TRINUCLEOTIDE AUA AND UAU FORMATION CATALYZED BY WHEAT GERM RNA POLYMERASE II

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The elongation of dinucleotides ApU and UpA to trinucleotides ApUpA and UpApU by wheat germ RNA polymerase II was studied at a medium ionic strength (60 mM-KCl). The catalytic mechanism of the first internucleotide bond formation consists in the binding of the primer dinucleotide followed by the binding of NTP ("ordered bibi" reaction), i.e. by an analogous mechanism as found for RNA polymerase holoenzyme from *E. coli*. In further experiments phosphonate analogues of dinucleotides ApU and UpA were used as the priming dinucleotides. It was shown that analogues U(c)pA and Up(c)A are very poor primers for the synthesis of corresponding trinucleotides; the elongation of analogues A(c)pU and Ap(c)U was not observed at all. The comparison of kinetic constants K_{ia}, K_{mA}, K_{mB} and V_{max} as well as the substrate properties of phosphonate analogues indicates the increased specifity of the wheat germ RNA polymerase initiation binding site in comparison with the *E. coli* holoenzyme.

It was recently established that the specifity and the efficiency of the transcription of eukaryotic genes is mediated by the complex interaction of RNA polymerase, protein transcription factors and regulatory DNA sequences[1,2]. However, our understanding of the catalytic properties of eukaryotic RNA polymerases I, II and III as well as the exact role of any of the transcription factors remains to be determined. The particularity of the eukaryotic transcription machinery is caused by the exceptional enzyme complexity[3,4].

RNA polymerase II which is responsible for the transcription of structural genes represents the most studied nuclear enzyme[5-7]. Nevertheless, our knowledge of active sites and of the catalytic mechanism of the first phosphodiester bond formation by RNA polymerase II is at present less complete than for *E. coli* RNA polymerase[8,9].

Recently a progress has been made in the study of the catalytic properties of RNA polymerase II in the selective initiation reaction[10-14] allowing some comparison with the well described *E. coli* enzyme[15-18]. For instance, it has been shown that purified wheat germ RNA polymerase II is able to catalyze the selective formation of dinucleotides on natural templates under specific conditions[10] as well as the elongation of priming dinucleotides to trinucleotides on the poly[d(A–T)]templa-

te[11,12]. The catalytic formation of those dinucleotides and trinucleotides termed "abortive synthesis" essentially depends on the presence of Mn^{2+} cations as the only divalent cation activator[10,11]. On the other hand, RNA polymerase from *E. coli* catalyzes this reaction in the presence of Mg^{2+}, or Mn^{2+} or both together[17,19]. The observation that Hela RNA polymerase II catalyzes abortive formation of trinucleotides using the adenovirus 2 major late promoter in the presence of Mg^{2+} (ref.[20,21]) points out the importance of further detailed study of the formation of the first internucleotide bond and of substrate binding sites on the enzyme by using the photoaffinity probes[22-24] and substrate analogues[25,26].

In this paper we have followed the selective synthesis of trinucleotides ApUpA and UpApU on the poly[d(A–T)]template using ApU and UpA as primer. Furthermore, the phosphonate analogues of dinucleotides which are substrates for *E. coli* holoenzyme were studied as potential substrates for the eukaryotic enzyme.

EXPERIMENTAL

Reagents: Nucleoside 5'-triphosphates (ATP and UTP), dinucleotide monophosphates (ApU and UpA) and 0-phosphonylmethylanalogues of ApU and UpA were used as described earlier[18]. [α^{32}P]ATP and [α^{32}P]UTP (both 15·17 TBq/nmol) were from Amersham. Poly[d(A-T)] was from Boehringer. All buffer components were research grade.

RNA polymease: Wheat germ RNA polymerase II was purified according to Jendrisak and Burgess[6] except for the following modifications: non-heat-stabilized fresh wheat germ was obtained directly from manufacturer; solid ammonium sulfate precipitation was performed for a period of 60 min; the DEAE-cellulose peak of RNA polymerase was immediately dialyzed against TGED buffer instead of the ammonium sulfate precipitation; and phosphocellulose PII was activated according to Greene et al.[27]. The final enzyme concentration was 0·53 mg of protein/ml and it had specific activity 292 units per mg according to standard assay using denatured calf thymus DNA as template[6]. The enzyme was stored in liquid nitrogen.

Reaction Assays and Analysis of the Products

RNA synthesis: The reaction mixture contained: 40 mmol l^{-1} Tris-HCl (pH 7·9), 1 mmol l^{-1} $MnCl_2$, 0·04 mmol l^{-1} EDTA, 0·5 mmol l^{-1} dithiothreitol, 0·9 mmol l^{-1} UpA, 8% (v/v) glycerol, 100 μmol l^{-1} ATP, 1 μmol l^{-1} [α^{32}P] labelled UTP (92·5 kBq per assay), 0·5 μg poly[d(A-T)] and 0·9 μg of enzyme protein; in some experiments 60 mmol l^{-1} KCl was present. The reactions were run at 35 °C for 30 min and terminated by mixing with two volumes of stop buffer (1 mmol l^{-1} EDTA, 80% deionized formamide and 0·1% xylene cyanol). The analysis of reaction products was performed on 15% polyacrylamide slab gels (40 × 20 × 0·02 cm) containing 7 mol l^{-1} urea and TBE buffer (89 mmol l^{-1} Tris, 57 mmol l^{-1} boric acid, 2·5 mmol l^{-1} EDTA, pH 8·8). The electrophoresis was conducted at 15 W until the dye migrated for 25 cm. The gels were autoradiographed at −70°C with X-ray films (Fotochema, Hradec Králové, Czechoslovakia) using Perlux intensifying screans (VEB Kali Chemie Berlin, G.D.R.). The RNA with slower mobility than xylene cyanol was quantified after its excision from gel by the Cerenkov counting.

Synthesis of trinucleotide UpApU: The reaction mixture (15 μl) contained 0·9 mmol l^{-1} UpA, 15 μmol l^{-1} [α^{32}P] labelled UTP (37 kBq per assay) and the other components of the reaction were the same as described above. The reactions were terminated by mixing with the same volume of lyophilized stop solution (9 mol l^{-1} urea, 0·05 mol l^{-1} EDTA, 0·1% bromphenol blue and 0·1% xylene cyanol) and analyzed on 24% polyacrylamide gels as described earlier[18]. The amounts of UpApU and UTP were determined after autoradiography by the Cerenkov counting.

Synthesis of trinucleotides using phosphonate dinucleotides: The ability of wheat germ RNA polymerase II to elongate phosphonate dinucleotides A(c)pU, Ap(c)U, U(c)pA and Up(c)A to the corresponding trinucleotides was studied at the same reaction conditions as described above for the synthesis of UpApU. The concentration of phosphonate dinucleotides was 2 mmol l^{-1} and various concentrations of elongation NTPs were used (5—50 μmol l^{-1}). The analysis of the reaction products was performed in the same way as mentioned above.

Verification of synthesized nucleotides: The structure of ApUpA, UpApU, U(c)pApU and Up(c)ApU trinucleotides was verified according to their RNase A and RNase T2 digestion pattern comparing radioactive products with authentic standards as described for similar reactions catalyzed by *E. coli* RNA polymerase[18].

Determination of Kinetic Constants for ApUpA and UpApU Synthesis

The reaction mixture (15 μl) contained: 40 mmol l^{-1} Tris-HCl (pH 7·9), 1 mmol l^{-1} $MnCl_2$, 60 mmol l^{-1} KCl, 8% (v/v) glycerol, 0·04 mmol l^{-1} EDTA, 0·5 mmol l^{-1} dithiothreitol, 0·05 μg poly[d(A-T)], 0·9 μg of enzyme protein and variable amounts of ApU and UpA and [α^{32}P] labelled ATP or UTP as indicated in the legends to Figs 1 and 2. The reactions were run at 35°C for 10—30 min to ensure the linear course of the product formation. The reaction products were analyzed by paper electrophoresis (Whatman 3 MM, 0·05 mol l^{-1} sodium dihydrogencitrate pH 3·8, 1 000 V). After the autoradiography the radioactive spots were excised and counted in 5 ml toluene–PPO–POPOP liquid scintillation coctail. The kinetic data were computed using least square procedure as described[18].

RESULTS AND DISCUSSION

The Effect of Reaction Conditions on the Synthesis of UpApU and RNA

The reaction conditions for abortive synthesis of trinucleotides catalyzed by wheat germ RNA polymerase II and RNA polymerase from *E. coli* are in general slightly different[10—18]. Essential is the presence of Mn^{2+} and a varying concentration of KCl (ref.[28]). Taking into account these published data we have chosen the concentration of 60 mmol l^{-1} KCl and 1 mmol l^{-1} $MnCl_2$ for the parallel investigation of poly[r(A—U)] synthesis and abortive production of UpApU.

The results are summarized in Table I. The gel electrophoresis of poly[r(A—U)] synthesis reveals the same pattern of the polymer formed by the wheat germ RNA polymerase II as was published elsewhere[11]. Although KCl at the concetration of 60 mmol l^{-1} inhibits UpApU formation by polymerase II to about 63% of the amount formed in its absence, it increases the overall poly[r(A—U)]synthesis by the factor of 2·3 and the length of the formed polymer as well.

Kinetics of UpApU and ApUpA Synthesis

The determination of kinetic constants was carried out as described in Experimental in the presence of 60 mmol l^{-1} KCl. The catalytic production of ApUpA and UpApU belongs to the group of two-substrate reactions. The initial velocity of their formation was measured as a function of the concentration of both substrates(priming dinucleotide and elongation NTP). The double-reciprocal plots and replots of the slopes and intercepts versus reciprocal ATP and UTP concentration are shown in Figs 1 and 2, respectively. The linearity of double-reciprocal plot (Figs 1*A* and 2*A*) shows an equilibrium ordered type of the reaction. The kinetic constants obtained from replots of double-reciprocal graphs (Figs 1*B* and 2*B*) are in agreement with initiation binding of priming dinucleotide followed by binding of the elongation NTP (ref.[29]). This mechanism is of the same type as observed for *E. coli* RNA polymerase holoenzyme[18,30]. The kinetic data K_{ia} (dissociation constant for dinucleotide primer), K_{mA} and K_{mB} (the Michaelis constants for dinucleotide and NTP, respectively) and V_{max} are summarized in Table II.

Properties of the Phosphonate Analogues of Dinucleotides

In order to obtain a more detailed information about the structure of the active site of wheat germ RNA polymerase II it was of interest to study the elongation of 5'-0- and 3'-0-phosphonylmethylanalogues of dinucleotides ApU and UpA which were studied previously as substrates for *E. coli* RNA polymerase[18,19]. In these experiments it was found that only the dinucleotides U(c) pA and Up(c) A can be elongated to corresponding trinucleotides which are then partialy resistant to RNase A and RNase T2 digestion as analogous products formed by *E. coli* holoenzyme[18]. The apparent kinetic constants for 25 μmol l^{-1} UTP are given in Table III including the data for UpA as a reference. The obtained kinetic data show that both analogues are poor substrates in the abortive synthesis reaction. However, the dinucleotides A(c) pU and Ap(c) U do not function as substrates for wheat germ RNA polymerase II under the conditions studied.

Comparison of the Catalytic Properties of Wheat Germ RNA Polymerase II and E. coli RNA Polymerase

Up to now a direct comparison of the catalytical properties during the specific initiation of eukaryotic and prokaryotic RNA polymerases was not possible[20,21].

However, the experimental model used enabled us to compare the catalytic properties of wheat germ RNA polymerase II and *E. coli* holoenzyme during the selective initiation since the same template is transcribed, identical products are formed and finally the reaction proceeds at a ionic strenght suitable for abortive synthesis with both types of enzymes. The results (Table II) show the following common properties

[a]TABLE I

Effect of KCl on RNA and UpApU synthesis catalyzed by wheat germ RNA polymerase II

KCl mmol l^{-1}	RNA[a] % of total radioactivity	UpApU pmol l^{-1} 30 min^{-1}
0	8	33·8
60	18	21·2

RNA with mobility slower than xylene cyanol dye.

TABLE II

Kinetic constants for elongation of (3′—5′)dinucleotides ApU and UpA to trinucleotides ApUpA and UpApU

Trinucleotide	K_{ia}	K_{mA}	K_{mB}	V_{max}
	mmol l^{-1}			pmol μg^{-1} min^{-1}
	Wheat germ RNA polymerase II			
ApUpA	0·280	0·390	0·016	0·62
UpApU	0·360	0·280	0·037	5·26
	E. coli holoenzyme[a]			
ApUpA	0·036	0·011	0·027	2·40
UpApU	0·104	0·036	0·034	200·80

[a] See ref.[18].

TABLE III

Apparent kinetic constants for elongation of (3′—5′)dinucleotides UpA, U(c)pA and Up(c)A to UAU trinucleotides for 25 μmol l^{-1} UTP

Dinucleotide	K_{mA}^{app} mmol l^{-1}	V_{max}^{app} pmol μg^{-1} min^{-1}
UpA	0·41	2·06
U(c)pA	1·69	0·05
Up(c)A	1·56	0·07

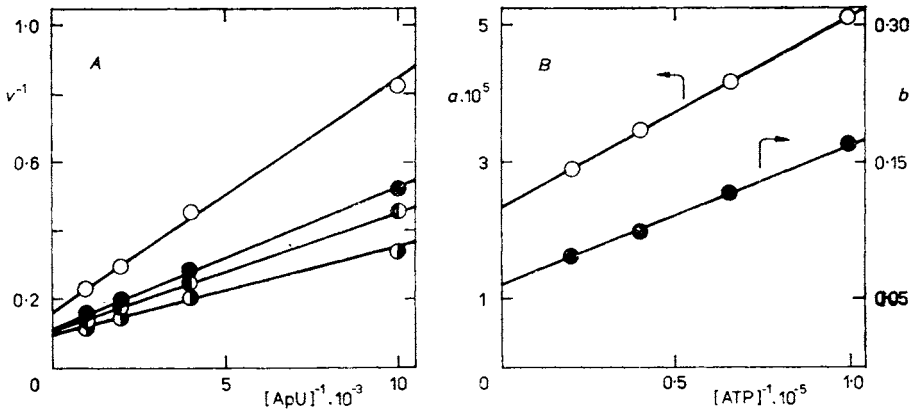

FIG. 1

Initial velocity pattern for ApUpA synthesis for 0·9 μg RNA polymerase II. A Double reciprocal plot (v^{-1} vs [ApU]$^{-1}$) for constant concentrations of 0·01 (○), 0·015 (●), 0·025 (◐), and 0·05 (◑) mmol l^{-1} ATP; v — initial velocity in pmol 30 min^{-1}; *B* Secondary plot of slopes (○), and intercepts (●), respectively, vs 1/[ATP]; a slope from the graph (*A*) in (pmol^{-1} 30 min/mmol^{-1} . . l); b intercept from the graph (*A*) in pmol^{-1} 30 min

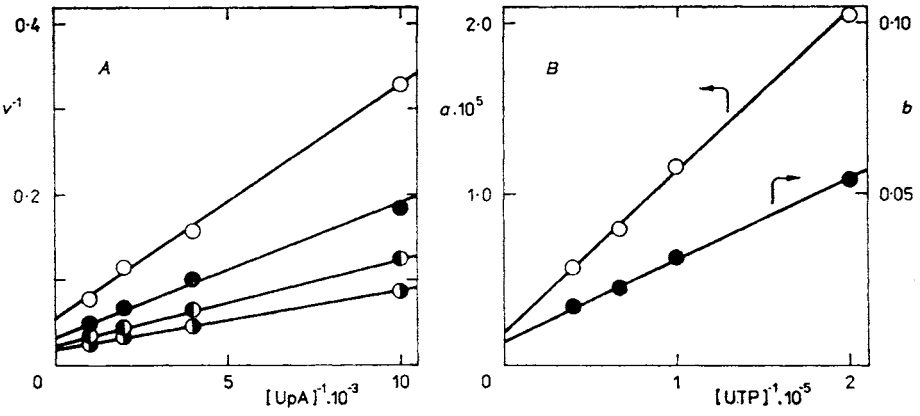

FIG. 2

Initial velocity pattern for UpApU synthesis for 0·9 μg RNA polymerase II. A Double reciprocal plot (v^{-1} vs [UpA]$^{-1}$) for constant concentrations of 0·005 (○), 0·01 (●), 0·015 (◐), and 0·025 (◑) mmol l^{-1} UTP; *B* Secondary plot of slopes (○), and intercepts (●), respectively, vs 1/[UTP]; v, a, and b — see Fig. 1

of *E. coli* holoenzyme and wheat germ RNA polymerase II: (i) the catalytic production of UpApU is substantially higher than ApUpA, (ii) the dissociation constant K_{ia} is considerably higher for UpA than for ApU and (iii) the Michaelis constant K_{mB} for UTP is considerably higher than for ATP. Nevertheless, in some properties both enzymes differ: (i) in the relation between K_{mA} for ApU and UpA, (ii) in the difference between the ratios of corresponding V_{max} values for UpApU and ApUpA (200/2·5 and 5·2/0·6, respectively) and (iii) in the fact that catalytic production of both trinucleotides is at least by one order of magnitude lower for the eukaryotic enzyme (Table II). To obtain further data on the difference between eukaryotic and prokaryotic polymerases concerning especially the initiation process the phosphonylmethyl analogues of priming dinucleotides were used. In this case only the synthesis of trinucleotides U(c)pApU and Up(c)ApU was observed; however, it was substantially less efficient in comparison with *E. coli* holoenzyme[18]. The inability of wheat germ RNA polymerase II to elongate A(c)pU and Ap(c)U to corresponding trinucleotides differs from the behaviour of *E. coli* holoenzyme but corresponds to that of the incomplete *E. coli* core enzyme[19].

These results point to an increased selectivity of active sites of eukaryotic polymerase reflected by the more stringent structure requirements for primer dinucleotides. The high specifity of eukaryotic polymerases is thus due not only to a more refined regulatory mechanism involving additional protein factors but also to a more specific architecture of the binding and active sites themselves.

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