Formation of Oligonucleotide−PNA−Chimeras by Template-Directed Ligation

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Abstract: DNA sequences have previously been reported to act as templates for the synthesis of PNA, and vice versa. A continuous evolutionary transition from an informational replicating system based on one polymer to a system based on the other would be facilitated if it were possible to form chimeras, that is molecules that contain monomers of both types. Here we show that ligation to form chimeras proceeds efficiently both on PNA and on DNA templates. The efficiency of ligation is primarily determined by the number of backbone bonds at the ligation site and the relative orientation of template and substrate strands. The most efficient reactions result in the formation of chimeras with ligation junctions resembling the structures of the backbones of PNA and DNA and with antiparallel alignment of both components of the chimera with the template, that is, ligations involving formation of 3′-phosphoramidate and 5′-ester bonds. However, double helices involving PNA are stable both with antiparallel and parallel orientation of the two strands. Ligation on PNA but not on DNA templates is, therefore, sometimes possible on templates with reversed orientation. The relevance of these findings to discussions of possible transitions between genetic systems is discussed.

Introduction

The difficulties of forming nucleotides under prebiotic conditions makes it unlikely that nucleic acids were the first genetic materials on the primitive earth.[1−3] It has therefore been proposed that one or more genetic systems composed of simpler monomers may have preceded the RNA world.[4] Genetic takeover would then have allowed the development of RNA-controlled life.[5] PNA (peptide nucleic acid)[6] is an example of the type of oligomer that might have preceded RNA, since it is simpler than RNA, but exhibits all of the important properties of nucleic acids with respect to information storage and potential for replication.[7−9] A continuous transition from one genetic system to another would be possible only if chimeras, that is, mixed molecules containing building blocks of both systems, could be formed. We have investigated this possibility by studying the template-directed formation of DNA−PNA chimeras.[10−12]

Design of the Study

The backbone repeats of DNA or PNA contain six bonds. Although the precise spacial arrangement of the ligation junction cannot be predicted for chimeras, a template-assisted ligation product with six backbone bonds at the junction would be expected to be minimally strained and, therefore, to be formed with maximal efficiency. The ligation of the 3′-phosphate group of DNA to the NH_2 group of PNA or of the 5′-OH group of DNA to the COOH group of PNA leads to a 3′-phosphoramidate or a 5′-ester linkage with a six-atom chimeric junction (Figure 1, b and c). Consequently, these ligations should be favored over the alternative ligations of a 5′-phosphate group of DNA and the NH_2 group of PNA or a 3′-OH group of DNA and the COOH group of PNA which lead to 5′-phosphoramidate or 3′-ester linkages with, respectively, eight or four bonds at the ligation junction (Figure 1, e and f).

A second important factor influences the efficiency of ligation reactions. There are eight logically possible arrangements of one DNA and one PNA substrate relative to a DNA template (Figure 2, upper panel), of which four involve parallel pairing of DNA strands and therefore should not be productive in template-directed ligation (Figure 2, b, d, f, h). Of the remaining four, two are energetically favored because they involve exclusively antiparallel arrangements of the substrates (Figure 2, a, c), while the last two are disfavored although not excluded, because they involve parallel binding only of the PNA substrate

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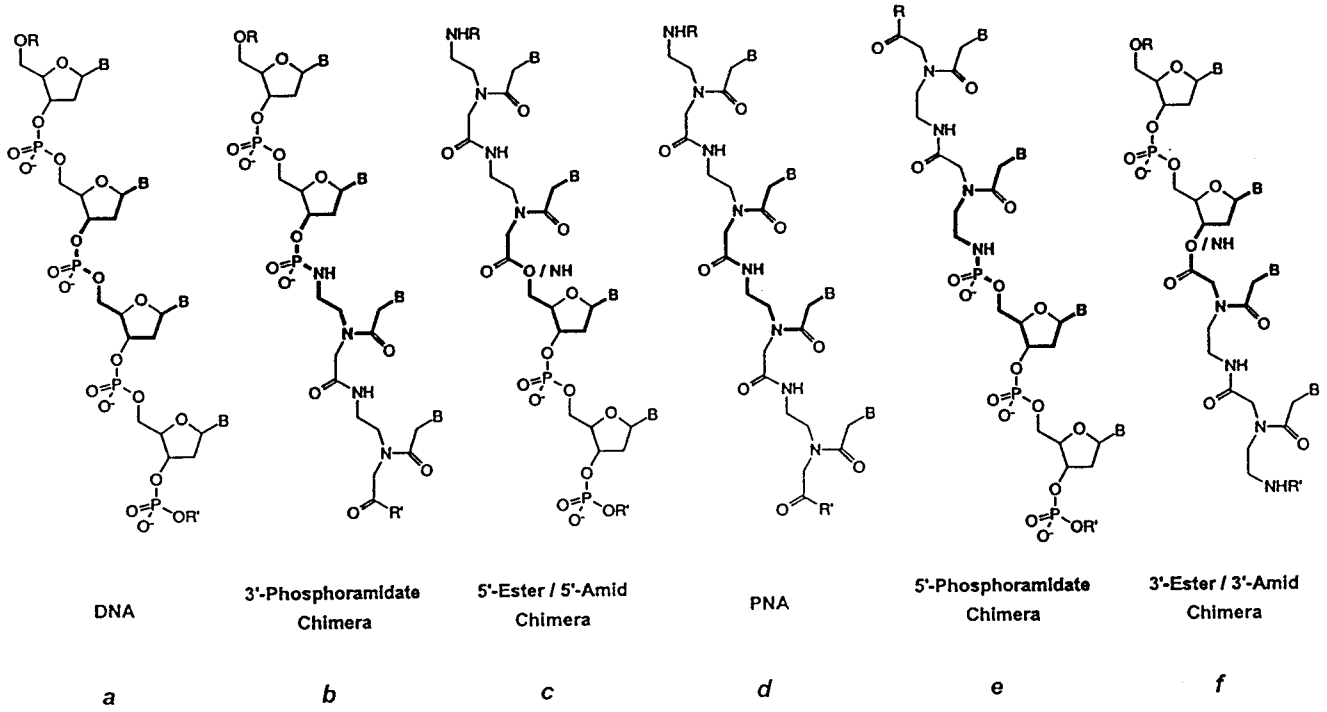

Figure 1. Structures of DNA, PNA, and chimeric ligation products.

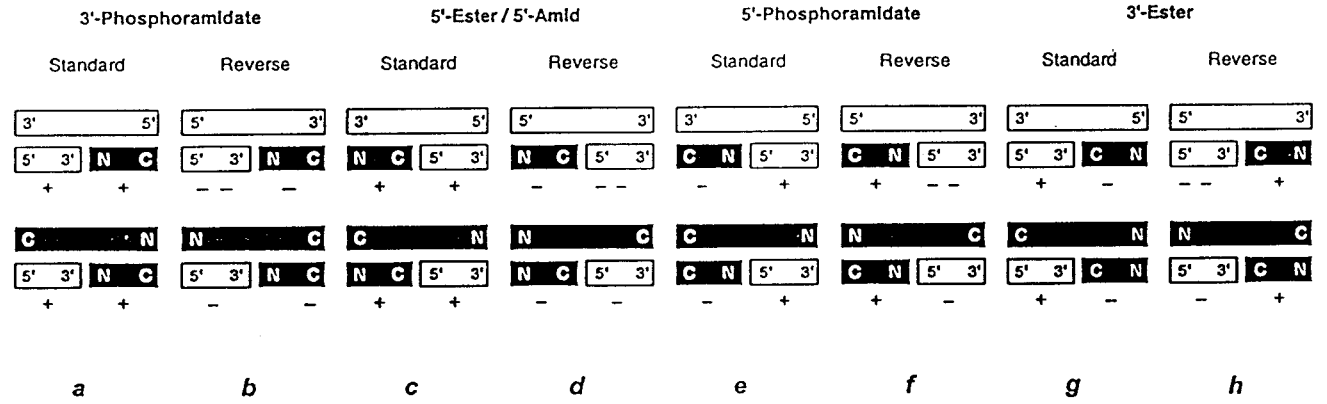

Figure 2. Evaluation of binding modes for all theoretically possible ligation reactions between DNA and PNA on DNA or PNA templates. DNA is represented by open bars and PNA by filled bars: (+) favored association, (−) disfavored association, (- -) forbidden association.

(Figure 2, e and g). We would, therefore, expect, other things being equal, that the most efficient ligation reactions would correspond to the orientations a and c in Figure 2. Less efficient ligation might occur for orientations e and g.

All eight possible arrangements can be realized on a PNA template (Figure 2, lower panels), because PNA is able to form complementary double helices with PNA and DNA in either the parallel or the antiparallel mode.[7] As in the previous case, orientations a and c in Figure 2 should be most stable because they involve antiparallel orientations of both substrates and e and g should be less stable because there is parallel pairing between PNA template and PNA substrate. However, with a PNA template, the remaining four arrangements which involve parallel binding between DNA substrate and PNA template cannot be excluded. Orientations f and h involve parallel binding of the DNA substrate and antiparallel binding of the PNA substrate and would therefore be expected to lead to intermediate stability, perhaps comparable to orientations e and g. The remaining orientations b and d with both substrates aligned in parallel orientation should be the least stable but might still support some ligation.

Here we present a detailed study of the template directed formation of DNA−PNA chimeras. We have studied the two structurally favored ligations, via the formation of 3′-phosphoramidate (Figure 1, b) and 5′-ester bonds (Figure 1, c) and the disfavored ligation via a 5′-phosphoramidate bond (Figure 1, e) using both PNA and DNA as templates. We did not investigate the formation of 3′-ester bonds.

For each type of bond formation, we first used template sequences which were chosen in such a way that the DNA substrate had to align antiparallel to the template to make reaction possible. This implies that in a reaction forming 3′-phosphoramidate or 5′-ester bonds both substrates must bind in antiparallel mode (Figure 2, a and c). Similarly, in reactions leading to the formation of 5′-phosphoramidate bonds, the PNA substrate must bind in the parallel mode (Figure 2, e).

To evaluate the potential of parallel orientation of strands in template-directed ligations involving PNA, we also investigated reverse ligation reactions, that is, reactions in which we used templates with the retrosequences. These reactions involve ligations with DNA substrates aligned parallel to the template as illustrated in panels b, d, and f of Figure 2. Finally, to

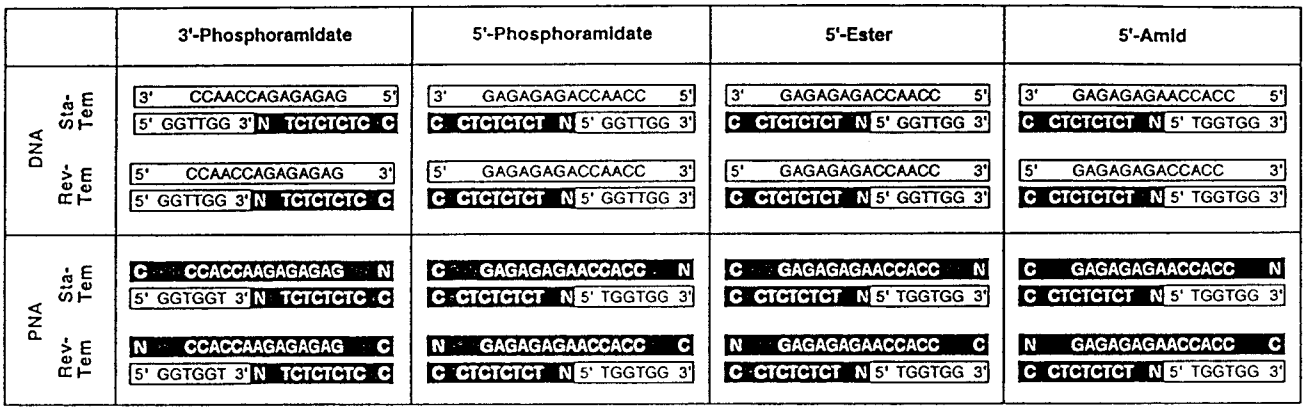

Figure 3. The simple ligation reactions that we studied. DNA is represented by open bars and PNA by filled bars.

overcome difficulties associated with the low nucleophilicity of the 5′-OH group, we studied the reaction of PNA with an oligodeoxynucleotide analogue in which the terminal 5′-hydroxyl group was replaced by a more nucleophilic amino group (Figure 1, c).

Results

Simple ligations were always performed using a 14-mer DNA or PNA template, an 8-mer PNA substrate and a [^{32}P]-labeled 6-mer DNA−substrate. Each type of ligation reaction was studied in imidazole buffer with 0.06 μM of [^{32}P]-labeled DNA substrate using different concentrations of DNA or PNA template (0, 0.1, 1, and 10 equiv) and PNA substrate (0, 0.1, 1, and 10 equiv). We used 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC) as activating agent in all our experiments. The reactions studied are illustrated in Figure 3.

The results refer to reactions on a standard template unless a reverse template is specified. In general, we found that no significant yield of ligation product could be detected in the absence of a template or, in the presence of a template, without activation. For each type of ligation, we observed that the rate of ligation depended on the concentration of DNA or PNA template and PNA substrate, but not always in a simple way. In preliminary experiments, we found that reaction on DNA templates proceeded best in the presence of salt, but reactions on PNA are favored in the absence of salt. All of the numerical data quoted, therefore, refer to reactions on DNA templates in the presence of 0.1 M NaCl or reactions on PNA templates in the absence of salt. The salt dependence of the yields was never large. The gel electrophoretograms of the products of typical reactions are presented in Figure 4B and a summary of the yields obtained in typical experiments are in Figure 5.

3′-Phosphoramidate Bond Formation. Ligation via a 3′-phosphoramidate bond is a very efficient reaction at both 25 and 4 °C (Figure 4B, a and d, and Figure 5). When 10 equiv of PNA or DNA template and 10 equiv of PNA substrate are used, the ligation reaction is so fast that it is almost complete after 4 h at 25 °C. We obtained 73% product on DNA and 70% on PNA templates. At 4 °C the reaction was slower giving a 52% yield after 4 h on either template; after longer times, the yields exceeded 80% on either template. Since the efficiency of conversion of the 3′-phosphate to the reactive imidazolide was between 80 and 90% (data not shown), the yield in these ligation reactions, based on activated substrate DNA, was almost quantitative. When we used 10 equiv of PNA substrate and only 1 equiv of template, the results were very similar. However, when the template and the PNA concentrations were both reduced to 1 equiv, the situation was more complicated. On a PNA template the final yield was reduced but is similar at 25 and 4 °C (46% and 49%). On a DNA template, the final yield is 86% at 4 °C but only 34% at 25 °C.

Surprisingly, the reaction on a DNA template shows catalytic turnover (Figure 4B, a, lanes 3 and 4). We obtained a yield of about 40% chimeric product after 7 days when we used 0.1 equiv of DNA template and 1 equiv of PNA substrate. This corresponds to the formation of about four ligated molecules on each template molecule. In an analogous experiment in which 0.1 equiv of PNA was used as template (Figure 4B, d, lanes 3 and 4) we did not observe catalytic turnover.

The reaction of the 3′-phosphate on a reverse DNA template did not give detectable yields of product under any conditions (Figure 4B, a, lane 10). This serves as a control showing that ligation is indeed completely dependent on the ability of the template to bring together the appropriate groups of the two substrates (Figure 3). Some retroligation was observed on a PNA template (Figure 4B, d, lane 10), but as expected, the reaction was much slower than on a standard template. After 4 h with 10 equiv of PNA substrate and 10 equiv of template, the yield was only 3% at 25 °C, compared to 70% on the standard template.

There is a significant side reaction between a PNA template and a DNA substrate when they hybridize to form a double helix (Figure 6). The amino group of the PNA attacks the phosphorimidazolide of the bound DNA to form a phosphoramidate bond. When 10 equiv of PNA template are used in the absence of PNA substrate, this reaction leads to 61% of side product after 14 d at 4 °C (Figure 4B, d, lane 7), but the reaction is progressively inhibited by increasing concentration of PNA substrate. When 10 equiv of PNA substrate are added, the yield of the side product is reduced to less than 3% (Figure 4B, d, lane 9).

5′-Phosphoramidate Bond Formation. The formation of 5′-phosphoramidate bonds (Figure 4B, c and f) is much slower than the formation of 3′-phosphoramidate in template-directed reactions (Figure 5). Furthermore, the reactions are much more sensitive to temperature. After 4 h, under optimal conditions (10 equiv of PNA substrate, 10 equiv of template), the yield of 5′-phosphoramidate-ligated product is only 6% on DNA and 4% on PNA at 4 °C. In the corresponding reaction of the 3′-phosphoramidate the yield is 52% in either case. However, at 4 °C after longer times (14 d) the 5′-phosphate gave a yield of ligated product of 65% on DNA and 19% on PNA. At 25 °C the reaction gives very little product on either a PNA or a DNA template even after 14 d.

The 5′-phosphoramidate ligation on a PNA template is the only example of a reaction for which a reaction mixture

A

lane	1	2	3	4	5	6	7	8	9	10	11
DNA	–	–	–	–	–	–	–	–	–	–	+
DNA*	+	+	+	+	+	+	+	+	+	+	–
Sta-Tem	–	–	0.1	0.1	1.0	1.0	10	10	10	–	10
Rev-Tem	–	–	–	–	–	–	–	–	–	10	–
PNA	–	10	1.0	10	1.0	10	–	1.0	10	10	10

Reaction Conditions:

0.06 μM DNA or DNA*

3'-/5'-Phosphoramidate: T = 4 °C, t = 14 d

5'-Ester: T = -20 °C, t = 28 d

B

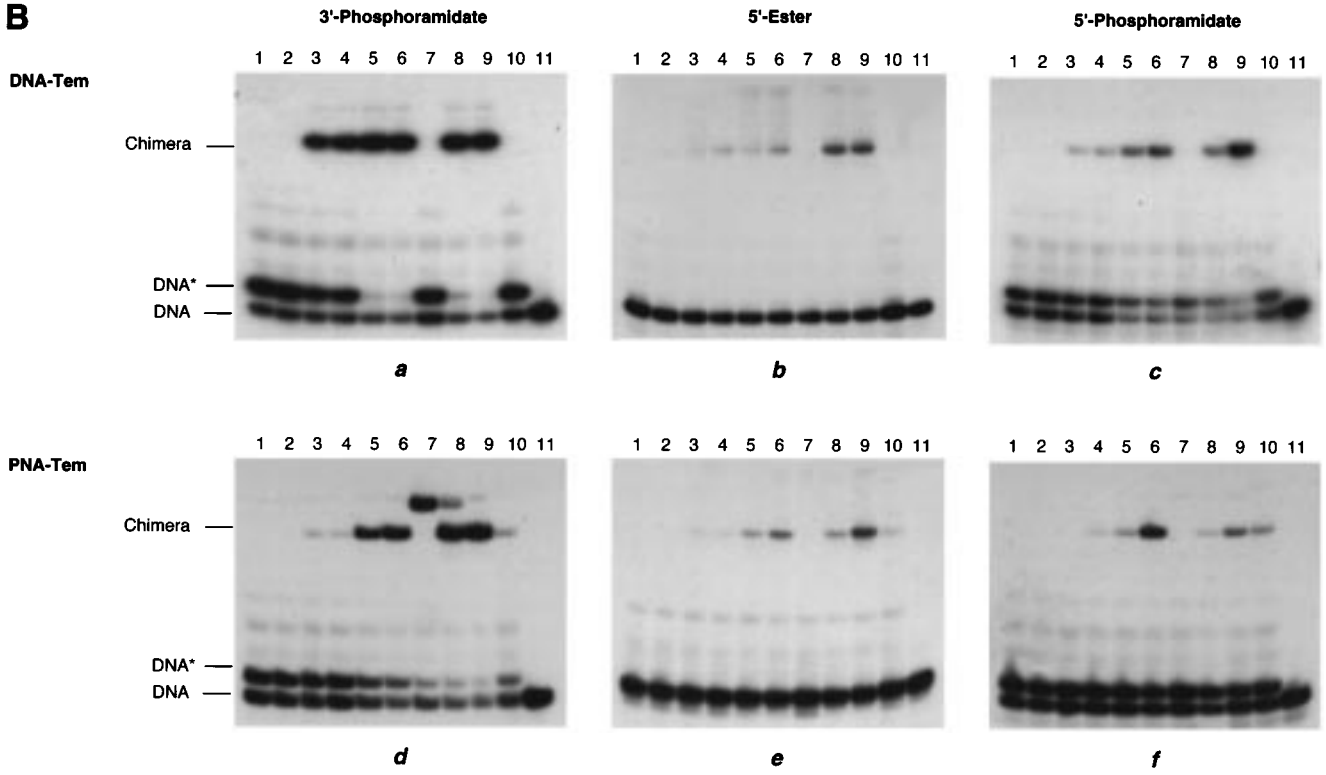

Figure 4. A. Reactions, conditions, and the concentrations of template and substrates employed in each of the six sets of reactions. B. Gel electrophoretograms of the products from each of the six sets of reactions.

containing 10 equiv of PNA substrate and 10 equiv of template gives lower yields than one containing 10 equiv of PNA substrate and only 1 equiv of template. After 14 d at 4 °C, we obtained 37% product with 1 equiv of template, but only 19% for the reaction with 10 equiv of template (Figure 4B, f, lanes 6 and 9).

No 5′-phosphoramidate products were formed on a reverse DNA template (Figure 4B, c, lane 10), but the reaction on a reverse PNA template (Figure 4B, f, lane 10) leads to the formation of small amounts of ligation products. When we used 10 equiv of template and 10 equiv of PNA substrate, the final yields of product at 25 °C are 4% on the standard PNA template versus 3% on the reverse PNA template. At 4 °C the final yields are 19% on the standard PNA template versus 9% on the reverse PNA template (Figure 4B, f, lanes 9 and 10).

A side reaction occurs between a reverse PNA template and the DNA substrate to produce a 5′-phosphoramidate chimera, analogous to the chimera produced by a 3′-phosphate and a standard PNA template (Figure 6). Although the reaction is inhibited by PNA substrate, we obtained a small amount of this side product even in the presence of 10 equiv of PNA substrate and 10 equiv of PNA template at 4 °C after 14 d.

5′-Ester Bond Formation. No product was detected when we attempted to ligate DNA with PNA via a 5′-ester bond at 25 or 4 °C. Substantial product formation is observed on DNA and PNA templates at −20 °C (Figure 4B, b and c). However, the reaction is slow and takes weeks for completion (Figure 5). The most efficient reaction gave yields of 33% and 25% on DNA and PNA templates, respectively, after 14 days. Attempts to preactivate the PNA as an acyl imidazolide did not result in improved yields. No product could be detected on a reverse PNA template.

5′-Amide Bond Formation. The 5′-amide ligations with a 5′-amino-terminated DNA substrate were performed to circumvent the problems associated with the low nucleophilicity of the 5′-OH in the ester ligations. The initial rate of amide bond formation is almost as great as that for 3′-phosphoramidate bond formation. The yields are 33% on DNA and 26% on PNA at 4 °C after 4 h (Figure 7, a) with 10 equiv of template and 10 equiv of PNA substrate. However, the presence of the amino functionality leads to the formation of very large amounts of side products after extended reaction times on both DNA and PNA templates (Figure 7, b).

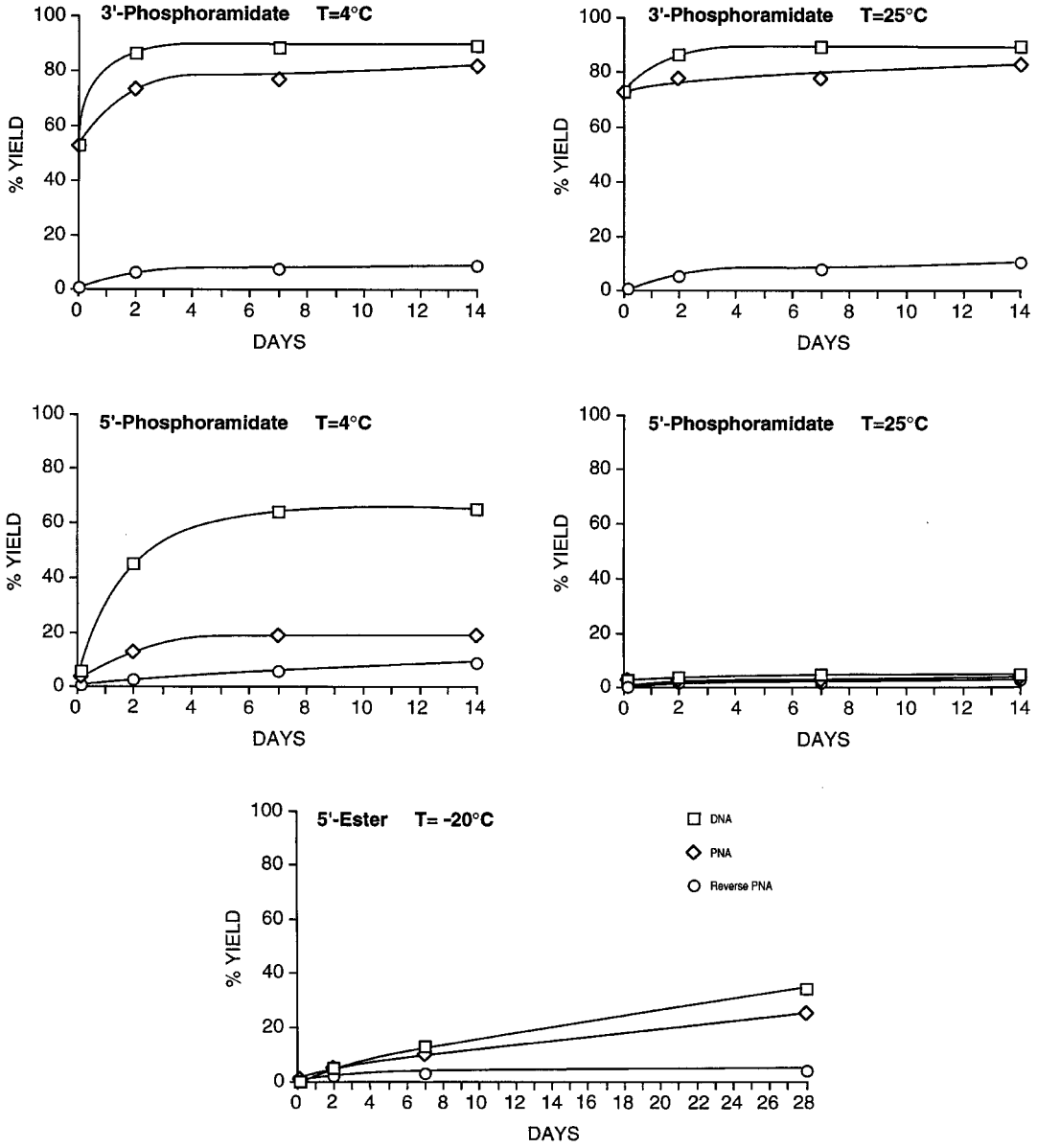

Figure 5. Time dependence of yields in simple ligations with 10 equiv of PNA substrate in the presence of 10 equiv of standard DNA, standard PNA, and reverse PNA templates.

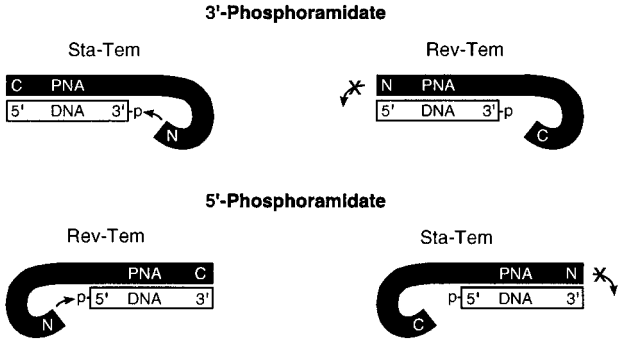

Figure 6. Orientations of PNA template and DNA substrates that permit formation of template−substrate chimeras.

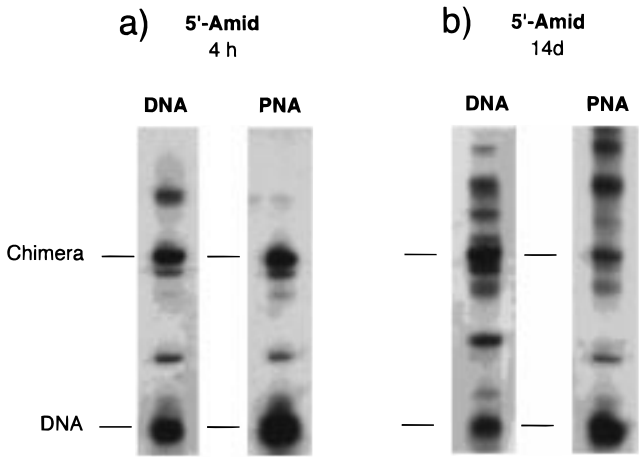

Figure 7. Gel electrophoretograms of products formed in ligations via a 5′-carboxamide bond after (a) 4 h and (b) 14 d.

Discussion

The initial rate of ligation of [^{32}P]-labeled DNA to PNA in the presence of a template depends on two factors: (1) the extent to which the [^{32}P]-labeled DNA is present in a ternary complex with the template and the substrate PNA and (2) the rate of ligation within the ternary complex. The results that we obtained after short incubation times can usually be explained in terms of these two factors alone. The final yield of ligation products depends also on competition between the ligation

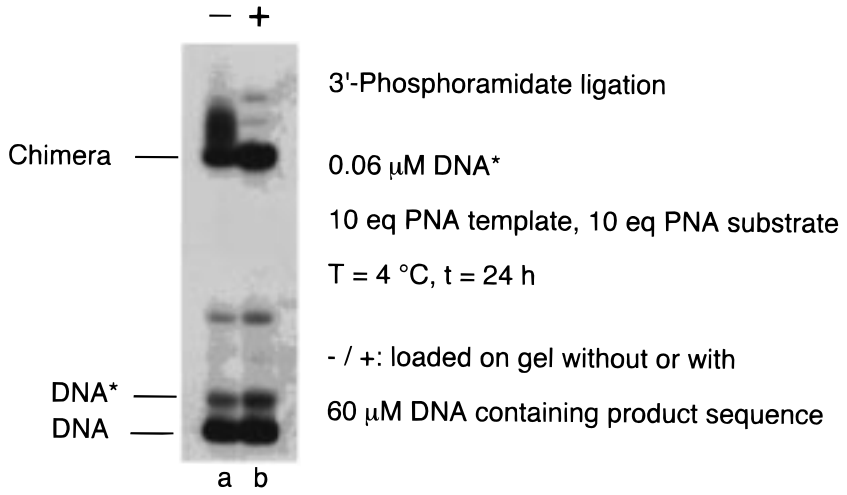

Figure 8. Gel electrophoretograms of products from a 3′-phosphoramidate ligation reaction using 0.6 μM DNA* with 10 equiv of PNA template and 10 equiv of PNA substrate at 4 °C for 24 h: (−) loaded with formamide after heating and chilling on ice; (+) loaded with formamide and 20 equiv of 60 μM DNA complementary to the template. DNA* denotes the phosphorimidazole of the labeled substrate, and DNA represents the deactivated substrate.

reaction and nonproductive hydrolysis of the activating agent and/or the activated substrates. Other things being equal, reactions that have high initial rates produce large yields, but the relation between initial rate and the yield may depend on temperature and substrate concentrations in a complicated way.

Our results demonstrate that all of the anticipated ligations can be performed successfully both on DNA and on PNA templates. There is usually little difference in efficiency between the two templates. Reverse DNA templates give no product in any case, because parallel binding of DNA duplexes does not occur. This latter finding is in agreement with a recent study which shows that phosphodiester/carboxamide PNA−DNA chimeras bind exclusively in the antiparallel mode to DNA.[13] Ligation on reverse PNA templates is possible. However, both DNA and PNA substrates have to bind in the unfavorable parallel mode to the reverse PNA template in 3′-phosphoramidate and 5′-ester or 5′-amide ligations. The reactions are, therefore, much less efficient than the reactions leading to 3′-phosphoramidate or 5′-ester bonds on standard templates.

One and only one of the substrates must align in the unfavored parallel mode to make 5′-phosphoramidate bond formation possible on either standard or reverse PNA templates (Figure 2, e and f). Consequently, ligation occurs with similar efficiency on both PNA templates. In this case, the efficiency of ligations is also affected by the unfavorable number of backbone bonds in the chimeric region (Figure 1, e). These two factors together account for the relatively low efficiency of 5′-phosphoramidate ligation relative to 3′-phosphoramidate ligation.

The dependence of the yield of 5′-phosphoramidate ligation product on the concentration of PNA template is unusual. The yield after 14 d in the presence of 10 equiv of PNA substrate is greater when only 1 equiv of template is used (37%) than in experiments with 10 equiv (19%) (Figure 4B, c and f, lanes 6 and 9). We believe that this is due to the weakness of the parallel association of the PNA substrate with the PNA template. If we assume that all of the [^{32}]P-labeled DNA substrate is bound to the template, then the proportion of the DNA substrate in productive ternary rather than nonproductive binary complexes will depend on the concentration of free PNA in solution. The presence of excess template will act as a "sink" for PNA substrate, reducing its concentration in free solution, and hence reducing the efficiency with which DNA substrate is incorporated into chimeras. This effect is only likely to be important for PNA substrate−template complexes of intermediate stability. If the interaction is weak, association of PNA with excess template will have little effect on the concentration of free PNA; if the association is strong, all of the DNA substrate will be present in ternary complexes, provided the total template concentration does not exceed the total PNA substrate concentration. Similar considerations would apply if DNA/template hybridization was incomplete, but the discussion would necessarily be more complicated.

The stability of double helices increases in the order DNA/DNA < DNA/PNA < PNA/PNA,[14] which suggests that chimeric products should bind more strongly to PNA than to DNA templates. This is confirmed by results of gel electrophoresis experiments; 10 M urea at room temperature was sufficient to dissociate chimeras from the DNA template strand. However, even when products were denatured in formamide by heating to 95 °C and chilling on ice, PNA complexes with chimeras were not completely denatured. In this case, we needed to add a large excess (200-fold) of an oligodeoxynucleotide complementary to the template prior to gel loading to dissociate chimeric products from their PNA templates. Figure 8 shows a smeared product band of the 3′-phosphoramidate chimera, generated on a PNA template, when no DNA (−) was added prior to electrophoresis. A sharp band is obtained when excess of DNA with the expected product sequence is added.

The high binding affinity of PNA templates also explains their inability to function catalytically: When we used 0.1 equiv of PNA template, we always observed product yields below 10%. DNA templates, perhaps surprisingly,[15,16] show catalytic turnover. This implies that in equilibrium, the substrates can partially replace the chimeric product on the DNA template. This difference between standard ligations which do not show catalytic turnover and ligation reactions via phosphoramidate bonds which do is presumably due in part to the loss of the electrostatic interaction between NH_3^+ and PO_4^- groups that occurs on ligation.

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The temperature dependence of template-directed ligations is complicated, because the temperature affects different factors determining the overall efficiency of a reaction. The ligation reaction should be faster at higher temperatures, but the same is true for the hydrolysis of the active intermediate. Furthermore, the ternary complex may be partially dissociated at higher temperature. Usually, the overall yield of products is larger at 4 °C than at 25 °C. Ligation via a 3′-phosphoramidate bond on PNA templates is an exception, since it gives similar product yields at 4 °C and at 25 °C. This indicates that, on account of the higher duplex stability of double helices involving PNA, there is little melting of the ternary complex at 25 °C.

Ligation via a 5′-ester bond produces a substantial yield of product only at −20 °C, while the analogous ligation to form an amide bond initially proceeds efficiently at 4 and 25 °C. This indicates that the failure of the ester ligation at higher temperatures is due to the lower nucleophilicity of the hydroxyl group. This allows hydrolysis by water molecules to compete effectively with ester-bond formation. At lower temperatures, hybridization is more nearly complete and the 5′-OH group of the DNA substrate in the ternary complex is confined to a configuration that favors reaction. We suspect that the carboxylate group is subjected to multiple rounds of activation and hydrolysis in the course of these reactions.

The amide ligation reaction, although it has a high initial rate, never gives good yields of the simple ligation product. Very large amounts of a series of side products with lower electrophoretic mobility than the ligation product accumulate at later times (Figure 7). They are most probably formed by the addition of further molecules of PNA to the first-formed ligation product via a "sliding" mechanism.

The formation of chimeras allows for a continuous transition from one informational polymer system to another. However, in the case of a PNA−RNA transition, the information accumulated in PNA could not necessarily be utilized by RNA. Prior to the evolution of protein synthesis, the information stored in a sequence is only useful insofar as it specifies a functionally useful polymer. A sequence that is, for example, catalytically active as PNA is unlikely to be active as RNA, since the two molecules in their single-stranded forms are likely to adopt completely different conformations and to present completely different backbone functional groups.[17] Chimera formation, therefore, could not transfer "useful" information from PNA to RNA. Chimera formation could, however, allow a transition to a superior information-storing polymer. RNA could first have evolved to serve as a template for PNA synthesis, and only later have acquired independent catalytic function.

We do not claim that PNA was a genetic material on the primitive earth, prior to the appearance in the RNA world, though this has sometimes been suggested.[18] However, we believe that our results likely illustrate a general phenomenon. Chimeric products are likely to form in sequence-dependent ligations whenever the structure of the junction of the chimeras is sufficiently similar to the structure of the backbone of the template. Our experiments do not address the fidelity of chimera formation in general, but they suggest that the specificity dictated by the strictly antiparallel orientation of the strands in nucleic acid double helices may not extend to other potentially informational systems.

Experimental Section

Materials. Imidazole, EDC, $NaIO_4$, cyclohexylamine, and di-*n*-propylmalonic acid were obtained from commercial sources. Polynucleotide kinase was purchased from New England Biolabs, terminal transferase was from Promega and γ-[^{32}P]-ATP and α-[^{32}P]-rCTP from Amersham. All PNAs were synthesized manually as described previously.[6,19] They were purified by RP-HPLC and characterized by electrospray MS. Oligonucleotides were synthesized on an Applied Biosystems automatic synthesizer. 5′-[^{32}P]-Derivatives were obtained using γ-[^{32}P]-ATP and polynucleotide kinase.[20,21] 3′-Labeling of oligonucleotides was performed by a modification of the procedure of Chu and Orgel.[22] Substrate (0.3 μM) oligonucleotide was treated with 1.2 equiv of α-[^{32}P]-rCTP and terminal transferase (0.75 U/μmol) for 30 min. The terminal cytosine of the product was then eliminated by oxidation with $NaIO_4$/cyclohexylamine/di-*n*-propylmalonic acid. The 3′-phosphorylated product was desalted by chromatography on oligonucleotide purification cartridges from ABI prior to gel purification. 5′-Amino-oligodeoxynucleotides were prepared according to Bruick et al.[23] All oligodeoxynucleotides were purified by electrophoresis on 8 M urea−20% polyacrylamide gels, followed by extraction with 0.5 M Tris·HCl containing 1 mM EDTA at pH 7.7 and chromatography on NENSORB columns or, with oligonucleotides shorter than heptamers, on OPC cartridges.

General Conditions for Simple Ligations. Ligations on DNA templates were carried out with 0.06 μM [^{32}P]-labeled DNA substrate together with appropriate amounts of PNA substrate and DNA template (Figure 4A) in the presence of 0.2 M imidazole buffer and 0.1 M NaCl. Ligations on PNA templates were carried out similarly, but omitting the NaCl.

All reactions were monitored by gel electrophoresis on 8 M urea−20% polyacrylamide gels. For reactions on DNA templates, aliquots of reaction mixtures were taken at appropriate times and loaded in 10 M urea/TBE (tris base, boric acid, EDTA) buffer. For ligations on PNA templates, aliquots of reaction mixtures were first incubated with 60 μM of DNA containing the anticipated product sequence ($\geq$200 equiv). Formamide/TBE buffer was then added, and the solution was heated to 95 °C for 3 min before chilling on ice prior to loading on the gel. Quantitation of yields was performed by autoradiography, excision of product bands from the gel, and scintillation counting.

Simple Ligations via Phosphoramidate Bonds. In experiments involving phosphoramidate bond formation, we used a modification of a published procedure[24] to generate the phosphorimidazolides: 0.24 μM [^{32}P]-labeled DNA was preactivated with 0.11 M EDC in 0.1 M imidazole buffer at pH 6.0 for 1 h. The imidazolide was then transferred to the reaction mixtures without intermediate purification. Ligations were performed in 0.2 M imidazole buffer (pH 7.7) at 25 °C or 4 °C.

Simple Ligations via Ester and Amide Bonds. Ester bond formation was achieved by in situ activation with 0.11 M EDC in 0.2 M imidazole buffer (pH 6.0) at 4 and −20 °C. Prior to loading on the gel, the 3′-phosphorimidazolide generated as a side product during the reaction was hydrolyzed at room temperature by overnight treatment with 0.1 M citric acid at pH 4.0. Ligation via amide bonds was achieved as described above for ester bonds either at 25 °C or 4 °C.

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