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Oligomerization of Ribonucleotides on Montmorillonite: Reaction of the 5′-Phosphorimidazolide of Adenosine

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The regiospecific formation of oligomers from unblocked monomers in aqueous solution is one of the central tenets in research on the origins of life on earth. Direct experimental support for this hypothesis has been obtained in studies of the condensation of the 5′-phosphorimidazolide of adenosine (ImpA) with itself and with P^1,P^2-diadenosine-5′,5′-pyrophosphate (AppA) in water in the presence of a montmorillonite clay. Oligomers of up to ten nucleotides in length are formed. Analysis of the trimers, tetramers, and pentamers formed from a 9:1 ImpA:AppA mixture has shown that 85% of the bonds formed are 3′,5′-linked and that any 2′,5′-linkages present are at the phosphodiester bond next to the 3′-terminus of the oligomers.

The observation of the catalytic role of RNA in processing RNA transcripts (*1*, *2*) suggests that RNA-like molecules (*3*) were the central biopolymers in the first life on earth. This postulate is strengthened by the observation of the catalytic elongation of a pentameric oligocytidylate [oligo(C)] by the ribozyme from a group I intron (*4*) and the efficient synthesis of the complementary oligoguanylate on tetrameric or longer oligo(C) templates (*5*). The relevance of these findings to the origins of life has always been tempered by the absence of a plausible prebiotic route to a 3′,5′-linked oligomer that would be amenable to elongation by ribozymes or template-directed replication (*6*, *7*). In previous research, mainly 2′,5′-linked oligoadenylates were formed from the phosphorimidazolide of adenosine (5′-ImpA). Reaction of 5′-ImpA in the presence of Zn^{2+} or Pb^{2+} resulted in the formation of tetrameric and pentameric 2′,5′-linked oligomers in yields of 25 and 35%, respectively (*8*, *9*). 2′,5′-Linked oligonucleotides, up to 16 units in length, are formed from the reaction of 5′-ImpA in the presence of UO_2^{2+} (*10*).

The conversion of monomers to dimers and trimers in the presence of Na^+-montmorillonite by reaction with a water-soluble carbodiimide [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC)] was reported previously (*7*). The condensation reactions of 5′-ImpA in the presence of Na^+-montmorillonite are reported here. Incubation of 1 ml of a 14.5 mM solution of 5′-ImpA in a solution (pH 8) of 0.2 M NaCl and 0.075 M $MgCl_2$ containing 50 mg of Na^+-Volclay (*11*) for 3 days at room temperature resulted in the reaction of all of the monomer and the formation of a 61% yield of oligomers. Analysis of the reaction products by high-performance liquid chromatography (HPLC) with an anion-exchange column (*12*) resulted in the detection of oligomers up to ten nucleotides in length (Fig. 1A). The absence of the P^1,P^2-diadenosine-5,5′-pyrophosphate (AppA), a reaction product of 5′-ImpA in the absence of Na^+-montmorillonite and the predominant product in the reaction of 5′-adenosine monophosphate in the presence of EDAC (*7*), suggested that the AppA formed was incorporated into the oligomeric products. This result was verified when a 9:1 mixture of 5′-ImpA:AppA gave less than a 1% recovery of AppA, as shown by reverse-phase HPLC analysis (*7*) when the reaction was performed under the same conditions. Anion-exchange HPLC (Fig. 1B) showed the formation of oligomers (67%) up to the decamer in length (*13*). Structural information on the reaction products from the 9:1 5′-ImpA:AppA reaction was obtained by collection of the respective peaks from the anion-exchange HPLC column and by the use of enzymatic and KOH hydrolyses to determine the structures of the oligomers in each fraction.

Hydrolysis with alkaline phosphatase (APH) was used to determine the percentage of each fraction with incorporated AppA groupings (*14*). The ratio of the area of the oligomer HPLC peak on ion-exchange chromatography after and before treatment with APH gave the proportion of oligomers with an incorporated AppA unit (Table 1). Comparison of retention times of the APH hydrolysis products with authentic samples of 3′,5′-A$(pA)_n$ made it possible to determine the amounts of 3′,5′-$(pA)_{n+1}$ (n = 2 and 3) in the trimer and tetramer fractions, respectively (Table 2). It followed from this determination that 2′,5′-ApA groupings are present only on the 3′-termini of the oligomeric chains and that the HPLC peaks with almost the same retention times as the 3′,5′-A$(pA)_n$ peaks were the corresponding $A^{3'}pA^{2'}pA$ and $A^{3'}pA^{3'}pA^{2'}A$ isomers in the trimer and tetramer fractions, respectively. The percentages of both isomers, obtained by HPLC analysis after treatment with APH, are given in Table 1.

Incubation of the separated fractions with ribonuclease T_2 (RNase T_2), an endonuclease that cleaves a 3′,5′-phosphodiester bond to the corresponding 5′-OH and 3′-phosphate (*15*), followed by analysis by anion-exchange chromatography and reverse-phase chromatography (*7*), resulted in the hydrolysis of the fraction and the detection of A, Ap, 2′,5′-ApA, pAp, pAppA, pAppAp, and $pAppA^{2'}pA$ (Table 1). Further hydrolysis of the RNase T_2 hydrolysate with APH resulted in the detection of A, AppA, 2′,5′-ApA, and

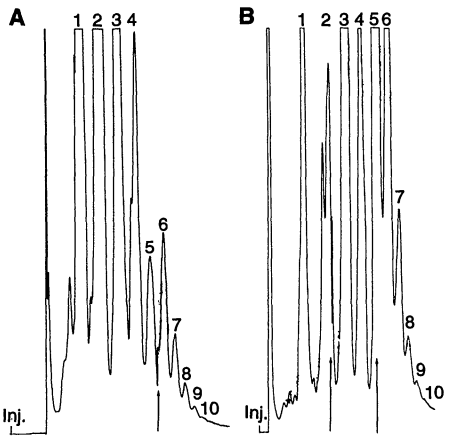

Fig. 1. (**A**) The anion-exchange HPLC separation of the oligomers formed from the reaction of ImpA on Na^+-montmorillonite. (**B**) The anion-exchange HPLC separation of the oligomers formed from the reaction of a 9:1 ImpA:AppA mixture on Na^+-montmorillonite. Arrows designate a change in recorder attenuation; Inj., sample injection. The numbers are the number of monomer units in the oligomers present in each peak.

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Table 1. Analysis of trimer, tetramer, and pentamer fractions by enzymatic and KOH hydrolysis; hydrolysis products are indicated as a percentage of the total. Fractions (0.3 ml) were incubated for 2 hours at 37°C at pH 4.5 with 0.03 units of RNase T_2. Further APH digestion of this fraction was performed by adjustment to pH 8 with NaOH and the addition of 0.18 units of enzyme and incubation for 8 hours at 37°C. Fractions were incubated with 1 M KOH for 50 hours at room temperature. HPLC analyses were performed on a HEMA-IEC B10 1000 Q column (Alltech) with a gradient of H_2O (pH 8) to which 0.4 M $NaClO_4$ (pH 8) was added (*12*). The HPLC equipment, with the addition of a Hitachi Autoinjector, was described previously (*7*). Fractions, ~1.6 ml, collected from the column were used directly in the hydrolytic studies. On the basis of APH hydrolysis, AppA incorporation in oligomers was 85% for the trimer, 94% for the tetramer, and 73% for the pentamer (incubation of 0.3 ml with 0.1 unit of APH at 37°C for 16 hours). The pAppA:pAppAp ratios from the KOH hydrolysis data and from the APH hydrolysis of the KOH hydrolysis products were 1.8 for the trimer, 0.62 for the tetramer, and 0.22 for the pentamer. Dashes indicate that the product was not detected.

Nucleotide	Trimer		Tetramer		Pentamer	
	RNase T_2	KOH	RNase T_2	KOH	RNase T_2	KOH
A	39	65	39	69	15	67
A2′pA	14	—	26	—	16	—
Ap	18	9	19	14	34	18
pAp, pAppA	20	12	9	6	14	4.3
pAppA2′pA	2	—	2	—	2	—
pAppAp	3	6	2	8	5	11

Table 2. Composition of the trimer, tetramer, and pentamer fractions. Compositions of each fraction were determined from the data given in Table 1 and by reversed-phase HPLC of the trimer fraction. AppA units bound to oligomers give the same incremental change to the HPLC retention times as do pA units; hence, they are considered to be monomeric units.

Trimer	%	Tetramer	%	Pentamer	%
3′,5′-pApApA	9	3′,5′-pApApApA	3	$(pA)_5$ isomers	27
pA3′pA2′pA	9	pA3′pA3′pA2′pA	9	$AppA(pA)_4$ isomers	17
AppA3′pA3′pA	65	$AppA(pA)_3$ isomers	54	$(Ap)_mAppA(pA)_n$ isomers ($m + n = 4$)	56
Ap3′AppA3′pA	6	$(Ap)_mAppA(pA)_n$ isomers ($m + n = 3$)	34		
Ap3′AppA2′pA	3				
AppA3′pA2′pA	9				

$AppA^{2'}pA$ by anion-exchange and reverse-phase chromatography. The identities of the reaction products, with the exception of $pAppA^{2'}pA$ and $AppA^{2'}pA$, were established by coinjection with authentic samples (*16*). The structure of $pAppA^{2'}pA$ is consistent with its HPLC anion-exchange (retention time of 18 min) and its APH-catalyzed conversion to $AppA^{2'}pA$ (retention time of 11 min) upon treatment with APH. The $AppA^{2'}pA$ structure is consistent with its resistance to RNase T_2 hydrolysis and its cleavage by venom phosphodiesterase (*15*).

Control experiments showed that RNase T_2 catalyzed the slow hydrolysis of the terminal phosphate groups of pAppA and pAppAp. Independent analysis of the proportions of these groups present was performed by hydrolysis with 1 M KOH (Table 1) (*17*). Because pAp and pAppA have the same retention times on ion-exchange HPLC, it was necessary to calculate the yield of pAppA from the ratio of oligomer cleaved by APH hydrolysis to that which was not cleaved (Table 1). The proportion of 5′-terminal AppA to internal AppA in the oligomers followed from the ratio of pAppA to pAppAp.

The detection of only 2′,5′-ApA and the absence of 2′,5′-pApA and 2′,5′-ApApA after RNase T_2 hydrolysis of the individual trimer, tetramer, and pentamer fractions established that the 2′,5′-links are present only in the phosphodiester bond next to the 3′-termini of the oligomer chains. This finding suggests that chain elongation on montmorillonite proceeds more rapidly if a 3′,5′-linked monomer unit is present on the 3′-terminus of the chain. The compositions of the trimer, tetramer, and pentamer fractions were determined from the hydrolysis data given in Table 1. It can be shown from the composition of the trimer fraction (Table 2) that 85% of the bonds formed are 3′,5′-linked.

The formation of RNA oligomers on a montmorillonite clay provides experimental support for the hypothesis that clays and other minerals may have catalyzed the polymerization of monomers to polymers on the primitive earth (*18*). The formation of about 85% 3′,5′-linkages demonstrates that mineral catalysis can result in bond formation with high regiospecificity (*19*). This finding also suggests that it was possible to form RNA oligomers with internal 3′,5′-links that were sufficiently long to be amenable to catalytic elongation by ribozymes from group I introns (*20*). The oligomers could also have served as templates for the template-directed synthesis of the complementary oligonucleotides. In addition, these short RNA units may have been ligated to longer ones by reaction of the 3′-OH in one chain with the 5′-AppA groups incorporated in another chain.

Dinucleoside pyrophosphates are formed readily from activated phosphomonoesters under prebiotic conditions (*21*), and it has been suggested that RNA oligomers formed under prebiotic conditions would be capped with pyrophosphate groups (*22*). Our findings suggest that pyrophosphates can also initiate the formation of RNA oligomers in the presence of montmorillonite to yield oligomers with capped and internal pyrophosphate groups. Pyrophosphate derivatives are intermediates in the enzymatic ligation of DNA and RNA (*23*), and it has been proposed that pyrophosphate groups may have been intermediates in the prebiotic ligation of RNA (*6*). The chemical ligation of DNA has been observed when other activating groups are used (*24, 25*).

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Cloning of a Subunit of Yeast RNA Polymerase II Transcription Factor b and CTD Kinase

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Yeast RNA polymerase II initiation factor b copurifies with three polypeptides of 85, 73, and 50 kilodaltons and with a protein kinase that phosphorylates the carboxyl-terminal repeat domain (CTD) of the largest polymerase subunit. The gene that encodes the 73-kilodalton polypeptide, designated *TFB1*, was cloned and found to be essential for cell growth. The deduced protein sequence exhibits no similarity to those of protein kinases. However, the sequence is similar to that of the 62-kilodalton subunit of the HeLa transcription factor BTF2, suggesting that this factor is the human counterpart of yeast factor b. Immunoprecipitation experiments using antibodies to the *TFB1* gene product demonstrate that the transcriptional and CTD kinase activities of factor b are closely associated with an oligomer of the three polypeptides. Photoaffinity labeling with 3′-*O*-(4-benzoyl)benzoyl-ATP (adenosine triphosphate) identified an ATP-binding site in the 85-kilodalton polypeptide, suggesting that the 85-kilodalton subunit contains the catalytic domain of the kinase.

Initiation of transcription by RNA polymerase II requires several accessory protein factors whose roles remain, for the most part, to be elucidated. Recognition of the TATA element of a promoter is effected by the binding of a factor termed TFIID, which may be assisted by TFIIA; other factors and RNA polymerase then bind to the complex. RNA polymerase II exists in cells in two forms that differ in the degree of phosphorylation of the CTD of the largest subunit. Phosphorylation of the CTD is thought to occur at every round of initiation and to be required for that process or for the transition to elongation (*1–5*); therefore, the CTD kinase might be an essential initiation factor. Yeast polymerase II initiation factor b copurifies with a CTD kinase (*6*, *7*). Factor b also copurifies with polypeptides of 85, 75, and 50 kD. We report the results of chemical cross-linking and molecular cloning experiments that identify the likely catalytic subunit and establish the association of transcription factor and CTD kinase with a single oligomeric molecule composed of the three subunits.

The photoactivatable ATP derivative 3′-*O*-(4-benzoyl)benzoyl-ATP (BzATP) (*8*) was used to identify ATP-binding subunits of factor b. When we incubated highly purified factor b with [α-^{32}P]BzATP and exposed the mixture to ultraviolet (UV) irradiation, the 85-kD polypeptide was labeled with ^{32}P (Fig. 1). The labeling was prevented by an excess of nonradioactive ATP and was dependent on UV irradiation. The 85-kD polypeptide therefore contains an ATP binding site and might be the catalytic subunit of the CTD kinase. Another possibility is that the 85-kD polypeptide has the DNA-dependent adenosine triphosphatase activity associated with factor b (*7*). The relation between the two activities is presently unclear.

The gene encoding the 75-kD subunit of factor b was cloned (*9*). Factor b was purified to near homogeneity (*6*), and the 75-kD polypeptide was subjected to tryptic digestion. The NH_2-terminal sequences of two peptides were obtained by sequential Edman degradation. Polymerase chain reaction with oligonucleotide primers derived from the peptide sequences gave an amplified fragment of 1 kb, which was used to probe yeast genomic libraries. Sequence analysis of the resulting genomic clones (Fig. 2) disclosed an open reading frame (ORF) of 1926 nucleotides encoding a polypeptide of 642 amino acids with a predicted molecular size of 73 kD. The sequences of both tryptic peptides were found within the ORF (Fig. 2). Analysis of yeast RNA (*10*) revealed a set of transcripts of 2.4 to 2.5 kb starting 11 to 35 bases upstream of the initiation codon. We have termed the gene *TFB1* (transcription factor b, subunit 1); the protein is designated TFB1.

The deduced protein sequence of TFB1 does not show any of the elements conserved in protein kinases, so the gene product is evidently not the catalytic subunit of the CTD kinase, as suggested by the affinity-labeling experiments. The 73-kD polypeptide may function in the regulation of kinase activity or in the interaction of the kinase with other components of the transcription machinery. The sequence of the polypeptide is unrelated to any other sequence in the Genbank database (release 70), nor does it contain any recognizable sequence motifs. However, in its native size and subunit composition, yeast factor b resembles the rat liver initiation factor δ (*11–13*) and the HeLa factor BTF2 (*14*, *15*). Moreover, the deduced amino acid sequence encoded by *TFB1* is similar to that of a 62-kD subunit of BTF2 (Fig. 2). The most significant region of similarity (42% identity, 62.5% similarity; $z = 14.7$ SD) (*16*) is in the region between amino acids 234 and 297; the similarity outside this region is only marginally significant (19% identity, 32% similarity; $z = 3$ SD).

For purposes of genetic analysis, we prepared a yeast strain with most of the coding sequence of *TFB1* (between the Acc I site corresponding to Ser^{31} and the Sal I-Acc I site corresponding to Asp^{528}) deleted. Sequences flanking the *TFB1* gene were inserted in the integrative *LEU2* vector pRS305 (*17*). After transformation of the diploid strain YPH501 (*17*), the replacement of *TFB1* sequences with plasmid sequences was confirmed by DNA blot hybridization. The heterozygous cells were then sporulated and tetrads were manually dissected (*10*). Only two spores of each true tetrad germinated and formed colonies on rich medium (and were both *leu*$^-$), which demonstrates that the *TFB1* gene is essential for viability.

To establish the relation between the 73-kD polypeptide encoded by the ORF and the polypeptides, transcription factor activity, and CTD kinase activity of factor b, antibodies were raised to the product of the cloned gene (*18*). The resulting antiserum reacted with a polypeptide of ~75 kD in a partially purified preparation of factor b (Fig. 3A); serum obtained from the same rabbit before immunization did not react with this polypeptide. To assess the association of the subunits and the biochemical activities, we covalently attached antibodies from immune and preimmune sera to Affi-Gel–protein A beads and used the antibodies to bind proteins from fractions containing factor b. Starting with a relatively crude fraction, a single step of immunoaffinity purification resulted in an eluted preparation containing almost exclusively the 85-, 73-, and 50-kD polypeptides (Fig.

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