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27 December 1984; accepted 22 February 1985

Template-Directed Synthesis of Novel, Nucleic Acid–Like Structures

Abstract. *In studying the origins of life, it is important to examine reactions of substrate mixtures that could plausibly have accumulated on the primitive earth. Nucleoside diphosphates would probably have been synthesized along with the standard nucleotides under prebiotic conditions. For these reasons, the template-directed reactions of activated derivatives of these diphosphates, alone or mixed with activated nucleotides, were investigated. An activated derivative of deoxyguanosine 3′,5′-diphosphate condensed efficiently on a polycytidylate template to give oligonucleotide analogues in which each 3′,5′-phosphodiester bond was replaced by a pyrophosphate linkage. Oligomers were formed even in the absence of a template, but much more slowly. Template-directed condensation occurred also with an analogous deoxyadenosine derivative on polyuridylic acid and with an analogous acycloguanosine derivative on polycytidylic acid.*

All naturally occurring nucleic acids have in common a backbone formed by joining the 3′- and 5′-OH groups of successive nucleosides via a phosphodiester bond. However, a double-stranded structure analogous to the Watson-Crick helix is sometimes stable even when the covalently linked backbone of one strand is interrupted or modified. Thus polycytidylic acid [poly(C)] (*1*) will form stable, double-helical complexes with a variety of monomeric derivatives of guanine, including guanosine and guanosine 5′-phosphate (*2*), while long, 2′,5′–linked oligoguanylic acids [oligo(G)'s] can be formed on poly(C) and form a double helix with the template (*3*).

These observations raise two general questions. Is the energy of base-pairing between G and C (or A and U), plus energy obtained by stacking base pairs on top of each other, sufficient to guarantee the stability of a variety of DNA-like double helices with one or both backbones very different from those familiar in biology? If so, can such oligonucleotide analogues be formed by template-directed synthesis?

As a first step toward answering these questions we have prepared and studied the template-directed reactions of the activated nucleotide analogues **1**, **2**, and **3** (Fig. 1) that can condense to form oligomers in which the phosphate group of the oligonucleotides is replaced by a pyrophosphate group. We chose these compounds because we have already shown that the formation of a pyrophosphate from a nucleotide and a nucleoside 5′-phosphoimidazolide in aqueous solution is a general and efficient reaction (*4*).

Phosphodiesterase I (snake venom phosphodiesterase), 2′-deoxyadenosine 3′,5′-diphosphate and 2′-deoxyguanosine 3′,5′-diphosphate were purchased from Pharmacia P-L. The 9-(1,3-dihydroxy-2-propoxy)methylguanine($\tilde{G}$) (*5*) was converted to the diphosphate with phosphoryl chloride by a modification of a published procedure (*6*), and its identity was confirmed by comparison in several chromatographic systems with an authentic sample (*5*). The diimidazolides (**1**, **2**, and **3**) were prepared by a standard procedure that has been used for the synthesis of 5′-phosphoimidazolides of nucleotides (*7*).

Prior to analysis by high-performance liquid chromatography (HPLC) on RPC-5 (*7*), samples of the reaction mixtures were incubated at *p*H 4 (room temperature) overnight to hydrolyze phosphoimidazolides. If the overnight hydrolysis with acid was omitted, a complex family of products, many of which contained one or more phosphoimidazolide groups, was obtained. Individual oligomers were collected from the RPC-5 column, desalted by dialysis, and degraded at room temperature with phosphodiesterase I (Pharmacia; 0.01 unit per 0.01 optical density unit of nucleotide, in 0.1*M* tris-HCl containing 0.01*M* $CaCl_2$, *p*H 8.5). The pdGp (*1*) obtained by phosphodiesterase digestion of isolated oligomers was identified by co-chromatography with authentic material on RPC-5.

Compound **1** condensed rapidly on a poly(C) template to form a homologous series of product oligomers with chain lengths up to about 20 (Fig. 2A). The ultraviolet absorption of guanine in the

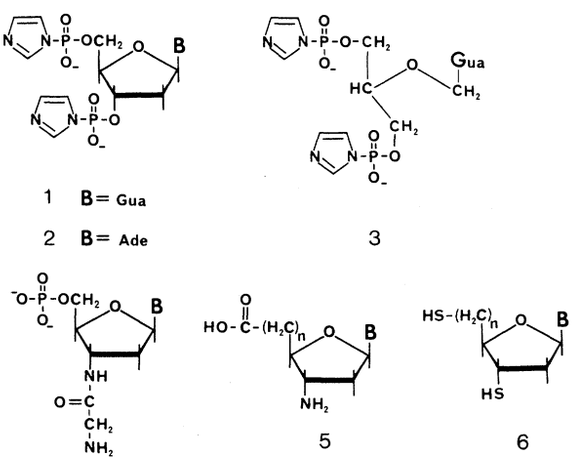

Fig. 1. The chemical names of the derivatives used are the following: **1**, 2′-deoxyguanosine-3′,5′-diphosphoimidazolide; **2**, 2′-deoxyadenosine-3′,5′-diphosphoimidazolide; **3**, 9-(1,3-dihydroxy-2-propoxy)-methylguanine-1,3-diphosphoimidazolide; **4**, 3′-glycylamido-2′,3′-dideoxynucleoside 5′-phosphate; **5**, homologues of 3′-amino-2′,3′-dideoxyuronic acids; **6**, homologues of 3′,5′-dithio-2′,3′,5′-trideoxypentafuranosylnucleosides. For derivatives **4**, **5**, and **6**, B is a nucleoside base.

products from preparative scale reactions was equivalent to about 70 percent of the cytosine in the template. The efficiency of the reaction (the ratio of the amount of G incorporated into products to the amount of C in the template) approached 100 percent after longer times. When the composition of the reaction mixture was varied, high yields of products were obtained with a salt concentration as high as 0.8*M* $MgCl_2$ and 1.0*M* NaCl or as low as 0.1*M* $MgCl_2$ and 0.1*M* NaCl. The yields of products decreased significantly in the absence of $MgCl_2$. However, if sufficient NaCl (1.0 to 5.0*M*) was present, the yields were still substantial.

The optimal *p*H for the oligomerization of ImpdGpIm (*1*) on a poly(C) template was about 6.5. At this *p*H, a triple-stranded rather than a double-stranded helix may be present. However, a large template effect was also observed at *p*H 8.1, a *p*H at which only double-stranded complexes of poly(C) and monomeric G derivatives are stable (*8*).

The time course of the non–template-directed oligomerization of **1** was very different (Fig. 2B). The reaction produced only traces of oligomers containing eight or more nucleotides after 3 days. At later times, longer oligomers accumulated until, after 2 weeks, the overall pattern of products was not very different from that obtained in the template-directed reaction. However, the yield of longer oligomers from the non-template reaction never exceeded 50 percent of the yield obtained within 3 days in the template-directed reaction. The slow, non–template-directed reaction apparently stopped because of nearly complete hydrolysis of the starting imidazolides (half-life about 2 days at 0°C).

Enzymatic digestion of individual product oligomers isolated from the reaction of **1** on poly(C) suggested that the mode of linkage was the expected internucleoside pyrophosphate. Incubation of the hexamer with phosphodiesterase I, which is known to cleave pyrophosphate bonds (*9*), produced a series of lower oligomers at early times. The average chain length decreased with increasing time. After long times the final product was the monomer pdGp. The length of time it takes to achieve complete degradation depends on the amount of substrate and the amount of enzyme; 0.01 absorbance unit of oligomer was degraded completely by 0.1 unit of enzyme in 1 hour (*p*H 8.5, 37°C, 0.01*M* Ca^{2+}).

The product oligomers obtained after 3 days from the self-condensation of the adenylic acid analogue, **2**, in the presence of poly(U) were comparable in length and yield (Fig. 2C) with those obtained in the poly(C)-G system. In the absence of template, very little oligomeric product was observed (Fig. 2D). The acycloguanylic acid analogue **3** also underwent a template-directed condensation (Fig. 2E), but less product was formed than in reactions of the deoxynucleotide analogues. As in the other systems studied, the non–template-directed reaction (Fig. 2F) produced only small amounts of oligomers within 3 days.

An initial impression that the distribution of product peaks was simpler in the presence of template, indicating a greater regiospecificity of reaction, needs to be substantiated by detailed structural studies. We believe that the first stage in the formation of pyrophosphate bonds is the hydrolysis of a proportion of the phosphoimidazole groups to free phosphate groups. In the second step, these free phosphates attack unhydrolyzed phosphoimidazolide groups to form pyrophosphate bonds (*4*). However, we cannot exclude the possibility that the direct condensation of two phosphoimidazole groups also contributes to the products.

In previous experiments on template-directed synthesis, specific, activated nucleoside 5′-phosphates were usually used as substrates, and 3′,5′-linked oligonucleotides were sought as products. However, it is unlikely that chemically pure substrates of this kind, for example 2-MeImpG (*1*), could have accumulated on the primitive earth. It is important to use the experience gained in experiments with specific substrates to design reactions that proceed efficiently with more plausibly prebiotic substrate mixtures.

The reaction of a nucleoside phosphate with a nucleoside phosphoimidazolide is general, and much more efficient than the corresponding reaction of a nucleoside. Non–template-directed syn-

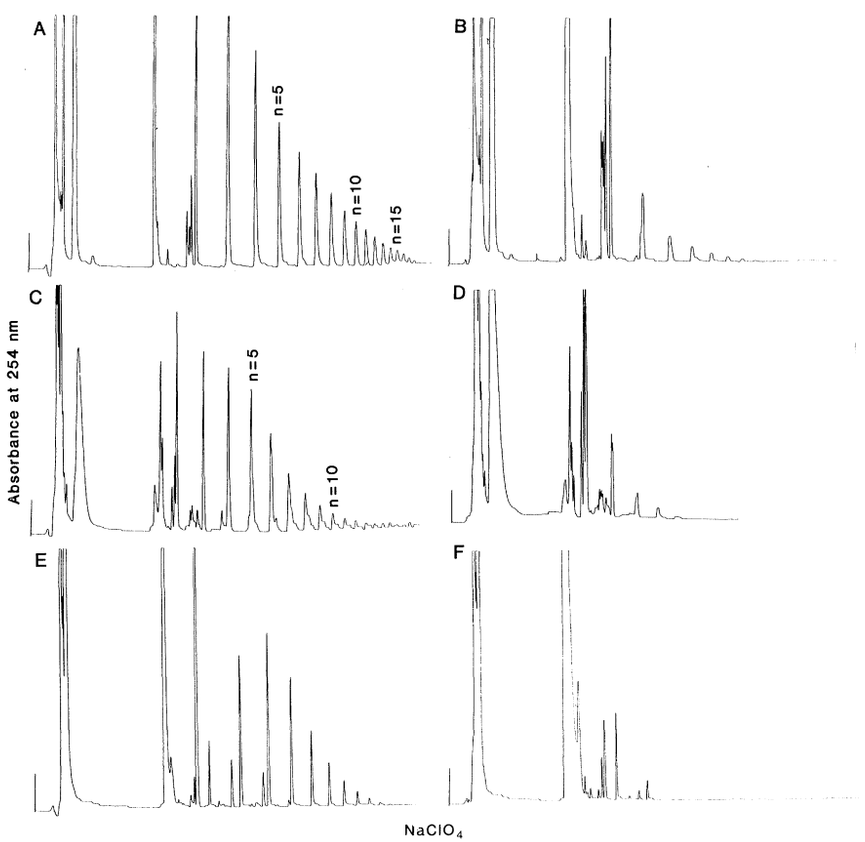

Fig. 2. HPLC analyses of reaction products from the self-condensation of 2′-deoxynucleoside-3′,5′-diphosphoimidazolides of G and A and of the corresponding derivative of an analogue of G in the presence and absence of polynucleotide templates. Reaction mixtures were prepared as previously described (*7*). (A) The reaction mixtures contained 0.1*M* ImpdGpIm, 0.1*M* poly(C), 0.2*M* $MgCl_2$, 0.1*M* NaCl, and 0.2*M* 2,6-lutidine-HCl (*p*H 6.5) and was kept at 0°C for 3 days. Chain lengths are given by *n*. (B) As (A), but without poly(C). (C) The reaction mixture contained 0.1*M* ImpdApIm, 0.1*M* poly(U), 0.2*M* $MgCl_2$, 0.1*M* NaCl, and 0.2*M* 2,6-lutidine-HCl (*p*H 6.5) and was kept at 0°C for 3 days. (D) As (C), but without poly(U). (E) The reaction mixture contained 0.1*M* ImpGpIm, 0.1*M* poly(C), 0.2*M* $MgCl_2$, 1.0*M* NaCl, and 0.2*M* 2,6-lutidine-HCl (*p*H 6.5) and was kept at 0°C for 3 days. (F) As (E), but without poly(C). Reactions were stopped by the addition of an excess of EDTA and, after overnight hydrolysis at *p*H 4, were analyzed on RPC-5 in 0.02*M* NaOH containing 0.002*M* tris-$HClO_4$ and with a linear gradient of $NaClO_4$ (0 to 0.04*M* over 60 minutes) at a flow rate of 1.0 ml/min.

thesis of short oligomers of deoxynucleosides linked by pyrophosphate bonds is, therefore, easier than the formation of equivalent oligonucleotides. Furthermore, template-directed reactions are, as we have shown, much less dependent on the choice of the activated substrate and the reaction conditions. Even the very simple and potentially prebiotic acyclonucleoside derivative ImpGpIm (*1*) was an adequate substrate.

In other experiments we have shown that poly(C) will direct the efficient copolymerization of 2-MeImpG and ImpdGpIm (*1*) at all ratios of the two substrates (*10*). Thus it seems plausible that a poly(C) template could bring about a copolymerization of components selected from a complex mixture of partially and fully phosphorylated G derivatives. If, as we expect, a poly(C) analogue in which pyrophosphate groups have partially or fully replaced the normal phosphate groups will itself act as a template for the condensation of G derivatives, it will indicate that template-directed synthesis is much less demanding than has been demonstrated previously.

Our results also suggest, but certainly do not prove, that a great variety of template-directed reactions may be possible with unconventional substrates. Substrates **4** and **5**, for example, in the presence of poly(C), might react to form amide-linked polymers when the phosphate is activated in **4** (*11*) or the carboxyl in **5**, while substrate **6** might form disulfide-linked oligomers on oxidation (Fig. 1). While such reactions may not be interesting in the context of the origins of life on the earth, they would greatly extend the scope of template-directed organic synthesis.

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References and Notes

1. Abbreviatons: A, adenosine; C, cytidine; G, guanosine; U, uridine; G, 9-(1,3-dihydroxy-2-propoxy)methylguanine; dG, 2'-deoxyguanosine; pN (N is A, G, or dG), the 5'-phosphate of N; 2-MeImpN, the 2-methylimidazolide of pN; Np, the 3'-phosphate of N; ImpNpIm, the 3',5'-diphosphoimidazolide of N; ImpGpIm, the 1,3-diphosphoimidazolide of G (compound **3**).
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20 August 1984; accepted 27 November 1984

Epizootic Carcinoma in the Winter Flounder, *Pseudopleuronectes americanus*

Abstract. *The winter flounder,* Pseudopleuronectes americanus, *is an esteemed food fish and has sustained an important commercial and recreational fishery for many years in the northeastern United States. Histopathologic examinations of hepatic tissues of winter flounder from Boston Harbor revealed a high prevalence of neoplasms. The lesions, designated as cholangiocarcinomas and hepatocarcinomas, were found in 16 of 200 fish examined and resembled those experimentally induced in rodents by exposure to carcinogens.*

Neoplasms have been noted in many marine fish species, and they affect virtually all organs (*1–3*). Most of the tumors were described incidental to the conduct of other studies and usually occurred in a single animal. Recently, epizootic neoplasia was noted in localized populations of several bottom-dwelling marine fishes from the U.S. East and West coasts. Hepatomas (adenomas, hepatocarcinomas, and cholangiocarcinomas) have been found in English sole (*Parophrys vetulus*) from the Duwamish River, a tributary of Puget Sound (*4, 5*), and in tomcod (*Microgadus tomcod*) from the Hudson River estuary (hepatocarcinomas) (*6*). The only other epizootic neoplasm in a marine fish in the United States is the neurilemoma of the bicolor damselfish (*Eupomacentrus partitus*) from the Florida Keys (*7*). Although specific carcinogens [polycyclic aromatic hydrocarbons (PAH's) and polychlorinated biphenyls (PCB's)] have been suggested as the inducers of the hepatomas of English sole and tomcod, it is unlikely

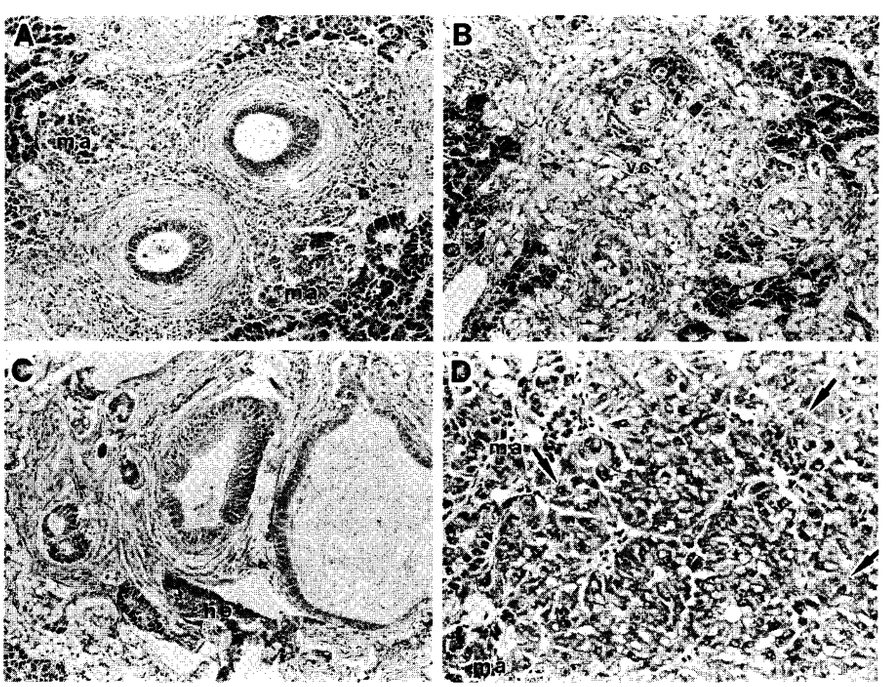

Fig. 1. (A) Pericholangitis. Leukocytes and macrophage aggregates (ma) surround atypical bile ducts (×20). (B) Vacuolated cells. Vacuolated cells (vc) form acini and are enveloped by fibrous tissue (×20). (C) Bile duct dilation and hyperplasia. Hyperplastic epithelium (he) is contiguous with vacuolated cells (×20). (D) Adenoma. Macrophage aggregates (ma) define outer edge of neoplasm. Note mitoses (arrows) (×40). Stain, hematoxylin and eosin.