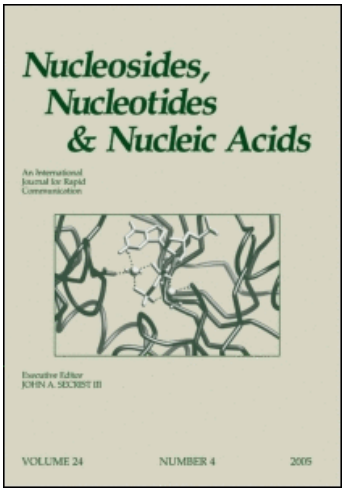

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THE ATP ANALOGUE CONTAINING A METHYLENE GROUP IN PLACE OF THE 5′ OXYGEN SERVES AS A SUBSTRATE FOR BACTERIOPHAGE T3 RNA POLYMERASE.

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ABSTRACT: Transcription reactions catalyzed by bacteriophage T3 RNA polymerase and using CTP, GTP, UTP and an analogue of ATP containing a methylene group in place of the 5′ oxygen yield RNA-like polymers. These products are degraded to the 5′-mononucleotides CMP, GMP, UMP, and the corresponding analogue of AMP by exposure to snake venom phosphodiesterase. By using appropriate DNA templates, a single residue of the AMP analogue can be located at the site adjacent to the cleavage position in the substrate strand of a hammerhead ribozyme. The modified substrate strand is completely resistant to cleavage by the ribozyme's catalytic strand.

The fact that polynucleotide phosphorylase can form polymers from an ADP analogue in which the 5′ oxygen is replaced by a methylene group[1,2] has led to the investigation of the corresponding nucleoside triphosphate analogues as potential substrates for the RNA polymerases. Methylene for 5′-oxygen substitution in oligonucleotides does not appear to make large changes in their conformations, since adenosine oligomers containing the substitution at every linkage readily participate in triple helix formation.[2] In addition, oligomers bearing this type of phosphodiester modification display total resistance to the action of those exo- and endonucleases which normally cleave the P-O(5′) linkage. If this combination of the two properties, specific helical binding and nuclease resistance, can also be demonstrated in oligomers of mixed sequences, methylene for phosphodiester oxygen substitution will be useful in the design of new molecules for application in "antisense" therapeutic strategies.

The new finding that the adenosine triphosphate analogue is accepted by an RNA polymerase permits the incorporation of the modified linkage at specific positions in RNA transcribed from a DNA template. In the example of a hammerhead ribozyme described below, the procedure has been used to locate a single modified linkage at the cleavage point

in the substrate oligomer. This should allow the preparation of milligram quantities of a ribozyme complex that will not undergo cleavage during subsequent structural analysis.

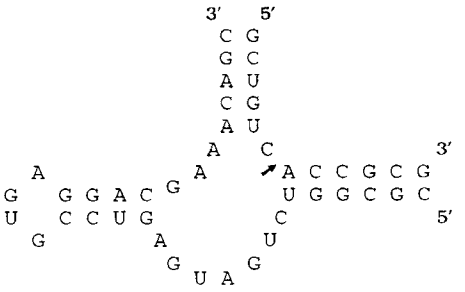
Synthesis of the ATP analogue (pppcA). The ATP analogue was prepared and purified using methods similar to those employed for the production of the corresponding ADP analogue.[1,2] 5′-Deoxy-5′-phosphonomethyladenosine and pyrophosphoric acid were condensed in the presence of the reagent 1,1′-carbonyldiimidazole using the method described for the conversion of dAMP to dATP.[3] The product was purified by paper chromatography in the descending mode on Whatman 3MM chromatographic paper, first with the solvent system 2-methylpropanoic acid/1 M ammonia (62.5 : 37.5, v/v; R_f = 1.1 relative to ATP) and then with 1-propanol/concentrated ammonia/water (55 : 10 : 35, v/v; R_f = 0.95 relative to ATP).

Synthesis and characterization of RNA. Preliminary transcription tests were performed using the MEGAscript T3 kit purchased from Ambion, Inc., with some modifications. Each analogue reaction (final volume 40 μL) contained 5 mM each of the ultrapure nucleotides CTP, GTP, and UTP (Pharmacia Biotech, Inc.), along with 1.3 mM pppcA, in place of the supplied nucleoside triphosphates. Other additions were 4 μL of the enzyme mix, 4 μL of the 10X transcription buffer, 160 U of extra bacteriophage T3 RNA polymerase, 2 μL of 100 mM dithiothreitol, and 1 μg of the supplied linear DNA template pTRI-Xef (carrying the gene for the 1.79 kilobase *Xenopus laevis* elongation factor 1-α mRNA). After incubation at 40° C for 16 h, the mixture was treated with 1 U of DNase I for 15 min at the same temperature.

The RNA product was separated from the unused triphosphates on a 0.7% agarose electrophoresis gel, recovered by electro-elution into 7.5 M ammonium acetate, and concentrated by precipitation with EtOH. The pellet was washed with 70% EtOH, and the isolated RNA was digested to completion with phosphodiesterase I from *Crotalus adamanteus* venom. Fractionation of the digest yielded only four nucleotide products: CMP, GMP, UMP and pcA (the AMP methylene analogue). The new HPLC system developed for the separation of nucleotide components derived from natural and modified RNA consists of an anion-exchange column (1 X 25 cm) of cross-linked polyethyleneimine-silica[4], with the elution solvent 0.05 M potassium phosphate (pH 6.0) / 30% MeOH at a flow rate of 1 mL / min. In this system all five nucleotides pcA, UMP, CMP, AMP, and GMP are completely separated; peak elution volumes (mL) are 22.7, 25.8, 31.7, 37.8, and 59.6, respectively. The normal mRNA was transcribed in a parallel reaction using 5 mM ATP in place of pppcA. In this case, the HPLC separation of the products deriving from the nuclease digest yielded the four regular nucleoside 5′-phosphates.

Specific incorporation of the analogue into a hammerhead ribozyme substrate. The nucleotide sequences of the catalytic and substrate strands of the ribozyme designed for this

work were chosen such that the desired bimolecular complex would be the only structure to form in solution. This structure is shown in the following figure along with the cleavage position as indicated by the arrow:



The 33-mer catalytic strand was constructed by chemical synthesis as previously described, using RNA monomers containing the new protection group *o*-nitrobenzyloxymethyl for the 2′-hydroxyl.[5] The dodecanucleotide substrate strands containing the regular -CpA- linkage or the analogue -CpcA- linkage were prepared by transcription reactions using a T3 promoter / template formed by the two synthetic DNA strands:

CGCGGTGACAGCTTTAGTGAGGGTAATT and AATTACCCTCACTAAAG.

Each transcription reaction (40 μL) contained 260 pmol of the 28-mer DNA strand, 260 pmol of the 17-mer DNA strand, 40 mM Tris-HCl (pH 8.0), 30 mM $MgCl_2$, 5 mM each of the three ultra pure nucleoside triphosphates (UTP, CTP, and GTP), 1.3 mM pppcA or 5 mM ATP, and 8 U of recombinant RNase inhibitor (Clontech). After incubation at 40° C for 15 min, 10 mM dithiothreitol, 240 U of T3 RNA polymerase, and 4 μCi of [α-^{32}P]CTP (800 Ci/mmol) were added to the mixture. Further incubation at 40° C was continued for 4 h, and the DNA was then degraded with DNase I as before. Electrophoresis of the two mixtures on a 15% denaturing polyacrylamide gel gave the two dodecanucleotides in about the same yield. The dodecamer products were recovered from the gel by electro-elution in dialysis bags (MWCO 6-8 K), exposed to the ribozyme catalytic strand (5 h, 40° C), and again subjected to electrophoresis on a 15% polyacrylamide gel. Subsequent autoradiography indicated that the dodecamer containing the analogue linkage is completely resistant to cleavage, while the normal dodecamer substrate is cleavable (at the C-A linkage) into the two hexamers, as expected.

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