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Solid phase-supported thymine dimers for the construction of dimer-containing DNA by combined chemical and enzymatic synthesis: a potentially general method for the efficient incorporation of modified nucleotides into DNA

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ABSTRACT

The ability to study the structure–activity relationships of the *cis-syn* thymine dimer, the major photoproduct of DNA, has been greatly aided by the availability of a building block suitable for its sequence-specific incorporation into oligonucleotides by standard automated DNA synthesis. Unfortunately, its usefulness is compromised by the fact that it takes six steps to synthesize in low overall yield and, as with all phosphoramidite building blocks, has to be used in great excess over the support in standard automated synthesis. To extend the usefulness of this building block, we have directly coupled it to standard A, C, G and T long chain alkylamine-linked controlled pore glass supports to yield a solid phase-supported dimer. We then demonstrate that 13mers containing a 3′-terminal d(T[*cis-syn*]TN) group synthesized with this support at 0.2 μmol scale can be efficiently incorporated into longer oligonucleotides by both primer extension with 3′→5′ exonuclease-deficient Klenow fragment or T4 polymerase and dNTPs or by enzymatic ligation with T4 DNA ligase to another oligonucleotide opposite a complementary template. The site specificity and integrity of the *cis-syn* thymine dimer after both primer extension and ligation was confirmed by *cis-syn* dimer-specific cleavage with T4 *denV* endonuclease V. This general approach should be applicable to the synthesis of many types of site-specific nucleic acid modifications and would be of particular use for those for which the required building blocks are expensive or difficult to make.

INTRODUCTION

Building blocks suitable for site-specific incorporation of modified nucleotides into oligonucleotides by solid phase automated phosphoramidite-based synthesis have made it possible to generate a wide variety of probes and tools for both molecular biologists and chemists (1–6). Often the building block can be synthesized economically in a few steps in large quantities, so that the large excess of building block recommended for standard automated synthesis protocols (10-, 50- and 150-fold excess for 10, 0.2 and 0.04 μmol scale syntheses respectively) is of no particular consequence. In other cases, however, the building block requires many steps to synthesize or involves costly isotopically enriched reagents or intermediates. In these cases it is quite wasteful and costly to prime the reagent delivery lines and use a large excess of building block, which cannot be recovered in an easily re-usable form due to the nature of the phosphoramidite chemistry involved. Furthermore, to ensure that sufficient building block is available for the anticipated uses, one often has to prepare more than is necessary and, once it is prepared, it often has a limited shelf-life. These problems are particularly acute for a building block which has been used to sequence specifically introduce the *cis-syn* thymine dimer **1** (Fig. 1) into a wide variety of DNA substrates for biophysical, repair, replication and *in vivo* mutagenesis studies (7–12). This building block (compound **2**, Fig. 1) can be prepared in eight steps from thymidine in an overall yield of ~1% (13–15), making it quite wasteful to incorporate into oligonucleotides by standard automated synthesis, particularly when one would like to incorporate it into a large number of different sequences. To circumvent the problems associated with using a thymine dimer building block for each synthesis, we have developed stable, solid phase-supported dimers that can be used to incorporate the dimer into any sequence context by a combination of synthetic and enzymatic steps. This new approach relies on the ability of nucleic acid polymerases and ligases to extend an oligonucleotide sequence to the 3′-side of a modification, (4,16–22) and standard automated DNA synthesis to extend to the 5′-side of a modification. In principle, this approach could also be used for the synthesis of a wide variety of site-specifically modified DNA and RNA molecules.

The basic strategy of this new approach is to first chemically synthesize an oligonucleotide bearing the modified nucleotide at or close to the 3′-end by standard automated synthesis utilizing a

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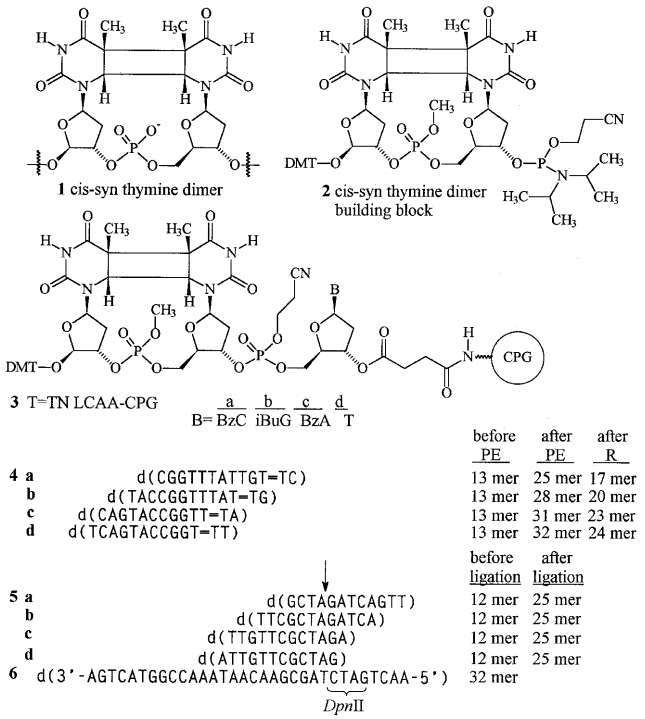

Figure 1. Structures of the *cis-syn* thymine dimer, the building block used to attach the dimer to the CPG-supported nucleosides, the resulting CPG-supported dimers and the oligonucleotides used in this study. Bz, benzoyl; iBu, isobutyrl; PE, primer extension; R, restriction cleavage by *Dpn*II at the site of the arrow; T=T, *cis-syn* thymine dimer.

solid support bearing the modified nucleotide. Then, in a second step, extend the 3′-end with either a polymerase and nucleotide triphosphates opposite a template or by chemical or enzymatic ligation to another oligonucleotide. The location of the modified nucleotide with respect to the 3′-end of the oligonucleotide, and hence the manner in which the modified nucleotide is to be attached to the solid support, is dictated by the ability of the polymerase or ligase to tolerate the modification. Direct attachment of the 3′-hydroxyl of a modified nucleoside to the support via the standard ester linkage would be suitable for modifications which cause little or no structural distortion, such as isotopomers. With more structurally distorting modifications, such as the *cis-syn* thymine dimer, it would seem to be more prudent to attach the phosphoramidite building block to A-, C-, G- or T-linked LCAA-CPG (long chain alkylamine controlled pore glass) supports. In this case the 3′-end of the oligonucleotide containing the modified nucleotide would be terminated with a normal nucleotide and, as will be shown for the *cis-syn* dimer, enables efficient primer extension and ligation.

MATERIALS AND METHODS

All oligodeoxynucleotides were synthesized at 0.2 µmol scale on an ABI 380B synthesizer by standard β-cyanoethyl phosphoramidite chemistry and purified on a preparative 2 mm thick × 165 mm long, 7 M urea, 1:19 cross-linked, 15% polyacrylamide gel at 300 V. The oligodeoxynucleotides were visualized by 254 nm light and desalted by precipitation with 1 vol. 0.3 M NaOAc and 3 vol. ethanol. 5′-End-labeling was with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs) in kinase buffer (70 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT) according to standard procedures (23), followed by phenol/chloroform extraction and precipitation with 1 vol. 0.3 M NaOAc and 3 vol. ethanol. All samples were separated by electrophoresis on a 0.4 mm thick × 375 mm long, 7 M urea, 1:19 cross-linked, 15% acrylamide gel at 1800 V. The DNA fragments were visualized by autoradiography with Kodak XAR-5 film at –70°C and quantified by densitometry on a 300A Molecular Dynamics Computing Densitometer. Bands corresponding to products of primer extension, ligation and cleavage by T4 endonuclease V and *Dpn*II were corrected for the relative amounts of –1 products in the primers.

Preparation of *cis-syn* thymine dimer-linked A, C, G and T solid supports

The *cis-syn* thymine dimer building block was synthesized using the same synthetic route as was used to synthesize dTpdU (15), which took seven steps starting from thymidine (Sigma). Building block **2** (4.4 eq.) was then coupled to 10 µmol CPG-supported deoxynucleosides (500 Å pore size; CPG Inc.) for 1 h using standard β-cyanoethyl phosphoramidite chemistry on an ABI 380B synthesizer to give **3a–d** in yields determined by trityl analysis of 90, 75, 87 and 52% respectively. These were then used to synthesize four 13mer primers **4a–d** at ~0.2 µmol scale.

Primer extension of the dimer-containing oligonucleotides 4a–d by exo$^-$ Klenow fragment

Reactions were carried out by incubating 2.6 pmol (0.13 µM) 5′-end-labeled primers **4a–d** with 26 pmol (1.3 µM) 32mer template **6**, 2 U exo$^-$ Klenow fragment and 100 µM dNTPs in 20 µl 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 50 mM NaCl at 25°C overnight. The extension reaction was then extracted with phenol/chloroform and ethanol precipitated. One third of the product mixture was incubated with 10 U *Dpn*II in 10 µl 100 mM NaCl, 50 mM bis Tris–HCl, pH 6.0, 10 mM $MgCl_2$, 1 mM DTT at 37°C for 1 h. Another third was incubated with 448 U T4 *denV* endonuclease V in 10 µl 32 mM Tris–HCl, pH 8.3, 10 mM EDTA, 100 mM NaCl, 0.1 mg/ml BSA at 37°C. After 1.5 h 20 µl 1.5 M aqueous piperidine was added and the mixture heated to 95°C for 30 min, then lyophilized and precipitated with ethanol.

Ligation of the dimer-containing oligonucleotides 4a–d

Approximately 3 pmol each of the four 5′-end-labeled 12mers **5a–d** and 3 pmol each of the four dimer-containing 13mers **4a–d** were annealed to the 32mer template **6** in 18 µl T4 DNA ligase buffer (55 mM Tris–HCl, pH 7.8, 11 mM $MgCl_2$, 11 mM DTT, 1.1 mM ATP, 28 µg/ml BSA). After annealing the mixtures were incubated at 0°C with 1 µl T4 DNA ligase (400 U) and an additional 1 µl 25 mM ATP. Aliquots were removed at the indicated times and T4 *denV* endonuclease V cleavage was carried out as described above for the primer extension reactions.

RESULTS AND DISCUSSION

The T=TC, T=TG, T=TA and T=TT LCAA-CPG supports, compounds **3a–d**, were prepared in ~52–90% yield using an automated synthesizer cycle to couple 4.4 eq. *cis-syn* dimer building block **2** to 10 µmol A, C, G and T supports respectively

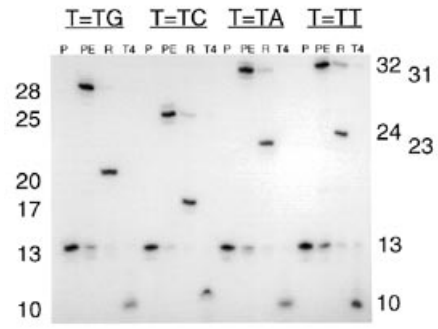

Figure 2. Autoradiogram of a denaturing polyacrylamide gel of 5′-end-labeled dimer-containing primers **4a–d** (lanes P), their primer extension products (lanes PE), and *Dpn*II- (lanes R) and T4 *denV* endonuclease V- (lanes T4) treated extension products.

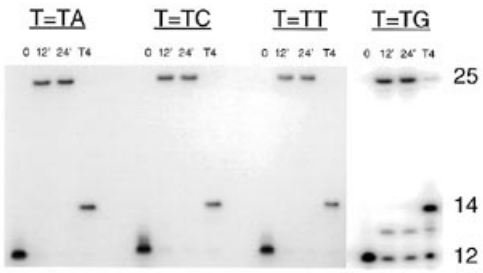

Figure 3. Autoradiogram of a denaturing polyacrylamide electrophoresis gel of the ligation reaction mixtures of the 5′-end-labeled oligonucleotides **4a–d**, for the indicated times (in hours), and the products of cleavage by T4 *denV* endonuclease V.

for 1 h. The supports were thoroughly washed with dry acetonitrile, dried under an argon stream and stored at 4°C. To determine whether oligonucleotides terminating at the 3′-end in T=TN could be extended by polymerases and ligated, 13mer primers **4a–d** were synthesized at 0.2 µmol scale, along with 12mers **5a–d** and a complementary 32mer template **6** (Fig. 1). A *Dpn*II restriction site was engineered into the template to enable cleavage of the primer extension products and thus circumvent the synthetically undesirable incorporation of additional nucleotides at blunt ends by 3′→5′ exonuclease-deficient polymerases (24,25). Non-templated addition of nucleotides is not an issue when the modified oligonucleotide is being incorporated into circular DNA duplexes by standard oligonucleotide-directed mutagenesis techniques (for reviews see 26,27; for some specific examples see 18,28,29).

When the dimer-containing primers **4a–d** were incubated with 3′→5′ exonuclease-deficient Klenow fragment of DNA polymerase I (exo⁻ KF) (30) and dNTPs opposite the 32mer template fully extended products were produced in yields of 94, 82, 82 and 78% (Fig. 2). In all cases only a full-length product was produced following cleavage of the extension products by *Dpn*II, indicating that no misalignment-mediated synthesis took place. The site and integrity of the *cis-syn* dimer in the extension products were confirmed by incubation with *cis-syn* dimer-specific T4 *denV* endonuclease V (31–33). This repair enzyme cleaves DNA at the site of a dimer by hydrolyzing the glycosidic linkage at the 5′-T of the dimer and by a process of β and δ elimination reactions, which can be driven to completion by heating with piperidine, produces a strand break (34,35). The ability of exo⁻ KF to extend the dimer-containing primers is in accord with the ability of Klenow fragment to incorporate a number of modified nucleotide triphosphates into DNA (4). The highly processive and exonuclease-proficient T4 DNA polymerase was also found to extend the dimer-containing primers in yields of >86%, though some 3′→5′ exonucleolytic degradation products were observed. Another highly processive but exonuclease-deficient enzyme, exo⁻ T7 DNA polymerase (Sequenase version 2.0) (36), did not extend the primers as well. When the 3′-terminal nucleotide was intentionally removed from the dimer-containing 13mers **4a–d** by the action of the 3′→5′ exonuclease activity of T4 polymerase none of the resulting primers terminating in a dimer could be extended by exo⁻ KF.

To determine whether or not ligation could also be used to prepare internal dimer-containing oligonucleotides, T4 DNA ligase and ATP were incubated with the four dimer-containing 13mers **4a–d**, along with 12mers **5a–d,** in the presence of the complementary 32mer template **6** (Fig. 3). The ligation was nearly quantitative in all cases, except for the dimer-containing primer terminating in G, which only led to 57% ligation after 24 h. Again, the site and integrity of the *cis-syn* dimer were confirmed by subsequent treatment with T4 *denV* endonuclease V.

CONCLUSION

In conclusion, we have shown that it is possible to construct *cis-syn* thymine dimer-containing oligonucleotides by way of a stable, easily manipulated solid phase-supported dimer. In this study excess thymine dimer phosphoramidite was used to prepare the support, but in cases where it is desirable to conserve the modified phosphoramidite, one or less equivalents could be used. Likewise, a Type II restriction enzyme site was engineered into the DNA product for the primer extension procedure, but to eliminate sequence restrictions within the modified oligonucleotide a Type IIS restriction enzyme site could have been used instead. Unlike Type II restriction enzymes, Type IIS restriction enzymes cleave sequence independently at a specific distance from their recognition site (37). In addition to being useful for incorporating *cis-syn* dimers into DNA, this methodology should also be applicable to a wide variety of modified nucleotides, though the exact nature of the modification may dictate how many nucleotides, if any, are required on the 3′-side of the modification to enable further extension by polymerases or ligation systems. With regard to scale, the procedures described would appear to be limited to <1 nmol based on the cost of commercially available enzymes and the unoptimized ratios of enzyme to substrate described herein. The true limit of the method may actually be in the range 10–100 nmol, however, as we have shown, photodamaged oligonucleotides can been ligated at a 2 nmol scale in >10% yield with only about four times the amount of DNA ligase described herein for 3 pmol (34). On the other hand, efficient non-enzymatic methods exist for synthesizing 5′-phosphorylated oligonucleotides (38) and for template-directed ligation of oligonucleotides (39–41), both of which have been used to prepare micromolar amounts of ligated products in purities suitable for NMR studies (42). Thus, as long as the modification is stable to the reagents involved, it might be possible to use a chemical ligation procedure in place of an

enzymatic one to prepare site-specifically modified oligonucleotides in the 1–10 μmol amounts required for NMR and crystallographic studies.

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