Magnetic Resonance Studies of the Conformation of Enzyme-Bound Adenylyl(3′→5′)uridine and Adenosine 5′-Triphosphate on RNA Polymerase from *Escherichia coli*†

Bruce L. Bean, Ruth Koren, and Albert S. Mildvan*

ABSTRACT: Kinetic studies of Mn(II)-activated RNA polymerase show that ApU stimulates the incorporation of [^{3}H]ATP into polyribonucleotides and that [^{3}H]ApU is itself incorporated into the polyribonucleotide product, indicating that ApU can function as an initiator of RNA synthesis. The paramagnetic effects of RNA polymerase-Mn(II) on the relaxation rates of the protons and phosphorus of ApU bound at the initiation site and of the protons of ATP bound at the elongation site have been investigated. The binary Mn(II)-ApU complex has also been studied as a control. Using correlation times determined from the frequency dependence of the longitudinal relaxation rates of water protons and of ApU protons in the enzyme complexes, distances from the enzyme-bound Mn(II) to the protons and ^{31}P of ApU (9.0–10.5 Å) and to the protons of ATP (4.0–5.7 Å) have been calculated. The smaller distances to ATP than to ApU strongly support the tight binding of the divalent cation activator Mn(II) at the elongation site. The distances from enzyme-bound Mn(II) to the protons of enzyme-bound ATP are indistinguishable from those in the binary Mn(II)-ATP complex. Upon binding to the enzyme-ApU-Mn(II) complex, ATP decreases the number of fast exchanging water ligands on Mn(II), indicating the displacement or occlusion of water ligands. The seven distances from the enzyme-bound Mn(II) to the protons and phosphorus of enzyme-bound ApU exclude direct coordination of ApU and can best be fit by a nonhelical S_1 conformation with glycosidic χ angles of 30° (adenosine) and 60° (uridine). Since an RNA A-type helical conformation for bound ApU is excluded by the distances, the enzyme may play a catalytic role in separating the newly synthesized RNA chain from the enzyme-bound DNA template as the growing RNA chain passes through the initiation site. Unlike the ternary complex, in the weak binary Mn(II)-ApU complex the distances suggest the direct coordination of a phosphoryl oxygen to Mn(II) and a helical P_3-type conformation for ApU. Mn(II) and its residual water ligands appear to be intercalated between the parallel planes of the bases which are 7.5 ± 1.0 Å apart.

All RNA polymerases require a divalent metal ion for catalytic activity (Stevens and Henry, 1964). Either Mg(II) or Mn(II) will activate RNA synthesis, with the paramagnetic ion Mn(II) stimulating synthesis at a rate twice that achieved with Mg(II) (Furth et al., 1962). The active site of this enzyme actually involves two sites, designated the initiation site and the elongation site. The initiation site appears to bind the initiator, a purine nucleotide or dinucleoside monophosphate (Downey and So, 1970) with the 5′-terminal base being either adenine or guanine (Chamberlin, 1974). The elongation or polymerization site can bind any of the four nucleoside triphosphate substrates, with a specificity determined by the DNA template.

In a previous paper (Koren and Mildvan, 1977), we have provided thermodynamic and kinetic evidence for a single tight Mn(II) binding site on the enzyme functioning at the elongation site. We now have extended this work through high-resolution NMR[1] by using the Mn(II) ion as a paramagnetic reference point to examine the structure and conformation of bound nucleotides, both at the initiation site, using the dinucleoside monophosphate initiator ApU, and at the elongation site, using ATP in the presence of the initiators ApU or GpU. A preliminary report of this work has been published (Mildvan et al., 1977).

Experimental Procedures

Materials

RNA polymerase was purified by the method of Burgess and Jendrisak (1975) from 5 pounds of *E. coli* (K12, ¾ log phase, grown on enriched medium) purchased from Grain Processing Co., Muscatine, Ill. The purified enzyme was at least 96% pure as judged by acrylamide gel electrophoresis. It contained the proper percentages of α, β, β', and σ subunits to within 4%, and gave a specific activity of 300 units/mg when assayed under the conditions of Burgess (1969). The enzyme was reassayed at the conclusion of each NMR experiment and it retained a minimum of 81% of its initial activity. Protein concentration was determined by UV absorption at 280 nm assuming an extinction coefficient of 0.65 mg^{-1} ml (Richardson, 1966) and a molecular weight of 500 000 (Chamberlin, 1974). Nucleic acid contamination of the enzyme was negligible as estimated by the ratio of 1.8 of its UV absorption at 280 and 260 nm.

Pancreatic ribonuclease was purchased from Worthington. Alkaline phosphatase and ribonuclease T_2 were purchased from Sigma, and inorganic pyrophosphatase (from yeast) was a gift of Dr. Barry Cooperman of the Chemistry Department, University of Pennsylvania.

The nucleoside triphosphate ATP and the dinucleoside monophosphate adenylyl(3′→5′)uridine (ApU), purchased from Sigma, were stored at −20 °C. The solid ATP or ApU

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[1] Abbreviations used: NMR, nuclear magnetic resonance; UV, ultraviolet; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance.

was dissolved in H_2O and neutralized to pH 7.5 at 2 °C with KOH. ATP was judged pure by thin-layer chromatography with 1.0 M LiCl as the solvent (Randerath and Randerath, 1964). Nucleotide concentrations were determined by UV absorption at 259 nm for ATP ($\epsilon = 15.4 \times 10^3$) (Dawson et al., 1969), and at 258 nm for ApU ($\epsilon = 23.2 \times 10^3$) (P-L Biochemicals, 1975). All other reagents were the highest grade obtainable. Trace metal contaminants were removed from buffer, enzyme, and nucleotide solutions by elution through Bio-Rad Chelex 100.

Methods

Kinetic Experiments. To confirm the functioning of ApU as an initiator of Mn(II)-activated RNA synthesis, as originally found with Mg(II) (Downey and So, 1970), kinetic experiments with Mn(II) were carried out. The enzyme was dialyzed overnight against 50 mM Tris-Cl (pH 7.5) and 150 mM KCl. The assay used measured the incorporation of [^{3}H]ATP or [^{3}H]ApU into an acid-insoluble product, and used poly(dA-dT)·poly(dA-dT) as a template. The reaction mixture, in 0.10 mL, contained 50 mM Tris-Cl (pH 7.5), 0.5 mM $MnCl_2$, and variable amounts of ATP, [^{3}H]ATP, ApU, [^{3}H]ApU, UTP, and template. The reaction was started by the addition of enzyme (30–90 pmol). The mixture was incubated at 24 °C for 30–65 min and was stopped by adding 2 mL of 1 M $HClO_4$ containing 20 mM sodium pyrophosphate. The acid-insoluble radioactivity was measured by the method of Loeb (1969).

Preparation of Radioactive ApU. Radioactive ApU, labeled with ^{3}H in the uridine portion, was prepared by incubating RNA polymerase (143 μg/mL); [^{3}H]UTP (0.5 mM); ATP (0.5 mM); poly(dA-dT)·poly(dA-dT) (0.20 mg/mL); in the presence of $MgCl_2$ (10 mM), KCl (150 mM), and Tris-Cl, pH 7.5 (50 mM), in a total volume of 10.0 mL at 27 °C. Inorganic pyrophosphatase (40 μg) was added after 90 min. The reaction was stopped after 150 min with 2.5 mL of NaEDTA (200 mM) and boiling for 5 min. The reaction mixture was then dialyzed for 3 days against 50 mM Tris-Cl containing 100 mM KCl.

The polyribonucleotide product $(ApUp)_N$ was degraded to the dinucleotide ApUp in a reaction mixture (total volume 3.5 mL) which contained: 50 mM Tris-Cl (pH 7.5), 50 μg of pancreatic RNase A, and 1.05 mg of $(ApUp)_N$. The mixture was digested for 16 h at 37 °C. The dinucleotide was isolated by chromatography on DEAE-cellulose and the 3′-phosphoryl group was removed with alkaline phosphatase in a reaction mixture (total volume 2.0 mL) which contained: 50 mM Tris-Cl (pH 7.9) and 45 μg of *E. coli* alkaline phosphatase.

Radioactive ApU was purified by DEAE-cellulose chromatography. The specific activity was 8.3 cpm per pmol. The identification of the product as ApU was established by RNase T_2 digestion in a reaction mixture (total volume 40 μL) which contained: 7.3 nmol of ApU, 20 mM ammonium acetate, and 0.3 unit of T_2 RNase.

The digestion mixture was separated by thin-layer chromatography (2-propanol–HCl–H_2O, 65:16.7:18.3). Identification of [^{3}H]uridine and AMP was established by comigration with appropriate markers. A review of these procedures has been published by Holley (1968).

Preparation of Enzyme Solutions for NMR Studies. The enzyme, which was stored at −70 °C in 10 mM Tris-Cl, pH 7.9, containing 50% (v/v) glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 100 mM NaCl (Burgess and Jendrisak, 1975), was diluted ten times with cold 70 mM Tris-Cl (pH 7.5) and 200 mM KCl, precipitated by adding 0.5 g/mL of solid $(NH_4)_2SO_4$, and centrifuged 20 min at 15 000 rpm. The pellet was redissolved in 0.05 M Tris-Cl (pH 7.5) and 150 mM KCl, passed through a Sephadex G-25 column, and concentrated by vacuum dialysis against this second buffer to a final concentration of 50 mg/mL. For the proton NMR experiments, the water solvent was replaced by D_2O using vacuum dialysis. Dilution with deuterated buffer and concentration by vacuum dialysis was repeated at least three times to ensure maximal deuteration.

Magnetic Resonance Measurements. The binding of Mn(II) to ApU was studied by EPR (Cohn and Townsend, 1954) and by measurements of the longitudinal relaxation rate ($1/T_1$) of water protons (Mildvan and Engle, 1972). The longitudinal ($1/T_1$) and transverse ($1/T_2$) nuclear magnetic relaxation rates of the protons and phosphorus of ApU and the protons of ATP were measured at 100 MHz (protons) and 40.5 MHz (^{31}P). In addition, the longitudinal and transverse relaxation rates of the protons of ApU were measured at 220 MHz, and the longitudinal and transverse relaxation rates of water protons were determined at 8, 15, 24.3, 40, and 100 MHz. The 40.5-MHz (^{31}P), 100-MHz (^{1}H), and 220-MHz (^{1}H) measurements of $1/T_1$ were made using the pulsed Fourier transform 90°–homogeneity spoil–τ–90° method of McDonald and Leigh (1973), and all other $1/T_1$ measurements were made with the 180°–τ–90° null point method (Carr and Purcell, 1954). All $1/T_2$ measurements were made by the spin-echo 90°–τ–180°–τ method (Carr and Purcell, 1954). The water proton measurements at 8, 15, 24.3, and 40 MHz were made on an NMR Specialities PS 60W spectrometer modified in this laboratory, and the 100-MHz and 220-MHz measurements were performed on the Varian XL-100 and HR-220 FT NMR spectrometers, respectively. Proton decoupling was used to simplify the ^{31}P resonance and to eliminate residual H_2O signals in ^{1}H measurements at 100 MHz. The temperature for the relaxation measurements was 24 °C and was maintained to ±1 °C by cooled nitrogen gas flow.

To determine the paramagnetic contribution to the relaxation rates of the protons and phosphorus of ApU and the protons of ATP, a Mn(II) solution was added stepwise to the sample containing the enzyme and substrate or to the substrate alone after measuring the diamagnetic relaxation rates in the absence of metal or in the presence of Mg(II) instead of Mn(II). Both types of diamagnetic measurements yielded identical results which were then subtracted from the relaxation rates observed with Mn(II) to yield the paramagnetic contributions to $1/T_1$ and $1/T_2$. The amount of enzyme–Mn(II)–substrate complex present was determined by subtracting the contributions due to the other Mn(II) complexes (Mn(II)–substrate and enzyme–Mn(II)) using the appropriate dissociation constants (Koren and Mildvan, 1977).

Theoretical Basis for Calculations. The calculations in this paper are based on the theory of Solomon and Bloembergen (Solomon, 1955; Solomon and Bloembergen, 1956; Bloembergen, 1957; Bernheim et al., 1959) for the paramagnetic effects on the nuclear spin relaxation rates of a magnetic nucleus which is bound near a paramagnetic species. The equations for the two relaxation rates are:

$$\frac{1}{T_{1M}} = \frac{2}{15}\frac{S(S+1)\gamma_I^2 g^2 \beta^2}{r^6}\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_s^2\tau_c^2}\right) + \frac{2}{3}\frac{S(S+1)A^2}{\hbar^2}\left(\frac{\tau_e}{1+\omega_s^2\tau_e^2}\right) \quad (1)$$

$$\frac{1}{T_{2M}} = \frac{1}{15}\frac{S(S+1)\gamma_I^2 g^2 \beta^2}{r^6}\left(4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{13\tau_c}{1+\omega_s^2\tau_c^2}\right) + \frac{1}{3}\frac{S(S+1)A^2}{\hbar^2}\left(\tau_e + \frac{\tau_e}{1+\omega_s^2\tau_e^2}\right) \quad (2)$$

where $1/T_{1M}$ and $1/T_{2M}$ are the paramagnetic contributions to the longitudinal and transverse relaxation rates of a molecule bound near the paramagnetic nucleus, S is the electron spin, γ_I is the nuclear gyromagnetic ratio, g is the electronic g factor, β is the Bohr magneton, r is the metal-nucleus distance, ω_I and ω_s are the nuclear and electron spin larmor precession frequencies, τ_c and τ_e are the correlation times for the dipolar and contact interactions, and A is the contact hyperfine coupling constant.

The measured values of the paramagnetic relaxation rates $1/T_{1p}$ and $1/T_{2p}$ are related to $1/T_{1M}$ and $1/T_{2M}$ by equations derived by Luz and Meiboom (1964) and Swift and Connick (1962), respectively:

$$\frac{1}{fT_{1p}} = \frac{q}{T_{1M} + \tau_M} + \frac{1}{T_{os}} \tag{3}$$

$$\frac{1}{fT_{2p}} = \frac{q}{\tau_M}\left(\frac{(1/T_{2M})^2 + 1/(T_{2M}\tau_M) + \Delta\omega_M{}^2}{(1/T_{2M} + 1/\tau_M)^2 + \Delta\omega_M{}^2}\right) + \frac{1}{T_{os}} \tag{4}$$

where f is the ratio of the concentration of the bound metal to the concentration of the ligand, q is the number of ligands bound at or near each metal ion, τ_M is the residence time of the ligand in the complex, $\Delta\omega_m$ is the chemical-shift difference between free and bound ligands, and $1/T_{os}$ is the contribution of outer sphere molecules which diffuse past the paramagnetic center. If $\Delta\omega_m \approx 0$, as is generally the case with Mn^{2+} complexes (Mildvan and Engle, 1972), then

$$\frac{1}{fT_{2p}} \approx \frac{q}{T_{2M} + \tau_M} + \frac{1}{T_{os}} \tag{5}$$

Now if the contact term, τ_M, and $1/T_{os}$ are negligible (Mildvan and Engle, 1972; Melamud and Mildvan, 1975) then

$$\frac{1}{fT_{1p}} = \frac{qC^6}{r^6} f(\tau_c) \tag{6}$$

where

$$C^6 = \frac{2}{15} S(S+1)\gamma_I{}^2 g^2 \beta^2 \tag{6a}$$

and

$$f(\tau_c) = \frac{3\tau_c}{1 + \omega_I{}^2\tau_c{}^2} + \frac{7\tau_c}{1 + \omega_s{}^2\tau_c{}^2} \tag{6b}$$

Equation 6 will be used in the calculations of q and r.

If the correlation time is dominated by the electron spin relaxation time T_{1e} of the Mn(II) ion, then it is given by (Bloembergen and Morgan, 1961):

$$\frac{1}{T_{1e}} = B\left[\frac{\tau_v}{1 + \omega_s{}^2\tau_v{}^2} + \frac{4\tau_v}{1 + 4\omega_s{}^2\tau_v{}^2}\right] \tag{7}$$

where B is a constant containing the electronic spin S and the zero-field splitting of Mn(II), and τ_v is a characteristic time for symmetry distortions of the complex. This frequency dependent correlation time has two limiting cases:

$$(1)\omega_s{}^2\tau_v{}^2 \gg 1 \tag{8}$$

Then $T_{1e} = \omega_s{}^2\tau_v/2B$ and the ratio of $1/fT_{1p}$ at two different NMR frequencies (ν_1 and ν_2) is given by (Fung et al., 1973):

$$\frac{1/fT_{1p}(\nu_1)}{1/fT_{1p}(\nu_2)} = \frac{1 + 4\pi^2\nu_2{}^2(\nu_2/\nu_1)^4\tau_s{}^2}{(\nu_2/\nu_1)^2[1 + 4\pi^2\nu_1{}^2\tau_s{}^2]} \tag{9}$$

with $\tau_s = T_{1e}$ at ν_1.

$$(2)4\omega_s{}^2\tau_v{}^2 \ll 1 \tag{10}$$

TABLE I: Incorporation of [^{3}H]ATP and [^{3}H]ApU into RNA.

[ApU] (μM)	fmol of ATP incorp[a] min^{-1} (pmol of enzyme)$^{-1}$	fmol of ApU incorp[b] min^{-1} (pmol of enzyme)$^{-1}$
0	449	0
18.2	505	
36.5	511	0.32
73.0	560	0.49
146		0.93

[a] In addition to ApU, the reaction mixture contained 50 mM Tris, 0.5 mM $MnCl_2$, 0.052 mg/mL poly(dA-dT)·poly(dA-dT), 106 μM [^{3}H]ATP, 104 μM UTP, and 30 pmol of enzyme. The mixture was incubated at 24 °C for 30 min. Incorporation of [^{3}H]ATP in the absence of enzyme was <2% of that measured in the presence of enzyme. The estimated error in the ATP incorporation is ±5%. [b] The reaction mixture contained 50 mM Tris, 0.5 mM $MnCl_2$, 0.10 mg/mL poly(dA-dT)·poly(dA-dT) sonicated for 30 s, 21.3 μM ATP, 21.0 μM UTP, 90 pmol of enzyme, and [^{3}H]ApU in place of unlabeled ApU at the indicated concentrations; it was incubated at 24 °C for 65 min. Incorporation of [^{3}H]ApU in the absence of enzyme was less than 22% of that observed in its presence. The estimated error in the ApU incorporation is ±10%.

In this case $T_{1e} = 1/5B\tau_v$ and is independent of frequency. The ratio of $1/fT_{1p}$ at two NMR frequencies gives

$$\frac{1/fT_{1p}(\nu_1)}{1/fT_{1p}(\nu_2)} = \frac{1 + 4\pi^2\nu_2{}^2\tau_c{}^2}{1 + 4\pi^2\nu_1{}^2\tau_c{}^2} \tag{11}$$

Water Proton Relaxation Rates. The correlation times τ_c for fast exchanging ligands in binary enzyme-metal and ternary enzyme-metal-substrate complexes are given by

$$1/\tau_c = 1/\tau_R + 1/T_{1e} + 1/\tau_M \tag{12}$$

where τ_R is the tumbling time of the complex, T_{1e} is the metal electronic spin relaxation time, and τ_M is the residence time of the ligands in the first coordination sphere of the metal ion (Peacocke et al., 1969). For water ligands on the enzyme, τ_R (estimated from the diamagnetic ratio T_1/T_2 in the presence of enzyme and Mg(II)) (Abragam, 1961) is quite long so that the correlation time for fast exchanging water molecules is given by $1/\tau_c = 1/T_{1e} + 1/\tau_M$. Since in macromolecular complexes of Mn(II) it is often found that $1/\tau_M \ll 1/T_{1e}$ (Maggio et al., 1975) as in the present case, then the correlation time for water molecules is only the metal electron spin relaxation time.

Since $1/fT_{2p}$ of water protons greatly exceeds $1/fT_{1p}$, then, from eq 3 and 5, τ_M contributes negligibly to $1/fT_{1p}$; i.e., $1/fT_{1p} = 1/T_{1M}$ (Nowak and Mildvan, 1972b). Hence the longitudinal proton relaxation rates of water were analyzed with eq 6 using $C = 812$ Å $s^{-1/3}$ and $r = 2.87 \pm 0.05$ Å for the Mn(II)-H_2O interaction (Mildvan and Engle, 1972) to yield q, the number of fast exchanging water ligands in the various Mn(II) complexes. The correlation times were found by taking the ratios of $1/T_{1p}$ at various frequencies

$$\frac{1/T_{1p}(\nu_1)}{1/T_{1p}(\nu_2)} = \frac{f(\tau_c(\nu_1))}{f(\tau_c(\nu_2))} \tag{13}$$

and fitting the data to eq 6 and 7.

For each complex, the values of τ_v and B were varied in a computer search until the measured and calculated ratios of $1/T_{1p}$ gave the best agreement at all frequencies. The calculated values of $f(\tau_c(\nu_1))$ were then used in eq 6 to yield q, as previously described (Maggio et al., 1975).

TABLE II: Water Proton Molar Longitudinal and Transverse Molar Paramagnetic Relaxation Rates of Various Mn(II) Complexes as a Function of Frequency.[a]

	$1/T_{1p}$ (1 M) (s^{-1})					$1/T_{2p}$ (1 M) (s^{-1})	T_{1p}/T_{2p}
	8 MHz	15 MHz	24.3 MHz	40 MHz	100 MHz	24.3 MHz	24.3 MHz
$Mn-H_2O$	12 900	9 550	8 370	7 860	6 610	84 500	10.1 ± 1.0
E-Mn	81 400	107 000	121 000	114 000	23 100	353 000	2.9 ± 0.3
Mn-ATP	13 300	10 900	13 200	11 800	9 490	58 100	4.4 ± 0.5
E(ApU)-Mn-ATP	83 800	92 500	51 800	72 800	41 900	207 000	4.0 ± 0.4
E-Mn-ApU[b]	39 400	60 400	69 000	66 500	45 400	363 000	5.3 ± 0.6

[a] The errors in these parameters are ≤12%. All solutions contained, in addition to the components of the complexes, 150 mM KCl and 50 mM Tris-Cl (pH 7.5). RNA polymerase (54 μM), $MnCl_2$ (20 μM), ApU (200 μM), and ATP (120–190 μM) were typical concentrations used. T = 21 °C. The molecular relaxivities were calculated from binary, ternary, and quaternary mixtures of enzyme, Mn(II), ATP, or ApU using the dissociation constants previously determined (Koren and Mildvan, 1977) and eq 14. [b] The dissociation constants of the enzyme-Mn(II)-ApU complex, for use in eq 14, were determined by a titration of the enzyme-Mn(II) complex with ApU measuring $1/T_1$ of water protons under conditions described previously (Koren and Mildvan, 1977). The titration was fit by computer (Reed et al., 1970) with a % standard deviation of 0.6% to yield $\epsilon_T/\epsilon_b = 0.78$, K_3 = [E-Mn][ApU]/[E-Mn-ApU] = 1.4 μM, and K_S = [E][ApU]/[E-ApU] = 2.6 μM.

For binary and ternary complexes of Mn(II), the paramagnetic relaxation rate $1/T_{1p}$ of the complex under investigation was found by subtracting from the observed $1/T_{1p}$ the relaxation rates of all other Mn(II) complexes. For example, with a ternary enzyme-metal-substrate complex

$$\left(\frac{1}{T_{1p}}\right)^{(1M)}_{obsd} = \left(\frac{1}{T_{1p}}\right)^{1M}_{f}\frac{[Mn]_f}{[Mn]_t} + \left(\frac{1}{T_{1p}}\right)^{1M}_{E\text{-}Mn}\frac{[E\text{-}Mn]}{[Mn]_t} + \left(\frac{1}{T_{1p}}\right)^{1M}_{Mn\cdot S}\frac{[Mn\cdot S]}{[Mn]_t} + \left(\frac{1}{T_{1p}}\right)^{1M}_{E\cdot Mn\cdot S}\frac{[E\cdot Mn\cdot S]}{[Mn]_t} \quad (14)$$

where $(1/T_{1p})^{1M}_i$ are the molar paramagnetic relaxation rates (or molar relaxivities) of the species indicated by the subscript i. The concentration of each complex was found from previously determined dissociation constants (Koren and Mildvan, 1977) using a program developed by Reed et al. (1970).

Relaxation Rates of the Protons and Phosphorus of ApU and of the Protons of ATP. Since $1/fT_{2p}$ greatly exceeded $1/fT_{1p}$ for the nuclei of ApU and ATP in their respective complexes with Mn(II) and RNA polymerase-Mn(II), $1/fT_{1p}$ was not limited by chemical exchange. Hence eq 6 was used to determine distances from Mn(II) to the protons and phosphorus of ApU and to the protons of ATP in these complexes. For these calculations, C = 812 Å $s^{-1/3}$ for protons, C = 601 for ^{31}P and q was experimentally found to be 1 by titrations (Koren and Mildvan, 1977). The correlation times were determined by studies of the frequency dependences of $1/T_{1p}$ of the protons of water in the same complexes, and also by the frequency dependence of $1/T_{1p}$ of the protons of ApU in the ternary enzyme-Mn(II)-ApU complex.

Results

Kinetic Evidence for Initiation by Adenylyl(3′→5′)uridine. Downey and So (1970) have shown that ApU stimulates the incorporation of ATP into acid-insoluble products by 75% with poly[d(A-T)] as template. The divalent cation used for their experiments was Mg(II). We have confirmed these findings with Mg(II) and have extended them to Mn(II). The results are exemplified in Table I, where a 24% increase in the amount of ATP incorporated is noted when 73 μM ApU is present in the reaction mixture. In other experiments in which the concentration of MnATP was widely varied from 11 to 400 μM, ApU (10–40 μM) produced a 74% increase in the maximal velocity of ATP incorporation and an 82% increase in the K_M (MnATP). This latter effect on the K_M is in part responsible for the smaller stimulation of ATP incorporation by ApU at subsaturating levels of MnATP.

In addition we have measured the incorporation of [^{3}H]ApU itself into acid-insoluble product and have found it to be linear with total [^{3}H]ApU concentration in the reaction mixture (Table I).

These results provide additional evidence for ApU being an initiator of RNA synthesis catalyzed by Mn(II)-activated RNA polymerase.

Frequency Dependence of Water Proton Relaxation Rates. We have previously shown (Koren and Mildvan, 1977) that RNA polymerase binds Mn(II) tightly (K_D = 9 μM) at a single site, and 100-fold more weakly at six additional sites. Moreover this difference in affinity between tight and weak sites is increased in the presence of ApU or ATP. Hence when the concentration of enzyme exceeds that of Mn(II), as in all of our magnetic resonance experiments, the occupancy of the weak sites is very small (<5%) compared with that of the tight site. Hence the predominant paramagnetic effects detected are plausibly assumed to be due to Mn(II) at the tight site. Table II lists the $1/T_{1p}$ values of water protons calculated with eq 14 for various Mn(II) complexes as a function of frequency, as well as the $1/T_{2p}$ values at 24.3 MHz. In all cases $1/T_{2p} \gg 1/T_{1p}$, indicating that the residence time (τ_M) of a water ligand in the paramagnetic environment is small compared with its relaxation time (T_{1M}). This point is further supported by the frequency dependence of T_{1p} for the binary E-Mn(II), the ternary E-Mn(II)-ApU, and the quaternary E-ApU-Mn(II)-ATP complexes (Table II).

The values of T_{1e} for various complexes at five frequencies are summarized in Table III along with the average values of q as determined from the data at all of the frequencies. The number of fast exchanging water ligands remaining coordinated to Mn(II) on the enzyme (q), while only approximate (Mildvan and Gupta, 1977), are appropriate for octahedral complexes of Mn(II). The T_{1e} values will be used later to find the correlation times for the interaction of enzyme-bound Mn(II) with the protons and phosphorus of the initiator ApU and the protons of the substrate ATP.

Mn(II)-Adenylyl(3′→5′)uridine Relaxation Rates. As a control for the enzyme studies, the longitudinal and transverse relaxation rates of six resolvable protons of ApU were measured at 100 MHz as a function of Mn(II) concentration. The peak assignments are those given by T'so et al. (1969) and are shown in Figure 1 which also exemplifies the T_1 measurements. Titrations measuring the effects of Mn(II) on the relaxation rates of the protons and ^{31}P of ApU are exemplified in Tables IV and V, respectively. A summary of the

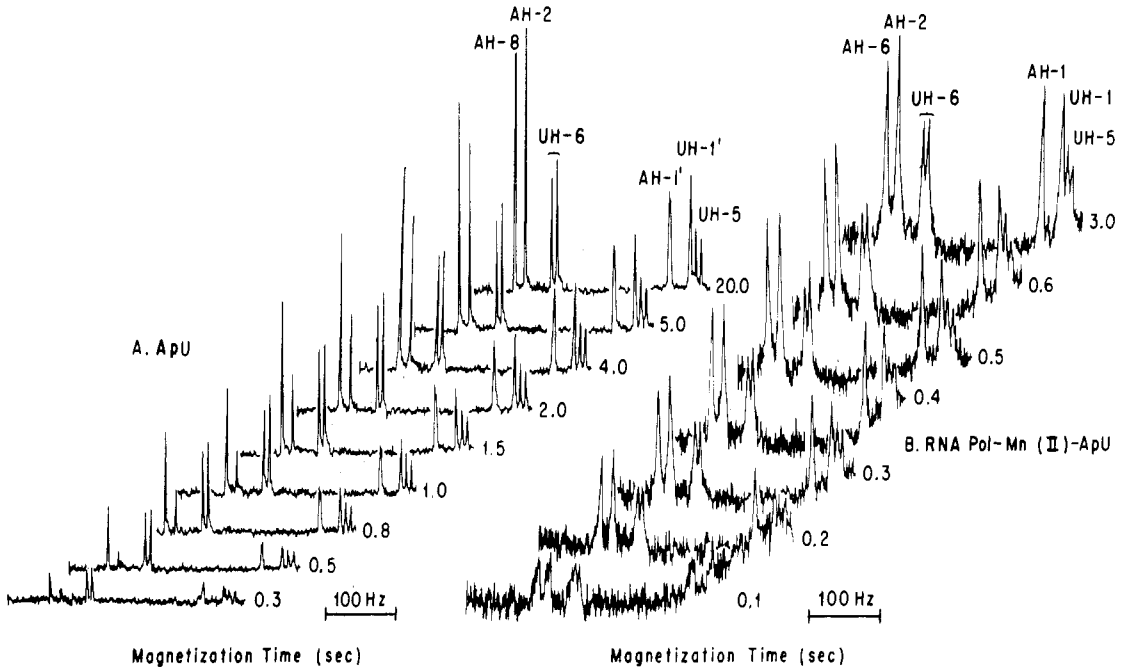

FIGURE 1: Longitudinal magnetic relaxation of the assigned proton resonances of (A) adenylyl(3′→5′)uridine and of (B) adenylyl(3′→5′)uridine bound in a complex with RNA polymerase and Mn(II). The NMR samples which were 99% deuterated contained: (A) 50 mM Tris-HCl, pH 7.5 (in H_2O), 150 mM KCl, 9.86 mM ApU (T = 24 °C); (B) 50 mM Tris-HCl, pH 7.5 (in H_2O), 150 mM KCl, 19.2 mM ApU, 74.6 μM enzyme, 16.2 μM $MnCl_2$ (T = 21 °C). In both A and B, spectra are the result of 100 transients at 100 MHz.

TABLE III: Frequency Dependence of the Electronic Spin Relaxation Time of Mn(II) and the Number of Fast Exchanging Water Ligands for Various Enzyme Complexes Determined from Water Relaxation Rates.[a]

Complex	B (s^{-2})	τ_v (s)	T_{1e} (s) 8 MHz	15 MHz	24.3 MHz	40 MHz	100 MHz	q
E-Mn(II)	5.0×10^{19}	2.5×10^{-12}	1.6×10^{-9}	1.7×10^{-9}	1.9×10^{-9}	2.5×10^{-9}	6.4×10^{-9}	2.2 ± 0.3
E-Mn(II)-ApU	1.3×10^{20}	3.2×10^{-12}	5.2×10^{-10}	5.7×10^{-10}	6.7×10^{-10}	9.3×10^{-10}	2.9×10^{-9}	3.1 ± 0.6
E(ApU)-Mn(II)-ATP[b]	2.5×10^{20}	5.0×10^{-13}	1.6×10^{-9}	1.6×10^{-9}	1.6×10^{-9}	1.6×10^{-9}	1.8×10^{-9}	1.8 ± 0.5[b]

[a] The errors in q represent twice the standard error of the value of q calculated from T_{1p} and T_{1e} at each of the five frequencies. B and τ_v are the best fit parameters of eq 7. [b] The biphasic nature of the $1/T_{1p}$ values of the water protons in the E(ApU)-Mn-ATP complex (Table II), which may exceed the overall error of three experiments, raises the possibility of two macromolecular complexes of Mn(II). Assuming two E(ApU)-Mn-ATP complexes, separate analyses of the $1/T_{1p}$ data at low frequencies (8-24.3 MHz) and high frequencies (24.3-100 MHz) were made, yielding extreme q values of 0.7 and 2.4, respectively. Both of these values are below that of the E-Mn-ApU complex, as concluded by assuming a single E(ApU)-Mn-ATP complex.

$1/fT_{1p}$ and $1/fT_{2p}$ values at 24 °C is presented in Table VI. Since $1/fT_{1p}$ and $1/fT_{2p}$ are comparable, it is possible that there was a significant contribution to $1/fT_{1p}$ from the exchange term τ_M. This possibility was eliminated by measuring the temperature dependences of $1/fT_{1p}$ (Mildvan and Cohn, 1970) and by the large $1/fT_{2p}$ value of ^{31}P (see later). The Arrhenius plots of ln $1/T_{1p}$ vs. $1/T$(K) for each proton and for the ^{31}P had positive slopes from 4 to 44 °C with activation energies of 3.7 ± 1.3 kcal/mol indicating that the $1/T_{1p}$ values were not dominated by chemical exchange and could be used for distance measurements.

The $1/T_1$ and $1/T_2$ relaxation rates of the phosphorus nucleus of ApU were also measured at 40.5 MHz (Figure 2, Table V), as a function of Mn(II) concentration. The chemical shift of the phosphorus was 0.87 ppm upfield from an 85% H_3PO_4 standard. Table VI gives the $1/fT_{1p}$ and $1/fT_{2p}$ values at 24 °C. Since $1/fT_{2p} \gg 1/fT_{1p}$ and was not limited by chemical exchange as established by its temperature dependence, we were justified in using $1/fT_{1p}$ for distance measurements to ^{31}P. Moreover, the order of magnitude greater value of $1/fT_{2p}$ of ^{31}P than of $1/fT_{1p}$ of all of the protons of ApU indicates the latter values all to be in the fast exchange case and also appropriate for distance calculations.

In order to determine the Mn(II)-proton and Mn(II)-phosphorus distances, two additional pieces of information are needed: the correlation time τ_c and the dissociation constant K_D of the Mn(II)-ApU complex. Very little frequency dependence of $1/T_{1p}$ was expected for this binary complex, so the correlation time was calculated from the measured enhancement $\epsilon_b = 2.9 \pm 0.5$ of water protons at 24.3 MHz in the same complex assuming $q = 5$ water ligands for Mn(II)-ApU, $q = 6$ for Mn(II)-H_2O, and τ_c (Mn(II)-H_2O) = 3×10^{-11} s (Bloembergen and Morgan, 1961). The result was $\tau_c = 1.3 \times 10^{-10}$ s, which is comparable to the tumbling time $\tau_c = 1.7 \times 10^{-10}$ s calculated from Stokes' Law. Accordingly, optical (Warshaw and Tinoco, 1966; Jaskunas et al., 1968) and diamagnetic NMR studies (Ts'o et al., 1969) show no aggregation but some internal stacking of ApU under conditions similar to those used here. The K_D of the Mn(II)-ApU complex of 225 ± 50 mM was determined by EPR and was found to be inde-

TABLE IV: Proton Relaxation Rates (in Units of Seconds^{-1}) of ApU in Titrations of ApU and the ApU–RNA Polymerase Complex with $MnCl_2$.[a]

Expt	[ApU] (mM)	[$MnCl_2$] (μM)	[Enzyme] (μM)	AH-8				AH-2				UH-6			
				$1/T_1$	$1/fT_{1p}$[b]	$1/T_2$	$1/fT_{2p}$[b]	$1/T_1$	$1/fT_{1p}$[b]	$1/T_2$	$1/fT_{2p}$[b]	$1/T_1$	$1/fT_{1p}$[b]	$1/T_2$	$1/fT_{2p}$[b]
I	9.86			0.72		1.04		0.23		0.54		1.37		0.51	
	9.52	46.4		2.56	0.932×10^4	2.22	5975	1.66	7241	1.77	6228	1.98	3088	2.30	9064
	9.48	80.5		4.43	1.29×10^4	3.86	8213	2.83	7572	2.38	5358	2.90	4456	2.42	5562
		Av:			11110 ± 1790		7094 ± 1120		7400 ± 170		5793 ± 1900		3772 ± 680		7313 ± 1750
II	20.2		58.7	1.84		5.20		1.36		4.68		2.71		6.03	
	19.8	19.6	57.5	2.70	869	7.93	2758	2.28	929	10.01	5384	3.46	758	8.97	2970
	19.4	38.5	56.4	3.29	731	14.42	4646	3.07	861	12.26	3819	3.93	615	12.41	3214
		Av:			800 ± 70		3702 ± 940		895 ± 40		4600 ± 780		687 ± 70		3092 ± 120

Expt	[ApU] (mM)	[$MnCl_2$] (μM)	[Enzyme] (μM)	AH-1′				UH-1′				UH-5			
				$1/T_1$	$1/fT_{1p}$[b]	$1/T_2$	$1/fT_{2p}$[b]	$1/T_1$	$1/fT_{1p}$[b]	$1/T_2$	$1/fT_{2p}$[b]	$1/T_1$	$1/fT_{1p}$[b]	$1/T_2$	$1/fT_{2p}$[b]
I	9.86			0.76		3.17		0.64		2.39		0.88		1.65	
	9.52	46.4		1.14	1924	3.89	3646	0.99	1822	3.28	4506	1.91	5216	2.52	5420
	9.48	80.5		1.86	3203	4.49	3844	1.72	3174	4.16	5155	3.11	6483	4.57	8504
		Av:			2563 ± 640		3750 ± 510		2498 ± 680		4830 ± 500		5849 ± 630		6962 ± 1540
II	20.2		58.7	2.50		10.59		1.61				2.36			
	19.8	19.6	57.5	2.86	364	13.09	2525	2.59	990			3.27	919		
	19.4	38.5	56.4	3.21	358	12.85	1138	3.23	816			3.52	584		
		Av:			361 ± 30		1830 ± 700		903 ± 90				752 ± 170		

[a] Conditions are given in Figure 1. The average errors in the individual measurements of $1/T_1$ are ±7% and for $1/T_2$ are ±20%. [b] The normalization factor $f = [Mn]_b/[ApU]_T$ for the binary Mn–ApU complex was calculated using the dissociation constant K_1(Mn–ApU) = 225 ± 50 mM (see text). In the presence of RNA polymerase, all of the bound Mn^{2+} was in the enzyme complex since its dissociation constant (K_A' = 4.1 μM) is 5.5×10^4-fold lower than that of the binary Mn–ApU complex (K_1 = 225 mM) (Koren and Mildvan, 1977), while the concentration of ApU is only 175 times that of RNA polymerase–ApU.

TABLE V: ^{31}P Relaxation Rates of ApU in Titrations of ApU and the ApU-RNA Polymerase Complex with $MnCl_2$.[a]

Expt	[ApU] (mM)	[$MnCl_2$] (μM)	[Enzyme] (μM)	$1/T_1$[c]	$1/fT_{1p}$[b,c]	$1/T_2$[c]	$1/fT_{2p}$[b,c]
I	7.50			0.191		0.50	
	7.36	5.30		0.327	5958	6.58	2.66×10^5
	7.37	9.90		0.445	5970	13.70	3.10×10^5
					Av: 5964 ± 10		$2.88 \pm 0.11 \times 10^5$
II	9.93		57.3	0.237		2.01	
	9.87	6.00	57.1	0.479	398	7.03	8.26×10^3
	9.81	12.50	56.7	0.787	432	10.72	6.84×10^3
	9.74	19.0	56.3	0.942	361		
					Av: 397 ± 24		$7.6 \pm 0.8 \times 10^3$

[a] Conditions are given in Figure 2. The average errors in the individual measurements of $1/T_1$ are ±7% and for $1/T_2$ are ±11%. [b] The normalization factors were calculated as described in Table IV. [c] Relaxation rates in s^{-1}.

pendent of temperature over the range 4 to 44 °C. This weak binary binding was exploited for the ternary enzyme measurements, where there was no correction necessary for the Mn(II)–ApU complex. The distances of the protons and the phosphorus of ApU from the Mn(II) nucleus are listed in Table VI. The two protons H-8 and H-2 of the adenine ring are closest to the metal, whereas the H-1′ proton of each ribose ring is farthest away. The distance from the phosphorus (3.8 ± 0.3 Å) indicates either a highly distorted inner sphere complex or a rapidly equilibrating mixture in solution of 20% inner sphere complex at a distance of 2.9 Å and 80% second sphere complex at a distance of 6.1 Å (Mildvan and Grisham, 1974).

Enzyme–Mn(II)–Adenylyl(3′→5′)uridine Relaxation Rates. In the presence of RNA polymerase, the paramagnetic effects of Mn(II) on the relaxation rates of the six resolvable protons (Table IV) and of the phosphorus of ApU (Table V) were quite different. The concentrations of Mn(II) were selected such that in every experiment at least 85% of the metal

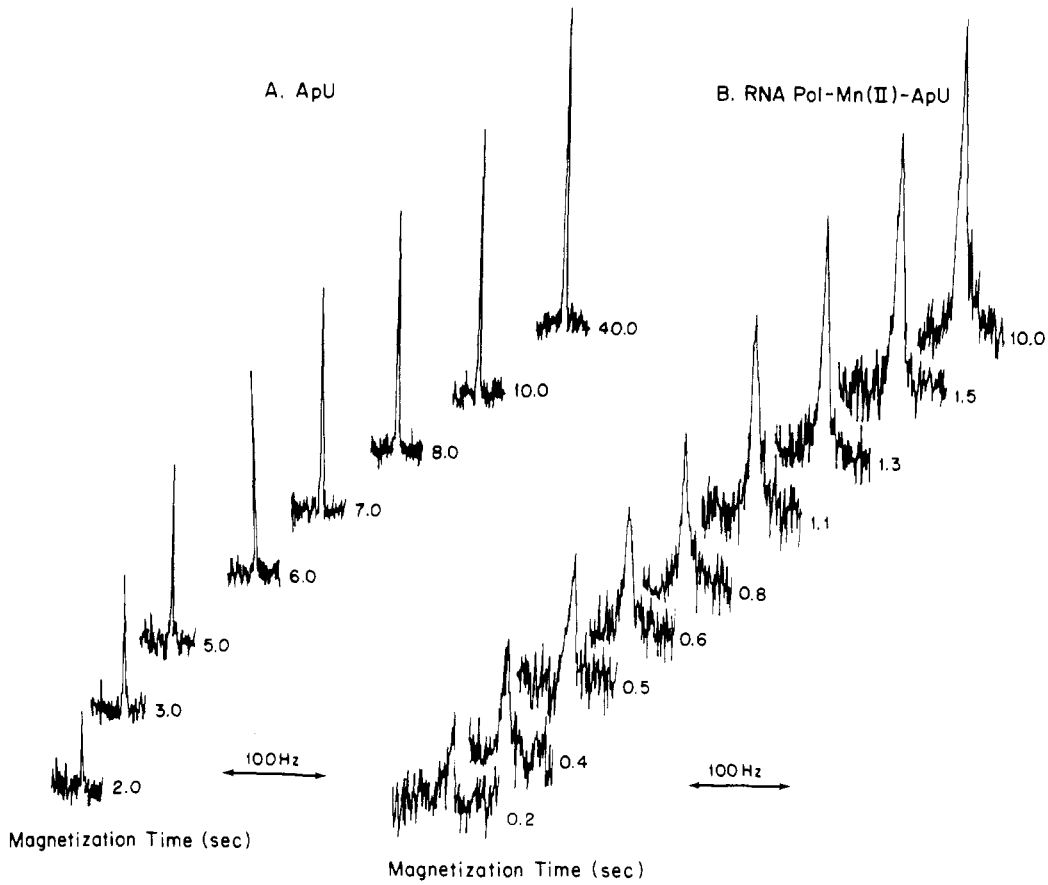

FIGURE 2: Longitudinal magnetic relaxation of the phosphorus resonance of (A) adenylyl(3′→5′)uridine and of (B) adenylyl(3′→5′)uridine bound in a complex with RNA polymerase and Mn(II). The NMR samples, which were 40% deuterated, contained: (A) 50 mM Tris-HCl, pH 7.5, 150 mM KCl, 7.50 mM ApU (T = 24 °C); (B) 50 mM Tris-HCl, pH 7.5, 150 mM KCl, 9.74 mM ApU, 56.3 μM enzyme, 19.0 μM $MnCl_2$ (T = 21 °C). A resulted from 10 transients and B involved 100 transients at 40.5 MHz.

TABLE VI: Paramagnetic Effects of Mn(II) on the Longitudinal and Transverse Relaxation Rates of the Protons (100 MHz) and Phosphorus (40.5 MHz) of Adenylyl(3′→5′)uridine.

Proton	$1/fT_{1p}$ (s^{-1})	$1/fT_{2p}$ (s^{-1})	r (Å)[a]
AH-8	10300 ± 2200	9000 ± 3600	4.7 ± 0.3
AH-2	7800 ± 2100	4200 ± 2200	4.9 ± 0.3
UH-6	3700 ± 1400	6500 ± 2800	5.6 ± 0.4
AH-1′	2400 ± 600	4100 ± 1500	6.0 ± 0.4
UH-1′	2400 ± 600	4500 ± 1200	6.0 ± 0.4
UH-5	5600 ± 1400	8000 ± 2600	5.2 ± 0.3
^{31}P	5900 ± 1800	280000 ± 60000	3.8 ± 0.4

[a] Calculated using τ_c of water protons (1.3×10^{-10} s) in same complex from data at 24.3 MHz. The $f(\tau_c)$ values were 3.8×10^{-10} s for protons and 3.9×10^{-10} s for ^{31}P.

was bound to the enzyme. No correction for binary Mn(II)-ApU was needed because of its extremely high dissociation constant (Table IV). $1/T_{1p}$ values of the protons (Figure 1) were determined at seven different enzyme concentrations as summarized in Table VII and were found to be independent of enzyme concentration, in agreement with theoretical calculations using the known dissociation constants (Koren and Mildvan, 1977). The diamagnetic $1/T_1$ did not change when 1 mM $MgCl_2$ was added, instead of $MnCl_2$, indicating that we were observing only a paramagnetic effect upon the addition of Mn(II). The transverse relaxation rates of four of the protons are also summarized in Table VII. The $1/fT_{2p}$ values of the two doublets of the uracil ring protons were not measured due to phasing problems (McLaughlin et al., 1973). In all cases $1/fT_{2p}$ is at least threefold greater than $1/fT_{1p}$, and the maximum error introduced into $1/T_{1M}$ by neglecting any exchange contribution is ≤30%, which translates into an uncertainty in the distances of less than 5% due to the sixth power relationship of eq 6. Hence the protons are in the fast chemical exchange region, and the $1/fT_{1p}$ values which are similar for all of the protons of ApU (Table VII) can be used for distance calculations.

Again the calculation of the Mn(II)-proton distances requires a determination of the correlation time τ_c. We used three separate methods which yielded comparable values (Table VIII). The first method assumes $1/\tau_c = 1/\tau_R + 1/T_{1e}$ ($1/\tau_M$ is insignificant), where τ_R is calculated from the diamagnetic T_1/T_2 ratio (Abragam, 1961) (first subtracting out the diamagnetic $1/T_1$ and $1/T_2$ of ApU alone, which accounts for any possible paramagnetic effects due to dissolved oxygen) (Bacon and Reeves, 1973) and T_{1e} is calculated from the frequency dependence of the water relaxation data of the same complex. The second method involves measuring $1/T_{1p}$ of the ApU protons at 100 MHz and 220 MHz in the ternary enzyme-Mn(II)-ApU complex and using eq 9 and 11, which make the extreme assumptions of a maximal frequency dependence for $\tau_c(\omega_s^2\tau_\nu^2 \gg 1)$ and no frequency dependence for τ_c (either $\omega_s^2\tau_\nu^2 \ll 1$ or $\tau_R \ll T_{1e}$). Only the H-2 proton of the adenine ring had a measurable paramagnetic effect at 220 MHz. The τ_c and $f(\tau_c)$ values obtained by the two methods are listed in Table VIII, which shows the close agreement in $f(\tau_c)$ between

TABLE VII: Paramagnetic Effects of Enzyme-Bound Mn(II) on the Longitudinal and Transverse Relaxation Rates of the Protons and Phosphorus of Adenylyl(3′→5′)uridine.[a]

[E] (μM)	Range of Mn(II) concn (μM)	$1/fT_{1p}$ (s^{-1}) AH-8	AH-2	UH-6	AH-1′	UH-1′	UH-5	^{31}P
73.2	0.2–74.3	900 ± 140	1340 ± 60	380 ± 120			1380 ± 200	
57.8	39.9	970 ± 130	1380 ± 150 450 ± 100[b]		800 ± 110	350 ± 130	620 ± 340	
56.4	19.6–38.5	770 ± 60	910 ± 60	630 ± 100	360 ± 60	880 ± 90	590 ± 50	
48.0	40.1	1060 ± 90	1970 ± 210	500 ± 160	480 ± 110	650 ± 160		
40.1	38.5	440 ± 60	1250 ± 70		270 ± 40	640 ± 200		
37.9	40.3	620 ± 140	1300 ± 40	550 ± 120	510 ± 60	540 ± 90	1020 ± 200	
28.0	40.5	790 ± 50	1150 ± 100	440 ± 150	560 ± 80	800 ± 150	1390 ± 60	
56.3	2.0–19.0							390 ± 30
Av $1/fT_{1p}$ (s^{-1})		790 ± 220	1300 ± 400	500 ± 100	500 ± 200	640 ± 190	1000 ± 400	
$1/fT_{2p}$ (s^{-1})		3600 ± 830	4100 ± 2300		1900 ± 1200	2700 ± 1600		7900 ± 800
r (Å)		9.8 ± 0.9	9.0 ± 0.9	10.5 ± 0.9	10.5 ± 1.1	10.1 ± 1.0	9.4 ± 1.0	9.3 ± 0.8

[a] Concentrations of ApU, Tris-Cl, and KCl are as given in Figures 1 and 3. The frequencies used are 100 MHz and 40.5 MHz for ^{31}P. [b] Frequency is 220 MHz for protons.

TABLE VIII: Correlation Times of the Protons and Phosphorus of Adenylyl(3′→5′)uridine Complexed with Enzyme-Bound Mn(II).

	AH-8	AH-2	AH-1′	UH-1′	^{31}P
τ_R	2.6×10^{-9}	2.5×10^{-9}	2.0×10^{-9}	4.3×10^{-9}	2.9×10^{-8}
T_{1e} (s)	2.9×10^{-9}	2.9×10^{-9}	2.9×10^{-9}	2.9×10^{-9}	2.9×10^{-9}
$\tau_c(1/T_R + 1/T_{1e})$ (s)	1.4×10^{-9}	1.4×10^{-9}	1.4×10^{-9}	1.4×10^{-9}	2.6×10^{-9}
$f(\tau_c)(1/\tau_R + 1/T_{1e})$ (s)	2.4×10^{-9}	2.4×10^{-9}	2.4×10^{-9}	2.4×10^{-9}	5.4×10^{-9}
$1/T_{1p}(100)/1/T_{1p}(220)$		3.07 ± 0.80			
$\tau_c(100/200)$ (s)		5×10^{-10} [a]–3×10^{-9} [b]			
$f(\tau_c)(100/220)$ (s)		1.4×10^{-9} [a]–2.4×10^{-9} [b]			
$f(\tau_c)_{av}(100/220)$ (s)		$(1.9 \pm 0.5) \times 10^{-9}$			

[a] Determined by assuming maximum frequency dependence of T_{1e} (eq 8). [b] Determined by assuming no frequency dependence of T_{1e} (eq 10).

the two methods. A third approximation to τ_c can be found by using the paramagnetic T_{1p}/T_{2p} ratio for protons but not for ^{31}P (Nowak et al., 1973) and this gave values of 3.2×10^{-9} and 1.9×10^{-9} s for τ_c and $f(\tau_c)$, respectively, again quite close to the $f(\tau_c)$ value found by the other two methods. This agreement, and the absence of nonexponential relaxation behavior argue against any significant contribution of spin diffusion (Kalk and Berendsen, 1976) to the relaxation rates of ApU under our conditions.

The correlation function $f(\tau_c)$ determined with the greatest precision from the water relaxation-plus-diamagnetic T_1/T_2 ratio method was used together with the $1/fT_{1p}$ values (Table VIII) to calculate the Mn(II)–proton distances which were all very similar (Table V).

The paramagnetic relaxation rates were also measured for the phosphorus nucleus of ApU (Figure 2, Table V) and are summarized in Table VII. Little chemical shift of the resonance was expected and none was detected, consistent with the large dilution factor (Gorenstein et al., 1976). In this case $1/T_{2p} \gg 1/T_{1p}$ so the Mn(II)–phosphorus distance can be calculated. The diamagnetic T_1/T_2 ratio for the ^{31}P nucleus of ApU on the enzyme (again subtracting diamagnetic relaxation rates of ApU alone) was used as previously described (Nowak and Mildvan, 1972a; Fung et al., 1976) to calculate a rotational correlation time of 2.9×10^{-8} s (Table VIII), which is an order of magnitude greater than the rotational time for the protons, indicating that the motion of the phosphorus atom on the enzyme is hindered to a much greater extent than that of the protons. However, the phosphorus retains some rotational freedom on the enzyme since the rotation time of the entire protein molecule of molecular weight 500 000 is estimated from the Stokes law to be 1.2×10^{-7} s. The range of distances from the enzyme-bound Mn(II) to the phosphorus and protons of bound ApU (9.0–10.5 Å) indicates that the ApU molecule is far from the Mn(II) binding site. Moreover, the Mn(II) to phosphorus distance of 9.3 ± 0.8 Å limits the possible conformations of ApU on the enzyme, a topic which will be discussed later.

Kinetic Properties of Binary Mn(II)–ApU and Ternary RNA Pol–Mn(II)–ApU Complexes. The largest value of $1/fT_{2p}$ for the binary Mn(II)–ApU complex gives a lower limit for $k_{off} \geq 9000$ s^{-1}, the rate constant for dissociation of this complex (Table VI). This rate constant, when divided by the dissociation constant of 225 mM, gives a value of $k_{on} \geq 40\,000$ M^{-1} s^{-1}. With the ternary RNA Pol–Mn(II)–ApU complex $k_{off} \geq 4000$ s^{-1} and using the dissociation constant of 1.4 μM (Table II) we find $k_{on} \geq 2.9 \times 10^9$ M^{-1} s^{-1} which is consistent with the formation of a diffusion controlled complex.

Enzyme–Mn(II)–Adenosine Triphosphate Relaxation Rates. The distances from Mn(II) to the protons of ATP at the

elongation site of the enzyme were examined by blocking the initiation site with a saturating concentration of either ApU or GpU. The paramagnetic relaxation rates of the H-8, H-2, and H-1′ protons of ATP were measured, and the observed rates were corrected for the predominant binary Mn(II)-ATP complex with the use of eq 14 and the known dissociation constants (Tables IX and X). The greatest fraction of Mn(II) in the desired enzyme-initiator-Mn(II)-ATP complex was 10% using ApU as the initiator and 4% with GpU as initiator. The concentrations of [MS] and [EMS] were calculated using the dissociation constants of Tables IX and X derived from water relaxation data. The relaxation rates of the protons in the binary Mn(II)-ATP complex were taken from Sloan and Mildvan (1976). A typical titration is shown in Table IX where it may be seen, by comparing $(1/fT_{1p})_{obsd}$ with $(1/fT_{1p})_{MnATP}$ that the presence of RNA polymerase causes a two- to three-fold enhancement of the effect of Mn(II) on the protons of ATP. Hence, although the enzyme-(ApU)-Mn-ATP complex contains only 10% of the Mn(II), it is responsible for 50 to 69% of the observed paramagnetic effect of Mn(II).

The average $1/fT_{1p}$ values of ATP in the quaternary enzyme-initiator-Mn(II)-ATP complex are listed in Table X.

The correlation time was calculated from the water relaxation data which gave T_{1e} and from the diamagnetic T_1/T_2 ratio which gave τ_R. The correlation time found ($\tau_c = 8.8 \times 10^{-10}$ s) was then used (assuming an uncertainty of 50% in $f(\tau_c)$) together with the longitudinal relaxation rates to calculate the Mn(II)-proton distances, which are also listed in Table X. The Mn(II)-proton distances previously found in the binary Mn(II)-ATP complex in solution (Sloan and Mildvan, 1976) are also given in Table X for comparison. The distances in the binary Mn(II)-ATP and quaternary enzyme-initiator-Mn(II)-ATP complexes do not differ significantly. Hence, it is unlikely that any drastic conformational changes in the adenine and ribose portions of ATP occur when ATP binds to the enzyme.

Discussion

By comparing the range of distances of the enzyme-bound ApU and ATP molecules from the bound Mn(II) ion (Tables VII and X), it is clear that the tightly bound Mn(II) site is significantly closer to the bound ATP than to the bound ApU. The presence of ATP at the elongation site rather than at the initiation site under these conditions is ensured by its order of magnitude greater affinity for the elongation site (Koren and Mildvan, 1977) and by the presence of a saturating concentration of a dinucleoside monophosphate which specifically blocks the initiation site. Hence, the distances from Mn(II) to ApU (Table VII) and to ATP (Table X) on RNA polymerase provide further evidence for the tightly bound Mn(II) occupying the active site for elongation, as previously suggested from a comparison of kinetic and binding data (Koren and Mildvan, 1977).

Another indication of the location of the Mn(II) site is given by the changes in the number of fast exchanging water ligands (q) when ApU and ATP are added to the enzyme-Mn(II) complex (Table III). It appears that, upon addition of ApU, q increases by ~1, indicating that either an additional water ligand exists on Mn(II) or that a previously immobilized water molecule has been freed, to exchange rapidly, by the addition of ApU. Upon the addition of ATP to the enzyme-(ApU)-Mn(II) complex, q decreases by 1 or 2, indicating the displacement or immobilization of water ligands and providing further evidence for the proximity of ATP to the tight Mn(II)

TABLE IX: Proton Relaxation Rates of ATP in a Titration of the ApU–RNA Polymerase–ATP Complex with $MnCl_2$.[a]

[ATP] (mM)	[$MnCl_2$] (μM)	[Enzyme] (μM)	[ApU] (mM)	H_8 $1/T_1$	H_8 $(1/fT_{1p})_{obsd}$	H_8 $(1/fT_{1p})^b$ MnATP	H_8 $(1/fT_{1p})^c$ E–Mn–ATP	H_2 $1/T_1$	H_2 $(1/fT_{1p})_{obsd}$	H_2 $(1/fT_{1p})^b$ MnATP	H_2 $(1/fT_{1p})^c$ E–Mn–ATP	H_1' $1/T_1$	H_1' $(1/fT_{1p})_{obsd}$	H_1' $(1/fT_{1p})^b$ MnATP	H_1' $(1/fT_{1p})^c$ E–Mn–ATP
6.10	0	93.2	0.588	4.25		1.12×10^4		5.32				6.21			
6.03	1.18	92.1	0.581	8.44	2.14×10^4	1.12×10^4	11.3×10^4								
5.89	3.46	90.0	0.568	23.6	3.29×10^4	1.12×10^4	22.8×10^4								
5.76	5.63	88.0	0.555	29.7	2.60×10^4	1.12×10^4	15.9×10^4					8.68	2.53×10^3	1.6×10^3	1.09×10^4
5.73	10.11	87.6	0.552					9.09	2.14×10^3	1.2×10^3	1.06×10^4	13.24	2.98×10^3	1.6×10^3	2.54×10^4
5.69	16.79	87.0	0.549					14.06	2.96×10^3	1.2×10^3	1.88×10^4	12.60	2.17×10^3	1.6×10^3	0.73×10^4
		Av:				$16.7 \pm 4.1 \times 10^4$					$1.47 \pm 0.41 \times 10^4$				$1.45 \pm 0.72 \times 10^4$

[a] Conditions are otherwise as given in Figure 1. The average error in the individual $1/T_1$ measurements is ±9%. Relaxation rates are in s^{-1}. [b] The values of $(1/fT_{1p})_{MnATP}$ under similar conditions are from Sloan and Mildvan (1976). [c] The value of $(1/fT_{1p})$ for the ATP protons in the enzyme complex was calculated from $(1/fT_{1p})_{obsd}$ using the equation (Mildvan and Engle, 1972): $(1/fT_{1p})_{obsd} = ([MnATP]/[Mn]_t)(1/fT_{1p})_{MnATP} + ([E–Mn–ATP]/[Mn]_t)(1/fT_{1p})_{E–Mn–ATP}$. The distribution of $[Mn]_t$ between the binary MnATP and quaternary ApU–enzyme–Mn–ATP complexes was calculated as 90.2 and 9.8%, respectively, using the dissociation constant of Mn(II) of 13.2 μM from the binary complex and 1.76 μM from the quaternary enzyme complex determined under the same conditions (Koren and Mildvan, 1977). Under these conditions, the dissociation constant for Mn(II) from the GpU–enzyme–Mn–ATP complex was 7.0 μM. The $(1/fT_{2p})_{E–Mn–ATP}$ values for the H_8 and H_2 protons in the ApU complex were calculated in the same way to be $1.8 \pm 0.7 \times 10^5$ s^{-1} and $2.4 \pm 0.4 \times 10^5$ s^{-1}, respectively, from this titration.

TABLE X: Paramagnetic Effects of Enzyme-Bound Mn(II) on the Longitudinal Relaxation Rates of the Protons of ATP in the Presence of ApU or GpU.[a]

Parameter		Complex	H-8	H-2	H-1′
Correlation times	τ_R (s)	E(ApU or GpU)-Mn-ATP	1.9×10^{-9}	1.9×10^{-9}	1.4×10^{-9}
	T_{1e} (s)[b]	E(ApU or GpU)-Mn-ATP	1.8×10^{-9}	1.8×10^{-9}	1.8×10^{-9}
	τ_c (s)[b]	E(ApU or GpU)-Mn-ATP	9.2×10^{-10}	9.2×10^{-10}	7.9×10^{-10}
	Av τ_c (s)	E(ApU or GpU)-Mn-ATP	8.8×10^{-10}	8.8×10^{-10}	8.8×10^{-10}
	$f(\tau_c)$ (s)	E(ApU or GpU)-Mn-ATP	2.0×10^{-9}	2.0×10^{-9}	2.0×10^{-9}
Relaxation rates	$1/fT_{1p}$ (s^{-1})	E(ApU or GpU)-Mn-ATP	$(13.0 \pm 4.4) \times 10^4$	$(1.9 \pm 1.5) \times 10^4$	$(1.7 \pm 0.9) \times 10^4$
	r (Å)	E(ApU or GpU)-Mn-ATP	4.0 ± 0.4	5.6 ± 0.9	5.7 ± 0.7
	$1/fT_{1p}$ (s^{-1})	MnATP	$(11.2 \pm 2.6) \times 10^3$	$(1.2 \pm 0.3) \times 10^3$	$(1.6 \pm 0.5) \times 10^3$
	r (Å)	MnATP	4.5 ± 0.5	6.4 ± 0.5	6.2 ± 0.6

[a] The Mn(II)-proton distances in the binary Mn(II)-ATP complex (Sloan and Mildvan, 1976) are listed for comparison. [b] Separate analyses of the $1/T_{1p}$ data on water protons assuming two E(ApU)-Mn-ATP complexes (see Table III) yielded at most an 85% greater value of τ_c, a 20% greater value of $f(\tau_c)$, and a 3% greater distance. The latter is well within the uncertainty in the distances.

site. On DNA polymerase I the terminal phosphate of dTTP was directly coordinated to the enzyme-bound Mn(II) (Sloan et al., 1975).

The conformation of ATP bound at the elongation site of RNA polymerase, although incompletely defined by only three distances from the enzyme-bound Mn(II) and by the presence of an excess of the binary Mn(II)-ATP complex, is indistinguishable from that of the binary complex, in which χ the conformational angle at the glycosidic bond is 90° (Sloan and Mildvan, 1976). This conformational angle is similar to that found in double helical B-DNA, but not in A-DNA, RNA double helices (25°), or in RNA-DNA hybrid helices (Milman et al., 1967; O'Brien and MacEwan, 1970; Arnott et al., 1975). Hence as previously established for DNA polymerase I (Sloan et al., 1975), RNA polymerase may bind that conformation of the substrate at the chain elongation site which can form Watson-Crick base pairs with the DNA template. However, this is clearly not the case at the initiation site (see below).

The conformations of ApU in the binary Mn(II)-ApU and ternary enzyme-Mn(II)-ApU complexes obtained from model building studies have been compared with seven conformations of dinucleoside phosphates which were derived by holding the individual nucleoside conformations rigid with their conformational angles equal to those found in known crystal structures of nucleosides and nucleotides, and systematic rotation about the phosphodiester linkages, in accord with energy considerations and steric effects (Kim et al., 1973). These seven conformations are denoted P_1, P_2, P_3, A_1, A_2, S_1, and S_3 where P stands for parallel, A for antiparallel, and S for skew with respect to the orientations of the two ribose rings. The P_3 conformation is that found in the RNA double helix (Kim et al., 1973).

The conformation of ApU in the binary Mn(II)-ApU complex determined from model building (Figure 3) is a variant of the P_3 conformation in which the distance between the parallel planes of the bases is increased by a factor of ~2 from a stacking distance of 3.4 Å to a value of 7.5 ± 1.0 Å to permit intercalation of the Mn(II) together with its water ligands. The water ligands probably donate hydrogen bonds to the nitrogens of the purine and pyrimidine rings. The intercalation of ethidium bromide between the bases of 5-iodo-UpA results in a comparable base separation of 6.8 Å (Tsai et al., 1975). Direct coordination of Mn(II) occurs only at a phosphodiester oxygen but the large Mn(II) to phosphorus distance (3.8 Å) suggests either distortion of the phosphorus to a trigonal bipyramidal

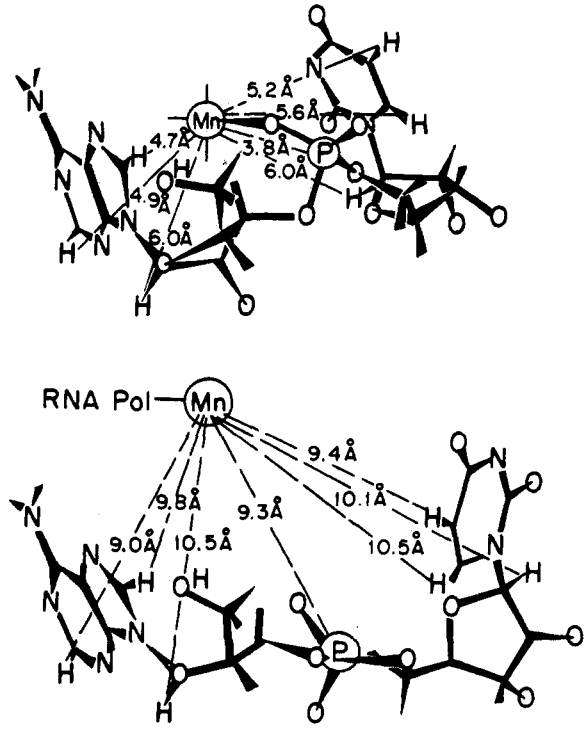

FIGURE 3: Conformations of adenylyl(3′→5′)uridine in its binary complex with Mn(II) (upper) and in its ternary complex with RNA polymerase and Mn(II) (lower), consistent with the indicated distances.

structure or the rapid equilibration of 20% inner sphere at a distance of 2.9 Å with 80% second sphere complex at a distance of 6.1 Å (Mildvan and Grisham, 1974). Either of these alternatives is consistent with the high dissociation constant of the binary Mn(II)-ApU complex. The position of the divalent cation Mn(II) thus appears to be different from that of the monovalent cation Na(I) in the crystalline Na-ApU complex, where the monovalent cation is not between the two stacked bases, and they are able to assume their usual 3.4-Å interbase distance (Seeman et al., 1976). The distances are consistent with C(3′)-endo conformations for both ribose rings, and the glycosidic angles between each base and ribose portion are 30° for adenine and 60° for uracil which fall into the range for the antiorientation (Lee et al., 1976). A similar conclusion has been reached from studies of coupling constants of dipurines and dipyrimidines (Lee et al., 1976).

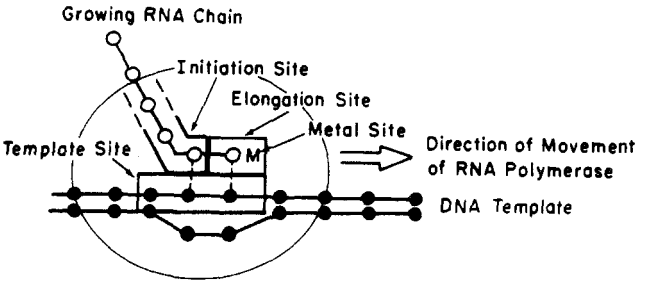

FIGURE 4: Schematic arrangement of sites on RNA polymerase, consistent with the magnetic resonance data. Passage of the growing RNA chain through the initiation site could separate the RNA from the DNA template.

On the enzyme, none of the atoms of bound ApU are close enough to the bound Mn(II) to be directly coordinated. The most likely conformation of ApU consistent with our distances is the S_1 conformation (Figure 3), with the probabilities for the other conformations decreasing in the order $S_1 \gg A_2 \gg P_1, P_2 \gg P_3, A_1, S_3$. The most likely S_1 conformation is not helical but, rather, is more appropriate for loop structures (Kim et al., 1973). The least likely P_3 conformation of bound ApU, which may be ruled out, constitutes part of the RNA double helix. The χ angles are approximately 30° at the adenosine portion and 60° at the uridine portion. Upon relaxing all constraints in the conformational angles, a variant of the A_1 conformation with $\chi = 120°$ at adenosine and $\chi = -30°$ at uridine provides an alternative fit to the distances. Such atypical structures have been suggested as components of the tRNA structure (Jack et al., 1976). Again, the helical P_3 conformation does not fit. Although subsequent changes in the conformation of enzyme-bound ApU during catalysis when the template is present cannot be excluded,[2] the nonhelical conformation of the bound initiator (Figure 4) may well be relevant to the catalytic mechanism since it would permit the initiator site to facilitate the release of the growing RNA chain from the DNA template. A linear model for chain elongation may operate (Figure 4) in which succeeding nucleotides added to the growing RNA chain may break their hydrogen-bonded interactions with the DNA template by passing through the initiation site as the enzyme advances along the DNA template. Such a model would account for the growing RNA strand projecting outward from the DNA template during transcription (Miller and Beatty, 1969). An alternative and simpler model assumes that the stability of the DNA duplex over the DNA-RNA hybrid alone causes the release of the RNA strand with no direct participation of the enzyme (Chamberlin, 1965). Supporting evidence is the observation that a homopolymer DNA-RNA hybrid and a single stranded DNA homopolymer constitute the enzymatic products when the hybrid is more stable than the double helical DNA synthetic homopolymer originally used as the template. However, such data are also consistent with the model of Figure 4 if it is assumed that the growing RNA chain, which has been separated from the template chain by passing through the initiation site, may recombine with the DNA on the enzyme, if thermodynamically favorable, to form the DNA-RNA hybrid. Since DNA-RNA hybrid helices are often found to be more stable than DNA-DNA helices (Thomas et al., 1976), a mechanism for catalysis of RNA-DNA chain separation on RNA polymerase seems necessary. Hence the enzyme initiator site may well have more than the single function of initiating RNA synthesis. It may, in addition, catalyze the separation of the newly synthesized RNA chain from the enzyme-bound DNA template.

[2] We point out that substrates assume "helical" conformations (i.e., assume the χ angle of B-DNA) on DNA polymerase in absence of template (Sloan et al., 1975). Further, the presence of template does not alter the affinity of the RNA polymerase-Mn(II) complex for the initiator ApA (Koren and Mildvan, 1977).

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