The effects of 5R-5,6-dihydro-5-hydroxythymidine on duplex DNA stability and structure

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ABSTRACT

An improved method for the chemical synthesis of oligodeoxynucleotides containing 5R-5,6-dihydro-5-hydroxythymidine (1) at a defined site is reported. UV melting studies carried out on duplexes containing 1 synthesized in this manner correlate with previously reported enzyme inhibition experiments, as well as computational studies. The melting experiments suggest that 1 destabilizes duplex DNA, but that the lesion preferentially base pairs to deoxyadenosine. These experiments also suggest that the presence of 1 in a duplex disrupts base pairing at the 5’-adjacent nucleotide and results in the thermally preferred misincorporation of purines opposite the 5’-deoxyadenosine stacked above 1 at this position. Despite the disruptions in base stacking, the UV melting experiments and enzymatic ligation/electrophoretic migration assays are consistent with the predicted macroscopic duplex structure containing intrahelical nucleotides.

INTRODUCTION

The C5-hydrates of thymidine (1 and 2) are one of several dihydrothymidine lesions that are produced in nucleic acids when these biopolymers are subjected to a variety of damaging agents (1–4). The consequences of the individual lesions on the biological function of DNA are highly variable. For instance, thymidine glycol (3) is a potent block of polymerase enzyme activity in vitro, whereas dihydrothymidine (4) has no effect on these enzymes (5–7). A structural rationale for these disparate effects was provided by computational experiments in which it was determined that the substitution pattern at the C5 position of the dihydrothymidines was a significant factor in determining how the replication of oligonucleotides containing these lesions was affected (8,9). The prediction that the C5-methyl group in the C5-hydrates adopts a pseudoaxial orientation (Scheme 1) was supported by recent studies on oligonucleotides containing 1 at a defined site (10). These experiments also supported the proposal that the hydrogen bonding pattern presented by 1 to Klenow exo⁺ was essentially unaltered from that exhibited by thymidine. Like thymidine glycol, 1 strongly inhibits Klenow exo⁺, but does not alter the fidelity of this enzyme significantly in vitro.

The inhibition of polymerase enzymes by 1 and 3 is reflected in the number of repair enzymes that excise these lesions from DNA (11–14). Recognition of DNA lesions by repair enzymes is sometimes attributed to a macroscopic deformation in the duplex structure (15). For example, in the case of thymidine glycol (3), the lesion is extrahelical (16,17). However, not all lesions occupy or induce other nucleotides to adopt extra-helical conformations within duplex DNA and are still excised by repair enzymes (18–22). The effect of 1 on the structure of DNA has been examined at the computational level. Molecular dynamics experiments predict that the nucleotides are intra-helical and that the lesion is base paired to the member opposite it in the duplex (8). Structural disruption induced by 1 is local in nature, with no macroscopic changes in the structure of the duplex. Furthermore, while base pairing at the 5’-adjacent nucleotide is disrupted, the monomeric components remain intrahelical. Herein, we present the first non-computational characterization of the effect of 5R-5,6-dihydro-5-hydroxythymidine (1) on the structure and stability of duplex DNA containing this lesion via thermal UV melting measurements on duplexes containing this lesion incorporated at a defined site in a chemically synthesized biopolymer. We also attempt to address the effects of 1 on the macroscopic structure of DNA by gel electrophoresis.

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RESULTS

Improved synthesis of oligonucleotides containing 5R-5,6-dihydro-5-hydroxythymidine (1)

Recently, we determined that the reported method for the synthesis of oligonucleotides containing 1 produced biopolymers containing randomly distributed N-allyl groups (10,23). The N-allyl groups were postulated to result from ‘back biting’ during the Pd(0)-mediated deprotection (24). Although the undesired modifications had no detrimental effect on the investigations concerning the effects of 1 on DNA polymerase activity, we suspected that other studies on biopolymers containing thymidine C5-hydrate (1) could be compromised by the presence of N-allylated nucleotides.

Consequently, we applied an alternative deprotection strategy for the synthesis of oligonucleotides containing 1. Potassium carbonate in methanol has been used to remove relatively labile protecting groups in oligonucleotide synthesis and organic synthesis in general (25,26). After determining that the alkali-labile dihydropyrimidine ring in 1 was stable to these conditions, we synthesized the appropriate phosphoramidite (9) in which the tertiary hydroxyl group was protected as the respective acetate. The acetate is labile to the K$_2$CO$_3$/MeOH deprotection conditions. Phosphoramidite 9 was prepared from the previously reported bis-silyl protected thymidine C5-hydrate (5, Scheme 2) and incorporated into oligonucleotides using standard automated cycles and ‘fast deprotecting’ phosphoramidites as synthons for the three native nucleotides containing exocyclic amines (27,28). Following treatment with K$_2$CO$_3$/MeOH, the deprotected oligonucleotide solution was acidified using acetic acid and then purified by gel electrophoresis. Electrospray mass spectral analysis of 10 and 11a clearly showed that all of the protecting groups were cleaved (Fig. 1). The only minor impurity detected in the biopolymers was material which retained some of the acetyl group. The 30mer (10) is longer than any material prepared previously using the Pd(0) deprotection method and comparison of this biopolymer with any of those clearly shows a remarkable improvement in the homogeneity of the product (10).

UV melting studies on the thermodynamics and thermal stability of DNA duplexes containing 5R-5,6-dihydro-5-hydroxythymidine (1)

Studies on the effects of 1 on Klenow exo− during translesional synthesis revealed that the dihydropyrimidine lesion presented a hydrogen bonding pattern to the enzyme that is comparable to that utilized for recognition of the native nucleotide. As mentioned above, these observations were predicted in previous computational experiments (8,9). UV melting temperatures of 12a and 13a are consistent with these computational predictions (Table 1). The presence of 1 in the dodecameric duplexes reduces the $T_{m}$ compared to that observed for the comparable duplex containing thymidine by between 8 and 12°C. However, the qualitative trend of mispairing on $T_{m}$ is the same for thymidine and 1, supporting the contention that the lesion presents a hydrogen bonding pattern comparable to that of the native nucleotide. Interestingly, the UV melting studies also correlate with the 36-fold increase in the misinsertion frequency of dA opposite the dA on the 3’-side of 1 compared to when thymidine opposite deoxyadenosine occupies the respective site (Table 2) (10). The $T_{m}$ for the dA·dA mispair is anomalously high (as reflected by the smaller $\Delta T_{m}$) in the duplex containing 1. Overall,
the destabilization of duplex DNA containing 1 in place of thymidine is gleaned from a Van’t Hoff plot which indicates a disrupted, less ordered helix (Table 3).

Examination of DNA curvature induced via the presence of 5R-5,6-dihydro-5-hydroxythymidine (1)

Qualitative evidence for a macroscopic change in the structure of a duplex containing 1 was examined using gel electrophoresis. A number of DNA lesions (e.g. 3) are believed to induce deformations in DNA structure (16,17). A similar structural effect of 1 was probed for using the enzymatic ligation, gel electrophoresis method (Fig. 2) (29–32). Carefully desalted duplexes (16 and 17) were ligated for 24 h and analyzed by non-denaturing gel electrophoresis. The duplexes were designed such that any bend induced by 1 in the duplex would be in-phase with the periodicity of the ligated duplex and, hence, would be expected to migrate more slowly through a non-denaturing gel than a similar unbent duplex (32). In order to facilitate detection of small degrees of bending manifested by slight differences in migratory aptitudes of longer oligomers of 16, the ligation mixtures were loaded on the gel a second time (Fig. 2), 2 h after their initial introduction to the gel. This enabled us to improve the resolution of the longer oligomers (Fig. 2, lanes 1 and 2), but not at the expense of the shorter ligated species (Fig. 2, lanes 2 and 4). The ligated duplexes (up to >20 decameric units long) containing thymidine or 1 embedded in a track of deoxyguanosines were indistinguishable by this method. In addition, the migration of ligated 16 was identical to that of ligated 18 (data not shown). The latter duplex has been shown previously to not give rise to bent DNA upon oligomerization (32). Finally, the relative gel mobility of the ligated duplexes was also unaffected by carrying out the electrophoresis at 4°C (data not show).

Table 1. The effect of 5R-5,6-dihydro-5-hydroxythymidine base pairing on UV melting temperature

<table>
<thead>
<tr>
<th>Base pair</th>
<th>(T_m^a) (°C)</th>
<th>(T_m^b) (°C)</th>
<th>(\Delta T_m^c) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T·A</td>
<td>52.6 ± 0.5</td>
<td>49.0 ± 0.4</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td>dG·X</td>
<td>46.2 ± 0.6</td>
<td>38.0 ± 0.9</td>
<td>8.2 ± 0.7</td>
</tr>
<tr>
<td>dC·X</td>
<td>36.0 ± 1.2</td>
<td>26.8 ± 0.9</td>
<td>9.2 ± 0.3</td>
</tr>
<tr>
<td>T·X</td>
<td>40.3 ± 0.4</td>
<td>27.7 ± 0.6</td>
<td>12.6 ± 0.8</td>
</tr>
</tbody>
</table>

\(^a\)Melting temperatures \((T_m)\) are the average of three runs obtained via cycling (heating/annealing) one sample within the cavity of the UV-VIS spectrophotometer.

\(^b\)Duplexes 15a-d were used. \(T_m\) obtained using 2.75 µM of each oligonucleotide.

\(\Delta T_m = T_m(\text{dA-T}) - T_m(\text{dA-X})\).

\(^c\)Duplexes 14a-d were used. \(T_m\) obtained using 2.75 µM of each oligonucleotide.

Table 2. The effect of 5R-5,6-dihydro-5-hydroxythymidine on base pairing at the 3′-adjacent site

<table>
<thead>
<tr>
<th>Base pair</th>
<th>(T_m^a) (°C)</th>
<th>(T_m^b) (°C)</th>
<th>(\Delta T_m^c) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dA·X</td>
<td>53.4 ± 0.5</td>
<td>42.3 ± 0.4</td>
<td>11.1 ± 0.1</td>
</tr>
<tr>
<td>dA·dA</td>
<td>41.8 ± 0.7</td>
<td>38.0 ± 1.2</td>
<td>3.8 ± 0.5</td>
</tr>
<tr>
<td>dA·dG</td>
<td>49.6 ± 0.5</td>
<td>34.8 ± 1.3</td>
<td>7.8 ± 0.2</td>
</tr>
<tr>
<td>dA·dC</td>
<td>40.2 ± 0.2</td>
<td>29.2 ± 0.8</td>
<td>13.1 ± 0.4</td>
</tr>
</tbody>
</table>

\(\Delta T_m = T_m(\text{dA-A}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-T}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-A}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-T}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-A}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-T}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-A}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-T}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-A}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-T}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-A}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-T}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-A}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-T}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-A}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-T}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-A}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-T}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-A}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-T}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-A}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-T}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-A}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-T}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-A}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-T}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-A}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-T}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-A}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-T}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-A}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-T}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-A}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-T}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-A}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-T}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-A}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-T}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-A}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-T}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-A}) - T_m(\text{dA-X})\).

\(\Delta T_m = T_m(\text{dA-T}) - T_m(\text{dA-X})\).
DISCUSSION

The above experiments were carried out in order to gain some perspective on how 1 affects the structure of the duplex DNA in which it is produced. The UV melting experiments and gel retardation studies are in remarkable agreement with previous molecular dynamics experiments (8,9). The UV melting experiments suggest that 1 presents a typical thymidine-like hydrogen bonding pattern and that the duplex is enthalpically destabilized. As was the case for duplexes containing the abasic site analogue 19, some of the enthalpic destabilization is compensated for by the larger degree of disorder in the modified duplex (33). There is also a correlation between the fidelity of enzymatic extension past the lesion and the thermal stability of duplexes containing the resulting 5'-adjacent base pair (34). This could be fortuitous and is not observed in all duplexes containing DNA lesions (35,36).

The disorder is believed to be induced by the pseudoaxial C5-methyl group in 1, which perturbs base stacking at the 5'-adjacent nucleotide. Molecular dynamics calculations predict a weakening in base pairing at this site (8). The preferential incorporation of deoxyadenosine at this position and the smaller depression in the Tm of the duplex containing a dA·dA base pair adjacent to 1-dA (14a) compared to the duplex containing thymidine base paired with dA (14d) are consistent with these predictions. The predicted deformation and weakening of the dA·T base pair might be expected to facilitate accommodation of the dA-dA base pair, which requires that the bases push apart (37). These experimental observations are also consistent with comparable experiments on oligonucleotides containing abasic site analog 19, which cannot form hydrogen bonds to a nucleotide opposite it (33). Presumably, the preferential incorporation (thermal and enzymatic) of dA opposite dA at the site adjacent to 1 and across from an abasic site is attributable to the greater ability of this purine nucleoside to base stack in a helix (38,39).

Despite the predicted weakening of the 5'-adjacent base pair, 1 is not expected to increase the flexibility or introduce discrete bends in duplex DNA (8). This is in contrast to solution NMR studies on 3, which reveal that this lesion adopts an extrahelical position and introduces a kink in the host duplex (16,17). We investigated the possibility that 1 may give rise to increased flexibility or even a defined bend in the biopolymer, using the useful (albeit imperfect) enzymatic ligation assay (29–32,40). Unlike other lesions, such as exocyclic adducts, ligated duplexes containing 1 showed no evidence of DNA bending/curvature using this assay (41,42). Oligomers of a duplex containing 1 migrated at the same rate through a non-denaturing gel as did comparable ligation products of a duplex (17) containing thymidine, as well as a duplex (18) which has been shown to not induce DNA curvature (32). Without detailed solution phase NMR data, these experiments are interpreted to imply that 1 is intrahelical in 14b, as predicted by molecular dynamics calculations (8).

**Figure 2. Autoradiogram of ligated duplexes 16 and 17. Lanes 1 and 3, 17; lanes 2 and 4, 16. Lanes 1 and 2 were loaded 2 h prior to lanes 3 and 4.**

CONCLUSIONS

UV melting experiments and electrophoretic migration studies on duplexes containing 1 support previous computational experiments concerning this lesion. The present experiments support a general structure of duplex DNA containing 1 in which the lesion selectively base pairs with a deoxyadenosine opposite it in the helix, but disrupts base stacking in the 5'-direction. The disruption of base stacking at the adjacent base pair results in a duplex that is better able to accommodate mispaired nucleotides at the 5'-adjacent base pair to 1 than an unmodified duplex. Consequently, a duplex containing a mispaired deoxyadenosine opposite another deoxyadenosine is relatively favored on account of its inherent ability to form stable π stacking interactions.

MATERIALS AND METHODS

General methods

All reactions were carried out in oven-dried glassware under an argon atmosphere, unless noted otherwise. Pyridine, disopropylylamine and CH2Cl2 were distilled from CaH2 and THF was distilled from potassium-benzenophenone ketyl. Oligodeoxynucleotides with or without modification were synthesized on an ABI 380B synthesizer using standard protocols on a succinato long chain alkylamine controlled pore glass support purchased from Glen Research. Phenoxyacetyl- and isobutyryl-protected phosphoramidites purchased from Pharmacia were used in the preparation of C-5-hydrate-containing oligonucleotides.
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Acetate 6

Thymidine hydrate 5 (0.27 g, 0.55 mmol) was co-evaporated with pyridine (3 × 5 ml). DMAT (13 mg, 0.11 mmol) was added to the resultant oil and the mixture was dissolved in pyridine (5 ml). Acetic anhydride (62 μl, 0.66 mmol) was added via syringe to the solution and the reaction mixture was stirred at 25°C overnight. Pyridine was removed under reduced pressure and the residue was dissolved in EtOAc (25 ml) and washed sequentially with 10% HCl (5 ml), saturated NaHCO3 (5 ml), water (10 ml) and brine (8 ml). The organic extract was dried over Na2SO4, filtered and concentrated to afford crude 6. Acetate 6 (0.18 g, 60%) was obtained on silica gel chromatography (90:10–30:70 hexanes:EtOAc gradient): IR (thin film) 2954, 2929, 2858, 1737, 1714, 1472, 1251, 1120 cm–1; 1H NMR (CDCl3) δ 8.16 (br s, 1 H), 6.30–6.26 (m, 1 H), 4.34–4.32 (m, 1 H), 3.92 (d, J = 11.7 Hz, 1 H), 3.82–3.69 (m, 3 H), 3.59 (d, J = 11.7 Hz, 1 H), 2.10 (s, 3 H), 2.08–1.90 (m, 2 H), 1.59 (s, 3 H), 0.93–0.89 (m, 18 H), 0.12–0.07 (m, 12 H); 31P NMR (CD3OD) δ 168.6, 164.9, 155.1, 151.1, 139.6, 107.7, 75.6, 72.3, 63.2, 42.5, 37.5, 25.8, 25.7, 21.9, 20.8, 17.9, 17.9, 14.7, 4.9, 5.6, 5.7; LRMS (FAB) 604 (M+); HRMS (FAB) calculated for C33H36O9N2P 604.2281, found 604.2277.

Diol 7

Acetate 6 (0.17 g, 0.33 mmol) was dissolved in THF (5.6 ml) at 0°C and a solution of TBAF (0.19 g, 0.72 mmol) in THF (0.7 ml) was added dropwise to the mixture. The reaction mixture was warmed to room temperature over 4 h. After 0.5 h, THF was removed under reduced pressure. Trityl derivative 8 (0.12 g, 90%) was obtained on silica gel chromatography (90:10 CH2Cl2:MeOH): IR (thin film) 2961, 1742, 1708, 1504, 1363, 1251, 1120 cm–1; 1H NMR (CDCl3) δ 169.5, 169.2, 158.5, 151.0, 149.5, 149.2, 149.0; LRMS (FAB) 806 (M+1); HRMS (FAB) calculated for C42H54O10N4P 805.3578, found 805.3591.

Phosphoramidite 9

Diisopropylethylamine (133 μl, 0.76 mmol) was added to a solution of trityl compound 8 (0.11 g, 0.19 mmol) in CH2Cl2 (2.0 ml) at 0°C. N,N-Diisopropylamino cyanoethylphosphoramidite chloride (58 mg, 0.24 mmol) was added to the reaction mixture dropwise. The reaction mixture was allowed to warm to 25°C. The reaction mixture was quenched with saturated Na2CO3 and the mixture was extracted with EtOAc (3 × 10 ml). The organic extract was dried with Na2SO4, filtered and concentrated under reduced pressure. Phosphoramidite 9 (114 mg, 75%) was obtained by flash chromatography on silica gel (1:1 EtOAc:hexanes): IR (thin film) 2961, 1742, 1708, 1504, 1363, 1249, 1172, 1124, 1032 cm–1; 1H NMR (CDCl3) δ 6.75–6.25 (m, 9 H), 6.86 (d, J = 8.8 Hz, 4 H, one diastereomer), 6.85 (d, J = 8.4 Hz, 4 H, second diastereomer), 6.31 (t, J = 7.0 Hz, 1 H), 4.69–4.64 (m, 1 H), 4.06 (dd, J = 11.3 Hz, 2.9 Hz, 1 H), 3.98 (d, J = 11.3 Hz, 1 H), 3.82 (s, 6 H, one diastereomer), 3.81(s, 6 H, second diastereomer), 3.80–3.30 (m, 5 H), 2.66 (t, J = 6.2 Hz, 2 H), 2.48 (t, J = 6.2 Hz, 2 H), 2.29–2.08 (m, 2 H), 1.97 (s, 3 H, both diastereomers), 1.50 (s, 3 H, one diastereomer), 1.49 (s, 3 H, second diastereomer), 1.28–1.05 (m, 12 H); 31P NMR (CDCl3) δ 149.2, 149.0; LRMS (FAB) 806 (M+1); HRMS (FAB) calculated for C33H36O9N4P 805.3578, found 805.3591.

General procedure for deprotection and cleavage of thymidine C5-hydrate oligonucleotides from the solid support

Oligonucleotides containing thymidine C-5 hydrate were treated with 0.05 M K2CO3 in anhydrous methanol for 3 h at 25°C. The supernatant was treated with 2 molar equivalents of acetic acid and then concentrated to dryness. Gel electrophoresis on 20% polyacrylamide gels afforded the purified oligonucleotide.

DNA melting experiments

Samples (total volume 400 μl) for the thermal denaturation studies contained a 1:1 ratio of complementary oligonucleotides (1–7 μM each). Solutions were prepared by the addition of appropriate volumes of stock solutions of the two complementary oligonucleotides to 200 μl of 2× PIPES buffer (20 mM PIPES, pH 7.0, 20 mM MgCl2, 200 mM NaCl), followed by dilution with distilled, deionized water to a volume of 400 μl. The complementary oligonucleotides were hybridized by heating the samples to 90°C, followed by slow cooling over 14–16 h to room temperature. The melting studies were carried out in 1 cm path length quartz cells on a Beckman DU 640 UV-VIS spectrophotometer equipped with a thermoprogrammer. Absorbance was monitored while the temperature was ramped at a rate of 0.5°C/min over a range of 45°C (25–70°C). Melting temperatures were calculated by computer fit of the first derivative of absorbance with respect to 1/θ (43).

DNA curvature experiments

Oligonucleotides 11a–c were labeled with [γ-32P]ATP, while their complementary oligonucleotides were phosphorylated with ATP (overnight at 37°C). Following purification by gel electrophoresis, the labeled oligonucleotides were hybridized in ligase buffer to their complementary oligonucleotides by heating the mixture to 90°C and slow cooling (overnight) to 25°C. The duplexes obtained (16–18) were ligated with T4
DNA ligase over 24 h at 4°C; an additional quantity of ATP was added to the ligation buffer to obtain an ATP concentration of 2.5 mM (total). Ligated material was loaded on 8% non-denaturing polyacrylamide gels. Each ligation reaction was loaded twice (2 h apart) in order to facilitate analysis of oligomers of significantly different length. The ligation ladders were visualized autoradiographically.

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