Sequence-Specific Cleavage of DNA via Nucleophilic Attack of Hydrogen Peroxide, Assisted by Flp Recombinase†

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ABSTRACT: Hydrogen peroxide is capable of effecting the cleavage of a specific phosphodiester bond in DNA, when used in concert with the recombinase enzyme Flp from *Saccharomyces cerevisiae*. This cleavage is *not* caused by oxidative damage of the DNA backbone but instead is the result of nucleophilic attack by peroxide. A single phosphorus–oxygen bond is broken in the reaction. Cleavage of DNA by peroxide also occurs with an inactive mutant of Flp in which the active site nucleophile tyrosine has been replaced by phenylalanine. Besides providing information on the mechanism of strand cleavage by Flp, these results may contribute to the development of new synthetic DNA cleavage reagents that act by hydrolytic and not radical chemistry.

The phosphodiester backbone of DNA is notoriously resistant to hydrolytic cleavage. As a consequence, many small molecules that are used to degrade DNA cleave the deoxyribose backbone via radical chemistry (Stubbe & Kozarich, 1987). Enzymes, of course, are capable of efficiently hydrolyzing the phosphodiester bonds of DNA. We report here a system in which hydrogen peroxide acts as a nucleophile to effect the cleavage of a specific phosphodiester bond in DNA. We perform this reaction using hydrogen peroxide in concert with the Flp recombinase of *Saccharomyces cerevisae*, which activates the phosphodiester for attack by the diffusible small nucleophile. While Flp is capable of cleaving DNA on its own, the reaction we report occurs at a Flp binding site that is not cut by the enzyme under normal circumstances. Furthermore, peroxide-mediated cleavage of the DNA backbone takes place with a Flp mutant that lacks the active site tyrosine which in wild-type Flp attacks the DNA backbone to form a covalent deoxyribose-3′-phosphotyrosyl intermediate (Evans et al., 1990; Gronostajski & Sadowski, 1985). Besides providing new information on the pathway of DNA cleavage by Flp, we show here how to construct a reagent which makes a single, specific nick in one strand of a DNA duplex.

EXPERIMENTAL PROCEDURES

Materials. The wild-type Flp protein and a mutated version, Flp(Y343F), were expressed in *Escherichia coli* (Jayaram, 1985) and purified (Parsons et al., 1990). Radiolabeled DNA molecules containing the Flp recombination target sequence (Figure 1) were prepared from plasmid pJ3 (Jayaram, 1985) by standard methods (Maniatis et al., 1982).

In Situ Cleavage Reactions. A mixture containing free DNA and DNA with one, two, or three Flp binding sites occupied was generated by incubating 45 ng of Flp with 30 fmol of DNA (bottom strand radiolabeled at the 3′ end; see Figure 1A) in a buffer consisting of 50 mM Tris[1]·HCl (pH 7.5), 60 mM NaCl, 200 μg/mL bovine serum albumin, 100 μg/mL calf thymus DNA, 1 mM dithiothreitol, and 2% glycerol at 30 °C for 30 min. These four species were separated on a 5% polyacrylamide gel (Andrews et al., 1987). The gel was immersed in 10 mM Tris·HCl (pH 8.0) and 1 mM EDTA. Sodium ascorbate, Fe(II)·EDTA, and hydrogen peroxide (or each of the reagents individually) were added to final concentrations of 1 mM, 100 μM, and 0.3% (Dixon et al., 1992). After 4 min, thiourea was added to 10 mM. Gel slices containing each of the four DNA species were excised. DNA was recovered (Maxam & Gilbert, 1980) and electrophoresed on an 8% polyacrylamide denaturing gel.

Solution Cleavage Reactions. Flp and DNA were incubated in a buffer containing 50 mM Tris·HCl (pH 7.5), 10 mM EDTA, 75 mM NaCl, 12% PEG(6000), and 16% glycerol at 0 °C for 10 min. Hydrogen peroxide was added to 0.6% and incubation was continued at 30 °C for 20 min. The reaction was stopped by addition of SDS to 0.5%. Samples were incubated with proteinase K and prepared as described (Prasad et al., 1987) before electrophoresis on a 10% polyacrylamide denaturing gel.

Other Methods. Cleavage of DNA by the hydroxyl radical was performed as described (Dixon et al., 1992). Chemical sequencing ladders were generated according to Maxam and Gilbert (1980).

RESULTS AND DISCUSSION

Flp, a member of the Int family of recombinases, catalyzes a site-specific recombination reaction involving the 2-μm plasmid of yeast. An active Flp site consists of a pair of DNA sequences to which the enzyme binds, separated by an eight base pair long strand-exchange region (Figure 1A). Only the

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[1] Abbreviations: bp, base pair(s); EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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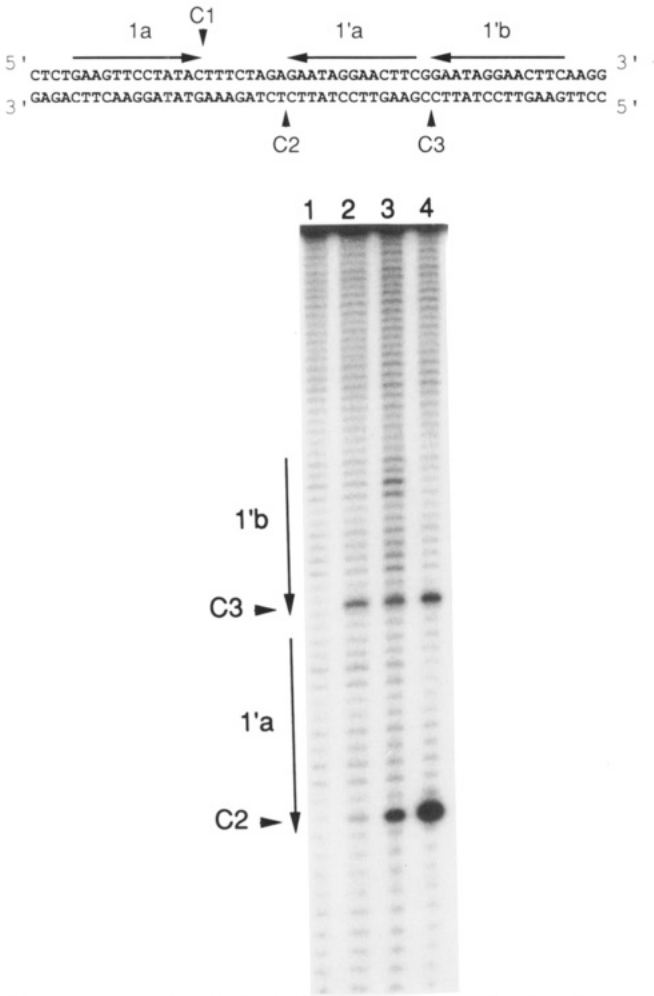

FIGURE 1: (A, top) An 80-bp DNA fragment was used as the substrate for Flp; 56 bp of the fragment are shown. This fragment, derived from the yeast 2-μm circle (Jayaram, 1985), contains three 13-bp Flp binding sites. Sites 1a and 1′a abut the strand cleavage and exchange points C1 and C2. A third Flp binding site, 1′b, is not required for Flp recombination. Cleavage at C3, which we observed in our experiments, has not previously been reported. (B, bottom) Cleavage at the boundaries of the essential and nonessential Flp binding sites. Flp–DNA complexes were separated by gel electrophoresis. The native gel was exposed to hydroxyl radical cleavage reagents. DNA from each complex was isolated and run on a denaturing polyacrylamide gel. The autoradiograph of the gel is shown. Lanes 1–4: DNA isolated from unbound and singly, doubly, and triply shifted Flp–DNA complexes, respectively. Binding and cleavage sites are marked. Bands were assigned using a Maxam–Gilbert sequencing lane prepared from the same DNA (not shown).

Flp enzyme itself is required for recombination between two such sites, in contrast to other recombinase systems that involve several proteins (Craig, 1988). During the recombination reaction, Flp cleaves the phosphodiester backbone on opposite DNA strands at the edges of the strand-exchange region. The positions of cleavage are marked C1 and C2 in Figure 1A. In the 2-μm plasmid the active Flp site (1a plus 1′a) is flanked by a single half-site (1′b) (Figure 1A). While Flp binds with reasonable affinity to the flanking half-site 1′b, the enzyme is unable to cleave adjacent to this half-site (Andrews et al., 1987; Prasad et al., 1987).

DNA Cleavage in Situ by Peroxide–Flp. In the course of a series of footprinting experiments on the Flp–DNA complex, we performed a hydroxyl radical footprinting reaction (Tullius & Dombroski, 1986), within a polyacrylamide mobility-shift electrophoresis gel. In this experiment we diffused iron(II) EDTA, sodium ascorbate, and hydrogen peroxide into a gel on which had been separated free DNA and the three Flp–DNA complexes (presumably singly, doubly, and triply occupied) (Andrews et al., 1987) that occur at the site shown in Figure 1A. An unexpected result of this experiment was the observation of DNA cleavage at the edge of the 1′b (flanking) Flp half-site (Figure 1B), a cleavage event that had heretofore not been seen. This cleavage site is marked C3 in Figure 1. The unusual cleavage occurs at the same nucleotide with respect to the 1′b Flp binding site as for the normal Flp reaction at the 1′a site (Figure 1A).

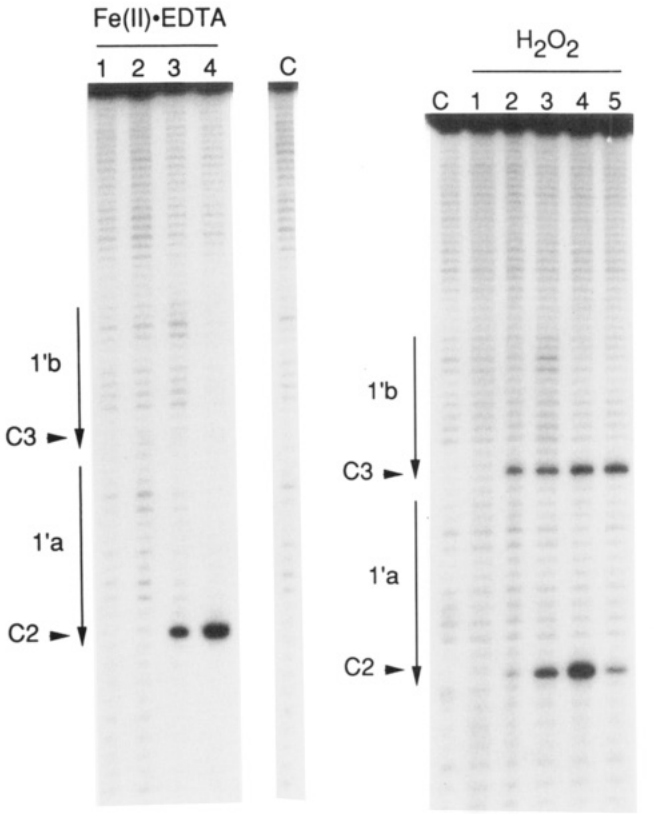

FIGURE 2: Flp acts in concert with hydrogen peroxide to cleave DNA at site C3. Flp–DNA complexes were formed using DNA that was radiolabeled at the 3′ end of the bottom strand (see Figure 1A) and were separated by electrophoresis on a native polyacrylamide gel. The gel was exposed to the reagent of interest. DNA was recovered and electrophoresed on a denaturing polyacrylamide gel. (A, left) The native gel was treated with 100 μM Fe(II)·EDTA for 4 min. Lanes 1–4: DNA recovered from unshifted and singly, doubly, and triply shifted bands, respectively. Lane C: DNA that was electrophoresed on the native gel but was not exposed to Fe(II)·EDTA. (B, right) Lane C: control DNA that was not run on a native gel. Lanes 1–5: DNA recovered from a native gel after the gel was treated with 0.3% hydrogen peroxide for 4 min. Lanes 1–4: unbound and singly, doubly, and triply bound DNA, respectively. Lane 5: DNA fully occupied by Fly(Y343F).

We next performed experiments to determine which of the added footprinting reagents were necessary for the unusual cleavage at C3. While iron(II) EDTA was inactive (Figure 2A), addition of hydrogen peroxide alone to the Flp–DNA complex in the gel gave cleavage adjacent to site 1′b (Figure 2B). The peroxide-mediated cleavage reaction was independent of the presence of the active site tyrosine (Tyr343) of Flp. The step-arrest mutant Flp(Y343F), which cannot cleave the DNA substrate in the normal Flp reaction (Prasad et al., 1987; Evans et al., 1990), is able to elicit the peroxide-induced cleavage at the edges of sites 1′a and 1′b (Figure 2B, lane 5).

DNA Cleavage in Solution by Peroxide–Flp. To characterize the reaction further, we performed cleavage of DNA with the peroxide–Flp reagent in solution. We used either wild-type Flp or the step-arrest Flp mutant Flp(Y343F) and a DNA substrate containing sites 1a, 1′a, and 1′b, radiolabeled at the 3′ end of the top strand and at the 5′ end of the bottom

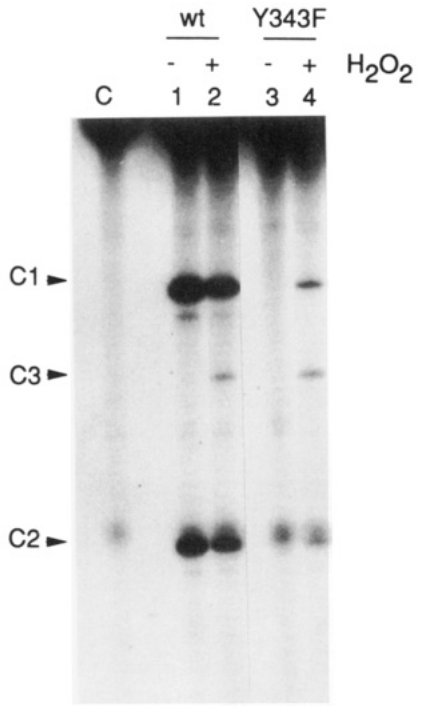

FIGURE 3: Flp and hydrogen peroxide in solution effect specific DNA cleavage. DNA that was radiolabeled at both the 3′ end of the bottom strand and the 5′ end of the top strand was preincubated with Flp (lanes 1 and 2) or Flp(Y343F) (lanes 3 and 4). Hydrogen peroxide was added to samples 2 and 4, and incubation of all samples was continued. Cleavage products was separated by electrophoresis on a denaturing polyacrylamide gel. Lane C: control untreated DNA. Cleavage sites are marked at the left of the autoradiograph. Cleavage at the unusual site C3 occurs only in the presence of hydrogen peroxide, for both wild-type Flp and Flp(Y343F) (lanes 2 and 4). Note that an artifactural band occurs in all lanes slightly above the position of the band representing cleavage at site C2. This artifactural band is the only band that is seen in lanes C and 3, as expected.

strand. With wild-type Flp alone (Figure 3, lane 1), cleavage at sites C1 and C2 (Figure 1A) is seen. Addition of hydrogen peroxide to wild-type Flp causes the appearance of a third product, representing cleavage at site C3 (lane 2). The Flp step-arrest mutant Y343F is incapable of DNA cleavage on its own (lane 3). However, addition of hydrogen peroxide to Flp(Y343F) results in cleavage at all three sites (lane 4). This experiment confirms the inference (Figure 2) that the active site tyrosine of Flp, which is essential for the normal Flp reaction, is not required for the peroxide–Flp DNA cleavage reaction.

Products of Strand Cleavage. How does hydrogen peroxide, combined with Flp, effect the unusual strand break at site C3? One mechanism for cleavage of DNA by hydrogen peroxide would invoke the intermediacy of free radicals produced by reaction of hydrogen peroxide with adventitious transition metal ions. The products of such a reaction would most likely be DNA strands left with phosphate at the ends adjacent to the deoxyribose that was attacked by the radical (Stubbe & Kozarich, 1987). A result of this reaction would be the elimination of the base and the deoxyribose of the nucleotide at the site of the reaction. Alternatively, nucleophilic attack on the phosphodiester, although heretofore unprecedented, would result in the production of one phosphate and one hydroxyl end and no net loss of deoxyribose or base from the DNA.

To determine the ends left on the DNA after the peroxide–Flp cleavage reaction, we compared the electrophoretic mobility on a high-resolution denaturing gel of the product of the peroxide–Flp cleavage reaction with the mobilities of DNA strands with known ends. The product of the reaction of the hydrogen peroxide–Flp(Y343F) reagent with a DNA molecule radiolabeled at the 3′ end of the bottom strand migrates between the products of hydroxyl radical-induced cleavage (Figure 4A), which are known to have 5′-phosphate ends (Stubbe & Kozarich, 1987; Tullius & Dombroski, 1986). This result is consistent with the Flp–hydrogen peroxide reaction leaving a 5′-hydroxyl end on the DNA strand, as does the normal Flp cleavage reaction.

DNA radiolabeled at the 5′ end of the bottom strand provides information on the identity of the 3′ end of the cleavage product (Figure 4B). The product of the normal Flp reaction has its 3′-phosphate covalently linked to Flp via Tyr343. Hence this product goes into the organic phase upon extraction with phenol–chloroform (lane 1) unless the reaction mixture is digested with proteinase K prior to extraction (lane 2). The electrophoretic mobility of the 3′-phosphotyrosyl DNA strand that is the product of proteinase K treatment is lower than that of a DNA strand of the same length having a 3′-phosphate end (lane 2). In contrast, both Flp (lanes 3 and 4) and Flp(Y343F) (lanes 7 and 8) combined with hydrogen peroxide yield a DNA cleavage product that comigrates with the appropriate band of a chemical sequencing ladder (Maxam & Gilbert, 1980) obtained from the same strand. Maxam–Gilbert chemistry leaves DNA with phosphate at both the 5′ and 3′ ends. Proteinase K treatment has no effect on the mobilities of the peroxide–Flp reaction products (compare lane 3 and 4 with lane 7 and 8). We conclude that the 3′ end of the product of peroxide–Flp cleavage is a free phosphate, not linked to protein.

Measuring the lengths of the DNA fragments produced after cleavage at site C3 demonstrates that no nucleotide is lost in the peroxide–Flp cleavage reaction. With 3′ radiolabeled DNA, sites C2 and C3 are exactly 14 nucleotides apart (Figure 4A; see also Figure 1A). With 5′ radiolabeled DNA, site C3 is again 14 nucleotides from site C2 (Figure 4B). If a nucleotide had been destroyed in the reaction, as would have occurred as the result of radical attack (Stubbe & Kozarich, 1987), these lengths would not be equal. Instead, we find that a single phosphodiester bond is broken in the reaction, between the two cytosines at site C3 (Figure 1A). Taken together, these observations provide strong evidence that the peroxide–Flp system cleaves DNA heterolytically and not by a radical-based mechanism.

The peroxide–Flp cleavage reaction is quite chemically clean. The fact that a single sharp band is seen at site C3 for both 3′ (Figure 4A) and 5′ (Figure 4B) radiolabeled DNA shows that no products other than strands having 3′-phosphate and 5′-hydroxyl termini at the site of cleavage are produced in the reaction.

Why is peroxide able to effect DNA cleavage when water, for example, is not? Peroxide is a stronger nucleophile than water, by virtue of the α-heteroatom effect (March, 1992). It is likely that the peroxide ion is the nucleophile in the reaction, as it is likely that it is the phenolate ion of tyrosine that is employed by the wild-type enzyme. The pK_a of hydrogen peroxide, 11.6, is lower than that of water and is, in fact, near the pK_a of the phenolic moiety of tyrosine, 10.1. In the case of proteins, Rana and Meares (1991) have shown recently that iron(II) EDTA, covalently attached to a protein, is able to activate hydrogen peroxide for nucleophilic attack on nearby peptide bonds, causing cleavage of the protein backbone.

CONCLUSIONS

We have shown here that DNA cleavage by Flp can be separated into two steps, activation of the phosphodiester and

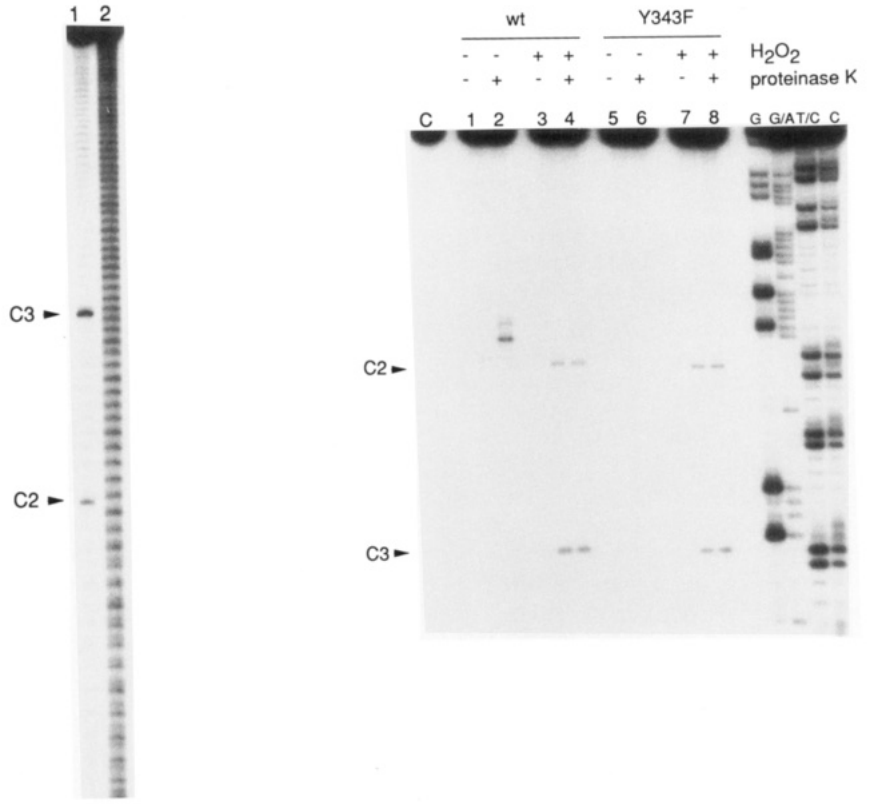

FIGURE 4: Characterization of the ends left on DNA after peroxide–Flp cleavage. (A, left) DNA cleaved by Flp(Y343F) plus hydrogen peroxide does not have 5′-phosphate at the end. The DNA used was radiolabeled on the 3′ end of the bottom strand (see Figure 1A). Lane 1: products of the Flp(Y343F)–hydrogen peroxide reaction. Lane 2: products of hydroxyl radical-induced cleavage. Shown is the autoradiograph of the denaturing gel on which these samples were electrophoresed. The prominent bands (C2 and C3) seen in lane 1 run between the products of hydroxyl radical cleavage, which are known to have phosphate at the 5′ end. The DNA sample run in lane 1 was recovered from a native gel after the gel was treated with 0.3% hydrogen peroxide for 4 min. DNA was cleaved with the hydroxyl radical and run in lane 2. (B, right) A phosphate group, not linked to Flp, occurs at the 3′ end of DNA cleaved by hydrogen peroxide–Flp. DNA radiolabeled at the 5′ end of the bottom strand (see Figure 1A) was used in this experiment. Lane C: untreated DNA. Lanes 1–4: DNA treated with wild-type Flp, plus the reagents indicated. Lanes 5–8: DNA treated with Flp(Y343F), plus the reagents indicated. The four lanes on the right contain the products of the indicated Maxam–Gilbert sequencing reaction. Bands C2 and C3 comigrate with the Maxam–Gilbert products, which are known to carry phosphate at the 3′ end.

nucleophilic attack. While it is not possible to observe separately these two steps with the wild-type enzyme, the step-arrest mutant Flp(Y343F) activates DNA for nucleophilic attack but is incapable of strand cleavage on its own. Hydrogen peroxide, however, can diffuse into the DNA–Flp(Y343F) complex and effect the second, strand cleavage, step. This cleavage can occur at an isolated Flp site which normally is not cleaved by the wild-type enzyme. Our results dovetail nicely with the recent discovery that the tyrosine that attacks a particular DNA phosphodiester comes not from the Flp that is bound nearest to that phosphodiester but instead from the Flp that is bound across the 8-bp strand-exchange region (Lee et al., 1992). Thus the natural reaction also can be thought of as employing a nucleophile in trans.

As a corollary to the observations we report here, our work shows that Flp(Y343F) can be used to make a sequence- and strand-specific, single-stranded nick in the DNA backbone, a reaction that is difficult to perform otherwise. The concept we demonstrate, that activation of a phosphodiester and its attack by a nucleophile can be performed by separate molecules, might profitably be applied to the design of new sequence-specific reagents for the hydrolysis of DNA.

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