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Catalyst for DNA Ligation: Towards a Two-Stage Replication Cycle**

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The chemical methods available for the ligation of complementary oligomers along a DNA template are complex, requiring transition metal and imidazole catalysis of the condensation reaction. We were interested in simplifying the required conditions by converting the DNA template itself into a better catalyst, and report a modified backbone linkage which greatly simplifies the reaction. As related backbone linkages have been prepared by chemically reading the DNA

sequence, this reaction completes a replication cycle, simplifying the ubiquitous three-stage replication cycle, the central dogma of biology,^[1] to a two-stage chemical process.

Several laboratories have contributed to optimizing the reaction conditions for template-directed phosphodiester formation.^[2] In the BrCN, imidazole, NiCl₂ cocktail, BrCN has a half-life of several minutes in the aqueous media. Premixing the cocktail generates *N*-cyanoimidazole, the reagent required to activate ligation.^[2d,e] While more stable than BrCN under the reaction conditions, displaying a half-life of several hours, *N*-cyanoimidazole will chemoselectivity ligate a nick site along a DNA template. Many aromatic drugs are known to bind tightly within the narrow minor groove of DNA, particularly A/T tracts,^[3] including netropsin, distamycin, Hoechst 33258, and the bis-amidine compounds benenil and pentamidine, and do so by displacing specific well-ordered H₂O molecules known to line this cavity.^[4] It is therefore possible that *N*-cyanoimidazole associates with the nick site better than BrCN to facilitate the activation and/or the ligation steps in the condensation.

In an attempt to replicate this effect by template modification, five synthetic templates, T_{N-H}, T_{N-pr}, T_{N-bu}, T_{N-im}, and T_{N-ea}, were compared with the native DNA (T_P) under ligation conditions (Figure 1). Each template was prepared

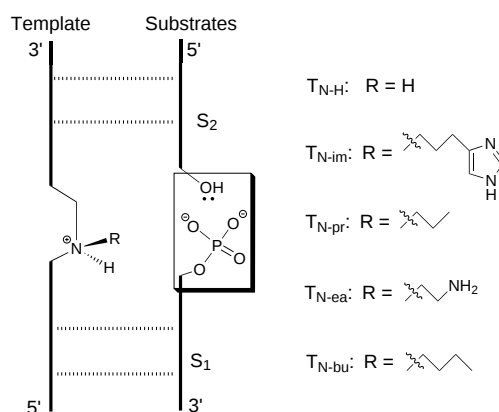


Figure 1. The ternary complex formed by template and complementary substrates, 8mer S₁ and 12mer S₂. The sequence of the template is HO-dCpCpGpTpTpCpGpTpTpTXXTpCpTpGpTpCpTpCpG-OH. S₁ is HO-dApCpGpApApCpGpGp-OH, and S₂ is HO-dCpGpApGpApCpApGpApApA-OH. X in the sequence represents the site of insertion of the various linkage structures. In Tp, X represents the native phosphodiester linkage of DNA.

with the appropriate synthetic amine thymidine dimer incorporated into standard solid-phase synthesis protocols.^[5d] The 20mer templates were designed such that the complementary DNA substrates, S₁ and S₂, displayed high binding affinity with the template. Thermal melting analyses indeed established that the melting temperature for all template–substrate duplexes T/S₁ (35 ± 2 °C) and T/S₂ (45 ± 2 °C) were very similar and > 10 °C above the reaction temperature. A 1:1:1 stoichiometry therefore generated significant and equivalent concentrations of the ternary complexes (T:S₁:S₂) for each reaction.

All modified templates and substrates were purified prior to reaction by reverse-phase chromatography, and purity was

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[**] We are grateful to Paul Gardner for assistance with solid-phase synthesis and the NIH (R21 RR12723) for support.

confirmed by denaturing PAGE. The 8mer S_1 was enzymatically 5'-phosphorylated by using T4 polynucleotide kinase and γ - ^{32}P -ATP. Figure 2a shows a typical phosphoimage analysis of the reaction after incubation for 24 h with three templates, T_p , T_{N-H} , and T_{N-pr} , using the full conditions optimized for DNA ligation (BrCN, imidazole, NiCl_2).^[2] The fastest running material is the 5'-phosphorylated 8mer substrate S_1 . In the presence of template, a single new higher molecular weight species grew in with time at the expense of S_1 intensity. The new band was assigned as the ligated product by electrophoretic comparison with synthetic 5'-phosphorylated 20mer.

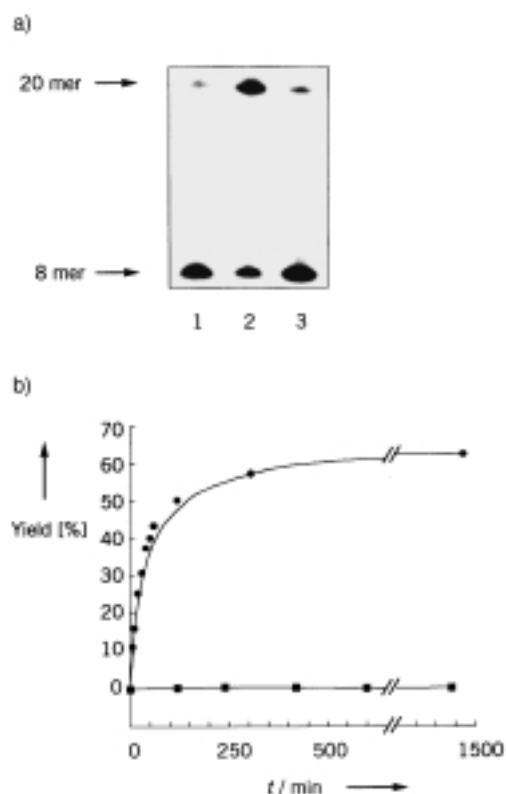


Figure 2. a) Autoradiograms of PAGE gels. Each coupling reaction was analyzed after 24 h under full DNA activation conditions (BrCN, imidazole, NiCl_2): Lane 1, T_p ; lane 2, T_{N-H} ; lane 3, T_{N-pr} . b) Time dependence of ligation reactions with templates T_p (■) and T_{N-pr} (●) using BrCN alone as the activating reagent.

Table 1 summarizes the initial rates of ligation obtained by catalysis with the different templates. Surprisingly, T_{N-H} was equally as good a catalyst as T_p . DNA strands containing a

Table 1. Initial rates of ligation.^[a]

Activation	T_p	T_{N-H}	T_{N-im}	T_{N-pr}	T_{N-ea}	T_{N-bu}
full conditions	1.1	1.2	15	22	1.6	21
without imidazole	n.d.	n.d.	23	34	n.d.	31
BrCN only	n.d.	n.d.	28	63	n.d.	76

[a] Ligation yields were determined from the percentage of radioactivity in the product band relative to that of the whole lane; initial rates are expressed as $10^{-1} \mu\text{M h}^{-1}$. The reaction conditions were full: 40 mM BrCN, 20 mM imidazole, 40 mM NiCl_2 ; without imidazole; and BrCN only. n.d. = not determined; the reaction yields in these cases were less than 4% after 24 h.

single amine linkage were previously shown to form less stable duplexes with complementary DNA,^[5d] and T_{N-H} was expected to be less preorganized for condensation at the nick site as a result of the flexibility of the amine linkage.^[3] While the template containing imidazole T_{N-im} was a better catalyst than T_p , giving a >10 -fold larger initial rate and significantly better yields under these activation conditions, both T_{N-pr} and T_{N-bu} were even better. Without imidazole added to the reaction, both T_p and T_{N-H} were inactive, while the catalytic activity of T_{N-im} , T_{N-pr} , and T_{N-bu} was further improved. Figure 2b compares the relative effectiveness of T_{N-pr} and T_p with BrCN alone. Under these conditions, T_{N-pr} and T_{N-bu} are exceptional catalysts, while DNA is inactive.

The data in Table 1 establish that the addition of *N*-alkyl substituents to the template dramatically increases the rate of ligation with BrCN alone. The ligation rates with *N*-cyanoimidazole were tested by premixing the cocktail for 5 h prior to addition of T and S. The T_p -catalyzed initial rate was elevated from 0.11 to $2.4 \mu\text{M h}^{-1}$, identical with the T_{N-pr} rate. Therefore, T_{N-pr} does not enhance the reaction of all dehydrating agents at the nick site, but opens the BrCN manifold.

The role of more polar groups in the R substituent was further explored through the preparation of the T_{N-ea} template. The *N*-ethylamine substituent is isosteric with the *N*-propyl, but likely contains a protonated primary amine in solution at pH 8.^[6] As shown in Table 1, the enhancement observed with this template was weak at best, the T_{N-ea} template giving a 14-fold lower initial rate than T_{N-pr} under the full activating conditions and inactive otherwise. In contrast, the more sterically demanding but neutral T_{N-bu} template showed catalytic activity equivalent with T_{N-pr} . The incorporation of more polar functional groups to aid in the catalytic steps may be possible, but minimally their placement must be carefully considered.

To test for the global effect of the backbone modification, the reaction site was shifted by a single base pair to either the 5' or 3' side of the modification by investigation of the 9mer+11mer and 7mer+13mer complementary substrate pairs. Under full activation conditions where observed initial rates could be compared and expressed relative to the DNA template, T_{N-pr}/T_p , both the 7 + 13 and 9 + 11 substrate pairs gave ratios of 1, while the 8 + 12 substrate pair was 20 (Table 1). Therefore, consistent with a B-DNA conformation found for related T_{N-H} templates,^[3d] the site of closest backbone-backbone approach in the duplex is offset by three base pairs in linear sequence, and a one base pair shift to either side eliminates the *N*-propyl enhancement. The effect of the *N*-propyl substituent is therefore local and site-specific, with no apparent global change in duplex structure contributing to catalysis.

While a greater understanding of the catalytic reactions of the $T_{N-alkyl}$ templates will require further structural and kinetic analyses, covalently anchoring the hydrophobic substituents to the backbone amine within the nick site opens the reaction manifold with BrCN. The alkyl substituent on the amine linker, as was seen with alkyl groups on the bases,^[7] should alter local groove solvation, minimally allowing BrCN access so as to open the new reaction manifold. Clearly such

backbone modifications can profoundly enhance ligation reactions and we suspect can be further extended by the appropriate placement of functional groups.

Our understanding of template-directed synthesis and sequence replication will benefit considerably from the discovery that such simple modifications to the backbone can open reaction manifolds inaccessible to DNA. DNA can be chemically translated into T_{N-H} backbones.^[5a, b] By exploiting features of the reading reaction, $>10^6$ fold amplification of the encoded sequence information has been achieved (Figure 3).^[5c, e] Here we complete the cycle, establishing that

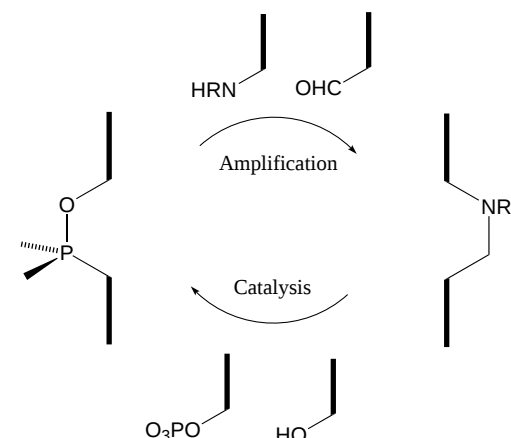


Figure 3. Proposed two-stage replication cycle showing both the amplification in the synthesis of the ethylamine-containing backbone and the use of that backbone to catalyze the synthesis of the original template.

the amplified product can catalyze the transfer of the encoded information back into DNA in an efficient, site-specific manner, and under conditions where DNA is inactive. For a single turn of this overall two-stage replication cycle, greater than the biological exponential growth limit could be achieved. Certainly, it is now possible to read the DNA-encoded information into different polymers that have unique catalytic properties, in this case to catalyze the synthesis of the initial DNA template. To the extent that these single ligation steps can be extended to multiple ligation or polymerization events, the design of strategies for molecular evolution and functional selection in materials other than natural biopolymers becomes possible.

Experimental Section

Thymidine dimers: The initial dimer was synthesized by a previously developed convergent approach.^[5c,d] Reductive amination of the 5'- and 3'-bis-silyl-protected dimer with the appropriate aldehyde gave the required tertiary alkylamines chemoselectively in 85% yield. Deprotection with $Et_3N \cdot 3HF$ followed by 5'-dimethoxytrityl and 3'-phosphorylation with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite gave the activated thymidine dimer for solid-phase synthesis. Thymidine dimers containing primary and secondary amines were protected by trifluoroacetylation prior to activation and cleaved at the end of the solid-phase synthesis in concentrated ammonia.

5'-Phosphorylation: In a typical procedure, the oligonucleotide (900 pmol), T4 polynucleotide kinase (30 U), γ - ^{32}P -ATP (10 pmol), ATP (1125 pmol,

1.25 equiv), $MgCl_2$ (10 mM), DTT (5 mM), and Tris-HCl (70 mM) at pH 7.6 were incubated in a total volume of 50 μ L at 37 °C for 30 min. The enzyme was heat inactivated at 65 °C for 20 min, and the oligonucleotide was purified by reverse-phase Sep-Pak filtration.

Ligation: Condensation reactions were initiated at 24 °C by the addition of a freshly prepared BrCN solution to give a mixture containing: both substrates and template (10 μ M), BrCN (40 mM), $NiCl_2$ (20 mM), imidazole (20 mM), and 0.5 M NaCl at pH 8 in Tris-HCl (20 mM) in a total volume of 30 μ L. Each reaction was quenched with urea (5 M) and analyzed by high-resolution denaturing 20% polyacrylamide gels. No product ($>1\%$) could be detected in the absence of template.

Received: February 17, 2000

Revised: June 10, 2000 [Z14726]

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