24-Me), 2.018 (s, 3 H, CH_3COO), 4.68 (m, 1 H, 3-H), 5.27 (m, 2 H, 22-, 24-H); MS 372 (M^+, 5), 357 (3), 330 (43), 315 (24), 301 (35), 270 (32), 243 (100), 201 (19); IR (cm^{-1}) 1733 (C=O), 972.7 (C=C trans);[56b] HRMS calcd for $C_{25}H_{40}O_2$ 372.3028, found 372.3013.

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Nonenzymatic Template-Directed Synthesis on Oligodeoxycytidylate Sequences in Hairpin Oligonucleotides

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Abstract: We have developed a novel method for studying template-directed synthesis in hairpin oligonucleotides. An unpaired segment at the 5′-terminus of the hairpin acts as an intramolecular template for the extension of the paired 3′-terminus. Products are analyzed by denaturing gel electrophoresis of [^{32}P]-labeled hairpins. Using this system, we have studied the synthesis of oligoguanylates on an oligodeoxycytidylate template. We find that guanosine 5′-phosphoro(2-methyl)imidazolide adds efficiently to a terminal riboguanylate residue at temperatures in the range 0-37 °C but not at 50 °C. At 0 °C, the half-time for addition of the first G residue is about 3 h, and the reaction rate is independent of pH in the range 6.5-8.0. The first addition reaction results in the formation of a predominantly 3′-5′-internucleotide bond. When the 3′-terminal riboguanylate residue is placed by a deoxyguanylate residue, the half-time for the first addition increases from about 3 to about 30 h.

Introduction

The replication of nucleic acids and the transcription of DNA are two of the fundamental processes that allow living systems to transfer and utilize genetic information. Both involve the syntheses of 3′-5′-linked polynucleotides on complementary templates, using nucleoside 5′-triphosphates as substrates. In contemporary living systems these syntheses are catalyzed by DNA and RNA polymerases. The base sequence of the template dictates the sequence of the polynucleotide product through Watson-Crick base pairing.

These biochemical processes can be modeled in much simpler chemical systems. Polyribo- or polydeoxyribonucleotides can act as templates and catalyze the nonenzymatic synthesis of complementary oligonucleotides from activated nucleoside 5′-phosphates. Poly(C) or poly(dC), for example, catalyzes the polymerization of guanosine 5′-phosphoro(2-methyl)imidazolide (2-MeImpG), producing a mixture of 3′-5′-linked guanylic acid oligomers (oligo(G)s) up to at least 30 nucleotides in length.[1] When the template reaction on a poly(C) template is carried out in the presence of the four ribonucleoside 5′-phosphoroimidazolides, only the complementary base, the guanosine nucleotide, is incorporated in the polymer in significant amounts.[2] Templates containing both cytidine and guanosine nucleotides direct the synthesis of the 3′-5′-linked complementary oligomers; r(CCGCC), for example, directs the synthesis of r(GGCGG)[3] and similarly d(CCCGCCCGCCCGCC) directs the synthesis of r-(GGCGGGCGGGCGGG).[4]

While the oligomerization of 2-MeImpG on poly(C) or an oligocytidylate template is a very efficient reaction, most other template reactions are less efficient. Polyuridylic acid directs the synthesis of oligo(A)s from adenosine 5′-phosphoroimidazolide, but the reaction is not very efficient and leads to products that are mainly 2′-5′-linked.[5] Poly(A) does not form a stable double helix with uridine mononucleotides, and it does not catalyze the polymerization of activated uridine 5′-phosphate derivatives, while oligo(G)s or poly(G) form very stable quadruplexes through Hoogsteen pairing and, therefore, do not influence the chemistry of monomeric activated cytidine 5′-phosphate derivatives. The template-directed reactions of nucleotides and oligonucleotides have been reviewed.[6]

Template-directed reactions have usually been carried out by incubating an aqueous solution of an activated ribonucleotide, often the nucleoside 5′-phosphoro(2-methyl)imidazolide (2-MeImpN), at a concentration of about 0.1 M with a roughly stoichiometric amount of template oligomer.[6] The high concentrations of activated mononucleotides that are required in these reactions lead to the synthesis of significant amounts of products that are formed independently of the template, mainly short oligomers containing phosphodiester and pyrophosphate bonds. In analyzing the products by HPLC, these side products tend to mask the low molecular weight products formed in the template-directed reaction. This masking effect becomes more serious as the concentration of the template is reduced. Another difficulty is that nonenzymatic template-directed reactions, unlike most enzyme-catalyzed processes, do not always initiate at a unique site on the template. Initiation can occur at internal positions on the template,

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(6) Joyce, G. F. *Cold Spring Harbor Symposium on Quantitative Biology*; Cold Spring Harbor, NY, 1987; Vol. LII, pp 41-51.

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(a)
I. CCCCCCCTAGTTTTTCTAGG
II. CCCCCCCTAGTTTTTCTAG*rG*
III. CCCCCCCTAGTTTTTTTCTAG*rG*
IV. TTTCCCCCCCTAGTTTTTCTAG*rG*
V. CCCCCCTAGTTTTTCTAG*rG*

(b)

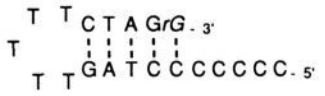

Figure 1. (a) Sequences of the hairpin oligonucleotide used in this work. (b) The hairpin structure formed by oligo II.

leading to the formation of a complex mixture of product oligomers.[4] These factors have complicated the analysis of the products formed in template-directed reactions.

Here, we introduce a new method for studying template reactions that overcomes many of these problems, the elongation of an oligonucleotide that adopts a hairpin structure (Figure 1b). In this system, the position of initiation is uniquely determined by the secondary structure of the hairpin oligonucleotide. The use of a [^{32}P]-labeled oligomeric substrate allows us to use extremely low concentrations of the template. Interference from products formed off the template is eliminated since they are not radioactive. Using this system, we have studied the template-directed addition of successive guanosine nucleotides to hairpin oligomers terminated with a ribo- or deoxyriboguanosine residue.

Results

Template Reaction on Hairpin Oligonucleotides. Oligonucleotide II (Figure 1a) forms the hairpin structure shown in Figure 1b. The single-strand segment of the hairpin, a sequence of five cytidine residues, is used as the template in directing the oligomerization of 2-MeImpG. The template reaction was carried out by incubating 5′-[^{32}P]-labeled II with a 0.1 M solution of 2-MeImpG. Figure 2 shows an autoradiogram of the gel electrophoretogram of the products from the reaction after different reaction times.

After 4 h, four new oligomers are formed (lane 2), we believe by the additions of one, two, three, and four guanosine nucleotides to the 3′-end of the hairpin. After 24 h, all of the starting material was converted to the product with four added guanosine residues (lane 5). The product of the third addition is just resolved from the product of the fourth addition by gel electrophoresis. The electrophoretogram suggests that the reaction stopped after 24 h, since after longer reaction time (5 days, lane 9) we did not observe any slower moving bands. We will show that the single band in lane 5 does correspond to a single oligonucleotide, but the single band in lanes 6–9 in fact corresponds to a mixture of oligomers with four and five added G residues. Incubation of the hairpin oligonucleotide II with 2-MeImpA under the same conditions did not give detectable amounts of oligomers longer than the starting material. In other experiments (data not shown) we substituted one or more of the residues in the single strand region of the hairpin with T, A, or G residues. In no case did the elongation reaction proceed significantly past the substitution. These results establish that the elongation reaction is template-directed.

The detailed analysis of the fourth and fifth additions is made difficult by the unusual dependence of the electrophoretic mobilities of the oligomers formed on their chain length. Usually the addition of successive residues of the same base to an oligomer in the size range with which we are concerned results in a series of products that give more or less evenly spaced bands on electrophoresis. However, it is well-recognized that change in the conformation of oligonucleotides profoundly effects their mobilities.[7] Thus, if the addition of bases results in a change of conformation, an anomalous dependence of the mobility on the chain length is to be anticipated.

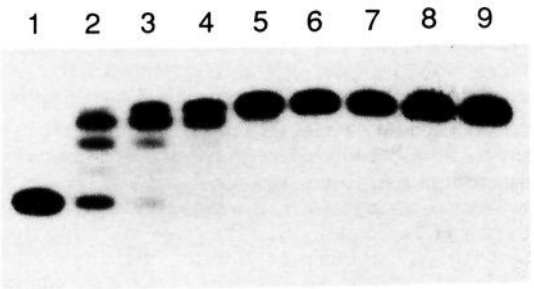

Figure 2. Autoradiogram of the gel electrophoretogram of products from the reaction of 2-MeImpG with 5′-[^{32}P]-labeled II after different periods of time: lane 1, starting material; lanes 2–9, after 4 h, 6 h, 10 h, 24 h, 2 days, 3 days, 4 days, and 5 days.

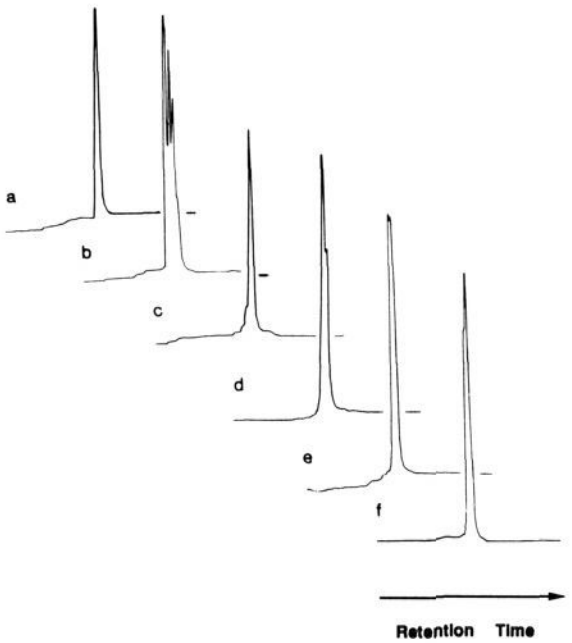

Figure 3. HPLC elution profiles of the products from the reaction of 2-MeImpG with oligo II after different reaction times: (a) starting material, (b) 4 h, (c) 30 h, (d) 2 days, (e) 4 days, and (f) 7 days.

Double-stranded stems are stabilized when they are connected by a short single-stranded loop. Thus it is not clear that the usual conditions of denaturing gel electrophoresis (8 M urea) will destabilize the hairpin structures. Our results are most easily explained if we suppose that in 8 M urea the starting oligomer and the first two adducts exist predominantly as open chain structures, but further addition of G residues results in the formation of a substantial proportion of faster-moving hairpin structures in the equilibrium mixture. The data reproduced in Figure 2, lane 2, for example, show that the first two additions result in substantial and roughly equal decreases in mobility. The third addition does not decrease the mobility as much as the first and second additions, while the fourth addition has only a slight effect on the mobility.

To determine unambiguously the nature of the product mixture after extended reaction times, we analyzed the products from a large scale reaction using an RPC-5 column at pH 12. Under such strongly basic conditions, all base pairs are disrupted, so retention times increase steadily with oligomer length. Profiles of the products formed after various times are shown in Figure 3. The results from HPLC analysis confirm and augment those obtained from gel electrophoresis. After 4 h reaction time four new peaks, corresponding to the oligomers from the first, second, third, and fourth additions are observed (Figure 3b). After 30 h, only one peak, that corresponding to the addition of the four guanosine residues, is present (Figure 3c). The peak corresponding to the product of the fifth addition appears after 48 h (Figure 3d). After 4 days the fifth addition was about 50% complete (Figure

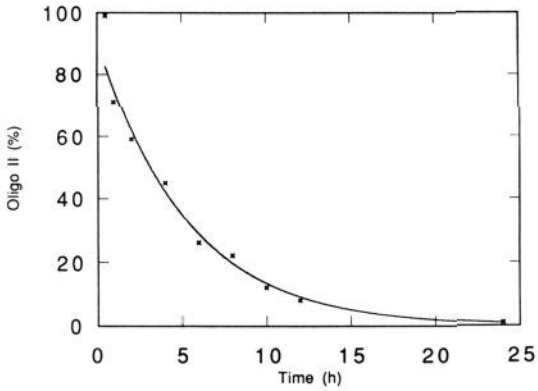

Figure 4. The rate of disappearance of starting material during the reaction of oligo II with 2-MeImpG.

3e). After 7 days, the product of the fifth addition became the major product (Figure 3f). These results clearly show that the single band on the gel in Figure 2, lanes 6–9, corresponds to a mixture of two oligomers. A study of the corresponding reaction on a hairpin molecule with only four cytidine nucleotides in place of five (Figure 1a, III), confirms that the last addition, in this case the fourth addition, is very slow (see below).

Template-directed addition of G residues to oligomer II is a pseudo-first-order reaction since a large excess of 2-MeImpG is used and very little hydrolysis occurs in the first few hours of the reaction. This permits us to determine the half-life of addition of G residues on an oligocytidylate template. Since the product from the first addition is converted to the second addition product, and so on, it is easiest to estimate this rate by following the disappearance of the starting material. In Figure 4 we plot the percentage of starting material that survives as a function of reaction time. The half-time for adding the first G residue is estimated to be about 3.0 h.

Characterization of the Products from the Template Reaction. The addition of a G residue to a 5′-[^{32}P]-labeled oligonucleotide II could occur at the 5′-terminus through a pyrophosphate linkage or at the 2′–3′-terminus through a 2′–5′- or a 3′–5′-phosphodiester linkage. Treatment of the reaction mixture with alkaline phosphatase resulted in the disappearance of all the product bands, demonstrating that addition to the 5′-terminus is negligible.

To determine the nature of the phosphate linkages we hydrolyzed the products with alkali and with two specific ribonucleases. The products from a reaction that had proceeded for 24 h (Figure 2, lane 5) were isolated by preparative gel electrophoresis. Partial alkaline hydrolysis of this product resulted in the appearance of four new bands, the fastest moving having a mobility somewhat greater than that of the starting oligomer (Figure 5a, lane 5). These bands must correspond to oligonucleotides terminated by a 2′- or 3′-phosphate confirming that the major product was obtained by the successive formation of phosphodiester bonds at the 2′–3′-terminus.

T1 ribonuclease is an enzyme that specifically cleaves 3′–5′-phosphate linkages in RNA adjacent to G residues to give a 3′-phosphate and a 5′-hydroxyl group at the cleavage site. When the major product formed after 24 h was treated with T1 ribonuclease, it was finally converted to a material with the same mobility as that formed by complete alkaline hydrolysis (Figure 5a, lane 7). This shows that the first addition results in the formation of an exclusively 3′–5′-linked product. In another experiment, the same product was digested with ribonuclease T1 for different periods of time. Surprisingly, we observed small amounts of an intermediate in which one G residue had been removed, but no intermediate corresponding to the removal of two or three G residues.

The product formed after 24 h was next hydrolyzed with ribonuclease H, an enzyme that cleaves 3′–5′-internucleotide bonds in RNA to generate a 5′-phosphate if, and only if, the substrate is hybridized to complementary DNA. The time course of the hydrolysis is illustrated in Figure 5b. First, it is clear that the

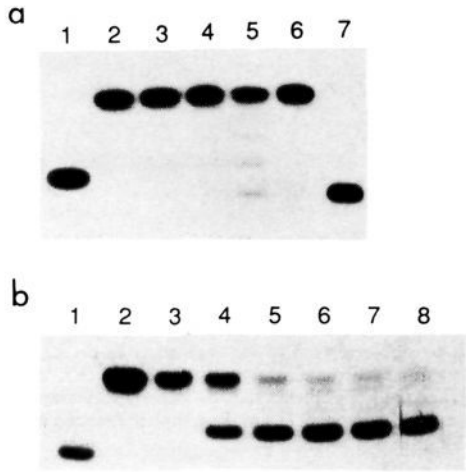

Figure 5. Electrophoretograms of the products from (a) alkaline and T1 hydrolysis of the products isolated from the reaction of 2-MeImpG and oligo II after a 24-h reaction time: lane 1, [^{32}P]-labeled oligo II; lanes 2, 3, and 4, the purified product; lane 5, alkaline hydrolysis; lane 6, control; lane 7, T1 ribonuclease hydrolysis. (b) Ribonuclease H digestion of the purified product: lane 1, [^{32}P]-labeled oligo II; lane 2, purified product; lane 3, control; lanes 4–8, the sample after being treated with RNase H for 5, 15, 20, 40, and 60 min.

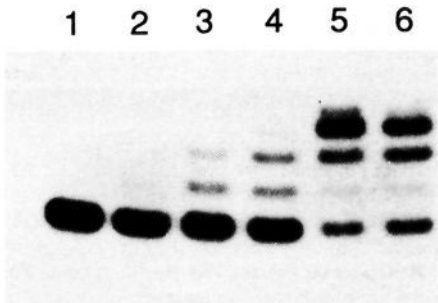

Figure 6. Autoradiogram of the products from the reaction of [^{32}P]-labeled oligo II with various concentrations of 2-MeImpG. The reactions were analyzed after 4 h. Concentrations of 2-MeImpG are 0.0001 M (lane 1), 0.005 M (lane 2), 0.01 M (lane 3), 0.02 M (lane 4), 0.05 M (lane 5), and 0.1 M (lane 6).

main product is different from that obtained by exhaustive digestion with ribonuclease T1. It has the same mobility as the product from the first G addition to the hairpin. This indicates that the second G residue is mainly attached by a 3′–5′-bond. The presence of small amounts of undegraded starting material and of some intermediate size products even after extensive degradation indicates that the product is not entirely 3′–5′-linked throughout its length.

The results of ribonuclease digestion of the major product from the 24 h reaction mixture do not allow us to draw firm conclusions about the isomeric character of the phosphodiester bonds that are formed during the third and fourth additions of G. Little is known about the ability of ribonucleases to hydrolyze 3′–5′-linked bonds in hairpin structures. Since they act only on single-stranded RNA, they can degrade only the denatured form of the hairpin. The formation of a single intermediate, that in which just one G residue is removed, by ribonuclease T1 perhaps indicates that hairpins with fewer G residues denature more easily and are therefore degraded rapidly to the final product. It is striking that ribonuclease T1 degrades the linkage to the first added G residue, while ribonuclease H hydrolyzes the second linkage but not the first.

Concentration, pH, and Temperature Dependence of the Reaction. The products from the reaction of II with different concentrations of 2-MeImpG are illustrated in Figure 6. No reaction could be observed with 0.0001 M of 2-MeImpG (lane 1). The rate of the reaction increases with the concentration of 2-MeImpG in the range 0.005–0.05 M (lane 2–lane 5), but does

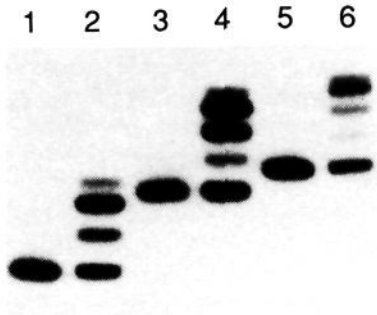

Figure 7. Electrophoretogram of the products from the addition of 2-MeImpG to various hairpin oligonucleotides: lane 1 (V), lane 3 (III), lane 5 (IV), and the [^{32}P]-labeled starting materials. Lanes 2, 4, and 6 are the corresponding products from the template reaction of these oligonucleotides after 4 h reaction time.

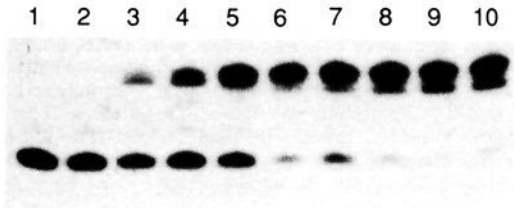

Figure 8. Electrophoretogram of the products from the reaction of the template with deoxyguanosine at the 3′-end (oligo I) analyzed at different periods of time: lanes 1–10, after 4 h, 6 h, 20 h, 1 day, 2 days, 3 days, 4 days, 5 days, 7 days, and 8 days.

not increase further at higher concentrations (lane 6). In other experiments (data not shown) we find that the rate of the reaction and the nature of the products is almost independent of pH in the range 6.5–8.0. The reaction proceeds efficiently at temperatures up to 37 °C but scarcely at all at 50 °C.

Template Reactions on Related Hairpin Oligomers. To confirm that our results are of general applicability and not restricted to a single template, we carried out reactions on a series of templates related to II (Figure 1a, oligos III–V). The results illustrated in Figure 7 for reactions terminated after 4 h show that increasing the loop size from T_5 to T_7 (Figure 1a, oligo III) has very little effect on the reaction (lane 4). The same is true when the 5′-terminus of the template is extended by a series of 3 T residues (Figure 1a, oligo IV) (lane 6). Shortening the template by one C residue (Figure 1a, oligo V) has a more profound effect—now 2 rather than 3 residues are added rapidly, and the third addition has only just begun after 4 h (lane 2). The third addition is complete after 24 h (data not shown). HPLC analysis (data not shown) established that the fourth addition is slow, and is only about half completed after 4 days.

The Reactions of a Template Terminated by a Deoxyribonucleotide. Oligomer I differs from II only in the substitution of a deoxyguanosine residue for the 3′-terminal riboguanosine residue (Figure 1a). The time course of the reaction of 2-MeImpG with I is illustrated in Figure 8. By plotting the percentage of surviving radioactive starting material against time (Figure 9), the half-time of addition of the first G residue is determined to be about 30 h, compared with approximately 3 h for the corresponding addition to oligomer II.

Discussion

The use of hairpin structures to study template-directed synthesis is novel. The template, in this work a pentamer C_5, is the 5′-terminal single-strand segment of the hairpin (Figure 1b). The first reaction is the addition of 2-MeImpG to the rG at the 3′-terminus of the hairpin—the 3′-terminal base-paired segment of the hairpin can be considered as an intramolecular primer. Subsequent steps in the reactions involve the successive addition of further G residues to the primer.

The ability of oligonucleotides to form stable hairpin secondary structures (Figure 1b) is the key to the design of our experiments.

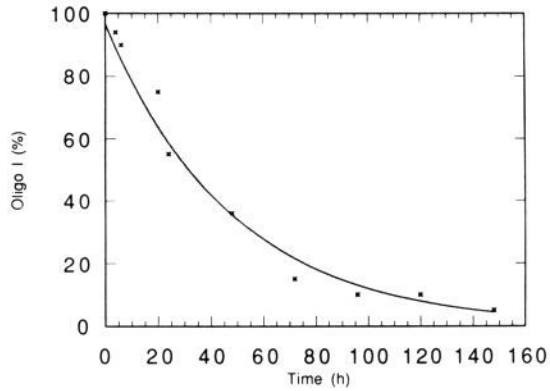

Figure 9. The rate of disappearance of starting material during the reaction of oligo I with 2-MeImpG.

The hairpin structures, because they involve intramolecular base-pairing, form at arbitrarily low concentrations. This allows us to carry out and analyze template-directed reactions using very low concentrations of the template (10^{-8} M). The enormous excess of 2-MeImpG in the reaction mixture guarantees that the oligomerization is a pseudo-first-order reaction, provided the time of reaction is short compared with the hydrolysis half-life of 2-MeImpG.

In most earlier studies of template reactions, HPLC was used to analyze products. Interference from products that are formed independently of the template, for example, short oligomers containing phosphodiester and pyrophosphate bonds, complicated the product analysis. To overcome this problem in the study of template reactions of self-complementary GCGCGCGC templates, [^{32}P]-labeled oligomers were used as primers, and the products were analyzed by autoradiography.[8] The use of an intramolecular primer, as in the present experiments, is a powerful extension of the method. Template reactions on 5′-[^{32}P]-labeled hairpins produce radioactive oligomers longer than the starting oligomer. The products usually give rise to well-defined bands with lower mobility than that of the starting material in autoradiograms of the gel (Figure 2). The products from side reactions that do not involve the hairpin are not radioactive and so do not interfere with the analysis of templated products.

The nucleotide sequence of the hairpin has been chosen to guarantee a unique secondary structure, so all template-directed reactions initiate at a unique site on the template. This eliminates the problem of random initiation that leads to the production of oligomers with different lengths. These features of the reactions of the hairpins have enabled us to extend our analysis of several important aspects of oligo(C) template-directed synthesis.

The addition of 2-MeImpG to the 3′-terminus of the hairpin II is surprisingly fast. The half-time for addition of a single residue is only about 3 h at 0 °C. This corresponds to the addition of about four residues per day. Clearly long oligo(G) structures could be formed efficiently in a few days, even at 0 °C, on appropriate templates.

Extension of oligo(G) primers on poly(C) has been studied extensively by Kanavarioti and White,[9] but under conditions somewhat different from those used in our experiments. The rate of extension that they measured varied greatly from primer to primer but was always substantially lower than that reported here. The extension of a G_8 template on poly(C) in 0.05 M 2-MeImpG, for example, had a half-time for the first addition of G of about 50 h. Extrapolation of Kanavarioti's data suggests that the corresponding half-time for extension of a G_{12} primer in a solution containing 0.05 M 2-MeImpG would have been about 8 h.

(7) Efcavitch, J. W. In *Gel Electrophoresis of Nucleic Acids. A Practical Approach*; Rickwood, D., Hames, B. D., Eds.; Oxford University Press: New York, 1990; pp 146–147.
(8) Ng, K.-M. E.; Orgel, L. E. *J. Mol. Evol.* **1987**, *29*, 101–107.
(9) (a) Kanavarioti, A.; White, D. H. *Origins Life* **1987**, *17*, 333–349. (b) Kanavarioti, A.; Chang, S.; Alberas, D. J. *J. Mol. Evol.* **1990**, *31*, 462–469.

The addition of the penultimate and final residues to the hairpin are much slower than the addition of the first three residues. We have attributed similar observations made when we oligomerized 2-MeImpG on short oligo(dC) templates[10] to the change in the environment of incoming 2-MeImpG residues that occurs as the end of the template is approached. Clearly 2-MeImpG substrates can be stabilized by stacking of additional monomers when the addition occurs at internal positions on the template, but this stabilization is not available for the addition of the final residue.

Comparisons with experiments in which short oligo(C)s or oligo(dC)s were used as templates are particularly revealing.[10] The interpretation of results from these experiments was greatly complicated by "sliding" of the product oligo(G)s on the oligo(C) templates, and by processes involving the bridging of two template oligo(C)s by a single product oligo(G). This led to the production of oligomers longer than the templates. The use of stem-loop templates eliminated the possibility of sliding. The very low concentrations of template make trimolecular processes involving two template molecules extremely improbable. When sliding is eliminated and the template concentration is reduced, we see no evidence of primer extension beyond the end of the template. The final product corresponds to copying of the five terminal C residues at the 5′-terminus of the hairpin by addition of five G residues to the 3′-terminus.

The rates of the template-directed reactions depend on two factors, namely the extent that the site on the template adjacent to the 3′-terminus of the primer is occupied and the intrinsic rate of reaction between the primer and an adjacent activated monomer. Since the latter is independent of concentration, the concentration dependence of the reaction is determined by the occupancy of the monomer site. Our results show that the rate of reaction increases up to 0.05 M 2-MeImpG but does not increase further at higher concentrations. This suggests that the monomer site is fully occupied at 0.05 M 2-MeImpG. The stacking of G residues on the primer is cooperative, so no simple relationship between rate and concentration is to be anticipated. Our results differ from those of Kanavarioti et al.[9] who reported that, at 1 °C, the rate of the reaction is still increasing with concentration at least up to a concentration of 0.05 M. Presumably the presence of a poly(C) template (0.03 M) reduced the concentration of free 2-MeImpG in their experiments. No such reduction occurs with our experiment, since the concentration of template is in the 10^{-8} molar range.

The pH dependence reveals some new features of the reaction. Our result shows that the reaction is not affected by the pH within the range of 6.5–8.0, while previous study on oligomerization of 2-MeImpG on a poly(C) template indicates that the reaction products change dramatically when the pH of the solution is lowered below 7.5.[1] We attributed this change to the formation at pHs below 7 of the well-known triple helix involving one poly(C) chain and one protonated poly(C) chain.[11] When templates are used at very low concentrations, triple strands cannot form, so the nature of the products does not change over the pH range 6.5–8.

The course of the template-directed addition reaction at 0 °C is unaffected when the loop size is increased from 5 to 7 (Figure 1a, III). This is not surprising since, although 7-residue loops are somewhat less stable than 5-residue loops,[12] the secondary structure of the hairpin with a 7-residue loop should still be stable at 0 °C. The addition of three T residues to the 3′-terminus of the hairpin also has little effect on the reaction. This strongly suggests that the inefficiency of the terminal additions is not simply due to the absence of a phosphodiester bond at the 5′-terminus of the hairpin but rather to the absence of further C residues on which G residues can stack cooperatively.

When the 5′-terminal single-stranded template is reduced from five to four C residues, we find that the third and fourth additions are slow, rather than the fourth and fifth. The only reasonable explanation is that the rate of reaction is determined by the distance to the 5′-terminus of the template strand.

Oligodeoxynucleotides and oligoribonucleotides are comparably efficient as templates, but activated deoxyribonucleotides are much less efficient than activated ribonucleotides as substrates.[10,13] Consequently, activated ribonucleotides have been used in most studies of template-directed reactions. Our experiments now provide quantitative data showing that the rate of addition of 2-MeImpG to a terminal ribonucleotide is about 10 times as rapid as the addition to a terminal deoxynucleotide. We believe that this is related to the lower pK_a of the hydroxyl groups of a ribonucletide (12.5) compared to that of a deoxynucleotide (14.0). However, we cannot exclude the possibility that differences in the nucleophilicity of the un-ionized alcohols and differences in conformation also contribute to the difference in reactivity.

Much evidence has been cited suggesting that, in the course of chemical evolution, RNA preceded DNA as an informational macromolecule.[14,15] Studies of chemical reactivity have provided some of the strongest evidence by showing that ribonucleotides are far more reactive in forming polymers than deoxyribonucleotides under potential prebiotic conditions. Adenosine, for example, undergoes template-directed reaction on poly(U) far more efficiently than deoxyadenosine.[13] The present experiments provide quantitative data for the first time; the ribo-terminated oligomer reacts about 10 times as rapidly as the corresponding deoxyribo-terminated oligomer. Since template-directed oligomerization of activated nucleotides always competes with hydrolysis, activated deoxynucleotides would be very much less efficient than activated ribonucleotides in generating long oligomers in nonenzymatic reactions.

The procedures described in this paper have been adapted to study synthesis on template strands including two or more bases, for example, C and G. The results of this work will be described in future publications.

Experimental Section

Materials and Methods. All chemicals were reagent grade and were used without further purification. Ribonuclease T1, ribonuclease H, and alkaline phosphatase (from calf intestine) were obtained from Boehringer, Mannheim. Guanosine 5′-phosphoro(2-methyl)imidazolide (2-MeImpG) was prepared according to a published procedure.[16] Solutions of 2-MeImpG were freshly prepared for each experiment.

Oligodeoxyribonucleotide (I) was synthesized on a 391A DNA synthesizer (Applied Biosystems). Oligomers (II, III, IV, V) terminated by a ribonucleotide at their 3′-ends were synthesized and deprotected following the standard DNA synthesis procedures but using a solid support derivatized with a ribonucleotide (Biosearch). The terminal acetyl protecting group of the ribonucleotide was removed together with the base protecting groups and the phosphate protecting groups during the ammonium hydroxide step in deprotection. All oligonucleotides were purified by preparative gel electrophoresis and were 5′-end labeled with [^{32}P]phosphate following a standard procedure.[17]

HPLC analysis of reaction mixtures containing oligonucleotides was performed on an RPC-5 column (4.6 × 250 mm) as previously described.[16] Polyacrylamide gel electrophoresis (20%, 8 M urea) was run on a 16 × 28 cm dimension gel plate at 1200 V for about 2 h. Gel loading buffer was prepared by mixing appropriate amounts of formamide (900 μL), xylene cyanol (25 μL, 2%), bromophenol blue (25 μL, 2%), and Tris/boric acid/EDTA buffer (50 μL). The buffer was stored at 0 °C.

Reaction Conditions. Template reactions were carried out in Eppendorf tubes in 20-μL volumes. The following procedure was used in most experiments. First, 20 μL of 1 M NaCl, 4 μL of 1 M $MgCl_2$, and a solution of 5′-[^{32}P]phosphate-labeled oligonucleotide (about 120000 cpm, ca. 0.1 pmol) were added to the tube. The solution was evaporated to dryness, and the residue was redissolved in 8 μL of 0.5 M 2,6-lutidine·HCl buffer (pH 8 at 0 °C) and 9 μL of water. The tube was heated on

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a water bath at 50 °C for 3 min, cooled for 3 min, and finally brought to 0 °C on an ice-bath. Next, a freshly prepared solution of 2-MeImpG (3 μL, 7.1 OD/μL, 0 °C) was added to the tube. The solution was stirred, and the reaction mixture was allowed to stand at 0 °C. After an appropriate time, an aliquot (1 μL) of the solution was transferred to a tube containing 50 μL of water and 1 μL of 0.5 M EDTA (pH 8). Samples (10 μL) of the resulting solution were evaporated to dryness, redissolved in 5 μL of water, mixed with 5 μL of loading buffer, and subjected to electrophoresis on polyacrylamide gel.

Reactions were also carried out at 25, 37, and 50 °C using 2,6-lutidine buffer at pH 8. A few experiments were carried out using essentially the same procedure but which other buffers at various pHs. We also studied the concentration dependence of the reaction in 2,6-lutidine buffer at pH 8 and 0 °C.

Determination of the Half-Times for the Addition of the First Guanosine Nucleotide to Templates I and II. Aliquots (1 μL) of the reaction mixture were withdrawn at appropriate times and transferred to a tube containing 50 μL of water and 1 μL of 0.5 M EDTA (pH 8) and then subjected to electrophoresis on polyacrylamide gel as described above. Bands on the gel were sliced out, and the amounts of radioactivity that they contained were quantitated on a scintillation counter. Half-lives for the first addition of a G residue to the template were estimated by plotting as a function of time the ratio of counts remaining in the band corresponding to starting material to the total counts applied to the gel. No allowance was made for the small amount of hydrolysis of 2-MeImpG that occurs during the earlier stages of the reaction, so the half-lives are approximate.

Isolation of Reaction Products. Reaction products were isolated by ethanol precipitation in the presence of a cold tRNA carrier. The tRNA carrier solution contains tRNA (0.4 mg/mL), ammonium acetate (0.3 M), and EDTA (0.1 mM). It was stored in a freezer at 0 °C.

The reaction mixture (20 μL) was allowed to stand for 24 h at 0 °C, and then 200 μL of tRNA carrier solution and 750 μL of cold ethanol were added. The resulting solution was cooled in acetone/dry ice for 1 h and then centrifuged for 10 min at 0 °C. The supernatant was removed, and the residue (60000 cpm) was dried using a Savant Speed-Vac. The residue was then redissolved in water (5 μL) and loading buffer (5 μL) and separated by electrophoresis on a polyacrylamide gel. The gel was run at 1200 V for about 2 h and visualized by autoradiography on a Kodak film (X-OMAT AR). Bands on the gel corresponding to the major products were sliced out and then soaked in water (200 μL) overnight at room temperature. The supernatant was pipetted off, and the gel was washed with water (2 × 50 μL). These washings were combined with the supernatant and dried on a Savant Speed-Vac. The residue was redissolved in a solution of tRNA mixer (200 μL) and ethanol (95%, 750 μL). The solution was precipitated in a dry ice/acetone bath to give the product (ca. 50000 cpm). The product contains unlabeled yeast tRNA (ca. 1.6 μg of tRNA/1000 cpm).

Alkaline Hydrolysis of Products. A sample of the major product purified as described above (1000 cpm, containing about 1.6 μg unlabeled tRNA) was incubated in sodium hydroxide (5 μL, 0.05 M) on a water bath (55 °C). After 20 min, the reaction was stopped by addition of acetic acid (2.5 μL, 0.1 M). The solution was mixed with the loading buffer (10 μL) and then applied on a polyacrylamide gel.

Ribonuclease T1 Digestion of Products. An aliquot of the purified product (1000 cpm) was dissolved in Tris/EDTA buffer (10 μL, Tris·HCl, 10 mM, pH 7.4; EDTA, 1 mM). The solution was heated in a water bath (90 °C) for 3 min and transferred to a water bath at 55 °C. T1 ribonuclease (1 μL, 100 units) was added, and the solution was incubated for 1 h at 55 °C. The resulting solution was mixed with loading buffer (10 μL) and then analyzed on a polyacrylamide gel.

Ribonuclease H Digestion of Products. The purified product (ca. 10000 cpm) was dissolved in a Tris buffer (20 μL) containing Tris·HCl (0.05 M, pH 8.0), magnesium chloride (0.025 M), potassium chloride (0.1 M), dithiothrietol (1 mM), and sucrose (5% w/v). The solution was incubated at 55 °C for 3 min and then at 32 °C for another 30 min. Ribonuclease H (1 μL, 2 units/μL) was added, and the reaction mixture was left at 32 °C. At appropriate times an aliquot of the solution (4 μL) was withdrawn for analysis by electrophoresis on a polyacrylamide gel.

HPLC Analysis of Products. The reaction was set up as described above but using unlabeled oligonucleotide (1 OD). At appropriate times, an aliquot of the solution (1 μL) was withdrawn and dissolved in 1 mL of water containing 1 mM EDTA. One hundred microliters of this solution was analyzed on an RPC-5 column following a previously described procedure.[16]

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Conformation-Specific Detection of Guanine in DNA: Ends, Mismatches, Bulges, and Loops

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Abstract: DNA oxidation promoted by a square-planar complex of nickel(II) (**1**) in conjunction with $KHSO_5$ provided an excellent method for selectively detecting guanine residues that did not adopt a standard Watson–Crick duplex structure. Sites of modification were indicated by a diagnostic strand scission of DNA induced by subsequent treatment with piperidine. The specificity and, consequently, the utility of this nickel-based reagent were demonstrated through the use of defined oligonucleotide targets. All guanine residues of a random coil reacted readily under the described conditions while the other residues, adenosine, cytidine, and thymidine, remained inert. Most importantly, guanine residues were protected from modification when held within a duplex of complementary paired and stacked bases. This property then allowed for the reliable identification of mispaired, bulged, looped, and terminal guanines from otherwise helical regions of DNA. In addition, the predicted asymmetry of base stacking in a loop structure was confirmed by preferential derivatization of specific guanine residues.

Only a limited set of chemical reactions are currently available for identifying the structural heterogeneity of large DNA and RNA fragments. The physical techniques that have yielded such a wealth of information on oligonucleotide models[1] are rarely applicable to systems of high molecular weight. Accordingly, efforts continue to focus on the design of new reagents for nucleoside modification in order that further details on the variable conformation of polynucleotides may be examined.[2] Our recent

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