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Modified DNA fragments activate *NaeI* cleavage of refractory DNA sites

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ABSTRACT

Endonuclease *NaeI* cleaves DNA using a two-site mechanism. The DNA-binding sites are nonidentical: they recognize different families of flanking sequences. A unique *NaeI* site that is resistant to cleavage resides in M13 double-stranded DNA. *NaeI* can be activated to cleave this site by small DNA fragments containing one or more *NaeI* sites. These activators are not practical for genetic engineering because unphosphorylated activators that are consumed during the cleavage of substrate give ends that may interfere with subsequent ligations. We show that a DNA fragment containing phosphorothioate linkages at the *NaeI* scissile bonds (S-activator) is not cleaved by *NaeI*, even though this S-activator binds to the substrate site. The S-activator activates *NaeI* to cleave M13 DNA under conditions that completely exhaust unsubstituted activator. These results demonstrate that activation is not coupled to cleavage of activator, that *NaeI* reverts to its inactive state soon after dissociation of the EA complex, and that S-activator makes for a nondepletable activator during prolonged incubations.

INTRODUCTION

Several type II restriction enzymes, including *EcoRII* [1], *NaeI* [2,3], and *BspMI*, *HpaII*, *NarI*, and *SacII* [4], cleave their DNA substrates by means of a two-site mechanism. Occupation of one site either allosterically increases binding of substrate at the other site (*HpaII*, *NarI*, and *SacII*) or enables cleavage at the first bound site (*NaeI* and *BspMI*) [4]. The two *NaeI* DNA-binding sites are not identical: they recognize different families of sequences—activator and substrate [5]. The families of sequences are differentiated by the sequences flanking the core *NaeI* recognition sequence GCC/GGC. The unique *NaeI* recognition site in M13 double-stranded DNA is resistant to cleavage. *NaeI* endonuclease can be activated to cleave this site by activator DNA containing a susceptible *NaeI* site: the activator DNA can be either a natural or a synthetic duplex with or without phosphorylated ends [2].

Synthetic activator DNAs may be useful to overcome the resistance of DNA cleavage sites in genetic engineering, in DNA fingerprint analysis, and in the analysis of restriction fragment length polymorphisms (RFLPs), where complete cleavage is important. To be useful in genetic engineering the fragment must not interfere with subsequent ligation steps. Either an unphosphorylated DNA fragment must be used or one with ends blocked in some other fashion (e.g., 3′ deoxy ends). In addition, it is important that the activator not be cleaved to regenerate phosphorylated ends.

Phosphorothioate analogs have found many applications for studying enzyme functions and mechanisms. These substrate analogs have been used as reversible and irreversible inhibitors, transition state analogs, suicide substrates, and spectroscopic probes (for review, see ref. 6). Phosphorothioate containing DNAs have also been used to study restriction enzymes [7–9] and have had practical applications in restriction enzyme technology [10]. Many DNAs containing phosphorothioate substitutions are resistant to cleavage by their cognate restriction enzyme [7–9].

We investigated the cleavage and activation properties of DNA fragments containing phosphorothioate-substituted linkages (S-activator) at the scissile bonds in the *NaeI* recognition sequence. Here, we report that the phosphorothioate-containing strand was not cleaved by *NaeI* whether one or both of the strands of S-activator contained the phosphorothioate substitution: the unsubstituted strand in the heteroduplex activator was cleaved. S-Activator homoduplexes activated *NaeI* to cleave its resistant recognition sequence in M13 DNA under conditions that exhausted the unsubstituted activator (O-activator). Although not cleaved, S-activator was shown by competition experiments to bind to the substrate site. In addition to implying the utility of these activators, our results demonstrate that cleavage of the scissile bond of the activator DNA is not required for *NaeI* activation.

MATERIALS AND METHODS

M13mp18 double-stranded DNA was from Bethesda Research Laboratories (MD). Oligodeoxyribonucleotides 1) GGGTGCCGGCAGGG, 2) CCCTGCCGGCACCC, 3) GGGTGCC-S-GGCAGGG, and 4) CCCTGCC-S-GGCACCC

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were synthesized with an Applied Biosystems model 380A programmable DNA synthesizer according to methods provided by Applied Biosystems and using the manufacturer's reagents. The phosphorothioate DNA analogs were synthesized according to Applied Biosystems by sulfurization of a phosphite linkage introduced during automated synthesis. Therefore, the phosphorothioate is a mixture of the Rp and Sp diastereomers. Oligodeoxyribonucleotides were purified by polyacrylamide gel electrophoresis, crush eluted, phenol extracted, alcohol precipitated, and desalted through Sephadex G-25.

Oligodeoxyribonucleotides 1 and 3 were end labelled with (α-^{32}P)rATP (6000 Ci/mMole, 10 mCi/ml, NEN) using T4-polynucleotide kinase (New England Biolabs), incubated at 65° for 20 min after labeling, desalted through Sephadex G-25, and annealed to the complementary unlabeled oligodeoxyribonucleotide.

NaeI endonuclease was obtained from New England Biolabs (MA). Cleavage reactions were performed in 20 mM NaCl, 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol and bovine serum albumin (0.1 mg/ml) at 37° in 10 μl with the units of enzyme and length of time of reaction indicated. One unit of *NaeI* activity is defined as that amount of enzyme required to completely digest 1 μg of Adeno-2 DNA in 1 hr at 37°C in the above reaction buffer. Cleavages of M13 double-stranded DNA were assayed with ethidium-bromide stained 1% agarose gels using TAE gel buffer (40 mM Tris-base, 20 mM sodium acetate, and 1 mM EDTA, adjusted to pH 7.4 using acetic acid). Cleavage of activator was assayed by electrophoresis through a 20% polyacrylamide gel and autoradiography. For analysis of single-strand cleavage, the reaction products were mixed with an equal volume of a formamide/dye solution, heated to 100° for 10 min and put on ice and electrophoresed on a 20% polyacrylamide TBE (90 mM Tris-base, 90 mM boric acid, 2.5 mM EDTA) gel containing 7 M urea and the products visualized by autoradiography. Amounts of cleavage were quantitated by densitometric analysis of the autoradiograms exposed to be within the linear range of the film.

RESULTS

A DNA fragment that activates restriction enzyme cleavage of DNA without being consumed in the process should be useful for genetic engineering. Phosphorothioate-substituted linkages have been shown to block DNA cleavage by several restriction enzymes [7–9]. Therefore, we determined whether phosphorothioate-substituted linkages block *NaeI* cleavage, and compared the ability of *NaeI* to cleave O-activator and S-activator homoduplex DNA. We found that O-activator was completely cleaved with 0.8 units of *NaeI* in 30 min (Fig. 1), whereas S-Activator was not cleaved even after 4 hrs of incubation with this same amount of *NaeI*.

Only DNA fragments containing cleavable recognition sequences have generally been reported to act as activators [1, 2, 4]: for example, M13 DNA will neither activate its own cleavage nor that of other resistant DNAs [2]. Therefore, S-activator, which was shown above not to be cleaved by *NaeI* (Fig. 1), was studied for its ability to activate *NaeI* cleavage of M13 DNA substrate. S-Activator, which contains an *NaeI* recognition site with a phosphorothioate substitution in the scissile bond, was compared with O-activator for its ability to activate *NaeI* cleavage of DNA. The *NaeI*-activating abilities of these two DNA fragments were found to be similar (Fig. 2): 0.2 μg of M13 DNA was completely linearized, over a range of activator concentration from 0.013 to 1.3 μM, whether or not the phosphorothioate substitution was present at the scissile bonds. A small amount of residual supercoiled M13 DNA remained when the greatest concentration of O-activator was used (Lane 4): this is consistent with the ability of activator, by means of its *NaeI* recognition site, to competitively inhibit substrate cleavage when added at high concentration [5].

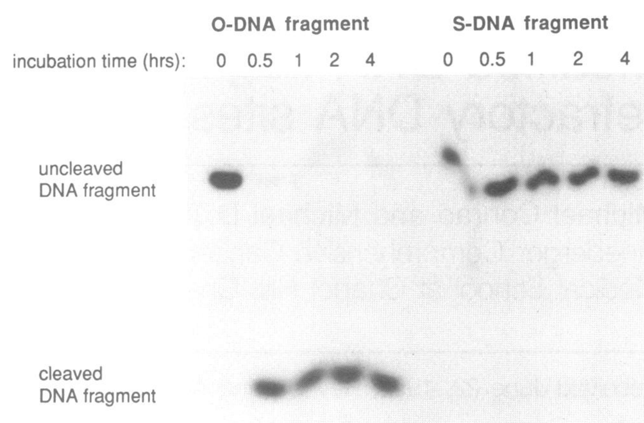
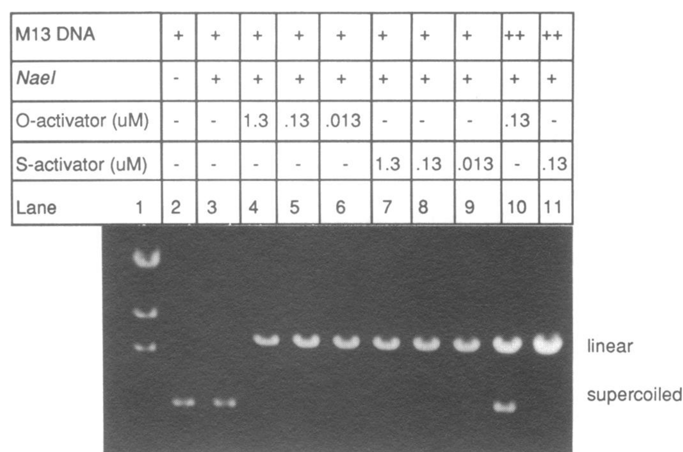


Figure 1. Endonuclease *NaeI* cleavage of phosphoro (O) and phosphorothioate (S) homoduplex DNA fragments. Oligodeoxyribonucleotide 1 was radiolabeled and annealed to its complement and the duplex at 0.13 μM was reacted with 0.8 U *NaeI* for the time indicated. The S-oligodeoxyribonucleotide 3 was radiolabeled and annealed to its phosphorothioate DNA complement and the duplex reacted as above. Reaction products were separated on a 20% polyacrylamide nondenaturing gel and autoradiographed.



M13 DNA	+	+	+	+	+	+	+	+	++	++
NaeI	-	+	+	+	+	+	+	+	+	+
O-activator (uM)	-	-	1.3	.13	.013	-	-	-	.13	-
S-activator (uM)	-	-	-	-	-	1.3	.13	.013	-	.13
Lane 1	2	3	4	5	6	7	8	9	10	11

Figure 2. Cleavage of M13 DNA by *NaeI* activated by phosphoro and phosphorothioate DNA fragments. Cleavage products were visualized by ethidium-bromide staining after electrophoresis on a 1% agarose gel. Lane 1, HindIII digest of lambda DNA (BRL mol. wt. markers); Lane 2, 0.2 μg M13 DNA; Lane 3, M13 DNA incubated for 30 min with 0.8 U *NaeI*; Lanes 4–6, same as Lane 3, but with the indicated conc. of O-activator (note the cleavage of M13 DNA); Lanes 7–9, same as Lanes 4–6, but with S-activator in place of O-activator; Lane 10, same as Lane 5, but with additional 0.2 μg of M13 DNA added after 30 min. of reaction time and the reaction allowed to proceed for another 30 min; Lane 11, same as Lane 10, but with S-activator instead of O-activator (note complete cleavage of added DNA).

Differences between S-activator and O-activator became apparent when an additional 0.2 μg of M13 DNA substrate was added to the cleavage reactions after 30 min of incubation with *NaeI*. The additional M13 DNA was not cleaved in 30 min by *NaeI* that had been activated by O-activator, whereas the additional M13 DNA was completely cleaved in 30 min by the *NaeI* that had received S-activator. Thus, S-activator had persisted through the first 30 min incubation with *NaeI* and was still present to activate *NaeI* to cleave the additional M13 DNA during the second 30 min incubation. These results demonstrate that S-activator can be used as a non-depletable activator for prolonged *NaeI* incubations.

Both O-activator and S-activator occupied the *NaeI* activator site to enable cleavage of M13 DNA. Can S-activator, which is not cleaved, bind to the *NaeI* substrate binding site? Competition between O- and S-activators for the substrate site was observed by reacting 0.13 μM radiolabeled O-activator with *NaeI* in the presence of increasing levels of either unlabeled O- or S-activator (Fig. 3). The amount of labeled O-activator cleaved decreased with increasing levels of competing activator. S-Activator competed about one-half to one-third as well for *NaeI* as O-activator. That is, over the linear range of the cleavage reaction, it took about two to three times as much unlabeled S-activator as unlabeled O-activator to reduce the amount of radiolabeled O-activator by a fixed amount.

To determine whether *NaeI* could nick a DNA fragment in which only one strand contained the phosphorothioate linkage at the scissile bond, the appropriate O/S-activator heteroduplex was constructed. NaeI cleavage of the O/S-activator and the O/O-activator were compared (Fig. 4). Reaction products were analyzed on a 20% polyacrylamide denaturing gel. The labeled unmodified strand of both activating DNA fragments was cleaved. The labeled unmodified stand of the O/O-activator homoduplex was completely cleaved within 30 min in agreement with the results analysed on a native gel shown in Figure 1. (We attribute the apparent low level of persistant uncleaved O/O-activator material apparent in Figure 4 to an excess of labeled strand.) Cleavage of the unmodified strand of the O/S-activator heteroduplex was significantly slower: 55% of the starting material was cleaved under our reaction conditions after 4 hrs (Fig. 4). No double-strand cleavage of the heteroduplex was observed when the reaction products were run under native-gel conditions (not shown).

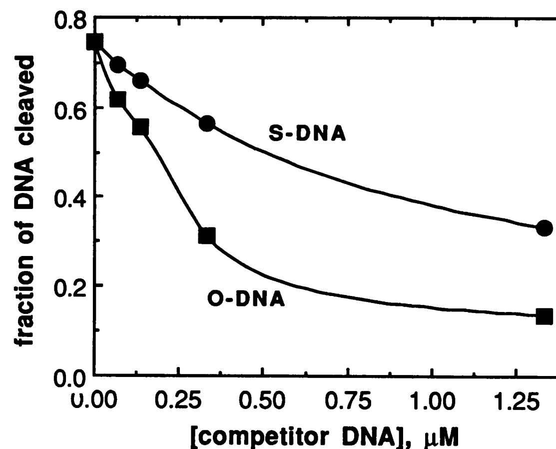


Figure 3. Inhibition by unlabeled O- and S-activators of *NaeI* cleavage of labeled O-activator. In 10 μl, labelled O-activator (0.13 μM) was reacted for 10 min with 0.8 U *NaeI* and the reaction inhibited by the addition of increasing [activator]. The experimental points show the fraction of labeled O-activator cleaved in the presence of (0, 0.066, 0.133, 0.333, and 1.33 μM) either unlabeled S-activator (●) or unlabeled O-activator (■). Reaction products were separated on a 20% polyacrylamide gel, the gel was autoradiographed, and densitometric measurements taken of the autoradiogram.

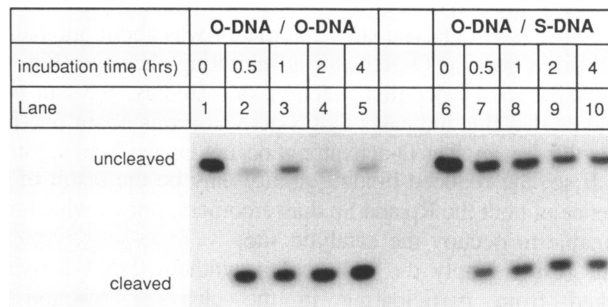


Figure 4. Interaction of *NaeI* with O/O-homoduplex activator and with a O/S-heteroduplex activator. In Lanes 1–5, 0.13 μM labeled oligodeoxyribonucleotide 1 was annealed to its DNA complement and reacted with 0.8 U *NaeI* in 10 μl for the time indicated. In Lanes 6–10, 0.13 μM labeled oligodeoxyribonucleotide 1 was annealed to its phosphorothioate DNA complement and reacted with 0.8 U *NaeI* in 10 μl for the time indicated. The reaction products were separated on a 20% TBE-urea denaturing gel and autoradiographed. The amount of cleavage was determined by densitometric analysis of the bands.

DISCUSSION

Resistant recognition sites exist for a group of type II restriction enzymes. This resistance can be overcome by the addition of exogenous activator DNAs containing cleavable sites. This discovery has potentially important implications for gene analysis and engineering. Since distant DNA cleavage sites must interact with the endonuclease to enable cleavage, one site may not be cleaved during RFLP analysis not because of a mutation at that site, but instead because of a mutation at the distant site. This distant mutation may be unrelated to the genetic polymorphism being studied. In addition, the inability to use some restriction sites in genetic engineering experiments could be overcome by activation of the appropriate enzymes.

We investigated the use of phosphorothioate substitutions in the scissile bonds of synthetic activator DNA. The purpose was to produce activators that could not be cleaved and so eliminate the ability of the activator to interfere in genetic engineering experiments. Many phosphorothioate DNAs are resistant to cleavage by their cognate restriction enzyme. Taylor et al. found that *AvaI* would cut neither the Rp nor the Sp diastereomer of a DNA homoduplex with a phosphorothioate linkage placed at the cleavage site [8]. *EcoRI*, however, can slowly cleave the Rp diastereomer of a homoduplex while the Sp diastereomer is not cleaved [7]. Twenty-nine restriction enzymes were tested with M13 double stranded DNA synthesized to contain phosphorothioate-modified minus-strand DNA[8]: seven of these enzymes exclusively nicked only the phosphate strand; six principally nicked the phosphate strand; and sixteen principally linearized the DNA.

We have expanded the study of the cleavage of phosphorothioate-containing DNA to *NaeI*, an endonuclease that fits a two-

site model for DNA cleavage [2, 3, 5]. The ability of a phosphorothioate containing homoduplex DNA fragment, S-activator, to be used by *NaeI* as activator and substrate was studied. S-Activator was not itself cleaved by *NaeI* but it did activate *NaeI* to cleave M13 DNA. This demonstrates that the susceptibility of the scissile bond to cleavage is not coupled to activation of *NaeI*. Our result implies that occupation of the *NaeI* activator site with all the proper contacts fulfilled is all that is required to induce the allosteric change in the protein that enables substrate cleavage. A similar conclusion was reached for *EcoRII*: activation of that endonuclease was achieved in the presence of *EcoRII* cleaved activator presumably held together by means of its sticky ends [11]. The dephosphorylated cleavage products, however, were unable to activate *EcoRII* [11], making this method of activation impractical.

The inability to cleave S-activator made for a persistent activator that was still present after all substrate in the reaction had been exhausted. The addition of new substrate was converted efficiently to product in the presence of S-activator, under conditions that completely exhausted O-activator. The inability of *NaeI* to cleave substrate once O-activator was exhausted demonstrated the lack of long-term memory of *NaeI*. That is, our results imply that *NaeI* must have the activator site occupied to cleave DNA: the allosteric change in *NaeI* is reversed upon dissociation of the EA complex.

Although S-activator was not cleaved by *NaeI*, it did inhibit cleavage of labeled O-activator. This shows that S-activator can occupy the *NaeI* catalytic site. S-activator was about one-half to one-third as good as O-activator at inhibiting cleavage (Fig. 3). In the absence of detailed reaction rate studies, we can only speculate that this indicates that S-activator was about one-half to one-third as good as O-activator at occupying the *NaeI* substrate site. If so, the reduced binding ability may be the result of the presence of both the Rp and Sp diastereomers, one of which may be unable to occupy the catalytic site.

Our results imply the utility of a synthetic DNA activator blocked from participating in the cleavage reaction by phosphorothioate analogs at the scissile bond. In principle, any analog that achieves the same lack of cleavage without greatly diminishing the affinity of the activator for the active site, will have equal utility. The resulting inexhaustible activator cannot participate in later steps required by genetic engineering experiment. We speculate that such activators will become useful tools to ensure cleavage of targeted sites, as it is realized that many restriction enzymes use a two-site mechanism to achieve DNA cleavage.

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