THE ENZYMATIC SYNTHESIS AND CODING PROPERTIES OF OLIGONUCLEOTIDES CONTAINING A 2',5' INTERNUCLEOTIDE LINKAGE AT THE 5' END

D. M. Brown and M. J. A. Tanner*

University Chemical Laboratories, Cambridge, England

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Earlier studies on the specificity of the primer requiring polymerization of ribonucleoside-5' diphosphates with polynucleotide phosphorylase suggested that when natural dinucleoside phosphates and higher homologues were used as primers for polymerization by the enzyme from E. coli, they were incorporated into polymer, but when those containing a 2'-5' internucleotide linkage were used as primer no incorporation into the polymer took place (Singer et al., 1960; Grunberg-Manago, 1963). This communication describes the incorporation of a dinucleoside phosphate containing a 2'-5' linkage into oligonucleotide by the polynucleotide phosphorylase from Micrococcus lysodieckticus, and the coding properties of the resulting oligonucleotides which contain a 2'-5' linkage at their 5' ends. Trinucleoside diphosphates containing only 2'-5' linkages are ineffective in the binding assay (Rottman and Nirenberg, 1966).

MATERIALS AND METHODS. Polynucleotide phosphorylase. A primer requiring preparation of polynucleotide phosphorylase from M. lysodieckticus was the generous gift of Dr. J. S. Anderson, and had been prepared by the methods of Singer and coworkers (Singer and Guss, 1962; Singer and O'Brien, 1963).

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*Present address: Division of Biology, California Institute of Technology, Pasadena, California.

Dinucleoside phosphates. A mixture of uridylyl(2',5')adenosine and uridylyl(3',5')adenosine was synthesized by a method based on that of Michelson (1959). The isomers were separated and purified on Biorad AG 1x2 by application of a gradient of triethylamine formate buffer, pH 5.2. The 2'-5' UpA was eluted prior to the 3'-5' UpA, and both compounds were electrophoretically pure. Pancreatic RNase digested the 3'-5' UpA to give Up and adenosine, as did spleen phosphodiesterase, and alkali; snake venom diesterase yielded pA and uridine. The 2'-5' UpA was resistant to pancreatic RNase and spleen phosphodiesterase while venom diesterase yielded pA and uridine and alkali gave Up and adenosine.

2'-5' Linked UAA, UAAA, UAAAA, UAU, and UAC. 15 μmoles each of 2'-5' UpA and ADP were present in a reaction mixture of total volume 0.5 ml, which contained 0.2 M Tris (OAc) pH 9.1, 0.03 M $Mg(OAc)_2$, 0.0001 M $CuCl_2$, 0.5 M NaCl and 0.2 mg of polynucleotide phosphorylase. After incubation at 37° for 72 hours, 100 μg of bacterial alkaline phosphatase (Worthington) was added, and the reaction mixture incubated at 37° for 1 hour. Preparative paper chromatography in n-propanol/NH_3/H_2O(55:10:35) separated the 2'-5' linked UAA and the homologous UAAA and UAAAA, each of which migrated as a single component on electrophoresis at pH 3.5.

Similar conditions were used for the addition of CDP and UDP onto 2'-5' UpA primer.

RESULTS AND DISCUSSION. Incorporation of 2'-5' dinucleoside phosphates. Uridylyl(2',5')adenylyl(3',5')uridine, uridylyl(2',5')adenylyl(3',5')adenosine, and uridylyl(2',5')adenylyl(3',5')cytidine, respectively, were obtained when 2'-5' UpA was used as a primer for the polymerization of UDP, ADP and CDP with polynucleotide phosphorylase under the equilibration conditions of Thach and Doty (1965). On electrophoresis at pH 3.5, the unnatural compounds had mobilities identical with those of the corresponding natural trinucleoside diphosphates, UAU (the gift of Dr. B. E. Griffin), UAA and UAC (Thach and Doty, 1965). Analysis with snake venom diesterase showed they had

their assigned composition and correct 5'-terminal residues (Table 1).

Table 1

Molar ratios of the products of venom digestion of 2'-5' linked trinucleoside diphosphates

	Trinucleoside diphosphate		
Product	UAA	UAC	UAU
U	1.0	1.0	1.0
pA	1.8	1.1	0.8
pC	-	1.0	-
pU	-	-	1.1

3 OD_{260} units of trinucleotide in 0.02 M NH_4HCO_3 pH 9.0 and 0.004 M $MgCl_2$ were incubated at 37° for 3 hours with 15 μg snake venom diesterase (Worthington). The products were separated by electrophoresis at pH 3.5, identified by comparison with markers of the major nucleotides and nucleosides, eluted and estimated spectrophotometrically at 260 mμ against suitable blanks.

The compounds were characterized by their reaction with various enzymes. They were all degraded with venom diesterase, but were all resistant to pancreatic ribonuclease and spleen phosphodiesterase under conditions which degraded natural UAA. The suggested structures are in accord with the known specificity of each enzyme (Razzell and Khorana, 1959, 1961; Witzel, 1963).

The comparative rates of ADP polymerization when primed by each UpA isomer are shown in Figure 1. Polymerization was carried with ^{3}H-ADP. The reaction with 2'-5' UpA primer is slower than that with the 3'-5' UpA isomer, but the extent of ADP incorporation is similar with both primers. The early part of the reaction (Figure 1 inset) reveals that although the initial rate of reaction is slower in the 2'-5' UpA primed case, the rates later become comparable, suggesting that the initiation of these chains is slower with the unnatural primer but as the 2'-5' linkage moves away from the enzyme catalytic site the rates become similar. Equilibration is also slower with the unnatural primer, as would be expected.

When various mixtures of the two UpA isomers were used to prime ADP polymerization, allowed to equilibrate until only trinucleoside diphosphate

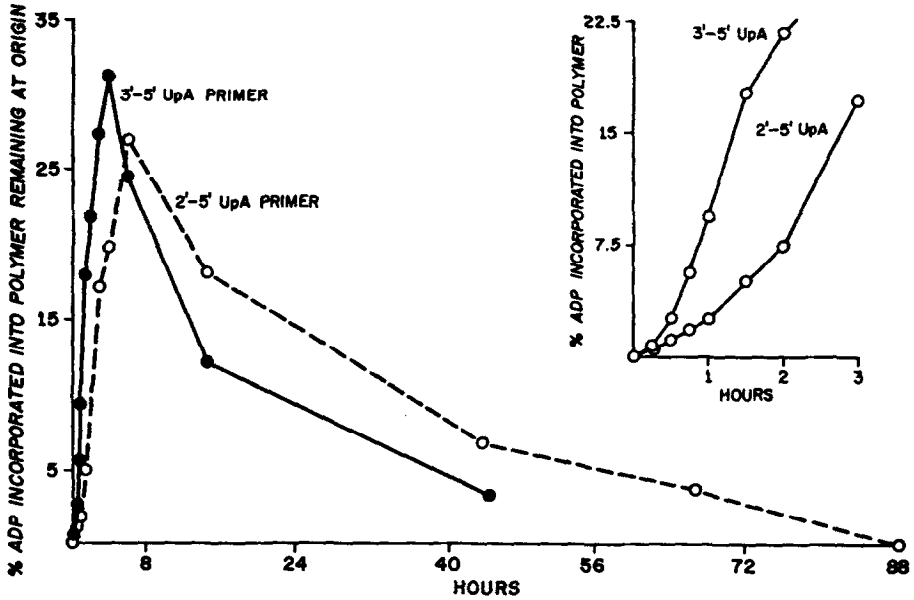

Figure 1. Kinetics of incorporation of ADP into polymer with natural and unnatural primers. Both reaction mixtures contained in 0.2 ml; 0.25 M Tris (OAc) pH 9.1, 0.025 M $Mg(OAc)_2$, 0.0001 M $CuCl_2$, 0.5 M NaCl, 0.11 mg polynucleotide phosphorylase and 5.5 OD_{260} units (1 μmole) of ^{3}H-ADP (Schwarz Bioresearch) of specific activity 13,600 cpm/OD_{260} unit. One reaction mixture was supplemented with 0.38 μmoles 2'-5' UpA and the other with 0.38 μmoles 3'-5' UpA. The mixtures were incubated at 37° and at the time indicated 15 μl aliquots of the reaction mixture were placed at the origin of a chromatogram, the reaction being stopped by the application of 5 μl glacial acetic acid to the spot on the chromatogram. All the samples were cochromatographed in n-propanol/NH_3H_2O (55:10:35), in which oligonucleotides of chain length greater than 6-7 remain at the origin. After chromatography an identical region around the origin of each sample was cut out and counted in a scintillation counter.

was present, and the relative proportions of the two UAA isomers produced estimated by pancreatic ribonuclease digestion, the 3'-5' UpA was found to be preferentially utilized. When the two UpA isomers were present in equal amounts approximately 13-fold more 3'-5' UpA was incorporated than 2'-5' UpA. Only when the input ratio (2'-5' UpA)/(3'-5' UpA) reached 6 did an equivalent amount of incorporation of both primers take place. The total trinucleotide produced decreased as the percentage of 2'-5' UpA in the primer was increased.

Table 2

Effect of unnatural oligonucleotides on the binding of tRNA to ribosomes

Oligonucleotides		OD_{260} units added	Yeast ribosomes Yeast tyr-tRNA μμmoles bound	E. coli ribosomes Yeast tyr-tRNA μμmoles bound	E. coli ribosomes E. coli tyr-tRNA μμmoles bound
None		--	1.21	2.60	0.44
Unnatural	UAU	0.42	1.60	3.41	0.69
Unnatural	UAC	0.36	2.20	3.69	0.52
Unnatural	UAA	0.56	1.26	--	--
Natural	UAU	0.30	4.96	6.32	1.03
Natural	UAC	0.25	5.46	6.36	0.84
Natural	UAA	0.30	1.85	3.16	0.45

Oligonucleotides		OD_{260} units added	Yeast lys-tRNA μμmoles bound
None		--	1.01
Unnatural	UAU	0.42	0.85
Unnatural	UAC	0.34	0.83
Unnatural	UAA	0.29	1.30
Unnatural	UAAA	0.19	1.42
Unnatural	UAAAA	0.20	1.65
Natural	UAA	0.15	1.01
Natural	UAAA	0.19	1.77
Natural	UAAAA	0.20	1.91
Natural	UAAAAA	0.20	2.20

Binding was carried out using the procedure of Nirenberg and Leder (Nirenberg and Leder, 1964). 3.05 OD_{260} units of washed E. coli ribosomes, and 2.05 OD_{260} units of washed yeast ribosomes were used as indicated. The assays with yeast ribosomes were carried out in the presence of 20 mM spermidine and 0.4 mM spermine (Brown et al.; Tanner) and 0.01 M Mg^{++}. When E. coli ribosomes were used binding was carried out at 0.03 M Mg^{++}.

The E. coli ribosomes and E. coli tRNA were the gift of Dr. B. F. C. Clark.

Binding properties of 2'-5' linked oligonucleotides. The effect of the 2'-5' linked UAA, UAU and UAC on the binding of yeast and E. coli ^{14}C-tyr-tRNA to yeast ribosomes and E. coli ribosomes is shown in Table 2.

The unnatural UAU and UAC show weak stimulations of the binding of tyr-tRNA. However the characteristics of binding are different from those of natural UAU and UAC (Brown et al.), in that optimal binding has a higher magnesium requirement, and the optimum position is reached faster during incubation. In addition, even large amounts of trinucleotide do not saturate the binding of yeast tyr-tRNA in contrast to the saturation observed with the natural trinucleotides. The data in Table 2 also show that the unnatural UAU and UAC do not stimulate the binding of yeast lys-tRNA, although the unnatural UAA does slightly stimulate the binding of this tRNA. It may be that with these unnatural trinucleoside diphos-

phates the last two bases largely determine the specificity of binding. The data on the binding of UAA and higher homologues demonstrate that the presence of a 2'-5' link does not completely prevent the reading of later AAA codons. Further details of the coding properties of these yeast $tRNA^{lys}$ and $tRNA^{tyr}$ species will be published elsewhere (Brown et al.).

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