# Identification of Specific Nonbridging Phosphate Oxygens Important for DNA Cleavage by Human Topoisomerase I†

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ABSTRACT: Methylphosphonate-bearing oligonucleotides are characterized by the replacement of one of the nonbridging oxygen atoms with a methyl group. While neutralizing the negative charge associated with the phosphodiester at the point of substitution, the methyl group also imparts chirality to the phosphorus atom. Herein we report the synthesis of a number of oligonucleotides containing isomerically pure $S_p$ and $R_p$ methylphosphonates at single positions for the purpose of investigating the hydrogen-bonding contacts necessary for human topoisomerase I function. It was possible to correlate these data to the recent X-ray crystal structure of a truncated form of the enzyme and demonstrate a severe decrease of cleavage efficiency when any of the nonbridging oxygen atoms upstream from the cleavage site was removed. Also observed was increased cleavage for oligonucleotides substituted with methylphosphonates downstream from the cleavage site. These effects were shown to be due primarily to alteration of the binding of the modified DNA substrates by human DNA topoisomerase I.

The topoisomerases are ubiquitous cellular enzymes that are responsible for the overall maintenance of supercoiled DNA. Through the introduction of transient single-strand breaks in the phosphodiester backbone (Figure 1), topoisomerase I is capable of relaxing superhelical stress by changing the DNA linking number (*1*). An active-site tyrosine residue is responsible for DNA nicking at the scissile phosphate bond (*2*), thus affording a transient covalent adduct. After strand passage, the DNA is resealed by a ligation reaction (*1*); in the process, the 5′-OH group of the acceptor DNA strand displaces the bound tyrosine residue by nucleophilic transesterification. By virtue of its central role in such processes as replication, transcription, and chromatin recombination, topoisomerase I has long been viewed as an attractive target for potential antiviral and anticancer therapies.

Recent efforts in our laboratory to further characterize human topoisomerase I have focused on investigating the extent to which the enzyme will tolerate modified cleavage and ligation substrates. The introduction of *iso*-DNA monomers in and about a high-efficiency human topoisomerase I cleavage site demonstrated a reasonable level of flexibility in the behavior of the enzyme, as many of the modified partial duplexes underwent acceptable cleavage and ligation reactions (*3*, *4*). The same was also true when the 5′-OH group was modified in the ligation reaction (*5*). An analogous examination of DNA oligonucleotides containing single 2′,5′-phosphate ester linkages as substrates for vaccinia topoisomerase I revealed that only substitution at the scissile phosphodiester disrupted the transesterification process to any significant degree, while all other substitutions were readily tolerated (*6*). Recently, these studies have been extended to investigate the ability of both human and vaccinia topoisomerases to effect cleavage of methylphosphonate-containing partial duplex substrates. A significant observation was recently reported in the context of the viral enzyme: the presence of the neutral methylphosphonate at the scissile phosphodiester revealed a latent endonuclease activity not previously observed (*7*).

Presently, we describe the synthesis of a number of human topoisomerase I partial duplex substrates containing single methylphosphonate substitutions in proximity to the high-efficiency cleavage site in both the scissile strand (Figure 2A) and the noncleaved strand (Figure 2B). Also described is the reverse DNA synthesis of the authentic oligonucleotide standard containing a 3′-methylphosphonate moiety that would result if an endonuclease activity similar to that observed for vaccinia topoisomerase I were present in the human enzyme. The results provide functional biochemical data that are fully consistent with the human topoisomerase I X-ray crystal structure (*8*) and demonstrate the importance of individual phosphodiester bonds in the efficiency of DNA substrate binding and cleavage.

## EXPERIMENTAL PROCEDURES

*General Methods and Materials.* Modified methylphosphonate-bearing oligonucleotides were synthesized as described previously (*7*, *9*). Unmodified oligonucleotides were purchased from IDT (Integrated DNA Technologies, Coralville, IA). All oligonucleotides were purified by reversed-phase HPLC.[1] Human topoisomerase I was purified in our group by Dr. Xiangyang Wang according to the procedure reported (*10*). T4 polynucleotide kinase was purchased from New England Biolabs (Beverly, MA). Proteinase K was obtained from Invitrogen (Carlsbad, CA). [$\gamma$-$^{32}$P]ATP (6000

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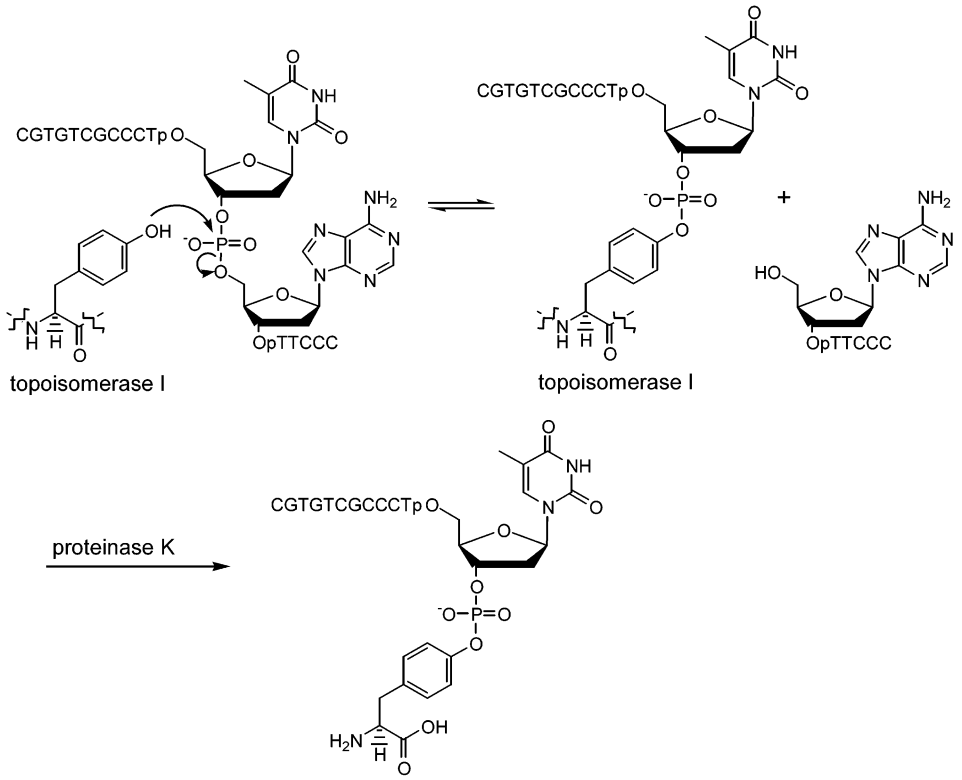


FIGURE 1: Mechanism of reversible DNA cleavage by human topoisomerase I, highlighting the transesterification process characteristic of the enzyme. Treatment of the (denatured) covalent binary complex with proteinase K affords an oligonucleotide *O*-linked to tyrosine.

Ci/mmol) was purchased from Amersham Biosciences Corp. (Piscataway, NJ).

*Oligonucleotide Substrates.* The modified oligonucleotides used in this study (Table 1) were synthesized on an Applied Biosystems ABI 391 DNA synthesizer using standard phosphoramidite chemistry (*11*, *12*) on a 1-$\mu$mol scale. Standard phosphoramidite bases and ancillary reagents were purchased from Transgenomic, Inc.; CPG support material was purchased from ChemGenes, Inc. The commercially available phosphoramidites were diluted in anhydrous acetonitrile to a concentration of 0.08 M. $N^4$-Acetyl-2′-deoxycytidine phosphoramidite was used in lieu of $N^4$-benzoyl-2′-deoxycytidine phosphoramidite to prevent ethylenediamine transamination during deblocking protocols, as reported by Hogrefe and co-workers (*13*). Stepwise coupling yields for unmodified phosphoramidites were estimated at 95% or greater by trityl cation assay at 498 nm (*14*). Coupling times were extended to 20 min for activated cassette insertion to maximize coupling efficiency. Methylphosphonate-containing oligonucleotides were cleaved and deblocked by the procedure reported by Hogrefe and co-workers (*13*, *15*) (i.e., suspension in concentrated $NH_4OH$ for 2 h at room temperature, filtration to remove CPG, and treatment of the concentrated supernatant and washings with ethylenediamine for 6 h). After concentration by vacuum centrifugation, the synthetic oligonucleotides were detritylated and purified by Nensorb chromatography according to the following procedure: the deblocked and cleaved crude oligonucleotide was applied to the activated column dissolved in 4 mL of 0.1 M TEAA, pH 7.0; the column was washed with 10 mL of 1:9 acetonitrile−0.1 M TEAA, pH 7.0, to remove failure sequences; the column was then washed with 25 mL of 0.5% aqueous trifluoroacetic acid, followed by 10 mL of 0.1 M TEAA, pH 7.0, to effect detritylation; finally, the purified oligonucleotide was eluted from the column by washing with 5 mL of 35% aqueous methanol. The oligonucleotides were purified further by reversed-phase HPLC (10 $\mu$m Econosphere $C_{18}$ in a 10 × 250 mm column, using a gradient elution of 95:5 → 65:35 0.05 M $NH_4OAc$−acetonitrile over a period of 40 min), and the DNA was recovered by lyophilization after desalting in a microconcentrator (Millipore, Bedford, MA). Analytical re-injections were performed to ensure oligonucleotide purity. MALDI-TOF mass spectral analysis was then performed to determine molecular weight (Table 1).

*Reverse Synthesis of Oligonucleotide **30**.* The necessary 5′-phosphoramidites and functionalized CPG resin were purchased from ChemGenes, Inc. Reverse oligonucleotide synthesis was performed on a 1-$\mu$mol scale according to the program included with the ABI 391 DNA synthesizer. Phosphoramidites were diluted in anhydrous acetonitrile to a concentration of 0.1 M. Stepwise coupling yields were estimated to proceed at 90% or greater on the basis of trityl cation assay (*14*) at 498 nm. Upon completion of the synthesis of the trityl-OFF oligonucleotide, the contents of the reaction column were emptied into a 10-mL disposable filtration column. The support-bound oligonucleotide was then exposed to 3 mL of 1 M methylphosphonic dichloride in pyridine for 1 h. After filtration, the CPG was then subjected to 3 mL of 1:1 pyridine−water for 30 min. The

[1] Abbreviations: HPLC, high-performance liquid chromatography; THF, tetrahydrofuran; TBAF, tetra-*n*-butylammonium fluoride; ESI, electrospray ionization; CPG, controlled pore glass; TEAA, triethylammonium acetate; EDTA, ethylenediamine tetraacetic acid; DMT, 4,4′-dimethoxytrityl; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin.

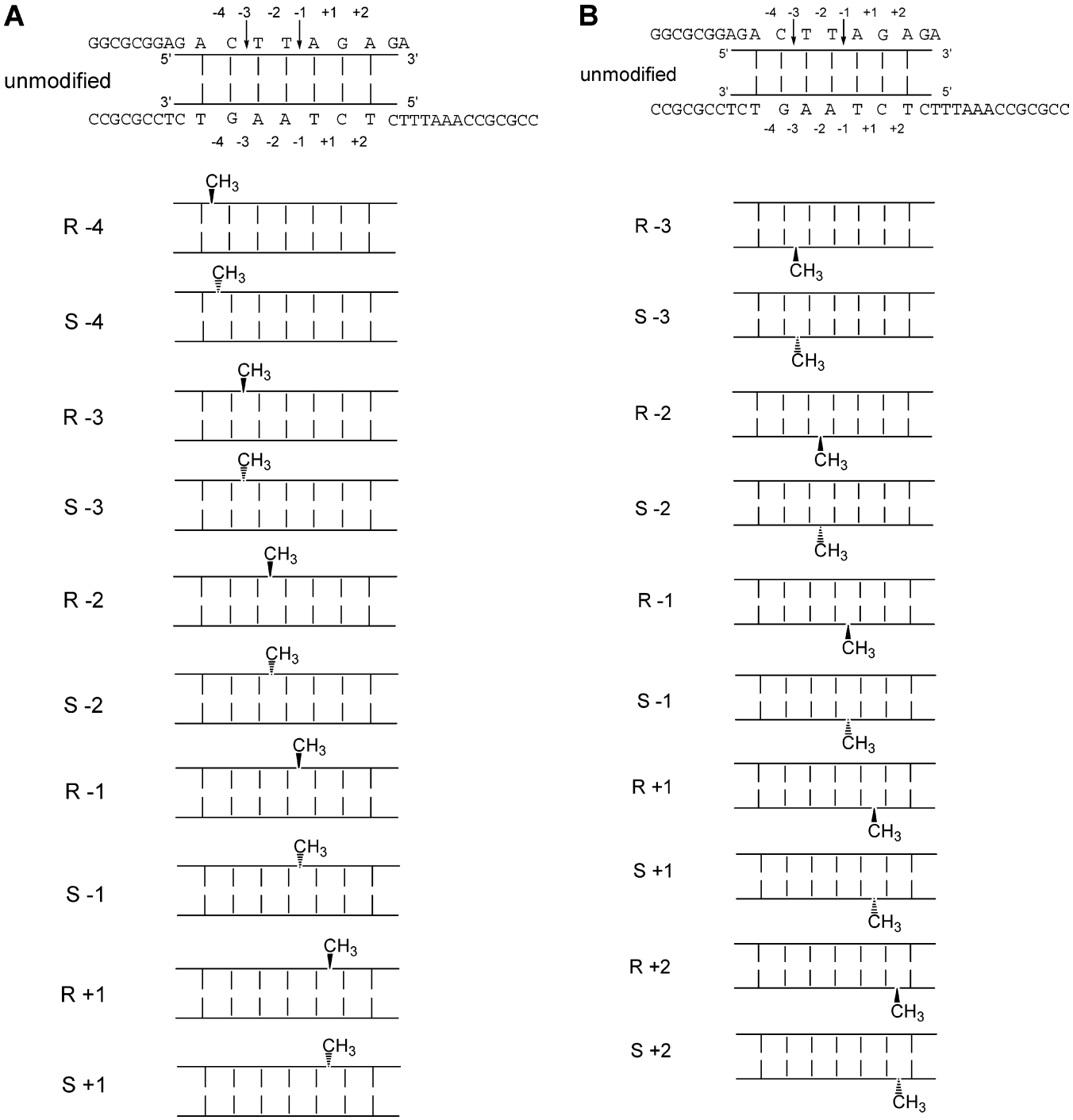


FIGURE 2: Partial duplex substrates containing single methylphosphonate linkages on the scissile (A) or noncleaved (B) strand. The positions of methylphosphonate substitution are indicated by a solid wedge ($R_p$ isomer) or a dashed wedge ($S_p$ isomer); the primary (−1) and secondary (−3) cleavage sites are indicated by arrows. The oligonucleotide pAATTTGGCGCGG was annealed to the partial duplex to facilitate cleavage by topoisomerase I (*5*).

support-bound oligonucleotide was then dried under diminished pressure and subsequently treated with 1 mL of 3:1 aqueous $NH_4OH$−ethanol at 60 °C for 12 h. The CPG was filtered and washed with two 1-mL portions of 1:1 acetonitrile−water. The combined filtrate was then concentrated under diminished pressure, and the residue was purified by reversed-phase HPLC (10 $\mu$m Econosphere $C_{18}$ in a 10 × 250 mm column, using a gradient elution of 95:5 → 65:35 0.05 M $NH_4OAc$−acetonitrile over a period of 40 min), and DNA oligonucleotide **30** was recovered by lyophilization after desalting in a microconcentrator. An analytical reinjection was performed to ensure oligonucleotide purity: yield, 2.64 mg (0.645 $\mu$mol); mass spectrum (MALDI-TOF), *m*/*z* 4091.6 (theoretical 4093.6).

*Electrophoresis.* Denaturing polyacrylamide gel (19% (w/v) acrylamide, 1% (w/v) *N*,*N*-methylenebisacrylamide, 7 M urea) electrophoresis was carried out at 500 V for 2−3 h. All gels were run in buffer (89 mM Tris−borate, pH 8.3, containing 1 mM EDTA). Denaturing polyacrylamide gel loading solution contained formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol. Gels were visualized by autoradiography and quantified utilizing a

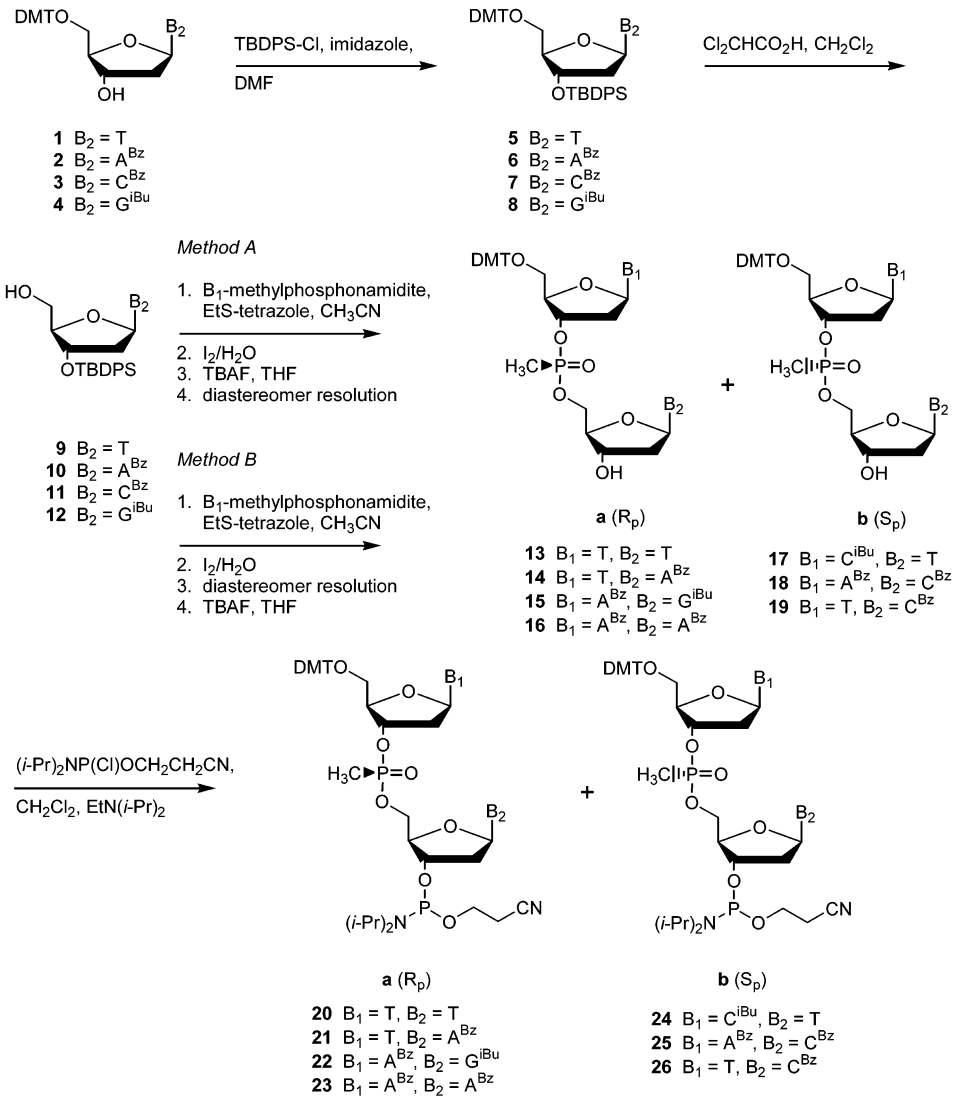


FIGURE 3: Synthetic procedures used to prepare the diastereomerically pure dinucleoside methylphosphonate cassettes; adapted from the procedure of Hogrefe et al. (*13*).

Molecular Dynamics 400 E Phosphorimager with ImageQuant version 3.2 software.

*Preparation of 5′-$^{32}$P End-Labeled Synthetic Oligonucleotides.* The synthetic oligonucleotide (10 pmol) was 5′-$^{32}$P-labeled in a reaction mixture (25 $\mu$L total volume) of 50 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$, 10 mM $\beta$-mercaptoethanol, and 0.01 mCi of [$\gamma$-$^{32}$P]ATP. The reaction was initiated by the addition of 10 units of T4 polynucleotide kinase. The reaction mixture was incubated at 37 °C for 1−2 h and terminated by heat treatment at 65 °C for 20 min to inactivate the enzyme.

*Hybridization of Substrates.* Oligonucleotides were hybridized in a reaction mixture (50 $\mu$L total volume) containing 10 mM Tris-HCl, pH 7.5, 40 mM NaCl, 5 mM $MgCl_2$, and 5 mM $CaCl_2$. The solution was heated to 80 °C for 5 min and cooled slowly to room temperature under ambient conditions (~3 h). Due to the low DNA concentration, hydbrization mixtures contained radiolabeled scissile strands (5′ $^{32}$pGG-CGC-GGA-GAC-TTA-GAG-A, 400 fmol) with a 100-fold excess of the noncleaved strand (5′ C-CGC-GCC-AAA-TTT-CTC-TAA-GTC-TCC-GCG-CC) and a 100-fold excess of a 12-mer oligonucleotide (5′pAA-TTT-GGC-GCG-G), which was used to "complete" the DNA partial duplex.

*Oligonucleotide DNA Binding by Topoisomerase I.* The 5′-$^{32}$P end-labeled DNA duplex (8 fmol) was treated with 25 ng of human topoisomerase I in a 10-$\mu$L reaction mixture containing 50 mM Tris-HCl, pH 7.5, and 100 ng/$\mu$L of BSA. The reaction mixtures were incubated at 37 °C for 30 min, adjusted to 5% glycerol, and then analyzed by 8% native polyacrylamide gel electrophoresis.

*Oligonucleotide DNA Cleavage by Topoisomerase I.* The 5′-$^{32}$P end-labeled DNA duplex (8 fmol) was treated with 25 ng of human topoisomerase I in a 10-$\mu$L reaction mixture containing 10 mM Tris-HCl, pH 7.5, 40 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 0.5 mM EDTA, and 0.5 mM DTT. The reaction mixtures were incubated at 37 °C for 2 h and then quenched by treatment with proteinase K (1 mg/mL containing 1% SDS, 37 °C, 60 min) and analyzed by 20% denaturing polyacrylamide gel electrophoresis.

*Oligonucleotide DNA Cleavage and Religation by Topoisomerase I.* The 5′-$^{32}$P end-labeled DNA duplex (8 fmol) was treated with 25 ng of human topoisomerase I and a 5′-OH acceptor oligomer (5′ $^{HO}$A-GAG-AAA-TTT-GGC-GCG-G) in 1000-fold excess relative to the radiolabeled oligonucleotide in a 10-$\mu$L reaction mixture containing 10 mM Tris-HCl, pH 7.5, 40 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 0.5

Table 1: Oligonucleotide Substrates Containing Methylphosphonates

| entry | oligonucleotide sequence[a] | coupling efficiency (%) | quantity isolated ($\mu$g) | parent ion observed[b] |
|---|---|---|---|---|
| 1 | GGCGCGGAGACTTpAGAGA ($R_p$) | 68 | 573 | 5609.43 |
| 2 | GGCGCGGAGACTTpAGAGA ($S_p$) | 78 | 404 | 5612.83 |
| 3 | GGCGCGGAGACTpTAGAGA ($R_p$) | 43 | 1140 | 5607.43 |
| 4 | GGCGCGGAGACTpTAGAGA ($S_p$) | 89 | 929 | 5613.28 |
| 5 | GGCGCGGAGACpTTAGAGA ($R_p$) | 80 | 50 | 5611.46 |
| 6 | GGCGCGGAGACpTTAGAGA ($S_p$) | 73 | 402 | 5614.09 |
| 7 | GGCGCGGAGApCTTAGAGA ($R_p$) | 66 | 482 | 5610.02 |
| 8 | GGCGCGGAGApCTTAGAGA ($S_p$) | 36 | 846 | 5609.34 |
| 9 | GGCGCGGAGACTTApGAGA ($R_p$) | 70 | 1640 | 5616.12 |
| 10 | GGCGCGGAGACTTApGAGA ($S_p$) | 45 | 752 | 5616.91 |
| 11 | CCGCGCCAAATTTCTCTpAAGTCTCCGCGCC ($R_p$) | 82 | 742 | 9033.81 |
| 12 | CCGCGCCAAATTTCTCTpAAGTCTCCGCGCC ($S_p$) | 79 | 893 | 9034.74 |
| 13 | CCGCGCCAAATTTCTCpTAAGTCTCCGCGCC ($R_p$) | 75 | 842 | 9033.38 |
| 14 | CCGCGCCAAATTTCTCpTAAGTCTCCGCGCC ($S_p$) | 75 | 747 | 9035.97 |
| 15 | CCGCGCCAAATTTCTCTApAGTCTCCGCGCC ($R_p$) | 87 | 693 | 9028.43 |
| 16 | CCGCGCCAAATTTCTCTApAGTCTCCGCGCC ($S_p$) | 64 | 927 | 9034.71 |
| 17 | CCGCGCCAAATTTCTCTAApGTCTCCGCGCC ($R_p$) | 58 | 745 | 9035.74 |
| 18 | CCGCGCCAAATTTCTCTAApGTCTCCGCGCC ($S_p$) | 60 | 918 | 9028.38 |
| 19 | CCGCGCCAAATTTCTpCTAAGTCTCCGCGCC ($R_p$) | 69 | 802 | 9033.85 |
| 20 | CCGCGCCAAATTTCTpCTAAGTCTCCGCGCC ($S_p$) | 85 | 1000 | 9028.76 |

[a] Sequences are written in a 5′ → 3′ direction; 'p' indicates the position of the methylphosphonate linkage. [b] Calculated molecular weight for human topoisomerase I scissile strand (18-mer), $M_r$ 5611.68; for noncleaved strand (30-mer), $M_r$ 9036.85.

mM EDTA, and 0.5 mM DTT. The reaction mixtures were incubated at 37 °C for 2 h and then quenched by treatment with proteinase K (1 mg/mL containing 1% SDS, 37 °C, 60 min) and analyzed by 20% denaturing polyacrylamide gel electrophoresis.

*Reaction Kinetics of DNA Cleavage by Topoisomerase I.* The 5′-$^{32}$P end-labeled DNA duplex (8 fmol) was treated with 25 ng of human topoisomerase I in a 10-$\mu$L reaction mixture containing 10 mM Tris-HCl, pH 7.5, 40 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 0.5 mM EDTA, and 0.5 mM DTT. The reaction mixtures were incubated at 25 °C for the time specified and then quenched by treatment with proteinase K (1 mg/mL containing 1% SDS, 37 °C, 60 min) and analyzed by 20% denaturing polyacrylamide gel electrophoresis. Gels were visualized by autoradiography, and the extent of covalent adduct formation (expressed as the percentage of the utilized 5′-$^{32}$P-labeled oligonucleotide that was attached to protein) was quantified by utilizing a Molecular Dynamics 400 E Phosphorimager with ImageQuant version 3.2 software. The observed rate constants ($k_{obs}$) were determined by normalizing the data to the end-point values (redefined as 100) and fitting to the equation $[100 - \%\ \text{cleavage}_{norm}] = 100\ e^{-kt}$.

*Reaction Kinetics of the DNA Religation by Topoisomerase I.* The 5′-$^{32}$P end-labeled DNA duplex (8 fmol) was treated with 25 ng of human topoisomerase I in a 10-$\mu$L reaction mixture containing 10 mM Tris-HCl, pH 7.5, 40 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 0.5 mM EDTA, and 0.5 mM DTT. The reaction mixture was incubated at 25 °C for 1 h to permit the covalent intermediate to form. Religation was initiated by simultaneous addition of NaCl to 0.5 M concentration and a 5′-OH 17-mer acceptor oligonucleotide at a 1000-fold excess relative to the radiolabeled oligonucleotide. The reaction was incubated at 25 °C for the time specified and then quenched by treatment with proteinase K (1 mg/mL containing 1% SDS, 37 °C, 60 min) and analyzed by 20% denaturing polyacrylamide gel electrophoresis. Gels were visualized by autoradiography, and the extent of religation (expressed as the percentage of the utilized 5′-$^{32}$P end-labeled oligonucleotide that was recovered as a 30-mer) was quantified by utilizing a Molecular Dynamics 400 E Phosphorimager with ImageQuant version 3.2 software. The observed rate constant ($k_{rel}$) was determined by normalizing the data to the end-point values (defined as 100) and fitting to the equation $[100 - \%\ \text{religation}_{norm}] = 100\ e^{-kt}$.

## RESULTS

*Dinucleoside Methylphosphonate Cassette Synthesis.* The synthesis of each dinucleoside methylphosphonate cassette required for synthesis of the modified partial duplex substrates for human topoisomerase I (Figure 2) is outlined in Figure 3. The synthetic strategy is based on the earlier work of Hogrefe and co-workers (*16*). The commercially available dimethoxytrityl-protected monomers **1**−**4** were protected on the 3′-hydroxyl functionality by treatment with *tert*-butyldiphenylsilyl chloride and imidazole in DMF for 3 days. DMT group removal was accomplished readily with dichloroacetic acid in methylene chloride to afford nucleosides **9**−**12**. Each of the requisite dinucleoside methylphosphonate cassettes was then prepared by admixture of the appropriate, commercially available 2′-deoxynucleoside 3′-methylphosphonamidite, EtS-tetrazole, and the corresponding nucleoside acceptor (**9**−**12**) in anhydrous acetonitrile. The coupling time was crucial to the success of the reaction and was limited to 20 min. The formed methylphosphonites were characteristically unstable and were oxidized immediately using a low-water-containing oxidizing mixture to minimize product decomposition, according to the protocols reported by Hogrefe and co-workers (*15*). Despite these optimized procedures, significant hydrolysis products were observed routinely and resulted in isolated yields <50%.

The activation of the isomerically pure dinucleoside methylphosphonate cassettes (**13**−**19**) was performed to accommodate standard phosphoramidite-based oligonucleotide synthesis protocols (*12*). Exposure to 3.0 mol equiv

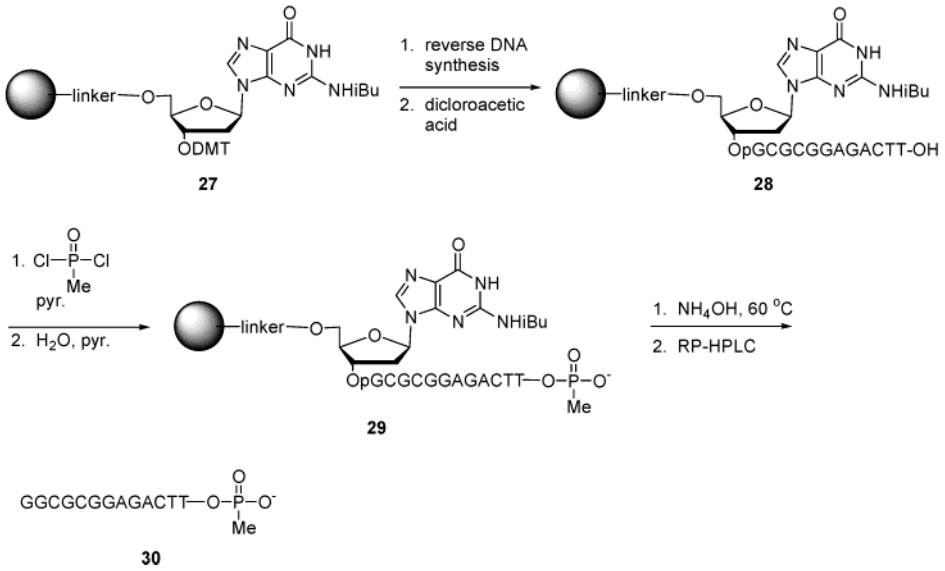


FIGURE 4: Synthesis of potential hydrolysis product **30** by reverse oligonucleotide synthesis.

of $\beta$-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite in methylene chloride and Hünig's base for 30 min, followed by chromatographic purification of the complete reaction mixture, afforded the desired functionalized dimers (**20**−**26**). Oligonucleotide synthesis on a 1-$\mu$mol scale to produce each sequence required for the topoisomerase I assays (Table 1) was then performed according to standard protocols, albeit with extended reaction times for cassette insertion to ensure maximum coupling efficiency. Also, $N^4$-acetyl-2′-deoxycytidine phosphoramidite was used in place of the more common benzoyl-protected monomer. A report by Hogrefe and co-workers suggests that typical methylphosphonate deprotection protocols will result in the transamination of $N^4$-benzoyl-2′-deoxycytidine (*15*); we did not observe this conversion to any significant extent in early synthesis attempts but used the alternative phosphoramidite monomer nonetheless. The use of reversed-phase HPLC rather than PAGE facilitated the purification of the desired oligonucleotides in superior yields. Included in Table 1 is the cassette coupling efficiency as determined by trityl cation absorbance at 498 nm (*14*), the quantity of each purified oligonucleotide obtained after desalting with microconcentrator, and the parent ion observed by MALDI-TOF mass analysis.

*3′-Methylphosphate-Terminating Oligonucleotide Construction Using Reverse DNA Synthesis Protocols.* Recently, Tian et al. reported a novel activity of vaccinia topoisomerase I observed upon cleavage of a DNA oligonucleotide duplex containing a methylphosphonate functionality at the site of cleavage. They found that a latent endonuclease activity was released when the nonbridging phosphate oxygen was replaced with a methyl group (*7*). In anticipation of a possible analogous activity residing in human topoisomerase I, we prepared the potential hydrolysis product using reverse oligonucleotide synthesis methods (*17*, *18*).

The synthetic scheme for the requisite oligonucleotide **30** is shown in Figure 4. The 5′-phosphoramidites and functionalized CPG resin needed for the synthesis are all commercially available. Once the desired sequence was completed with the final trityl group removed, the CPG was exposed to methylphosphonic dichloride in anhydrous pyridine, using a procedure adapted from Miller and co-workers for the construction of oligonucleoside methylphosphonates (*19*). The chloromethyl phosphonic ester intermediate was then hydrolyzed in 1:1 pyridine−water to afford the desired oligonucleotide terminating in 3′-methylphosphate. Deblocking and cleavage of the oligomer from the solid support was accomplished by exposure to concentrated aqueous ammonia at 60 °C for 12 h. The modified oligonucleotide was then purified by reversed-phase HPLC and finally desalted to give **30**; the molecular weight was verified by MALDI-TOF mass spectrometry.

*Human Topoisomerase I-Mediated Cleavage of Partial Duplexes Modified in the Scissile Strand.* The 5′-$^{32}$P end-labeled oligonucleotide modified scissile strands (Table 1, entries 1−10) were hybridized with an excess of unmodified noncleaved strand oligonucleotide. The partial duplex substrates were then incubated with human topoisomerase I at 37 °C for 2 h and then quenched by treatment with proteinase K to digest the covalently bound enzyme. Product formation was then analyzed by 20% denaturing polyacrylamide gel electrophoresis (Figure 5). Substrates having a methylphosphonate linkage upstream from the scissile phosphate all displayed nearly complete suppression of topoisomerase I-mediated transesterification. Of note is the partial shift of cleavage to the secondary site at the −3 position for substrates bearing modifications at the −1 and −2 sites (cf. lanes 7−10). Modification at the +1 site of the scissile strand resulted in cleavage by topoisomerase I with an efficiency approximately equal to that of the wild-type substrate for the $R_p$ methylphosphonate, and greater efficiency for the $S_p$ methylphosphonate (Figure 5, lanes 11 and 12). The rate of cleavage for the substrate containing the $S_p$ methylphosphonate was twice that of the unmodified substrate (Table 2). Clearly absent was any product resulting from latent hydrolysis activity analogous to that reported for vaccinia topoisomerase I (*7*), as demonstrated by the lack of a product band co-migrating with authentic standard **30** (lane 13).

*Human Topoisomerase I-Mediated Cleavage of Partial Duplexes Modified in the Noncleaved Strand.* The 5′-$^{32}$P end-labeled unmodified scissile strand shown in Figure 2B was hybridized with an excess of each of the oligonucleotides having a methylphosphonate linkage in the noncleaved strand

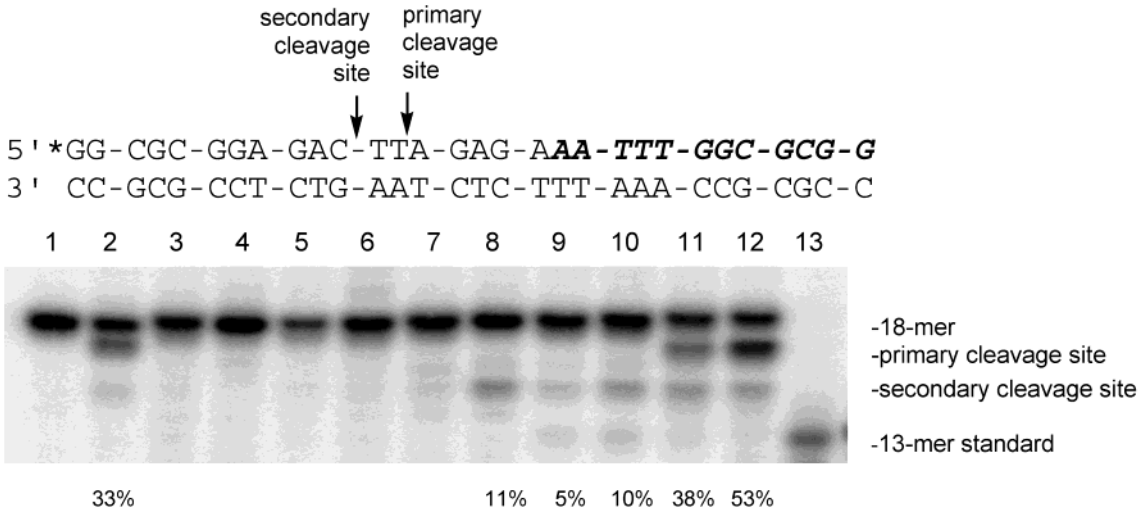


FIGURE 5: PAGE analysis of cleavage of the partial duplex substrates containing methylphosphonate substitutions in the scissile strand. Lane 1, DNA alone; lane 2, unmodified partial duplex + topoisomerase I; lane 3, $R_p$ −4 scissile substrate + topoisomerase I; lane 4, $S_p$ −4 scissile substrate + topoisomerase I; lane 5, $R_p$ −3 scissile substrate + topoisomerase I; lane 6, $S_p$ −3 scissile substrate + topoisomerase I; lane 7, $R_p$ −2 scissile substrate + topoisomerase I; lane 8, $S_p$ −2 scissile substrate + topoisomerase I; lane 9, $R_p$ −1 scissile substrate + topoisomerase I; lane 10, $S_p$ −1 scissile substrate + topoisomerase I; lane 11, $R_p$ +1 scissile substrate + topoisomerase I; lane 12, $S_p$ +1 scissile substrate + topoisomerase I; lane 13, 13-mer authentic standard **30**.

Table 2: Kinetic Parameters at Primary Cleavage Site 1 for Cleavage and Religation of Unmodified and Methylphosphonate-Containing DNA Duplexes by Human Topoisomerase I[a]

| | position of methylphosphonate | | kinetic data | | |
|---|---|---|---|---|---|
| | 18-mer | 30-mer | $k_{cl}$ ($\times 10^4$ $s^{-1}$) | $k_{rel}$ ($\times 10^4$ $s^{-1}$) | $K_{cl}^{calc}$ [b] |
| Wt | none | none | 27 ± 4 | 19 ± 1 | 1.4 ± 0.2 |
| R+1 | +1P | none | 21 ± 2 | - | - |
| S+1 | +1P | none | 39 ± 1 | - | - |
| R+1 | none | +1P | 25 ± 1 | 21 ± 1 | 1.2 ± 0.1 |
| S+1 | none | +1P | 60 ± 1 | 10 ± 1 | 6.0 ± 0.1 |
| R+2 | none | +2P | 21 ± 1 | 19 ± 1 | 1.1 ± 0.1 |
| S+2 | none | +2P | 58 ± 2 | 11 ± 1 | 5.3 ± 0.2 |

[a] Rate constants were obtained at 25 °C. The values for $k_{cl}$ and $k_{rel}$ were determined from single-turnover kinetic measurements, as described in the text, using irreversible conditions. [b] The values $K_{cl}$ were calculated from the ratio $k_{cl}/k_{rel}$.

(Table 1, entries 11−20). The partial duplex substrates were then incubated with human topoisomerase I at 37 °C for 2 h and then quenched by treatment with proteinase K to digest the covalently bound enzyme. Product formation was then analyzed by 20% denaturing polyacrylamide gel electrophoresis, and the results are shown in Figure 6. Substrates having a methylphosphonate linkage upstream from the scissile phosphodiester site (−1 to −3) were largely refractory to topoisomerase I-mediated transesterification (cf. lanes 3−8). In comparison, when substitutions were made at the +1 and +2 positions on the noncleaved strand, quantification of the cleaved bands demonstrated a cleavage efficiency comparable to that of the unmodified substrate when the $R_p$-methylphosphonates were present. There was an *increase* in cleavage efficiency of the modified substrates compared to the wild-type control for the $S_p$-methylphosphonates (cf. lane 2 and lanes 9−12). Thus, there was a slight stereochemical preference of the enzyme for cleavage of the partial duplexes containing $S_p$-methylphosphonate diastereomers at positions +1 and +2.

*Human Topoisomerase I-Mediated DNA Binding of DNA Partial Duplexes Modified in the Noncleaved Strand.* The

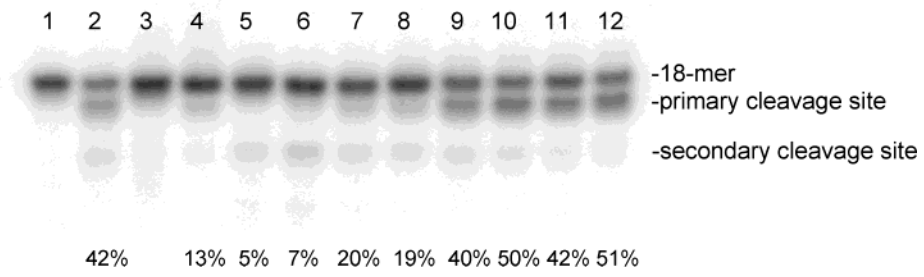


FIGURE 6: PAGE analysis of cleavage of the partial duplex substrates containing methylphosphonate substitutions in the noncleaved strand. Lane 1, DNA alone; lane 2, unmodified partial duplex + topoisomerase I; lane 3, $R_p$ −3 noncleaved modified duplex substrate + topoisomerase I; lane 4, $S_p$ −3 noncleaved duplex substrate + topoisomerase I; lane 5, $R_p$ −2 noncleaved duplex substrate + topoisomerase I; lane 6, $S_p$ −2 noncleaved duplex substrate + topoisomerase I; lane 7, $R_p$ −1 noncleaved duplex substrate + topoisomerase I; lane 8, $S_p$ −1 noncleaved duplex substrate + topoisomerase I; lane 9, $R_p$ +1 noncleaved duplex substrate + topoisomerase I; lane 10, $S_p$ +1 noncleaved duplex substrate + topoisomerase I; lane 11, $R_p$ +2 noncleaved duplex substrate + topoisomerase I; lane 12, $S_p$ +2 noncleaved duplex substrate + topoisomerase I. The cleavage efficiency, which is defined as the percentage of the cleaved substrate relative to total DNA, is shown at the bottom of each lane.

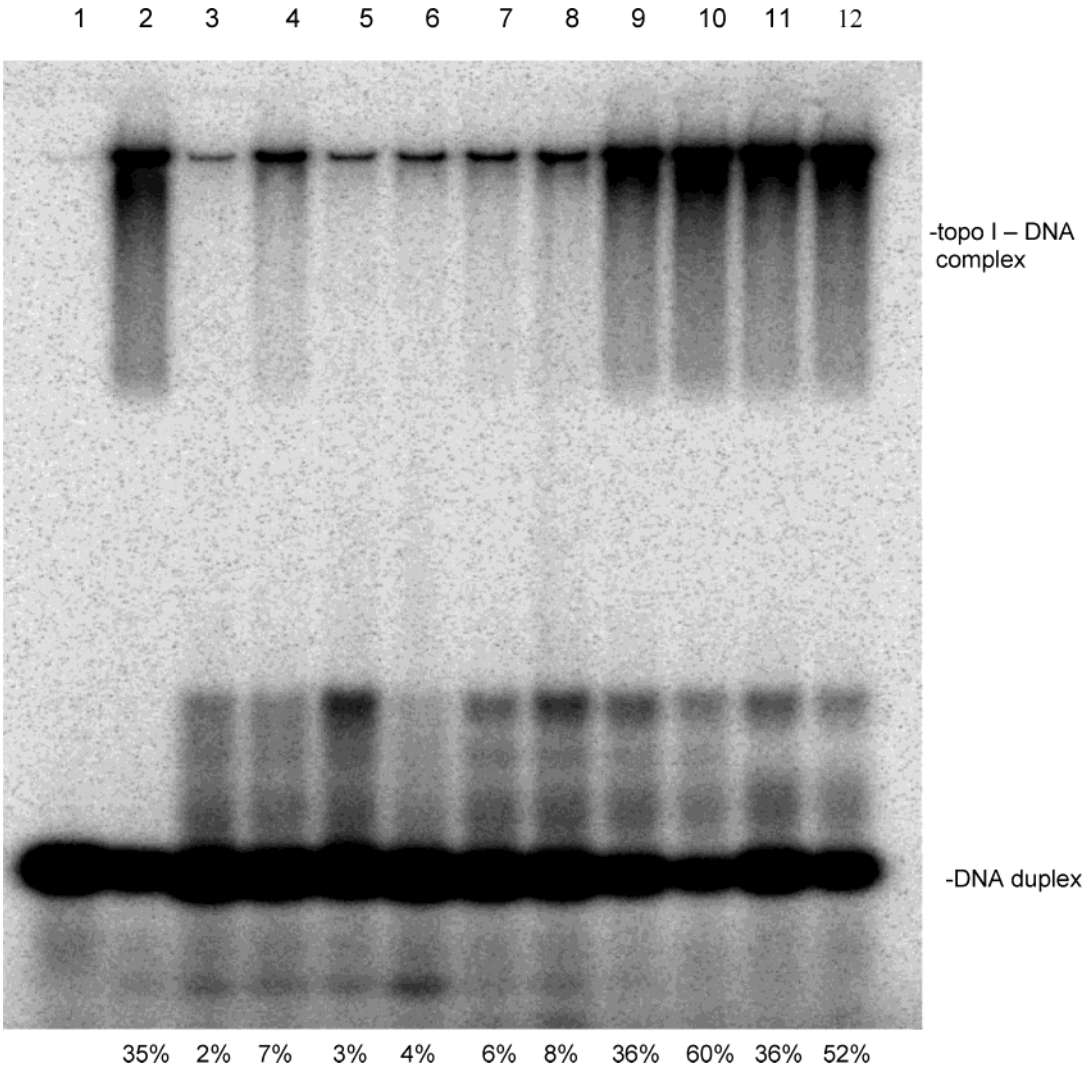


FIGURE 7: Human topoisomerase I-mediated DNA binding of DNA duplexes modified in the noncleaved strand. Autoradiogram of an 8% native polyacrylamide gel. The reactions were carried out as described in the Experimental Procedures. Lane 1, DNA alone; lane 2, unmodified DNA duplex + topoisomerase I; lane 3, $R_p$ −3 noncleaved modified duplex substrate + topoisomerase I; lane 4, $S_p$ −3 noncleaved duplex substrate + topoisomerase I; lane 5, $R_p$ −2 noncleaved duplex substrate + topoisomerase I; lane 6, $S_p$ −2 noncleaved duplex substrate + topoisomerase I; lane 7, $R_p$ −1 noncleaved duplex substrate + topoisomerase I; lane 8, $S_p$ −1 noncleaved duplex substrate + topoisomerase I; lane 9, $R_p$ +1 noncleaved duplex substrate + topoisomerase I; lane 10, $S_p$ +1 noncleaved duplex substrate + topoisomerase I; lane 11, $R_p$ +2 noncleaved duplex substrate + topoisomerase I; lane 12, $S_p$ +2 noncleaved duplex substrate + topoisomerase I. The binding efficiency, which is defined as the percentage of the DNA−topoisomerase I complex relative to the total DNA substrate, is shown at the bottom of each lane.

5′-$^{32}$P end-labeled unmodified scissile strand was hybridized with an excess of each of the oligonucleotides having a methylphosphonate linkage, as described in Experimental Procedures. The DNA duplex substrates (Figure 2B) were then incubated with human topoisomerase I at 37 °C for 30 min and analyzed by 8% native polyacrylamide gel electrophoresis, as shown in Figure 7. It was interesting to note that substrates having a methylphosphate linkage at or upstream from the scissile phosphodiester were barely bound by topoisomerase I (Figure 7, lanes 3−8), while those substrates bearing a methylphosphonate linkage downstream from the scissile phosphodiester were bound well (Figure 7, lanes 9−12). When the methylphosphonate group was at positions +1 and +2, the $R_p$ isomers had an enzyme binding efficiency comparable to that of wild-type DNA substrate; the $S_p$ isomers showed an increased binding efficiency (Figure 7). Thus, the DNA duplexes containing $S_p$-methylphosphonate diastereomers at positions +1 and +2 showed similar stereochemical preference of topoisomerase I binding as well as cleavage.

*Human Topoisomerase I-Mediated Religation of DNA Partial Duplexes Modified in the Noncleaved Strand.* In addition to DNA binding and cleavage activity, it was also interesting to study topoisomerase I-mediated DNA religation. As described above, the 5′-$^{32}$P end-labeled unmodified scissile strand was hybridized individually with an excess of each of the oligonucleotides having a methylphosphonate linkage. The DNA duplex substrates (Figure 2B) were then incubated with human topoisomerase I in the presence of a large excess of a 17-mer acceptor at 37 °C for 2 h and quenched by treatment with proteinase K to digest the covalently bound enzyme. Product formation was analyzed by 20% denaturing polyacrylamide gel electrophoresis (Figure 8). In common with the substrate cleavage reaction, topoisomerase I-mediated religation was observed primarily for those substrates having a methylphosphonate linkage

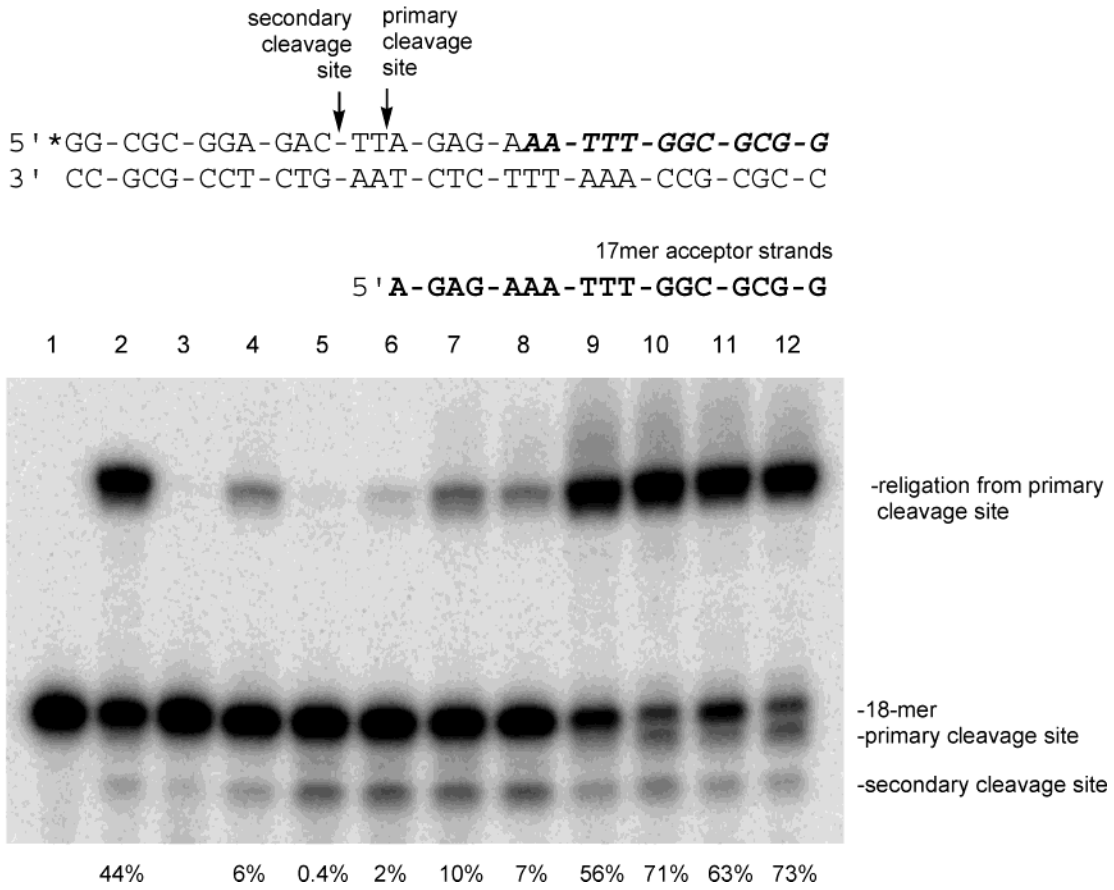


FIGURE 8: Human topoisomerase I-mediated cleavage and religation of DNA suicide duplexes modified in the noncleaved strand. Autoradiogram of a 20% denaturing polyacrylamide gel. The reactions were carried out as described in the Experimental Procedures. Lane 1, DNA alone; lane 2, unmodified DNA duplex + topoisomerase I; lane 3, $R_p$ −3 noncleaved modified duplex substrate + topoisomerase I; lane 4, $S_p$ −3 noncleaved duplex substrate + topoisomerase I; lane 5, $R_p$ −2 noncleaved duplex substrate + topoisomerase I; lane 6, $S_p$ −2 noncleaved duplex substrate + topoisomerase I; lane 7, $R_p$ −1 noncleaved duplex substrate + topoisomerase I; lane 8, $S_p$ −1 noncleaved duplex substrate + topoisomerase I; lane 9, $R_p$ +1 noncleaved duplex substrate + topoisomerase I; lane 10, $S_p$ +1 noncleaved duplex substrate + topoisomerase I; lane 11, $R_p$ +2 noncleaved duplex substrate + topoisomerase I; lane 12, $S_p$ +2 noncleaved duplex substrate + topoisomerase I. The extent of each reaction, defined as the percentage of the formation of recovered 30-mer relative to the total DNA substrate, is shown at the bottom of each lane.

downstream from the scissile phosphodiester (Figure 8). Quantification of the recovered 30-mer for the substrates with $R_p$ substitutions at +1 and +2 positions illustrated a reaction efficiency slightly greater than that of the unmodified substrate; there was strongly enhanced efficiency for the $S_p$ substitutions (Figure 8, lane 2 and lanes 9−12). Thus, these results also demonstrated the stereochemical preference for $S_p$-methylphosphonate diastereomers at positions +1 and +2.

*Kinetic Analysis of Topoisomerase I-Mediated Cleavage of Partial Duplexes Bearing Methylphosphonates at Positions +1 and +2.* In view of the results obtained with the noncleaved strand having methylphosphonates in the +1 and +2 positions, we sought to determine the kinetic parameters for cleavage of these species. Figure 9A shows a representative polyacrylamide gel electrophoresis analysis performed for each of the four modified partial duplex substrates having a methylphosphonate at position +1 or +2 on the noncleaved strand. An analysis was also performed on the wild-type substrate for comparison. In each case, the 5′-$^{32}$P end-labeled unmodified scissile strand shown in Figure 2B was hybridized with an excess of the noncleaved oligonucleotide strand, and the formed partial duplex was then treated with human topoisomerase I. The reactions were carried out at 25 °C in order to facilitate quantitation. Proteolysis with proteinase K, followed by polyacrylamide gel analysis, was then performed for each time interval tested. Visualization of the cleaved bands was performed using autoradiography to facilitate quantification and rate constant determination.

The time course of cleavage is shown in Figure 9B. The resulting kinetic parameters are summarized in Table 2. The $S_p$-methylphosphonate substitution at the +1 and +2 positions was remarkable in that the observed rates were more than twice that of the wild-type substrate, and the transformation proceeded significantly further as well. While the other modified partial duplex substrates all had overall cleavage efficiencies at least comparable to that of wild type, the rates of each $R_p$ isomer were nominally lower than that of the wild-type substrate.

*Kinetic Analysis of Human Topoisomerase I-Mediated Religation of DNA Partial Duplexes Bearing Methylphosphonates at Positions +1 and +2.* To determine whether the methylphosphonate modification on the DNA substrate affects the reaction equilibrium between topoisomerase I-mediated cleavage and religation, we investigated the religation rate constants for those species having methylphosphonate linkage at the +1 and +2 positions on the noncleaved strand and calculated the corresponding cleavage equilibrium constants $K_{cl}$ ($K_{cl} = k_{cl}/k_{rel}$). Figure 10A shows

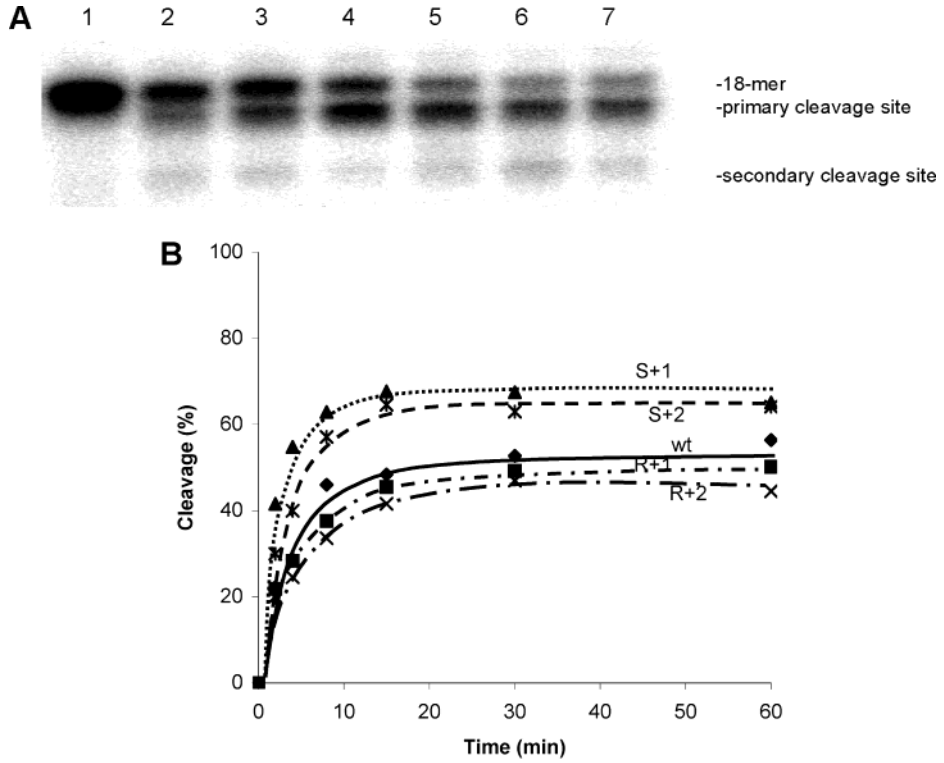


FIGURE 9: (A) Autoradiogram of a 20% denaturing polyacrylamide gel illustrating the time course of human topoisomerase I-mediated cleavage at 25 °C of the DNA substrate containing an $S_p$-methylphosphonate at position +2. The reactions were carried out as described in the Experimental Procedures. Aliquots were removed at the times indicated, digested with proteinase K, and applied to the gel. Lane 1, 0 min; lane 2, 2 min; lane 3, 4 min; lane 4, 8 min; lane 5, 15 min; lane 6, 30 min; lane 7, 60 min. (B) Time course of human topoisomerase I-mediated cleavage reaction with DNA duplexes containing methylphosphonate substitutions within the noncleaved strand.

a representative polyacrylamide gel electrophoresis analysis performed for each of the kinetic measurements. The DNA duplex substrate was first treated with human topoisomerase I at 25 °C for 1 h to permit accumulation of the enzyme−DNA covalent intermediate. After quenching of the cleavage reaction by addition of NaCl, the religation reaction was initiated by addition of a 5′-OH-terminated 17-mer acceptor in large excess. The reaction for each time interval was quenched by addition of SDS, followed by treatment with proteinase K, and then analyzed by polyacrylamide gel electrophoresis.

The time course of religation is shown in Figure 10B. The resulting religation rate constants are summarized in Table 2. The rate constants for religation for the substrates having $S_p$-methylphosphonate substitutions at the +1 and +2 positions were only about half that of the wild-type substrate, while the substrates with $R_p$-methylphosphonate substitutions had rate constants comparable to that of wild-type substrate. The results demonstrate that the reaction equilibrium was shifted toward cleavage for those substrates with an $S_p$ modification. However, little change was introduced by the $R_p$ modifications.

## DISCUSSION

To initiate DNA cleavage, topoisomerase I must bind to DNA (*20*), forming a noncovalent enzyme−DNA complex. Many factors could affect DNA−protein contacts, including hydrogen bonds or hydrophobic and ionic interactions. Direct hydrogen bonds between DNA-binding proteins and the backbone phosphates make up about half of all direct hydrogen bonds observed in protein−DNA complexes (*21*). Recently, in the crystal structures of human topoisomerase I and the noncovalent complex with DNA, it was proposed that the human topoisomerase I−DNA contacts are limited almost exclusively to protein−phosphate interactions (*8*, *22*), as described in Figure 11.

To further illustrate human topoisomerase I−DNA interaction, we modified the phosphodiester backbone by introducing a methyl group to replace one of the nonbridging oxygens (Figure 2). The methylphosphonate linkage neutralizes the negatively charged phosphate ester, introduces a hydrophobic residue, and imparts chirality to the phosphorus atom at the point of substitution. As a result, the topoisomerase I−DNA contacts could possibly be changed due to impaired hydrogen-bonding interactions as well as changed microenvironments in proximity to the point of substitution.

First reported by Miller and Ts'o in 1979 (*23*, *24*), the methylphosphonate linkage replaces one of the nonbridging oxygen atoms with a methyl group, effectively neutralizing the negatively charged phosphate ester at the point of substitution. Oligonucleotides constructed wholly from methylphosphonate monomers, denoted MATAGENS (*ma*sking *ta*pe for *gen*e expression), were among the earliest to be investigated as potential antisense agents. This was a consequence of some promising characteristics, including lack of degradation by cellular nucleases (*25*−*27*), rapid cell uptake (*28*−*30*), and high sequence selectivity at the level of hybridization (*23*, *28*). Unfortunately, the chirality about the phosphorus atom results in complex mixtures of diastereomers when oligonucleotides are prepared incorporating multiple methylphosphonamidite monomers. This problem is the subject of continuing study (*31*−*35*).

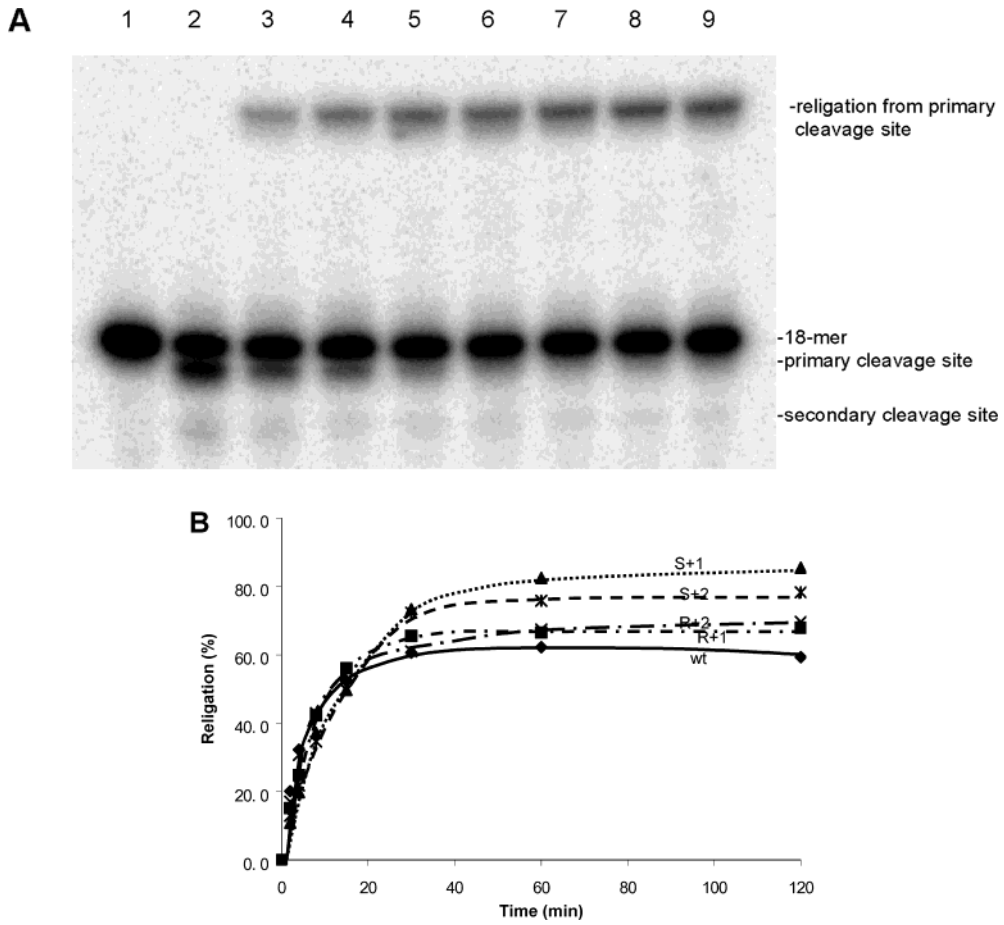


FIGURE 10: (A) Autoradiogram of a 20% denaturing polyacrylamide gel illustrating the time course of human topoisomerase I-mediated DNA religation at 25 °C (unmodified substrate). The reactions were carried out as described in the Experimental Procedures. The covalent topoisomerase I−DNA complex was permitted to accumulate for 1 h. The religation reaction was initiated by the simultaneous addition of NaCl and 5′-OH terminated 17-mer acceptor. Aliquots of the reaction were removed at the times indicated, digested with proteinase K, and utilized for gel analysis. Lane 1, DNA alone; lane 2, 0 min; lane 3, 2 min; lane 4, 4 min; lane 5, 8 min; lane 6, 15 min; lane 7, 30 min; lane 8, 120 min. (B) Time course of human topoisomerase I-mediated religation reaction with DNA duplexes containing methylphosphonate substitution within the noncleaved strand.

The synthesis of diastereomerically pure methylphosphonate-containing oligonucleotides has typically focused upon the coupling of suitably protected monomeric acceptor nucleosides to nucleoside methylphosphonamidites to produce diastereomeric dinucleoside methylphosphonates, which are then subject to an appropriate chromatographic protocol to resolve the $R_p$ and $S_p$ diastereomers (*36*−*38*). All 16 possible dinucleoside methylphosphonates have been prepared and reported (*13*, *15*, *16*, *36*−*38*). Reports that focus on oligonucleotides containing the two diastereomers suggest that the oligonucleotides containing $R_p$-methylphosphonates form more stable hybrid duplexes with target substrates when compared to those containing $S_p$-methylphosphonates, as measured by $T_m$ analysis. The observation has been suggested to result from the greater stacking ability of the $R_p$-methylphosphonates (*38*).

Our synthetic protocol for dinucleoside methylphosphonate synthesis was adapted from the procedure reported by Reynolds et al. in 1996 (*16*). The single change in protocol initially employed was the use of reversed-phase HPLC to resolve the diastereomeric mixture resulting after coupling of the monomeric components (Method A, Supporting Information). It has been reported that the diastereomers can be separated through the use of normal-phase flash columns, and we were able to implement this technique when appropriate in-line UV monitoring equipment was employed. Complete baseline resolution of the two isomers was still rarely observed, and incompletely separated fractions required additional purification.

Through the selective replacement of single pro-R or pro-S nonbridging oxygen atoms with a methyl group, it is possible to eliminate topoisomerase I−DNA hydrogen-bonding interactions at the point of substitution and thereby gain inferences about the effects of individual enzyme−DNA interactions. Obviously, this analysis depends on the assumption that the introduced methyl group is an acceptable replacement for the oxygen anion. Recently, the strategy was applied to the study of vaccinia topoisomerase I. Most surprising were the results obtained when isomerically pure methylphosphonate cassettes were introduced in lieu of the scissile phosphate itself (*7*). First, the change in transesterification rate was different for each diastereomer; introduction of the $S_p$-methylphosphonate linkage resulted in a rate decrease of 200 times compared to wild-type, whereas the rate decrease associated with the $R_p$ diastereomer was on the order of 3600. The results suggested a difference in stabilization for each nonbridging oxygen atom. Second, upon substrate cleavage by the enzyme, a rapid hydrolysis

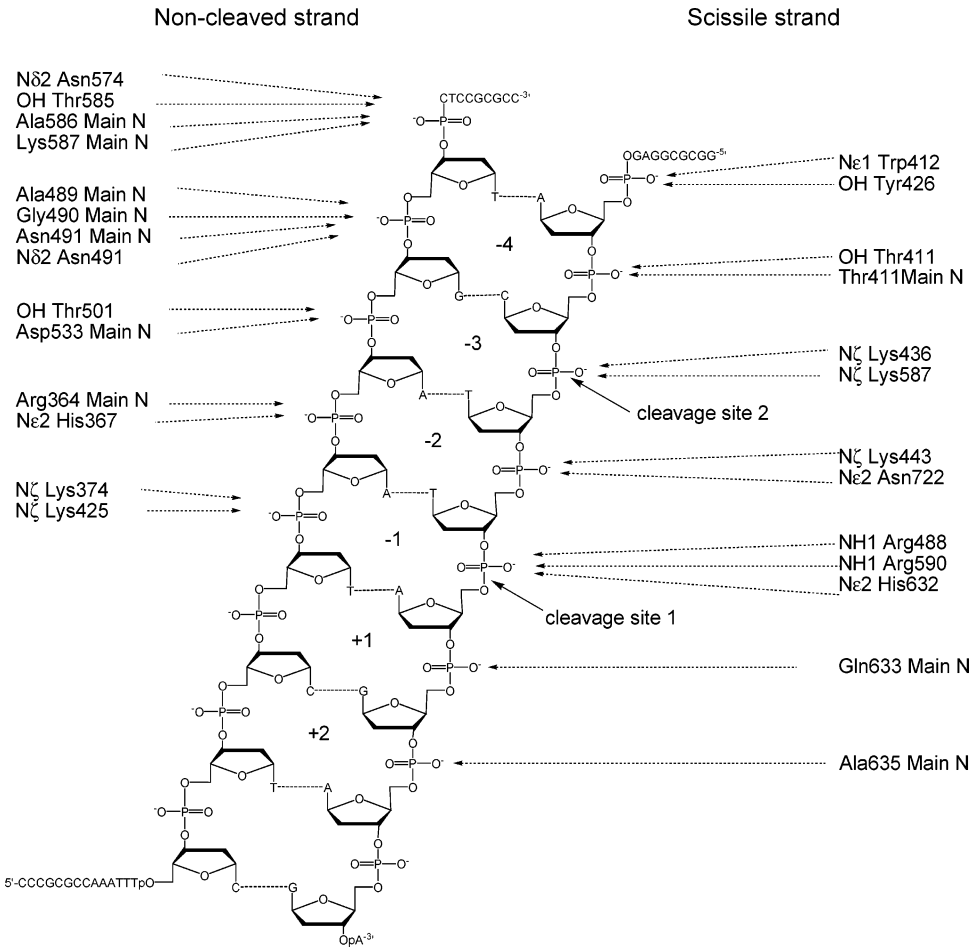


FIGURE 11: Hydrogen bond contacts to the partial duplex substrate of human topoisomerase I proposed by Redinbo et al. (*8*).

of the enzyme−DNA binary complex was observed, suggesting the presence of a latent endonuclease activity for vaccinia topoisomerase I that had not been recognized previously. It is well documented that methylphosphonate linkages in DNA are quite sensitive to even moderately basic conditions (*39*); exposure to such conditions causes the rapid hydrolytic decomposition of these linkages. In this context, it should be emphasized that the rapid hydrolysis observed in the presence of vaccinia topoisomerase I was enzyme-dependent; thus it can properly be attributed to an endonucleolytic activity of the enzyme rather than to simple chemical hydrolysis.

In view of the results obtained with vaccinia topoisomerase I, it was of interest to carry out similar experiments with human topoisomerase I. As shown in Figure 5, there was a complete lack of an analogous endonuclease activity associated with human topoisomerase I, as evidenced by the lack of product bands co-migrating with the synthetic oligonucleotide (**30**) whose composition is identical to the potential hydrolysis product. The divergence of results for the vaccinia and human topoisomerases I parallels the differences found between these enzymes when oligonucleotides containing 3′-deoxynucleosides at single positions were used as substrates (*3*, *4*, *6*).

The cleavage data for the partial duplexes containing methylphosphonate substitutions in the scissile strand are shown in Figure 5. Noteworthy is the very limited cleavage of any substrate containing a methylphosphonate at, or upstream from, the scissile phosphate bond. These results contrast with the observations made for the vaccinia topoisomerase I. For the viral enzyme, none of the modifications resulted in complete suppression of cleavage, although the cleavage rates were affected to varying degrees (*9*). These results argue for a requirement for hydrogen bond contacts between human topoisomerase I and its DNA substrate as a prerequisite for substrate cleavage. Hol and co-workers recently reported the crystallographic structure of a truncated human topoisomerase I bound to its duplex substrate (*8*). The arrangement of hydrogen bond contacts (Figure 11) is in very good agreement with our observations, although the extreme functional consequences of eliminating single hydrogen bonds could not have been anticipated. In essence, replacement of *any* of the nonbridging oxygen atoms upstream from and including the high-affinity cleavage site of human topoisomerase I was severely detrimental to substrate cleavage, although each of these positions was reported to make at least two and sometimes three contacts to the enzyme (*8*). The modest cleavage bands actually observed were due to cleavage at an upstream site previously described (*5*, *40*, *41*) and may reflect a limited capacity for the enzyme to bind to oligonucleotides containing methylphosphonates upstream from the cleavage site. This observation is similar to the results obtained when using substrate oligonucleotides having 3′-deoxynucleosides, as reported previously (*3*).

Also of interest was the effect of cleavage efficiency when a methylphosphonate was introduced at the position just downstream from the scissile phosphate, i.e., the +1 position

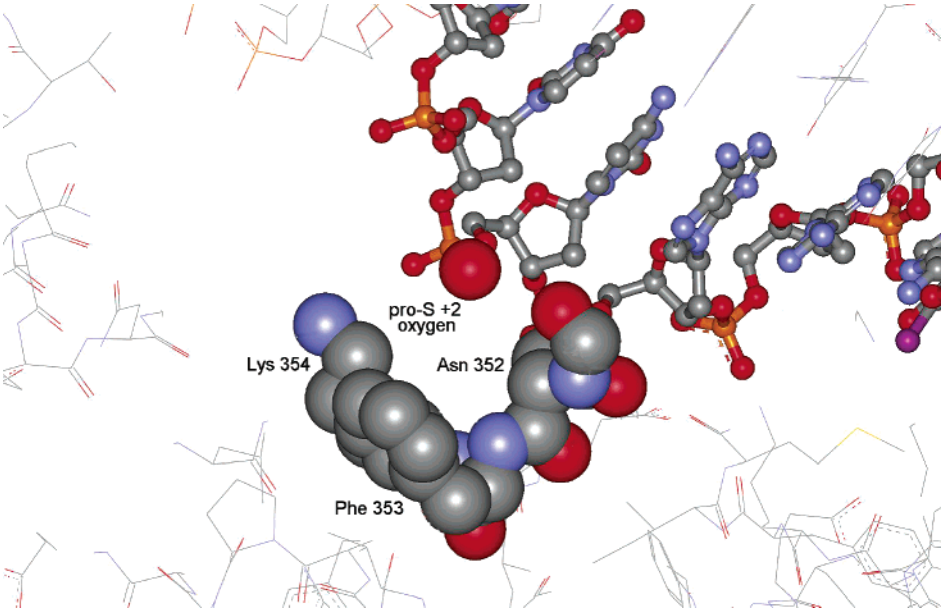


FIGURE 12: Putative hydrophobic pocket proximal to the pro-S +2 position of the noncleaved strand of the partial duplex substrate for human topoisomerase I. The putative pocket is composed of the side chain C atoms of Asn352, Phe353, and Lys354. Oxygens are shown in red, nitrogens are in blue, carbons are in gray, and phosphorus atoms are in orange. Atoms of interest are shown with their approximate van der Waals radii. The locations of the hydrogen atoms are not shown in the crystallographic data. The picture was produced using WebLab ViewerPro 4.0 (www.msi.com) and the Brookhaven Protein Data Bank coordinates 1K4S titled "Human DNA Topoisomerase I in Covalent Complex with a 22 Base Pair DNA Duplex".

(Figures 2A and 5). The crystal structure of topoisomerase I shows a single hydrogen bond contact between Gln633 and the +1 phosphate. Upon methylphosphonate modification at this site with the $R_p$ diastereomer, the cleavage efficiency was comparable to that of the wild-type substrate, while introduction of the $S_p$ diastereomer resulted in enhanced cleavage efficiency (Figure 5, lanes 11 and 12). Thus, it may be concluded that the +1 hydrogen bond contact is not critical to substrate binding by the enzyme and, as a result, does not diminish the facility of cleavage.

The cleavage of the oligonucleotides containing methylphosphonates in the noncleaved strand of the partial duplex substrate mirrored those obtained from the scissile strand. Indeed, all modifications in positions at, or upstream from, the scissile phosphate bond arrested cleavage, presumably by affecting substrate binding by the enzyme. Again, the crystal structure exhibits multiple hydrogen bond contacts between the enzyme and each of the upstream phosphates. Clearly, the introduction of a hydrophobic residue into a site intended for a charged O atom may further destabilize topoisomerase I−DNA substrate interaction. In the absence of such interactions as described for the +1 and +2 positions (*8*), the methylphosphonate substitutions were well tolerated; in fact, the analogues were cleaved with greater efficiency than the wild-type partial duplex. In the case of the $S_p$ +1 and $S_p$ +2 methylphosphonate substitutions, in particular, the cleavage rates were more than twice that of the unmodified substrate (Table 2).

The foregoing observations support a significant role for the phosphodiester backbone of DNA in topoisomerase I-mediated DNA cleavage. To confirm our conclusion that the suppression of DNA cleavage was due to diminished binding, two further experiments were carried out with DNA duplexes containing methylphosphonate substitutions in the noncleaved strand. The results from a binding assay (Figure 7) showed that, for those substrates having a methylphosphate linkage at or upstream from the scissile phosphodiester, topoisomerase I exhibited sharply diminished binding (Figure 7, lanes 3−8), while for those substrates bearing a methylphosphonate linkage downstream from the scissile phosphodiester bond, topoisomerase I bound to the modified DNA substrate (Figure 7, lanes 9−12). In addition, the results from a DNA religation experiment (Figure 8) showed that there was no significant religation of substrates having a methylphosphonate linkage upstream from the site of cleavage.

*The Existence of a Putative Hydrophobic Pocket.* Through replacement of the nonbridging oxygen atoms with a methyl group, an asymmetric center is introduced into the phosphodiester backbone of the DNA duplex. Our experiments demonstrated that the $R_p$ diastereomers at the +1 and +2 positions had DNA substrate binding efficiencies (Figure 7) as well as cleavage and religation activities (Figures 5, 6, and 8) comparable to those of the wild-type DNA substrate, while the $S_p$ diastereomers at these positions exhibited increased binding (Figure 7) and cleavage efficiencies (Figures 5, 6, and 8). By study of the kinetic parameters of the cleavage and religation reactions with the DNA substrate modified at the +1 and +2 positions (Figures 9 and 10, Table 2), it was demonstrated that the cleavage rate constants of the oligonucleotides containing $S_p$-methylphosphorates at the +1 and +2 positions were almost twice those of the unmodified substrate; however, the rate constants for religation were only half those of the unmodified substrate. Obviously, the equilibrium of the cleavage and religation reaction shifted toward cleavage due to the increased binding efficiency and the decreased religation activity of the DNA substrates having an $S_p$-methylphosphonate linkage. These results are consistent with stabilized topoisomerase I−DNA interaction due to the introduction of a hydrophobic residue into a site intended for a charged O atom. In fact, Figure 12 demonstrates the existence of a possible hydrophobic pocket surrounding the pro-S oxygen atom at the +2 position of

the noncleaved strand of the partial duplex. The side chains of Asn 352, Phe353, and Lys354 may contribute to this pocket. Introduction of the nonpolar methyl group at this position could thus lead to increased binding and cleavage efficiency.

In summary, the present results demonstrate the use of oligonucleotides containing methylphosphonates at single positions for a mapping of the cleavage requirement of human topoisomerase I. While modifications in all positions upstream from the scissile phosphate bond resulted in nearly complete suppression of cleavage activity, the downstream substitutions showed activity that was either comparable to or greater than that of the wild-type substrate. These findings are entirely consistent with the recent crystal structure reported for a human topoisomerase I construct (*8*).

## ACKNOWLEDGMENT

We thank Dr. Xiangyang Wang for the topoisomerase I used in this study.

## SUPPORTING INFORMATION AVAILABLE

Additional experimental procedures including $^{1}H$ and $^{13}C$ NMR data and high resolution mass spectrometry data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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