Selective chemical autoligation on a double-stranded DNA template

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ABSTRACT

We show that a double-stranded DNA segment serves as an effective template for spontaneously coupling short pyrimidine oligonucleotides containing terminal $-P(O)(O^-)S^-$ and $BrCH_2C(O)NH-$ groups. The efficiency of this autoligation depends markedly on proper base-pairing between the probe oligomers and the double-stranded target. This chemistry should be useful in designing highly selective probes for double-stranded polynucleotide segments.

INTRODUCTION

Synthetic oligonucleotides capable of forming triple stranded complexes with DNA are useful in a number of applications, including selective inhibition of protein–DNA interactions (1,2), induction of sequence-specific DNA cleavage (3), repression of gene transcription (4), and inhibition of expression of HIV-1 in infected human cells (5). Luebke and Dervan employed a 30-mer pyrimidine oligonucleotide as a template in circularizing *via* chemical ligation a 3.7 kilobase pair duplex DNA fragment (6). The reaction was carried out by activation with N-cyanoimidazole in the presence of zinc chloride and afforded in 7 hours a circular plasmid (15%, ligation at the termini of both strands of the duplex) and form II DNA (45%, corresponding to ligation of one strand).

We have recently shown that a pair of oligonucleotides in which one is terminated by a bromoacetyl group and the other by a phosphorothioate group undergo rapid and efficient autoligation in the presence of a single-stranded template oligonucleotide (7). This reaction can serve to enhance discrimination of oligonucleotide probes for a given target sequence (8). We report here that this chemistry is also applicable in autoligation of pyrimidine oligonucleotides on a duplex DNA template.

The oligomers employed in this study are indicated in Chart 1. Compounds **1a** and **2** were designed so that the phosphorothioate and bromoacetyl groups would lie in proximity when the oligomers bind *via* Hoogsteen base-pairing to the duplex template formed from **3** and **4**. Compounds **1b** and **1c** were prepared to examine the effect of nucleotide mismatches on the autoligation reaction.

Chart 1.

MATERIALS AND METHODS

General methods

Reversed phase high performance liquid chromatography (RP HPLC) was carried out on a Dionex chromatograph equipped with a Hewlett Packard Hypersil ODS 5μ 4.6 $\times$ 200 mm column using a 30 mM aqueous triethylammonium acetate buffer at pH 7, 1 ml/min flow rate, and a 1%/min gradient of acetonitrile. Melting curves were recorded on a Perkin-Elmer Lambda 2 UV/VIS spectrometer using solutions 1.0 M in NaCl, 15 mM in phosphate buffer at pH 7.05, and 4 μ M in each oligonucleotide. Changes in absorbance at 260 nm were followed while ramping the temperature at the rate of 1°C/min. Oligonucleotides were synthesized on a Millipore Expedite DNA synthesizer using β -cyanoethylphosphoramidite chemistry. They were isolated by RP HPLC with the DMT protecting group intact, and, following detritylation (80% aq. acetic acid, 30 min), were purified by RP HPLC.

Oligomers with 3'-phosphorothioate group (1a–c)

These compounds were prepared as described in reference 9. Briefly, Phosphate-ONTM amidite reagent (Cruachem) was coupled to dT-CPG (98% yield) in a syringe. Following detritylation, coupling with DMT-T phosphoramidite reagent, and

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oxidation with sulfur in carbon disulfide/pyridine, the loaded support was transferred to the DNA synthesizer for chain extension. To facilitate triplex formation at pH 7 (10), a 4-methyldeoxycytidine phosphoramidite reagent (Chem-Impex) was used in place of the standard deoxycytidine phosphoramidite reagent. After isolation by preparative HPLC, the oligomers eluted as clean peaks on RP HPLC (elution time: **1a**, 17.6 min; **1b**, 17.7 min; **1c**, 17.7 min) with an additional small peak (2–4% of the total) eluting at 18.3 min. The latter peak corresponds to the S–S product formed by oxidative coupling at the phosphorothioate groups; on addition of dithiothreitol these materials were converted back to oligomers **1a–c**.

5'-Bromoacetyl amino oligomer derivative (**2**)

5'-MMT-NH(TTTTTTTTTT) (RP HPLC elution time: 32.2 min) was prepared by chain extension of DMT-T-CPG by standard phosphoramidite chemistry and a final coupling with a phosphoramidite reagent prepared from 5'-monomethoxytrityl-amino-5'-deoxythymidine as described by Bannwarth (11). After cleavage from the support and isolation of the MMT-oligonucleotide, the MMT protecting group was removed by treatment with 80% aq acetic acid (90 min). Bromoacetylation was carried out (7,8) by adding 15 μ l of 0.4 M N-succinimidyl bromoacetate in acetonitrile to 5 A₂₆₀ units of the amino-oligonucleotide in 15 μ l of 0.2 M aq sodium borate (pH 8.5). Water (0.5 ml) was added after 30 min, the mixture was desalted on a NAP-5 column, and the bromoacetamido derivative (compound **2**) was isolated by RP HPLC (elution time: 19.1 min).

Ligation experiments

For the template directed reactions, an aqueous solution (1 M NaCl, 15 mM phosphate, pH 7.05) containing oligonucleotides **3** and **4** (4 μ M each) was heated at 80°C for 3 min and cooled to room temperature over a period of 30 min. After addition of one of the phosphorothioate terminated oligomer (**1a**, **1b**, or **1c**; 4 μ M final concentration), the solution was held at the temperature designated for the reaction (0, 20, or 30°C) for 1 h to permit hybridization; then oligomer **2** was added. At the end of the reaction period an aliquot of the solution was removed and analyzed by RP HPLC, which proved effective in separating the

reactant and product oligonucleotides. Ion exchange HPLC did not provide a satisfactory separation of the ligation product from oligomer **4**.

RESULTS AND DISCUSSION

Thermal dissociation experiments were carried out to gain information on the extent of association of the oligomers under the conditions employed in the ligation experiments. The template oligomers, **3,4**, formed a stable duplex (T_m 63°C). When one of the ligation components was added to the duplex mixture, the melting profile exhibited an additional break at a lower temperature, corresponding to dissociation of the third strand from a triple stranded complex. The T_m values for this dissociation were 32°C and 31°C, respectively, for the sequence-matching oligomers, **1a** and **2**, and 21°C for the mismatched oligomers, **1b** and **1c**.

The effect of a duplex template on ligation of oligonucleotides **1a** and **2** is demonstrated by the analytical RP HPLC profiles in Figure 1.

In absence of the template oligonucleotides very little coupling occurred over a period of 12 h at 0°C, as indicated in Figure 1A by recovery of oligomers **1a** (elution time 17.6 min) and **2** (19.1 min) and the presence of only a very minor peak in the region for the ligation product (~18 min) (Figure 1A). A large portion of the material eluting at ~18 min corresponds to a disulfide oxidation product of **1a** present initially in the reagent solution (**12**). We estimate that the extent of ligation over the 12 h period amounted to less than 4%. The profile for a reaction carried out for 12 h at 30°C was identical.

In contrast to these results, when the reaction was carried out in the presence of a duplex obtained from equimolar amounts of oligomers **3** and **4**, phosphorothioate **1a** and bromoacetyl-amino-oligonucleotide **2** reacted within 20 min to generated the bridged oligonucleotide, **5**, (Figure 1B) in >80% yield (13). A melting curve for the ligated mixture showed a break at 50°C for dissociation of the third strand from the triplex, in

Figure 1. RP HPLC of reactions at 0°C. (A) **1a**+**2** for 12 h; the major peaks correspond to **1a** (17.6 min) and **2** (19.1 min). (B) **1a**+**2**+**3**+**4** for 20 min; the peaks correspond to **3** (16.3 min), **1a** (17.6 min), **4** (18 min), **5** (18.5 min), **2** (19.1 min). (C) **1a**+**2**+**3** for 20 min; the major peaks correspond to template **3** (16.3 min) and reactants **1a** (17.6 min) and **2** (19.1 min); the minor peak at 18.3 min may be due to a disulfide oxidation product of **1a** and/or some ligation product.

Table 1. Extent of ligation in system **1**+**2**+**3**+**4**

Oligomer 1	Percent Ligation ^a		
	30°C	20°C	0°C
1a	72	72	80
1b	15	27	39
1c	24	38	44

^aReaction time, 20 min. See ref. 13 for quantitation.

Figure 2. RP HPLC of reactions for 20 min at 30°C; the peak assignments are the same in each case as in Figure 1B. (A) **1a**+**2**+**3**+**4**. (B) **1b**+**2**+**3**+**4**. (C) **1c**+**2**+**3**+**4**.

addition to the break above 60°C for dissociation of the duplex template.

Since a single stranded oligonucleotide can function as a template for ligation of complementary oligonucleotides appropriately derivatized with phosphorothioate and bromoacet-amido groups (7), we checked the possibility that this rapid coupling might have taken place on the single stranded purine oligonucleotide, **3**, rather than the duplex, **3,4**. In fact, this process is unlikely in the present system for two reasons: (a) the melting curve data show that very little oligomer **3** would be present in the single stranded form in 1.0 M aq. NaCl at 0°C, so in absence of catalytic turnover very little coupling on this template could take place; (b) 5-methylcytosine residues had been incorporated in **1a**, and guanosine units in **3**, such that complementarity of the bases and proximity of the reactive functional groups would only be achieved when these oligomers were aligned in parallel orientation, as in a triple stranded complexes. As an experimental test of the necessity for the pyrimidine oligonucleotide in this system, however, we examined ligation of **1a** and **2** at 0°C in the presence of the purine template, **3**, alone. The analysis of the products, presented in Figure 1C, show that very little coupling (a few percent at most) took place in 20 min when oligomer **4** was absent. When the time was extended to 17 h, the conversion to **5** was ~20%. This slow reaction can be attributed to partial binding of oligomers containing mismatches in the nucleotide sequence onto the single stranded target. We conclude that the rapid ligation represented in Figure 1B proceeds from a triple stranded complex derived by the association of oligomers **1a** and **2** with duplex **3,4**.

The effect of a nucleotide mismatch was examined by comparing the ligation efficiency for three different oligonucleotide phosphorothioate components. Compound **1a** is fully complementary to a section of the template; compound **1b** has a mismatch (dA in place dCme) near the ligation site; and compound **1c** has the mismatch (dA for dCme) several nucleotides removed from the ligation site. Data for the reactions of these compounds with oligomer **2** in the presence of template **3+4** at 0, 20, and 30°C are summarized in Table 1, and representative HPLC profiles are given in Figure 2 for reactions involving **1a-c** at 30°C. Three features are noteworthy. The coupling efficiency was markedly higher at all temperatures for the oligomer with the proper base pairing, **1a**. As in the case of coupling on a single stranded template (8), discrimination between the matched and mismatched oligomers was greater for reactions carried out at the higher temperatures, where binding of the mismatched oligomers is relatively poor. Finally, the mismatch in the nucleoside near the coupling site (**1b**) led to greater discrimination than the mismatch in one further removed (**1c**).

In conclusion, these experiments demonstrate a rapid, efficient, sequence dependent chemical autoligation reaction that takes place on a double stranded DNA template. This chemistry provides a means for transiently targeting a segment of DNA with a short oligonucleotide probe, for which binding is very sensitive to proper base sequence, and stabilizing the probe by coupling to an auxiliary oligonucleotide that binds to an adjacent DNA segment.

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12. Small amounts of a dimeric oxidation product [$\text{..OP(O)(O}^-\text{)S-SP(O)(O}^-\text{)O..}$], generated by atmospheric oxygen, generally accompany samples of terminal oligonucleotide phosphorothioates (Ref. 9). The small double peak ~18 min may be attributed to the disulfide product (early eluting) and the ligation product (later eluting). In accord with this assignment, the amount of the early eluting material did not increase over the 17 h period of the reaction; the elution time was the same as that for a sample of the oligomer–disulfide product prepared independently by oxidation by bubbling air through an aqueous solution, and the peak disappeared when the sample was treated with dithiothreitol.
13. Yields were calculated by dividing the area of the product peak, **5**, by the sum of the areas for peaks **1**, **2**, and **5**. In fact, for the case of **1a**, the HPLC profile indicates that **1a** must have been present in slight excess relative to **2** in the original mixture. On the basis that **2** is the limiting reagent, the conversion of **1a** and **2** to **5** is ~90%.