Toward a Mechanism-Based Fluorescent Assay for Site-Specific Recombinases and Topoisomerases: Assay Design and Syntheses of Fluorescent Substrates

Gagan Panigrahi,† Bao-ping Zhao,† Jiri J. Krepinsky, and Paul D. Sadowski*

Contribution from the Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario M5S 1A8, Canada

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Abstract: Site-specific recombinases and topoisomerases break and rejoin the phosphodiester bonds of DNA. Both classes of enzymes do so through the formation of a covalent intermediate involving a phosphodiester bond with a hydroxylated amino acid (usually tyrosine). We have previously shown that oligonucleotides that bear a 3′-phosphoryltyrosine residue linked to the phosphoryl group via a phenolic hydroxyl group are effective substrates for the assay of ligation by the FLP recombinase and mammalian topoisomerase I. In this article we describe the synthesis of oligonucleotides bearing several novel 3′-phosphoryl substituents. It is shown that oligonucleotides bearing a 3′-phosphoryltyrosine residue N-substituted on tyrosine with the bulky fluorescent groups dansyl and pyrene are ligated effectively by the FLP recombinase, and the dansyltyrosine derivative is used as effectively as the tyrosine adduct by mammalian topoisomerase I. The dansyl derivatives were completely stable during the syntheses; this underlines the potential usefulness of the dansylated class of compounds for the development of simplified assays and for mechanistic studies of breaking−joining enzymes.

Introduction

Topoisomerases and conservative site-specific recombinases catalyze the breaking and joining of DNA.[1,2] Topoisomerases are essential enzymes that relieve the superhelical strain introduced into DNA by the processes of DNA replication and transcription.[1] Site-specific recombinases catalyze a variety of genome rearrangements of episomal DNAs such as bacteriophages and plasmids. Both classes of enzymes share the property of conserving the energy of the phosphodiester backbone by forming a covalent phosphodiester bond between a nucleophilic amino acid on the protein (tyrosine or serine) and a phosphate terminus on the broken DNA (see Figure 1). In the first step of these reactions, breakage of the phosphodiester bond is accompanied by formation of a phosphate ester bond between one of the two DNA fragments and the hydroxylated amino acid in the enzyme active site (usually tyrosine). In the second step of the reaction sequence, the 5′-hydroxyl group of the 2′-deoxyribose on the other DNA fragment displaces the hydroxylated amino acid and reforms the phosphodiester bond.

In the case of topoisomerases, the breaking−joining reaction changes the linking number of the DNA. The conservative site-specific recombinases promote the joining of DNA molecules in novel recombinations. In addition, they possess either nonspecific or site-specific topoisomerase activity.[3,4] The FLP recombinase is a member of the integrase family of conservative site-specific recombinases. The enzyme is encoded by the autonomously replicating 2 μm plasmid of the yeast *Saccharomyces cerevisiae* and promotes the amplification of the plasmid.[5,6] *In vitro* studies have yielded considerable insight into the catalytic mechanism of the breakage-joining reaction of this protein.[7]

The active site of FLP[8,9] contains four residues that are absolutely conserved among all the integrase family members: arginine 191, histidine 305, arginine 308, and tyrosine 343. (The numbers refer to the amino acid residues of the FLP protein.) The catalytic triad of R191, H305, and R308 has been implicated in the activation of the scissile phosphodiester bonds of the *F*LP *r*ecognition *t*arget site (FRT site) for cleavage, and R191 and H305 have been implicated in the rejoining step of the reaction (ligation). The Y343 residue is the nucleophile that facilitates cleavage of the phosphodiester bonds and covalently attaches the protein to the 3′-phosphoryl group at the site of the break. The ligation stage of the reaction results from a nucleophilic attack of the 5′-hydroxyl group upon the phosphotyrosine bond with the resultant reclosure of the phosphodiester backbone and liberation of the protein.[7]

We have developed a series of substrates to assay the ligation step of the reaction. A nicked FRT-site substrate that contained a 5′-hydroxyl group and an adjacent 3′-phosphotyrosine group underwent efficient FLP-mediated ligation. This reaction did not require the nucleophilic tyrosine 343.[10] Analogous substrates were synthesized chemically, and the ligation reaction was extended to other site-specific recombinases and mammalian topoisomerase I.[11,12]

Since topoisomerases are the targets of agents useful in antimicrobial and antineoplastic chemotherapy,[13] we sought to

* Corresponding author (416) 978-6061 (tel), (416) 978-6885 (fax), P.sadowski@utoronto.ca (e-mail).

† The first two authors contributed equally to the experiments in this paper; order of listing is alphabetical.

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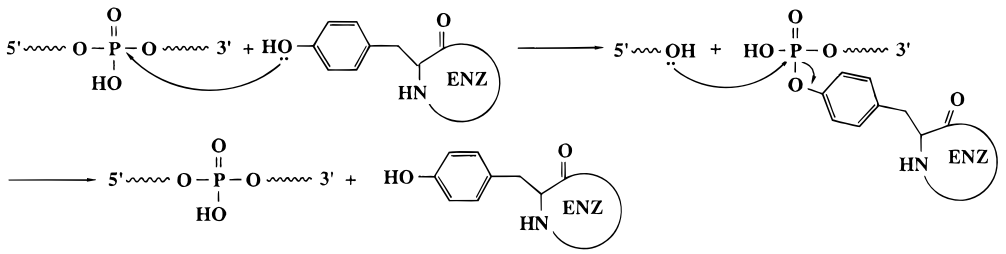

Figure 1. Chemistry of FLP- or topoisomerase I-mediated cleavage and ligation (top left). A tyrosine hydroxyl acts as a nucleophile to break a specific phosphoester bond to yield free a 5′-OH and a 3′-enzyme phosphotyrosyl intermediate (top right). A nucleophilic attack of a 5′-OH group upon the phosphotyrosyl linkage reforms the phosphodiester bond and liberates the enzyme (bottom).

develop fluorescent substrates that might be useful in high-throughput assays for screening novel therapeutic candidates available through recent advances in synthetic combinatorial chemistry. The development of such assays has at least three requirements: (i) the synthesis of the substrates must be compatible with commonly used automated methods of oligonucleotide synthesis; (ii) the bulky fluorescent groups must not interfere with the ligation activity of the enzymes; (iii) it must be possible to detect both products of the ligation reaction, namely, the elongated oligonucleotide as well as the liberated fluorescent compound. In this article we describe the synthesis of fluorescent tyrosine−oligonucleotide substrates for the FLP recombinase and mammalian topoisomerase I and the use of the substrates for the assay of these enzymes.

Experimental Section

Chemical Synthesis of 3′-Substituted Oligonucleotides. General Methods. Thin layer chromatography (TLC) was performed on silica gel $60F_{254}$ (Merck) or polyamide plates (Schleicher & Schuell) and visualized either by spraying with 50% aqueous sulfuric acid and heating at 200 °C or by inspection under UV light (Mineralight). Silica gel (230−400 mesh; Toronto Research Chemicals) was used for flash chromatography. All starting materials were dried overnight *in vacuo* (10^{-3} mmHg) over KOH or P_2O_5 prior to use, and the solvents were distilled from appropriate drying agents. Solutions were concentrated at 1 mmHg pressure in a rotary evaporator.

^{1}H NMR spectra (δ, ppm) were recorded at 500 MHz with a Varian 500 Unity Plus spectrometer at the NMR Spectrometry Laboratory (Director: Dr. A. A. Grey) of the Carbohydrate Research Centre, University of Toronto. Spectra were obtained in either $CDCl_3$ or CD_3OD containing a trace of TMS (0.00 ppm). All mass spectra were recorded with a VG Analytical ZAB-SE or Sciex API III spectrometer at the Mass Spectrometry Laboratory (Acting Director: M. Cheung) of the Carbohydrate Research Centre, University of Toronto. Relative intensities (%) of ion peaks are quoted in parentheses. Fluorescence spectra were recorded with spectrofluorometer MD 5020, Photon Technology International. The spectra were obtained in aqueous solutions.

Oligonucleotides were synthesized on an ABI 394 DNA/RNA synthesizer (Perkin-Elmer) using standard chemistry unless stated otherwise.

General Synthetic Procedures. We reported previously a scheme for the synthesis of 3′-phosphoryltyrosine-terminated oligonucleotides.[11,12] We followed this synthetic design outlined below with modifications noted (see steps 1−5 of Figures 2 and 3). Step 1: Removal of the dimethoxytrityl protecting group from the functionalized solid support (Teflon-based, Glen Research, or TentaGel-based, Rapp Polymere, Tübingen, Germany) to form the "activated support" (Figure 2, central column). Step 2: Coupling of protected tyrosine or its derivatives by an ester bond to the 5′-hydroxyl group obtained in step 1 and capping the unreacted 5′-hydroxyl group. Step 3: Removal of the *tert*-butyl group from the phenolic hydroxyl of tyrosine and its derivatives by a 1-h treatment with 1 mL of 50% trifluoroacetic acid in methylene chloride at room temperature. Step 4: Standard phosphoramidite synthesis of the oligonucleotide on a Perkin-Elmer ABI 394 DNA/RNA synthesizer. Step 5: Cleavage of the amino acid−oligonucleotide conjugates from the support and deprotection by ammonolysis. Step 6: Purification of the conjugates by a passage through Nensorb cartridges (DuPont) or by HPLC on a C_{18} column (Waters, 10 μm, 3.9 × 300 mm) using a gradient of acetonitrile and triethylamine acetate (0.1 M, pH 7). Electrospray mass spectrometry (ES-MS) was used to confirm the structure of the purified oligonucleotide conjugates.

Synthesis of Individual Substances. 3′-(*O*-Phosphotyrosylamide)-Terminated Oligonucleotides 1. These substances were prepared as described in ref 12 with the modifications described in the General Synthetic Procedures section above. Although these are actually tyrosine amides (see step 5 of General Synthetic Procedures and Results), for brevity we refer to the substituent as "tyrosine".

3′-(*O*-Phospho-*N*-dansyltyrosylamide)-Terminated Oligonucleotides 2. See Figure 2, left column.

***O-tert*-Butyl-*N*-dansyltyrosine (8).** A solution of dansyl chloride (270 mg, 0.74 mmol) in acetonitrile (30 mL) was added dropwise to a solution of *O-tert*-butyltyrosine (160 mg, 0.67 mmol) in lithium carbonate buffer (pH 9.5, 0.1 M, 60 mL) at 0 °C, and the mixture was stirred for 2 h. Acetonitrile was removed *in vacuo*, and the residual solution was acidified to pH 7 with 1 N HCl and extracted with ethyl acetate. The organic phase was dried over Na_2SO_4 and evaporated to dryness to yield a yellow solid residue, which was subsequently subjected to chromatography on a silica gel column. The title compound **8** (162 mg, 50%) was eluted with hexane:ethyl acetate (1:2). ^{1}H NMR ($CDCl_3$): 8.49 (d, J = 8.5 Hz, 1H), 8.20 (d, J = 8.8 Hz, 1H), 8.18 (dd, J = 7.3, 1.1 Hz, 1H), 7.52 (dd, J = 8.8, 7.5 Hz, 1H), 7.46 (dd, J = 8.5, 7.3 Hz, 1H), 7.19 (d, J = 7.5 Hz, 1H), 6.82 (d, J = 8.5 Hz, 2H), 6.70 (d, J = 8.5 Hz, 2H), 5.30 (d, J = 8.3 Hz, 1H), 4.18−4.14 (m, 1H), 2.90−2.88 (b, 8H), 1.28 (s, 9H). ES-MS: 469 [(M − H)$^-$, 100].

The *O-tert*-butyl-*N*-dansyltyrosyl-support **(9)**, *N*-dansyltyrosyl-support **(10)**, 3′-(*O*-phospho-*N*-dansyltyrosylamide)-terminated oligonucleotide, and 3′-(*O*-phospho-*N*-dansyltyrosylamide)-terminated oligonucleotide **2** were prepared according to General Synthetic Procedures (Figure 2, left column).

3′-(*N*-Phospho-*O-tert*-butyltyrosylamide)-Terminated Oligonucleotides 3. See Figure 3, right column. The oligonucleotide synthesis was initiated on the free amino group of the tyrosine.

***O-tert*-Butyl-*N*-Fmoc-tyrosyl-support (11).** This was prepared according to step 2 of the general procedure (Figure 3).

***O-tert*-Butyltyrosyl-support (12).** The Fmoc N-protective group was removed from the ^{t}Bu-Tyr-Fmoc Teflon-based solid support (**11**; 30 mg) by a 30-min treatment with piperidine in dimethylformamide (20%, 1 mL) at room temperature. The support was washed several times with DMF and anhydrous acetonitrile and air-dried. The oligonucleotide synthesis subsequently began at the free amino group of tyrosine as described for steps 4−6.[12]

3′-[*O*-Phospho-*N*-[4-(1-pyrenyl)butyryl]tyrosylamide]-Terminated Oligonucleotides 4 and 5. See Figure 3, left column. The *O-tert*-butyltyrosyl-support **(12**; 30 mg) was treated with succinimidyl 1-pyrenylbutyrate (10 mg; Molecular Probes, Inc.) in dimethylformamide (1 mL) for 12 h at room temperature to yield the 4-(1-pyrenyl)-butyramide of tyrosine (**13**). After several washes with DMF and anhydrous acetonitrile, this substance was air-dried and subjected to hydrolysis with trifluoroacetic acid, as described in step 3, to yield the *N*-[4-(1-pyrenyl)butyryl]tyrosyl-support. After the unreacted amino

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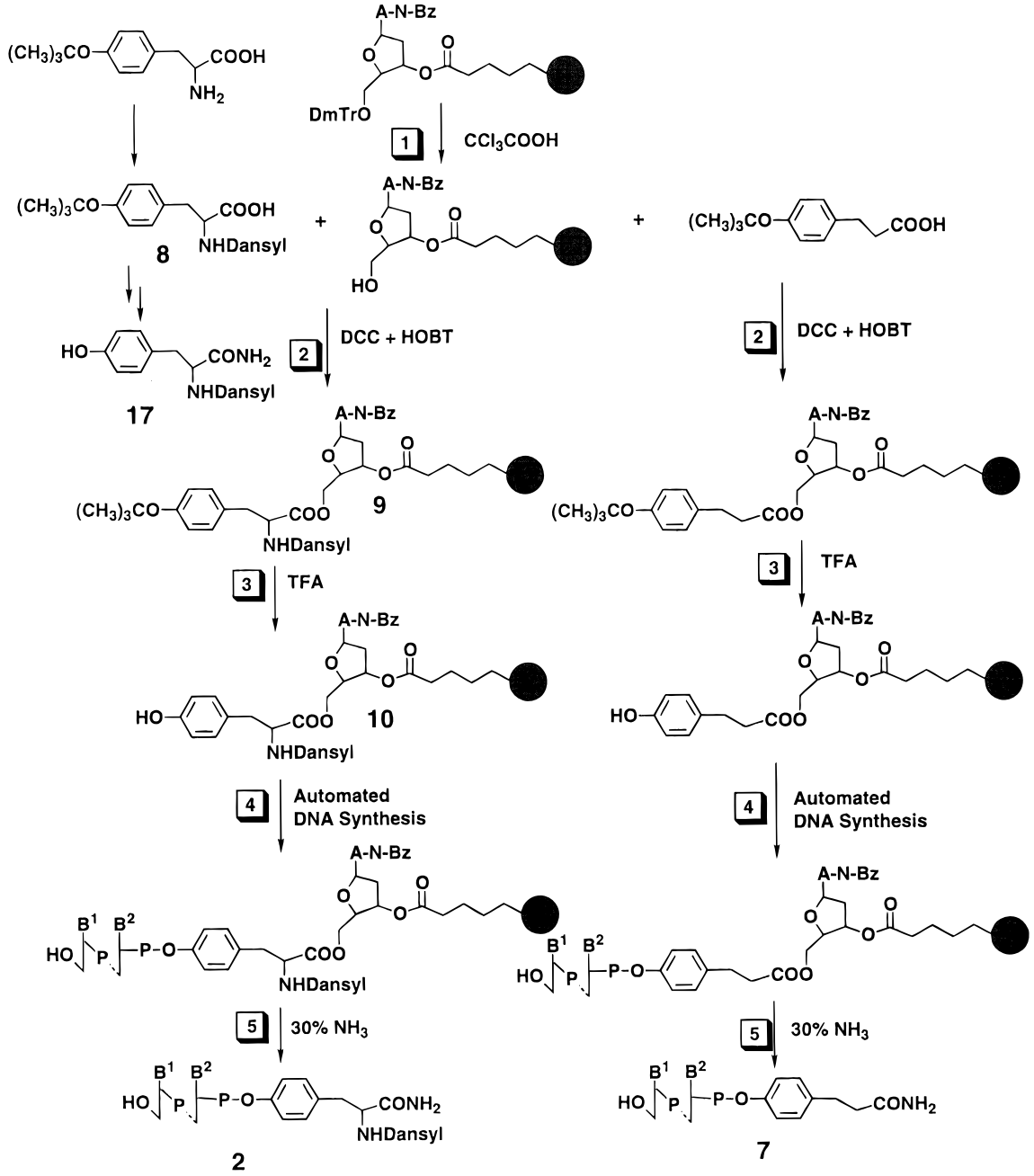

Figure 2. Schematic description of the step by step synthesis of the oligonucleotide conjugates **2** and **7**. The solid support may be either functionalized Teflon or TentaGel (cf. Experimental Section). The numbers in squares indicate the steps 1−5 (Experimental Section).

groups were capped with acetic anhydride, the protected oligonucleotides terminated with the 3′-*O*-phosphoryl-*N*-[4-(1-pyrenyl)butyryl]-tyrosyl-support (**14**) were prepared as described in step 4 and deprotected to yield a mixture of compounds **4** and **5** according to step 5. These two oligonucleotide conjugates were separated by reverse-phase HPLC (step 6). Compound **4** retained its fluorescence, but compound **5** did not fluoresce (see Discussion for more details).

3′-*O*-Phosphoryltyramine-Terminated Oligonucleotides 6. See Figure 4).

***N*-Fmoc-tyramine (15).** A solution of 9-fluorenylmethyl chloroformate (1.10 g, 4.24 mmol) in diethyl ether (10 mL) was added dropwise to a stirred suspension of tyramine (0.581 g, 4.24 mmol) in tetrahydrofuran (70 mL) at 0 °C. After the mixture was stirred for another 2 h, the organic solvents were removed *in vacuo*, and ethyl acetate (10 mL) was added. The solution was washed successively with 1 N HCl, water, and brine and dried over Na_2SO_4. The solids were removed by filtration, ethyl acetate was evaporated to dryness, and crude *N*-Fmoc-tyramine was obtained as a white solid. Chromatography on a silica gel column using hexane:ethyl acetate (3:1) as an eluent gave pure *N*-Fmoc-tyramine **(15**; 1.19 g, 78%). ^{1}H NMR ($CDCl_3$): 7.75 (d, J = 7.5 Hz, 2H), 7.57 (d, J = 7.3 Hz, 2H), 7.40 (t, J = 7.5 Hz, 2H), 7.31 (t, J = 7.5 Hz, 2H), 7.04 (d, J = 8.1 Hz, 2H), 6.77 (d, J = 8.1 Hz, 2H), 4.85 (s, 1H), 4.76 (b, 1H), 4.41 (d, J = 6.9 Hz, 2H), 4.21 (t, J = 6.9 Hz, 1H), 3.41 (q, J = 6.7 Hz, 2H), 2.74 (t, J = 6.7 Hz, 2H). EM-MS: 360 [$(M + H)^+$, 57], 182 [$(M - 179 + 2H)^+$, 53], 179 [$(M - 180)^+$, 100], 121 [$(M - 238)^+$, 56].

***N*-Fmoc-tyramine *O*-(2-Cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (16).** To a suspension of *N*-Fmoc-tyramine (150 mg, 0.42 mmol) in CH_2Cl_2 (5 mL) containing diisopropylethylamine (0.1 mL) was added dropwise a solution of 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (191 mg, 0.8 mmol) in CH_2Cl_2 (2 mL) at 0 °C. After the addition was completed, the reaction mixture was stirred for another 30 min, and subsequently a 5% aqueous solution of $NaHCO_3$ (10 mL) was added. The organic layer was separated, dried over Na_2SO_4, and evaporated to dryness to give amorphous compound **16**, which was subjected to chromatography on silica gel (60 g). The elution with hexane:ethyl acetate:triethylamine (2:1 + 1%) gave the pure compound **16** (156 mg, 60%). ^{1}H NMR ($CDCl_3$): 7.76 (d, J = 7.5 Hz, 2H), 7.57 (d, J = 7.3 Hz, 2H), 7.39 (t, J = 7.5 Hz, 2H), 7.30 (t, J = 7.3 Hz, 2H), 7.07 (d, J = 8.0 Hz, 2H), 6.98 (d, J = 8.0 Hz,

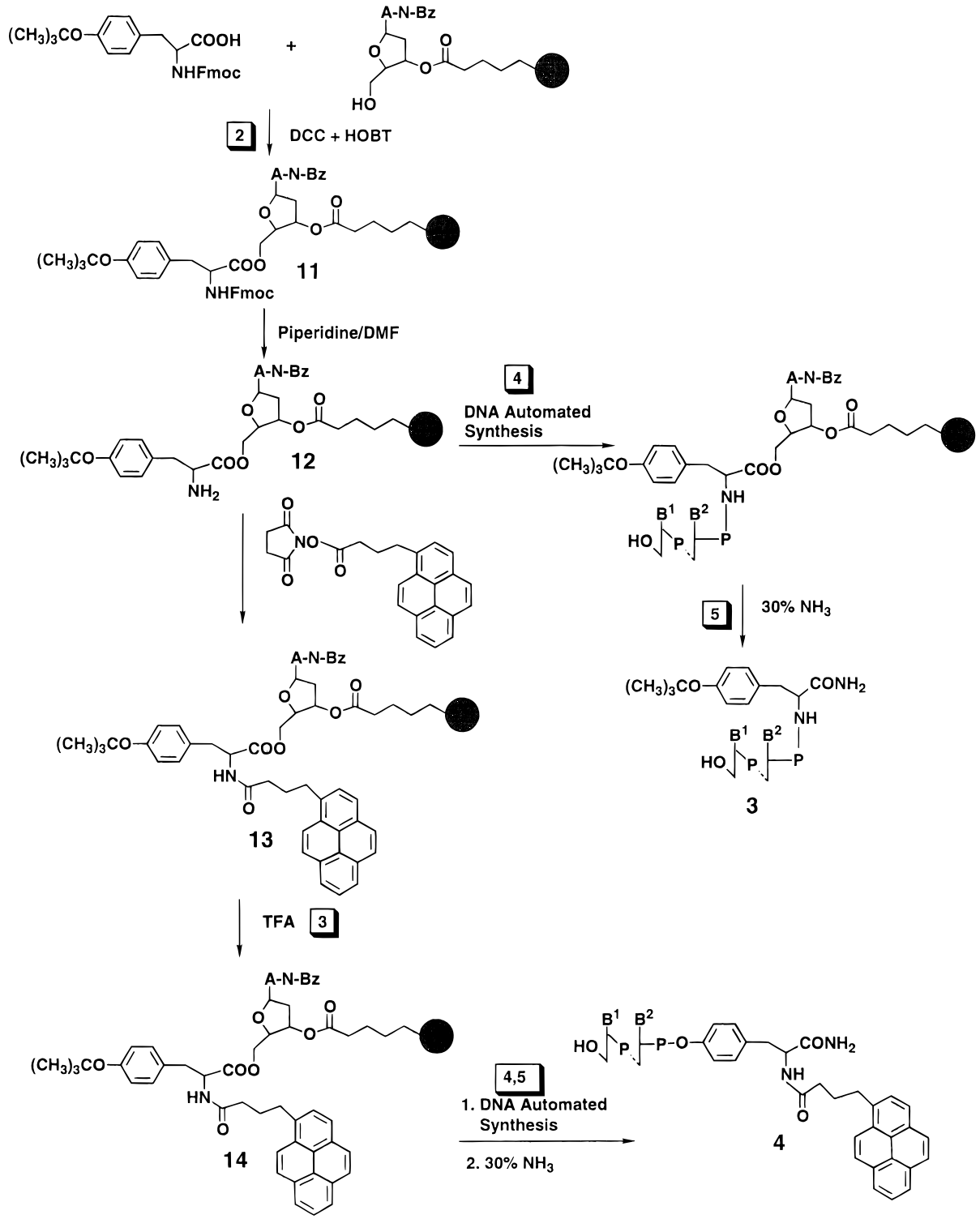

Figure 3. Schematic description of the step by step synthesis of the oligonucleotide conjugates **3** and **4**. The solid support may be both functionalized Teflon and TentaGel (cf. Experimental Section). The conjugate **5** accompanied the conjugate **4** (cf. Experimental Section and Discussion). The numbers in squares indicate the steps 1−5 (Experimental Section).

2H), 4.81 (b, 1H), 4.39 (d, J = 6.8 Hz, 2H), 4.20 (t, J = 6.8 Hz, 1H), 3.96−3.85 (m, 2H), 3.77−3.69 (m, 2H), 3.41 (q, J = 6.6 Hz, 2H), 2.75 (t, J = 6.6 Hz, 2H), 2.64 (td, J = 6.4, 1.5 Hz, 2H). ^{31}P NMR ($CDCl_3$): 147.3.

The oligonucleotides were synthesized in the 5′ → 3′ direction using 5′-CPG support and 3′-dimethoxytritylated 5′-phosphoramidites. The phosphoramidite of *N*-Fmoc-protected 4-(2-aminoethyl)phenol (**16**) was coupled in the sequence in the last cycle (see Figure 4), and this was followed by deprotection and purification as described in steps 5 and 6 of the General Synthetic Procedure.

3′-[[4-(Phosphoryloxy)phenyl]propionamide]-Terminated Oligonucleotides 7. See Figure 2, right column. The General Synthetic Procedure was followed (steps 1−6) using (4-*O*-*tert*-butylphenyl)-propionic acid.

Synthesis of *N*-Dansyltyrosine Amide (17). The coupling of *O*-*tert*-butyl-*N*-dansyltyrosine (210 mg) with TentaGel (S 30 000; 500 mg) in the presence of hydroxybenzotriazole (20 mg) and dicyclohexylcarbodiimide (400 mg) in dimethylformamide (5 mL) at room temperature was allowed to proceed for 48 h. The TentaGel was then collected by filtration and washed with dimethylformamide and dry acetonitrile.

To remove the *O*-*tert*-butyl group, TentaGel, to which *tert*-butyl-*N*-dansyltyrosine was linked (500 mg), was treated with trifluoroacetic acid in methylene chloride (50%, 5 mL) for 1 h at room temperature. The liquids were then removed by filtration, and the TentaGel was

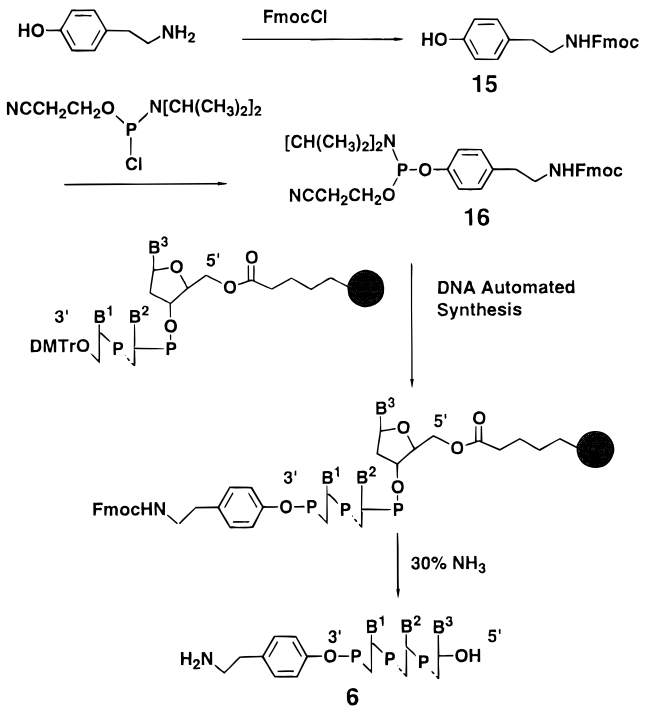

Figure 4. Schematic description of the synthesis of the oligonucleotide conjugate **6**.

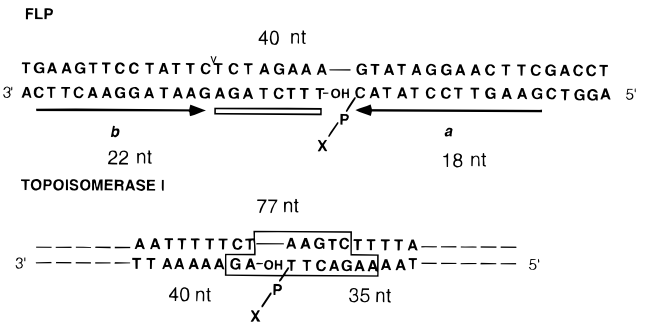

Figure 5. Sequences of the substrates for FLP and topoisomerase I. FLP: The horizontal arrows indicate the sequences of inverted symmetry elements to which FLP binds. The open box represents the core of the FRT site. The top strand consists of one oligonucleotide, 40 nucleotides in length, and the bottom strand consists of two oligonucleotides of 22 and 18 nucleotides in length. The caret indicates the phosphodiester bond of the top strand that is cleaved by FLP. Topoisomerase I: The minimum sequence needed for topoisomerase I-mediated cleavage is shown by the box. The top strand is 77 nucleotides in length, and the bottom strand consists of two oligonucleotides, 40 and 35 nucleotides in length.[11] The X represents the presence of 3′-phosphoryl modification.

washed sequentially with methylene chloride, water, and dry acetonitrile and treated with 30% aqueous ammonia for 2 h. *N*-Dansyltyrosine amide (**17**) was obtained by lyophilization of the mother liquors after the removal of TentaGel by filtration. ^{1}H NMR (CD_3COCD_3): 8.39 (d, J = 8.4 Hz, 1H), 8.16 (d, J = 8.8 Hz, 1H), 7.94 (dd, J = 7.3, 1.2 Hz, 1H), 7.42 (dd, J = 8.8, 7.5 Hz, 1H), 7.39 (dd, J = 8.4, 7.3 Hz, 1H), 7.12 (d, J = 7.5 Hz, 1H), 6.64 (d, J = 8.3 Hz, 2H), 6.32 (d, J = 8.3 Hz, 2H), 3.85 (m, 1H), 2.75 (s, 6H), 2.70 (dd, J = 13.89, 5.77 Hz, 1H), 2.55 (dd, J = 13.89, 7.69 Hz, 1H). HR-FAB-MS: calcd for $C_{21}H_{24}N_3O_4S$ [$(M + H)^+$], 414.1488; found, 414.1485.

Preparations of Synthetic Activated Substrates. Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer (Model 394) and purified either by the cartridge method or by HPLC (see above). For the FLP reaction a 22-nucleotide oligonucleotide was 3′ labeled with ^{32}P using terminal deoxynucleotidyl transferase and [^{32}P]ddATP (Figure 5, top panel). For the topoisomerase I reaction the oligonucleotides bearing a 3′ modification were 5′ labeled with ^{32}P using polynucleotide kinase (Figure 5, bottom panel). Each labeled oligonucleotide was then annealed with two other unlabeled oligonucleotides to form a duplex DNA substrate with a nick at the enzyme cleavage site and potential ligation site. Three oligonucleotides (50 pmol of radioactive oligonucleotide and 100 pmol of nonradioactive oligonucletide) were mixed in a 20 μL reaction containing 0.1 mM NaCl and 5 mM $MgCl_2$. The mixture was heated at 95 °C for 10 min and then slowly cooled to 25 °C. The substrates were passed through a G50 Sephadex column equilibrated with 10 mM Tris-HCl and 1 mM EDTA.

Preparation of FLP, Topoisomerase I, and Other Enzymes. Wild-type and mutant FLP proteins were prepared as described previously.[14] The purity of the wild-type FLP protein was 95%. The purity of the mutant proteins was 15−30%. Mammalian topoisomerase I was purchased from Promega. Polynucleotide kinase and terminal deoxynucleotidyl transferase were purchased from New England Biolabs.

Ligation Assays. The FLP and topoisomerase I reactions were done as described previously.[11,12] Where needed, 5 μg of proteinase K and 0.005% SDS were added to the reaction, and the incubation was continued at room temperature for 10 min. The products were analyzed by 8% denaturing gel electrophoresis.

Thin Layer Chromatography. The release of the fluorescent dansyltyrosine (**17**) from the 3′-phosphoryl end was monitored by thin layer chromatography. Typically, 100 pmol of substrate and 51 pmol of FLP were used in a reaction volume of 100 μL. The incubation was performed at room temperature for 1 h. The reaction mixture was extracted three times with ethyl acetate, the combined extracts were evaporated to dryness, and the residue was dissolved with 1 μL of water and spotted on a polyamide TLC plate (5 × 5 cm). The polyamide chromatogram was developed with 1.5% formic acid, and **17** exhibited R_f = 0.61. Compound **17** showed on the silica gel chromatogram developed with chloroform:methanol:glacial acetic acid, (15:4:1), R_f = 0.69.

Results

Ligation of 3′-Phosphoryl Derivatives by FLP. We have previously shown that enzymatically derived oligonucleotides bearing a 3′-phosphoryltyrosine residue were effective substrates to measure the ligation activity of the FLP recombinase.[10] Synthetic oligonucleotides bearing a 3′-phosphoryltyrosylamide (hereafter called "phosphoryltyrosine") were also effectively used by FLP, other site-specific recombinases, and mammalian topoisomerase I.[11,12]

We wished to broaden the spectrum of 3′-phosphoryl substituents for two reasons. First, the use of fluorescently tagged leaving groups might facilitate the development of simplified assays for recombinases and topoisomerases. Second, such substituents might provide further insight into the flexibility of the active site and mechanisms of action of such enzymes.

In addition to the 3′-phosphoryltyrosylamide **1**[11,12,15] as a control, we also made oligonucleotides bearing a 3′-phosphoryl group linked to *N*-dansyltyrosylamide **2** ("dansyltyrosine"), *N*-(4-butyrylpyrenyl)tyrosylamide **4** ("pyrenotyrosine"), phosphoryltyramine **6**, and (hydroxyphenyl)propionic acid **7**. All were linked to the 3′-phosphoryl end of the oligonucleotide via the phenyl hydroxyl group. We also synthesized an oligonucleotide in which the tyrosine was linked to the 3′-phosphoryl group via its amino group instead of the hydroxyl group to ensure that no unexpected enzymic reactions took place (see Figure 6 for a depiction of these structures). The synthesis of the pyrenyl derivative **4** resulted in the formation of a byproduct (**5**) in about equal amounts; **5** had lost its fluorophore, but its molecular size apparently did not differ significantly from **4** (see Discussion).

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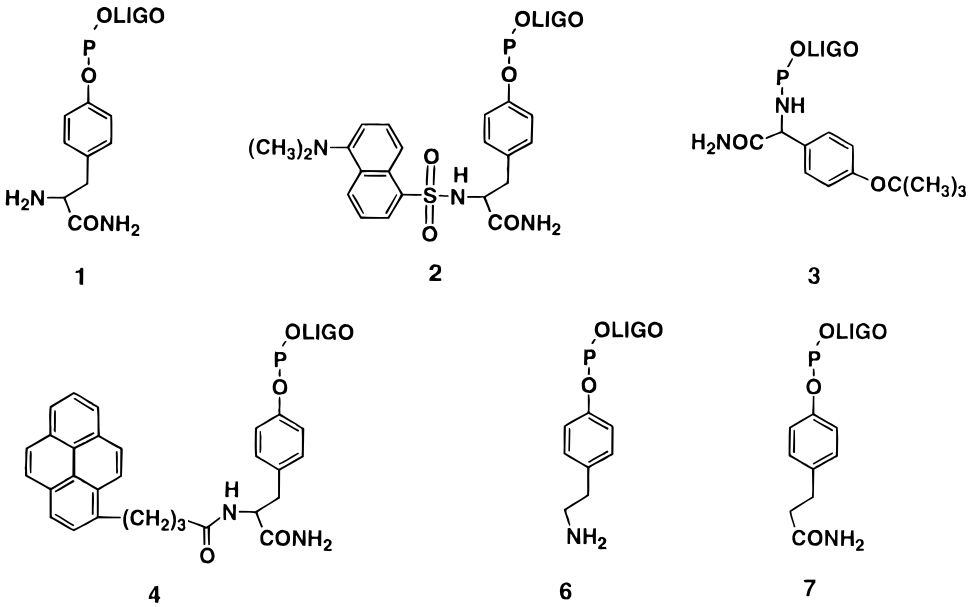

Figure 6. Structures of the 3′-phosphoryl substituents used in this study. The oligonucleotides were synthesized as described in the Experimental Section. The substituents are **1**, *O*-phospho-1−tyrosine amide; **2**, dansyltyrosine amide; **3**, N-phosphotyrosine; **4**, *O*-phospho-*N*-pyrenotyrosine amide; **6**, *O*-phosphotyramine; and **7**, *O*-(phosphohydroxylphenyl)propionate.

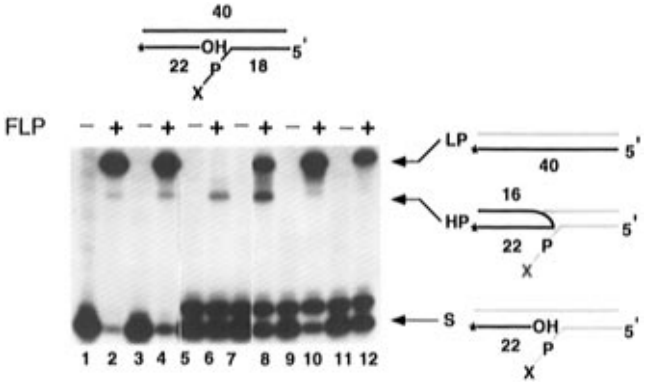

Figure 7. Ligation of 3′-phosphoryl-substituted oligonucleotides by FLP. The substrate is diagrammed at the top. The reaction mixtures contained 2.4 pmol of substrate and 17 pmol of FLP^+ and were incubated at room temperature for 20 min. The products were analyzed on an 8% denaturing polyacrylamide gel. LP = ligation product; S = substrate; HP = hairpin product which results from cleavage of the top strand by FLP and nucleophilic attack of the free 5′-OH group of the 3′-^{32}P-labeled bottom strand upon the FLP−oligonucleotide complex. The substrates bore the following 3′-phosphoryl substituents: lanes 1 and 2, -*O*-tyrosine **1**; lanes 3 and 4, -*O*-tyrosine-*N*-dansyl **2**; lanes 5 and 6, -*N*-tyrosine **3**; lanes 7 and 8, -*O*-tyrosine-*N*-pyrene **4**; lanes 9 and 10, -*O*-tyrosine-*N*-pyrene (pyrene modified) **5**; lanes 11 and 12, -*O*-tyramine **6**. Note that in lane 6 the hairpin product is formed even though the N-linked dansyltyrosine group is inactive for ligation. Some substrates (lanes 5−12) contained an unknown band that migrated behind the substrate band(s). This substance did not participate in, or interfere with, ligation.

These oligonucleotides were annealed to the two other oligonucleotides in order to assay ligation by the FLP recombinase (Figure 7). As shown previously, the tyrosylamide-terminated oligonucleotide **1** was ligated very efficiently (Figure 7, lane 2). The dansyltyrosine, both pyrenyltyrosine derivatives, and the tyramine-terminated substrates were also very effective substrates (Figure 7, lanes 4, 8, 10, 12). An oligonucleotide bearing a 3′-phosphoryl (hydroxyphenyl)propionate substituent (**7**) was also an active substrate for ligation (data not shown). Since these assays were done with enzymes in stoichiometric excess, the reactions reflect the final yields of products rather than rates. Only the N-linked, 3′-phosphoryltyrosine oligonucleotide failed to show any ligated product (Figure 7, lane 6). This shows the importance of the phosphodiester linkage to the aromatic leaving group. Note that all substrates showed detectable amounts of a hairpin ligation product[10] that resulted from cleavage of the top strand and ligation to the 3′-^{32}P-labeled bottom strand. This serves as a useful internal control for the enzyme activity.

Use of Synthetic Substrates To Assay Various Mutant FLP Proteins. Much information has been gained about the mechanism of action of the FLP protein by the study of mutations of several key residues of the protein. In order to learn whether the various substrates behaved differently from wild-type FLP protein, we assayed the ligation activity of several altered FLP proteins. The results are shown in Figures 8 and 9 and can be summarized as follows. The FLP Y343F[16] protein cannot cleave the FRT site because the nucleophilic tyrosine residue has been changed to phenylalanine.[17,18] FLP Y343F can effectively ligate the 3′-tyrosyl-oligonucleotide, although it cannot cleave and covalently attach to the substrate (Figure 8, lane 1). This mutant protein shows the same spectrum of activities as the wild-type protein (Figure 8, lanes 1−6). FLP Y343S (lanes 7−12) showed the same properties, although the levels of activity were uniformly reduced in this experiment. The arginine residue at position 308 has been implicated in activation of the scissile phosphodiester bonds of the FRT site for cleavage by tyrosine 343.[19] FLP R308K can perform efficient ligation of a substrate to which it can covalently attach.[20] It can cleave the tyrosine-terminated oligonucleotide about 10% as efficiently as FLP^+ protein and ligates this oligonucleotide to a similar extent (Figure 8, lane 13). Interestingly, FLP R308K shows some ligation of the dansyltyrosine (lane 14) and tyramine (lane 18) substrates but no activity with

(16) FLP Y343F means that the tyrosine residue at position 343 has been changed to phenylalanine. This nomenclature was applied to the other mutations as well.

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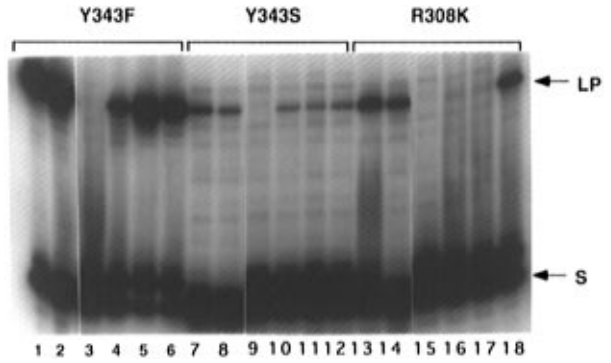

Figure 8. Ligation of various 3′-substituted substrates by mutant FLP proteins. The reaction conditions were the same as in Figure 7. The substrates were substituted at their 3′-phosphoryl termini with **1** (*O*-tyrosine, lanes 1, 7, 13), **2** (*O*-tyrosine-*N*-dansyl, lanes 2, 8, 14), **3** (*N*-tyrosine, lanes 3, 9, 15), **4** (*O*-tyrosine-*N*-pyrene, lanes 4, 10, 16), **5** (*O*-tyrosine-*N*-pyrene (modified), lanes 5, 11, 17), and **6** (*O*-tyramine, lanes 6, 12, 18). The proteins were FLP Y343F (lanes 1−6), 3.23 pmol; and FLP Y343S (lanes 7−12), 1.3 pmol; FLP R308K (lanes 13−18), 1.19 pmol.

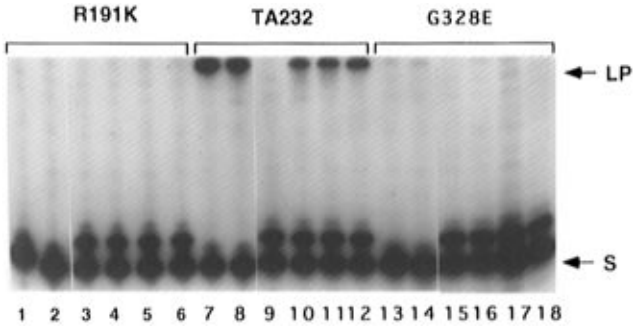

Figure 9. Reaction conditions and substrates were used as in Figure 8. Proteins used were FLP R191K (lanes 1−6), 5.95 pmol; FLP TA232 (lanes 7−12), 2.55 pmol; and FLP G328E (lanes 13−18), 3.4 pmol.

either of the pyrenoyl derivatives (lanes 16, 17). This implies that FLP R308K cannot cleave and attach to the latter substrates.

The other proteins (Figure 9) behaved as expected based on their known activities on the tyrosine-terminated substrates.[11] FLP R191K was ligation-defective with all substrates (lanes 1−6). FLP TA232, a DNA bending-defective protein, ligated all the substrates as well as FLP$^+$ (lanes 7−12), whereas FLP G328E, another bending-defective FLP, ligated none of the substrates (lanes 13−18).

Assay of Topoisomerase I Using Dansyltyrosine Derivatives. One objective of these studies was to develop fluorescent substrates that might be useful for screening of compounds that inhibit topoisomerase I. It was therefore important that the oligonucleotide bearing the bulky fluorescent adduct be as active in the ligation assay as that bearing only the tyrosine. We therefore compared the activity of the two oligonucleotides in a ligation assay of mammalian topoisomerase I.

As shown in Figure 10, the dansylated 3′-phosphorylated oligonucleotide was as effective a substrate as that bearing only the 3′-phosphoryltyrosine (cf. lanes 2−4 vs lanes 6−8). In these assays we also observed some radioactive material that remained in the wells of the denaturing polyacrylamide gel (lanes 3, 6, 7). We believe that this was a covalent complex between the enzyme and the DNA since the material was sensitive to proteinase K (Figure 10, lanes 4, 8). In these lanes we also observed the appearance of radioactive material that migrated slightly more slowly than the substrate (Figure 10, lanes 4, 8, ID). This material was due to the presence of oligopeptide(s)

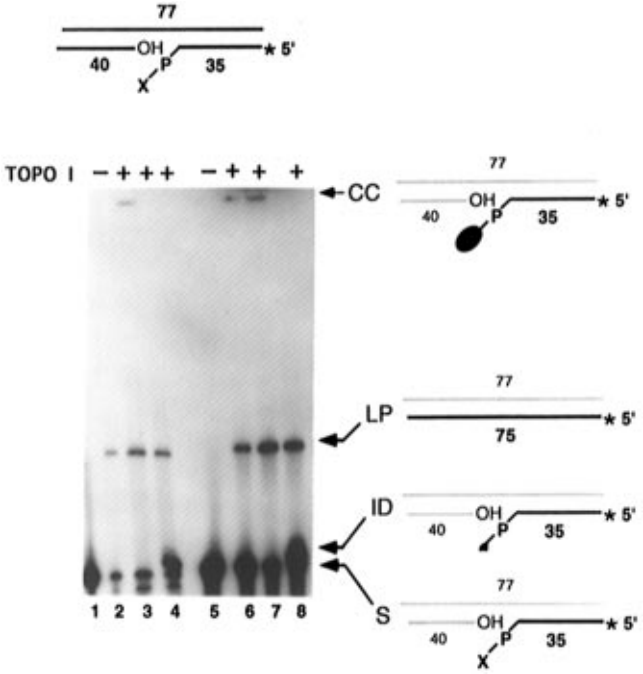

Figure 10. Ligation by mammalian topoisomerase I. The reaction mixtures contained either 3′-phosphoryl-*O*-tyrosine-terminated (lanes 1−4) or 3-phosphoryl-*O*-tyrosine-*N*-dansyl-terminated (lanes 5−8) oligonucleotides that were 5′ labeled with ^{32}P (*). The substrates are diagrammed at the top. The reaction mixtures contained 10 units (lanes 2, 6) or 20 units (lanes 3, 4, 7, 8) of topoisomerase I. Reaction mixtures in lanes 1−3 and 5−7 were concentrated and applied directly to the gel, whereas those in lanes 4 and 8 were treated with SDS and proteinase K prior to electrophoresis. The 8% sequencing gel was run for 20 min at 1500 V. CC, covalent complex; LP, ligation product; ID, incompletely degraded peptides.

that were incompletely removed from the 3′-phosphoryl terminus by proteinase K.

Fluorescence Spectroscopy. The excitation wavelengths of dansylated tyrosylamide **17** and the dansylated conjugate **2** were identical (340 nm), and the emission maximum was the same for both dansyl derivatives as well (545 nm). Although the ϵ value of dansyl is low (3400) in comparison with other fluorophores such as fluorescein, it was sufficiently high to be identified in **17** by thin layer chromatography on polyamide plates using an ordinary UV lamp for TLC (see below). The spectral properties of pyrene (excitation wavelength at 365 nm with maximum emission at 450 nm) are not more favorable (in fact, the Stokes shift is smaller than for the dansyl derivatives), and the synthesis of "pyrenylated" derivatives presents problems (see Discussion below).

Identification of the Fluorescent Product of Ligation. One product of FLP-mediated ligation is a lengthened ^{32}P-labeled strand that was detected by denaturing acrylamide gel electrophoresis. The other should be the free hydroxylated aromatic leaving group, i.e., the dansylated tyrosylamide **17**. In order to detect this product, a ligation reaction involving the N-dansylated tyrosine was scaled up; the products were extracted with ethyl acetate and analyzed by thin-layer chromatography. As expected, the fluorescent product that extracted into ethyl acetate showed the same $R_f = 0.61$ in 1.5% formic acid (polyamide plate) as the *N*-dansyltyrosine amide (**17**) standard. Thin-layer chromatography on a silica gel plate in chloroform:methanol:acetic acid (15:4:1) was also suitable for detection of *N*-dansyltyrosine amide (**17**) ($R_f = 0.69$). Similar experiments were done with the topoisomerase I ligation reaction and also showed that *N*-dansyltyrosine amide (**17**) was produced.

Inhibition by Camptothecin of Topoisomerase I-Mediated Ligation. To confirm the validity of the assumption that inhibitors of recombinases indeed would be detectable using substrates carrying a fluorescent label, camptothecin, a known inhibitor of topoisomerase I, has been included in the reaction mixture. As expected, the ligation reaction was suppressed (data not shown).

Discussion

Chemical Synthesis. The choices of the fluorescent reporter group were limited by the compatibility of such a group with the requirements of the synthesis and of the enzymic active site. Dansyl was the preferred choice since the sulfonamide functionality linking the fluorophore with tyrosine should be stable under both acid and basic conditions and because many common fluorophores with higher ϵ values (e.g., fluorescein) are much more difficult to use for preparations of analogous conjugates (they are incompatible with the automated synthesis of oligonucleotides, e.g., lactone in fluorescein).[21] The pyrene derivative, together with other structural variations, served to probe the requirements of the catalytic site.

3′-"Tyrosinylated" Oligonucleotides. The oligonucleotides were synthesized using 5′-dimethoxytritylated 3′-phosphoramidites on a derivatized tyrosine (or its analogues) linked to a solid Teflon support through a tyrosine carboxyl in the ester form using the protocol developed for the CPG (controled pore glass) support. Alternatively, we used TentaGel N which is a poly(ethylene glycol) grafted onto poly(styrene). TentaGel terminated with a hydroxyl group was used for the synthesis of both the tyrosinylated oligonucleotide **1** and the dansylated analogue **2** according to the protocol for automated oligonucleotide synthesis on TentaGel.[22,23]

A reporter group was usually attached to the amino group of the properly protected tyrosine by solution chemistry (Figure 2). Although in the case of tyrosylated oligonucleotides ammonolysis is completed in 1 h at room temperature, 2.5 h was needed in the presence of a bulky reporter group. In all cases the amino acid carboxyl becomes an amide (cf. Figures 2 and 3).[24]

The *tert*-butyl group with which the tyrosine hydroxyl was protected was removed by treatment with trifluoroacetic acid. This step was not compatible with the use of CPG solid support which is labile in the presence of strong acids; therefore, it had to be performed on Teflon or TentaGel.

In the case of pyrene-tagged conjugates, the reporter group was attached to the amino group of tyrosine that had already been linked to the solid support (cf. Figure 3). We have noted reduced stability of the pyrene ring system under the conditions of oligonucleotide synthesis. The expected fluorescing pyrenylated derivative **4** was accompanied by the nonfluorescing oligonucleotide derivative **5**. Both substances exhibited substantially prolonged elution times (35 and 33 min) on HPLC using a C_{18} reverse-phase column in trimethylamine acetate (pH 7), while the same nucleotide without the pyrene reporter group was eluted in 11 min. Clearly, the compound **5** is nonfluorescent because the conjugation within the pyrene ring system was interrupted. Although earlier observations[25] indicate sites of reactivity in pyrene and the exact chemical nature of this modification is presently under investigation, the activity of **5** in ligation assays underlines the ability of FLP to accommodate large variations in N-substituents of tyrosine (see below).

N-Linked oligonucleotide conjugates (phosphamides such as **3**) were synthesized as control compounds to confirm that they cannot be intermediates in the reactions catalyzed by recombinases. To our knowledge, such oligonucleotides have not been synthesized before.

3′-"Tyraminylated" Oligonucleotides. Since tyramine does not contain a carboxyl group to link tyrosine derivatives to a solid support, these oligonucleotides were synthesized on the CPG support in the 5′ → 3′ direction using 3′-dimethoxytritylated 5′-phosphoramidites (Figure 4). It is worth mentioning that although the reactivity of the amino group of tyramine with 9-fluorenylmethyl chloroformate was appreciably higher than that of the OH group of tyramine, the reaction had to be carefully controlled to avoid the additional derivatization of the phenolic hydroxyl group.

Ligation Assays. This study shows the feasibility of attaching bulky substituents to the 3′-phosphoryl terminus of oligonucleotides and using them to assay the ligation activity of the FLP recombinase and topoisomerase I.

The "ligation pocket" of the FLP protein is surprisingly flexible since it can accommodate a 3′-phosphoryl terminus that bears not only a tyrosyl group but also those that contain tyrosine that has been substituted with dansyl or pyrene groups. While the wild-type FLP protein is able to remove the tyrosine group and attach covalently to the 3′-phosphoryl terminus before it executes ligation (cleavage-dependent ligation),[20] a FLP protein that lacks the nucleophilic tyrosine 343 (FLP Y343F) is nevertheless able to carry out efficient ligation on all the 3′-substituted substrates tested. The fact that all the substrates behaved similarly to the tyrosine-substituted oligonucleotide with all the FLP proteins tested argues that the mechanism of ligation of all the modified substrates occurs by the same chemical mechanism. The importance of the phosphoryl linkage to the aromatic hydroxyl group was shown by the fact that the N-linked tyrosine derivative was inactive as a leaving group in the ligation reaction.

We extended the utility of the FLP ligation reactions by showing that mammalian topoisomerase I can also utilize the N-dansylated tyrosine derivative as a ligation substrate. We observed that these substrates also became covalently attached to the topoisomerase. It may be that the substrate first undergoes ligation, and then the protein recleaves and covalently attaches to the substrate. Alternatively the enzyme may cleave and covalently attach to the substrate without ligating it. Distinction between these two possibilities could make the substrates useful for studying the cleavage and ligation steps of the reaction separately.

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