CTP, GTP and ^{3}H-UTP (700 CPM/pmole) were brought to a final concentration of 0.2, 0.22, 0.22 and 0.05 mM, respectively.

The protocol for the assembly of the reaction components for this experiment is presented below, where the subscript indicates the time of incubation in minutes at 37°C for each successive segment of the reaction:

$\{E\sigma + T_7\Delta D_{111}\ \text{DNA} + \text{pppApU}\}_{10}\{C,G\}_1\{KCl\}_2\{A,U,C,G\}_{10}$ (as in Figure 2)

The reactions were terminated and assayed for radioactivity as previously described.

DETERMINATION OF THE KINETIC CONSTANT K_I FOR ⌜UpUp⌝

The reaction mixture (final concentration) was 40 mM Tris·HCl, pH 7.9; 0.1 ml EDTA; 80 mM KCl; 10 mM $MgCl_2$; 1.66 nM $T_7\Delta D_{111}$ DNA; 20 nM RNA polymerase holoenzyme; and either 0.04 or 0.08 mM pppApU. Various concentrations of

┌UpUp┐ were included in a preincubation mixture for 10 minutes prior to the addition of CTP and GTP to give a concentration of 20 μM. After the salt jump challenge, when KCl was increased to 0.38 M and incubated for 1 minute, the concentration of the substrates ATP, CTP, GTP and ^{3}H-UTP (700 CPM/pmole) were brought to a final concentration of 0.2, 0.22, 0.22 and 0.05 mM, respectively.

The protocol for the assembly of the reaction components for this experiment is presented below; again, the subscript indicates the time of incubation in minutes at 37°C for each successive segment of the reaction:

$$\{E\sigma + T_7\Delta D_{111} \text{ DNA} + \text{pppApU} + \text{┌UpUp┐}\}_{10}\{C,G\}_1\{KCl\}_2\{A,C,U,G\}_{10}$$ (as in Figure 3)

The reactions were terminated and assayed for radioactivity as previously described.

RESULTS

$T_7\Delta D_{111}$ DNA Template System

The inhibition of RNA polymerization during the transcription of the $T_7\Delta D_{111}$ (A_1 promoter AUCGA...) DNA template was examined in a system where the effect on initiation or elongation could be clearly separated. In Table I the effect of the cyclic dinucleotides is summarized. It can be seen that the cyclic dinucleotide ┌UpUp┐ inhibits the reaction when added prior to (experiment b-2) or simultaneously with (experiment a-3) the substrates required to form a stable ternary complex E•$T_7\Delta D_{111}$ DNA•pppApUpCpG, which is resistant to a salt jump challenge. The compound ┌UpUp┐, however, has no effect on the reaction when added during the elongation of the preformed stable ternary complex (experiment a-4).

The inhibition of RNA polymerization by ┌ApUp┐ was minimal with the $T_7\Delta D_{111}$ DNA template under all conditions tested (experiments b-3, a-5, a-6), and demonstrated no preferential inhibition for the initiation or elongation phase of the reaction.

Synthetic DNA Template Systems

The inhibition of RNA polymerization during the transcription of the synthetic DNA template poly(dA-dT)•poly(dA-dT) by ┌ApUp┐ is summarized in Table II. Little inhibition was observed when this cyclic dinucleotide was preincubated with the enzyme•DNA binary complex prior to the addition of substrate (experiment 1), however, substantial inhibition was observed when either one of the substrates (ATP or UTP) was contained in the preincubation mixture along with the cyclic dinucleotide (experiments 3 and 4).

Table I. Kinetic Studies using ⌜UpUp⌝ or ⌜ApUp⌝ with the $T_7\Delta D_{111}$ DNA as Template

	S	N'	KCl	N	% Velocity
(a)					
1)	pppApU,C,G	----	0.38 M	A,U	100
2)	pppApU	----	0.38 M	A,C,G,U	18
3)	pppApU,C,G,⌜UpUp⌝	----	0.38 M	A,U	35
4)	pppApU,C,G	----	0.38 M	A,U,⌜UpUp⌝	92
5)	pppApU,C,G,⌜ApUp⌝	----	0.38 M	A,U	82
6)	pppApU,C,G	----	0.38 M	A,U,⌜ApUp⌝	82
(b)					
1)	----	pppApU,C,G	0.38 M	A,U	100
2)	⌜UpUp⌝	pppApU,C,G	0.38 M	A,U	20
3)	⌜ApUp⌝	pppApU,C,G	0.38 M	A,U	78

The protocol for the assembly of the successive reaction segments are: S = components included in the preincubation mixture with the binary complex ($E \cdot T_7\Delta D_{111}$); N' = components added to the reaction mixture prior to the salt jump challenge where the mixture was made 0.38 M with KCl; and N = addition of remaining components required to complete the reaction subsequent to the salt jump challenge. Times of incubation for each segment and concentration of components added are all given in Methods.

Table II. Kinetic Studies using ⌜ApUp⌝ with a Synthetic DNA Template, poly(dA-dT)·poly(dA-dT)

	S	N	% Velocity
1)	----	A,U	100
2)	⌜ApUp⌝	A,U	94
3)	A,⌜ApUp⌝	U	52
4)	U,⌜ApUp⌝	A	51

Table II Legend - In experiment 3) ATP was included in the preincubation mixture along with the cyclic dinucleotide, and in experiment 4) UTP was included in the preincubation mixture along with the cyclic dinucleotide.

Table III. Kinetic Studies using ⌜UpUp⌝ with a Synthetic DNA Template, poly(dA)·poly(dT)

	S	KCl	N	% Velocity
1)	U	0.38 M	U	100
2)	U, ⌜UpUp⌝	0.38 M	U	35
3)	U	0.38 M	U, ⌜UpUp⌝	88

The interim concentration of UTP when added to segment "S" was 0.08 mM and 0.28 mM (final concentration) when added to segment "N" of the reaction.

The inhibition of RNA polymerase during the transcription of poly(dA)·poly(dT) by ⌜UpUp⌝ is summarized in Table III. Substantial inhibition was observed when the cyclic dinucleotide was added simultaneously with a low concentration (0.08 mM) of the substrate prior to the salt jump (experiment 2); however, only minimal inhibition was observed when the cyclic dinucleotide was added after the salt jump (experiment 3).

Kinetic Constants for pppApU and ⌜UpUp⌝

A K_m value of 58 μM for the dimer primer pppApU was determined from kinetic data (see Figure 2). A K_m value of 19 μM for pppApU was determined

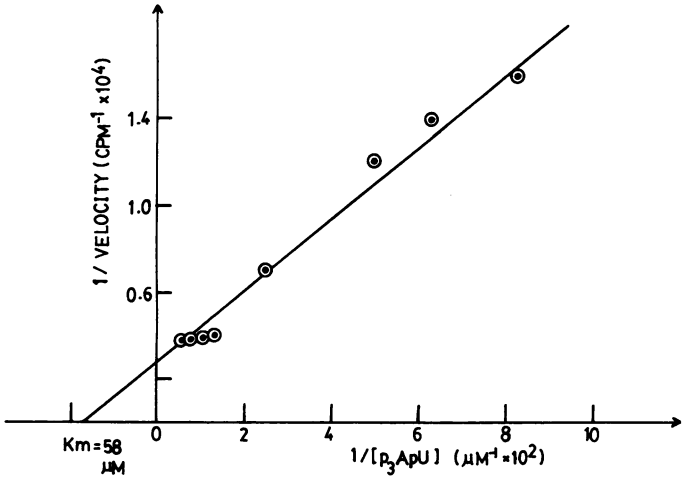

Figure-2 Lineweaver Burke plot of kinetic data for determination of K_M value for the dimer primer pppApU.

by Scheit (7), when all four nucleotides were present. The binding constant for the dimer primer is somewhat higher than for the nucleotide triphosphate substrates during elongation (K_m = 15 μM), but less than the K_m for the initiating substrate nucleotide triphosphate, $K_{m_{ATP}}$ = 150 μM.

The results of the kinetic inhibition studies with the cyclic dinucleotide ⌜UpUp⌝ are presented in Figure 3. A Dixon plot of the data (Figure 3a) shows competitive inhibition and yields a value for K_i of 28 μM. A secondary plot of the slopes of the primary plots vs. 1/pppApU is shown in Figure 3b. The line passes through the origin, characteristic of a linear competitive inhibitor.

DISCUSSION

In our model for the RNA polymerase enzyme, the active surface is represented as the cognate of a bis-(3'→5')-cyclic di(ribose monophosphate). The active surface, therefore, has two ribose binding sites and two catalytic sites which are positioned with a two-fold rotational symmetry, see Figure 1. Here, we have examined the possibility that a rigid bis-(3'→5')-cyclic dinucleotide can simultaneously bridge both sites in the initiation phase when neither binding site is occupied by a substrate molecule. We have observed that the bis-(3'→5')-cyclic dinucleotide ⌜UpUp⌝ is a linear competitive inhi-

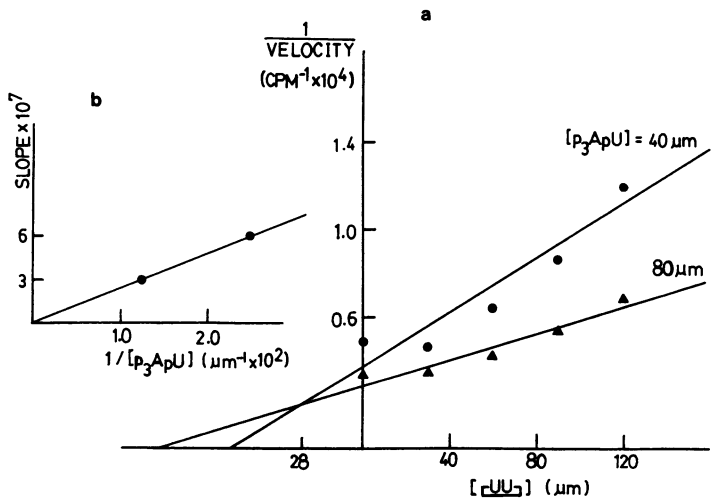

Figure-3 a) Dixon plot of kinetic data for determination of K_i value for the cyclic dinucleotide ⌜UpUp⌝ , b) secondary plot of primary plot slope vs. [pppApU].

bitor of the RNA polymerase (K_i = 28 μM), only during the initiation phase, prior to the formation of a stable ternary complex involving a tetrameric RNA product. No inhibition was observed when ⌜UpUp⌝ was added during the elongation phase, subsequent to the formation of a stable ternary complex.

The base dependency of these cyclic dinucleotides is indicated by the observation that ⌜ApUp⌝ (indistinguishable from ⌜UpAp⌝) does not demonstrate any substantial inhibition of the same system ($T_7\Delta D_{111}$ DNA, A_1 promoter = pppApUpCpGpA.....) during either the initiation or the elongation phase. The cyclic dinucleotide ⌜ApUp⌝ is, however, a potent competitive inhibitor of the polymerase when a synthetic DNA is used as a template poly(dA-dT)·poly(dA·dT), and either of the nucleotide triphosphates UTP or ATP is present during the reaction preincubation. A possible explanation for this latter observation is that the first substrate binding site (denoted i) is unique and is not employed during the elongation phase of the reaction. The additional unique properties of this first substrate binding site are: (1) K_m is an order of magnitude higher for the first substrate than for that same nucleotide triphosphate incorporated during elongation (7); and (2) the formation of the first phosphodiester bond is not inhibited by rifampicin (8,9). A speculative hypothesis is that the obligatory first substrate binding site (i) is the asymmetric starting point for establishing the 3'→5' polarity of phosphodiester bond formation, and that thereafter elongation involves only the symmetric active sites (e_1 and e_2). Such a model for initiation is depicted in Figure 4.

The model depicts the initiating site (i) which binds the first substrate and the symmetric elongation sites (e_1 and e_2) which bind the second and third substrates. The first phosphodiester bond forms then between substrates bound at i and e_1; the second phosphodiester bond forms between the product terminus substrate bound at e_1 and the incoming substrate bound at e_2. Commitment to elongation in this model would require the binding of the fourth substrate at e_1, and elongation would thereafter involve only the alternating use of the symmetric sites e_1 and e_2.

The formation of a stable ternary complex resistant to a salt jump or rifampicin challenge occurs coincident with the production of a bound tetrameric RNA product ($E\cdot T_7\Delta D_{111}$ DNA·pppApUpCpG). The abortive products released are exclusively the dimer (pppApU) and the trimer (pppApUpC) in this system (2). The longest abortive product released is a trimer which can be easily accounted for in this model for initiation. This enzyme-limited abortive product length is not consistent with results reported for abortive product

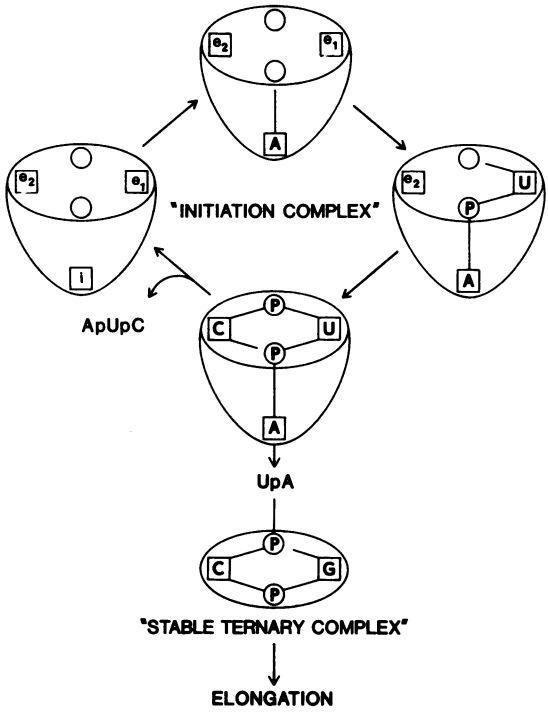

Figure-4 A scheme for the initiation sequence and formation of a stable ternary complex for the $T_7\Delta D_{111}$ A_1 promoter where the sequence of the transcript is pppApUpCpG------. The three binding sites are (i) the unique binding site only for the first nucleotide and (e_1 and e_2) the symmetric binding sites employed during the elongation phase.

lengths using other templates (poly(dA-dT) poly(dA-dT) (10), T_7 DNA (11) and Lac UV-5 (12)), and therefore is not a general property of the enzyme.

Rapid mix kinetic studies using $T_7\Delta D_{111}$ DNA as a template have suggested that the addition of the third base (CTP) modifies the amount of abortive dimer produced by acting as an allosteric agent (13). The reduction in the amount of abortive dimer produced may be accounted for by the favorable production of the abortive trimer pppApUpC.

The cyclic dinucleotides may thus bind to the unoccupied symmetric sites, e_1 and e_2, but not the initiation site, i. The lack of inhibition by $\lceil\underline{\text{ApUp}}\rceil \equiv \lceil\underline{\text{UpAp}}\rceil$ when $T_7\Delta D_{111}$ DNA is employed as a template suggests that base specificity (dictated by the template) may be a critical factor affecting the binding constant of the cyclic dinucleotides. The e_1 site would require (U)

and the e_2 site would require (C) for the A_1 promoter, therefore the e_2 site does not bind the purine base (A) in the case of ┌UpAp┐ and this cyclic dinucleotide is not an effective inhibitor of the polymerase. The cyclic dinucleotide ┌UpUp┐ positions a (U) at the e_1 site and the pyrimidine base (U) at the e_2 site which is accepted in place of the required (C). The obvious choice for a cyclic dinucleotide inhibitor for this promoter is ┌UpCp┐ and we are currently engaged in the synthesis of this compound.

The apparent requirement for occupancy of the (i) site by a nucleotide triphosphate (in the case of the synthetic templates lacking a promoter) in order to demonstrate inhibition by the cyclic dinucleotides is interesting and requires further investigation to be resolved.

Studies are also now in progress to attempt to derivatize differentially and thus identify the two functionally equivalent but topographically distinct active sites proposed by the model.

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