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Strand specific cleavage of phosphorothioate-containing DNA by reaction with restriction endonucleases in the presence of ethidium bromide

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ABSTRACT

A method for achieving strand specific nicking of DNA has been developed. Phosphorothioate groups were incorporated enzymatically into the (-)strand of M13 RF IV DNA. When such DNA is reacted with restriction endonucleases in the presence of ethidium bromide nicked DNA (RF II) is produced. All of the restriction enzymes tested linearised phosphorothioate-containing DNA in the absence of this dye. The strand specificity of the reaction was investigated by employing the ethidium bromide mediated nicking reaction in the phosphorothioate-based oligonucleotide-directed mutagenesis method. The mutational efficiencies obtained were in the region of 64-89%, indicating that these restriction enzymes hydrolyse the phosphodiester bond at the cleavage site of the unsubstituted (+)strand.

INTRODUCTION

Double stranded DNA containing phosphorothioate groups at certain positions in one strand is hydrolysed by some restriction endonucleases at a slower rate than unmodified DNA (1,2). In those cases where the final reaction product is nicked DNA it has been shown that cleavage occurs in the non-substituted strand (3,4). This observation has led to the phosphorothioate-based oligonucleotide-directed mutagenesis method which is explained in more detail in preceding publications (4-6).

One of the key steps in this method is strand specific cleavage of the (+)strand of M13 RF IV DNA by a restriction endonuclease. The DNA contains phosphorothioate groups only in the (-)strand which carries the desired mutation. The method is limited at present to eight restriction enzymes which produce nicked DNA . Several other enzymes tested previously in this laboratory were shown to linearise phosphorothioate DNA either after nicking had been completed (class II) or before complete formation of the nicked intermediate (class III) had occurred , thus,

rendering them unsuitable for use in this mutagenesis method (3). As these two groups of enzymes contain widely used restriction endonucleases including Eco RI, Bam HI, Hind III and Sac I, it seemed desirable to find conditions under which these enzymes would produce nicked DNA exclusively, thus extending the usefulness of the mutagenesis method.

Certain restriction endonucleases nick unmodified double stranded DNA without strand preference when the reaction is carried out in the presence of ethidium bromide (7,8). Based on these observations, it occurred to us that it might be possible to produce DNA nicked exclusively in the (+)strand by treating DNA containing phosphorothioate groups in the (-)strand with restriction endonucleases in the presence of ethidium bromide. If successful this should extend the applicability of the method provided that cleavage occurs specifically in the non-substituted strand.

We report here the efficient strand specific nicking of phosphorothioate-containing DNA by restriction endonucleases which normally linearise such substrates. This may be achieved by performing the enzymatic reaction in the presence of ethidium bromide.

EXPERIMENTAL

Materials and Methods

The enzymes Bgl I, Hind III, Pvu II and Sma I were obtained from Boehringer Manheim; Bam HI and Hpa II were from Gibco-BRL; Hgi AI, Sac I and exonuclease III were from New England Biolabs. The enzymes polynucleotide kinase and T7 gene 6 exonuclease were supplied by United States Biochemicals. Eco RI and DNA polymerase I were gifts from F. Grosse (Göttingen). The Klenow fragment, T4 DNA ligase and the phosphorothioate analogues, dNTPαS, used are described in the preceding paper (6). Nitrocellulose filters (13 mm in diameter, SM 11366) were supplied by Sartorious (Göttingen). Spun columns were prepared from Sephadex G50 DNA grade resin (Pharmacia) as described in (9). Single-stranded M13 DNA was prepared by the procedure of Nakamaye (4). The oligonucleotides mp2EM3 (5′-CGGCCAGTTGATTCGTAA-3′) and mp18EM3 (5′-CCGAGCTTTGATTCGTAATC-3′) were as described previously (3). Ethidium bromide was used as supplied by Sigma Chemie GmbH without further purification. The Geneclean kit was supplied by Bio 101 Inc. (La Jolla, California) and was used as directed. Analytical gel electrophoresis was performed exactly as desribed previously in 1% agarose slabs (3).

Ethidium Bromide Mediated Nicking Reactions

Preparation of DNA Substrates: M13 single-stranded DNA (10 µg) was annealed with calf thymus primer (10) by heating at 70°C for 5 min in 125 mM Tris·HCl, pH 8, and 125 mM NaCl in a volume of 40 µl. The solution was transferred immediately to a heating block at 37°C for 15 min.

Polymerisation of the primed template was carried out by adjusting the annealing solution to contain 50 mM Tris·HCl, pH 8, and 50 mM NaCl, 8 mM $MgCl_2$, 800 µM ATP, 200 µM each of the four dNTPs or with one dNTP replaced by its dNTPαS analogue as required at the same concentration, 10 units each of DNA polymerase I and T4 DNA ligase, in a total volume of 75 µl. The polymerisation was carried out at either 16°C overnight or at 37°C for 2 hours. The reaction was terminated by heating at 70°C for 10 min. The DNA was then precipitated with ethanol at -78oC (11). The DNA pellet was washed with cold 70% ethanol and resuspended in 200 µl of water. These stock solutions of DNA were then desalted using a spun column (9).

Restriction Enzyme Reactions

The buffer conditions used are outlined in Table I. Reactions were performed using 1 µg of either unmodified or the appropriate phosphorothioate-containing RF IV DNA as substrate. Ethidium bromide was added to either 5, 20, 40 or 80 µg/ml final concentration before addition of the specified amounts of enzyme. Control experiments were performed without ethidium bromide. Each reaction was carried out in a volume of 25 µl. Samples of 10 µl were removed from each reaction after the time period specified and analysed by agarose gel electrophoresis.

Mutagenesis Procedures

Mutational Systems: All mutagenesis reactions were performed on either M13mp2TAA or M13mp18TAA single-stranded DNA which produce colourless plaques on X-gal plates, when transfected into ΔM15 deficient cells. Using a mismatch primer to restore α-complementation, mutants were scored as blue plaques (4). The primer mp2EM3 converts the TAA stop codon of M13mp2TAA to a glutamine CAA codon by a single base change. The primer mp18EM3 also converts the TAA stop codon of M13mp18TAA to a glutamine codon.

Phosphorylation of Primer: The oligonucleotide primer, mp2EM3 or mp18EM3 (2 µl, 5 A_{260} units/ml) was treated with polynucleotide kinase (5 units) in 1 mM ATP, 100 mM Tris·HCl, pH 8, 10 mM $MgCl_2$ and 7 mM

Table I Buffer Conditions Used for Restriction Enzyme Reactions

Restriction Enzyme	dNTPαS[a]	Enzyme Units[b]	Salt Concentrations (mM) Tris·HCl	NaCl	$MgCl_2$	KCl	DTT
Bam HI	dGTPαS	27	5	150	6	-	-
Bgl I	dATPαS	36	10	65	10	-	1
Eco RI	dATPαS	30	100	50	5	-	5
Hgi AI	dCTPαS	6	25	75	5	-	2.5
Hind III	dATPαS	11	50	50	10	-	-
Hpa II	dCTPαS	12	10	-	10	10	1
Pvu II	dCTPαS	11	6	60	6	-	6
Sac I	dCTPαS	20	6	-	6	-	6
Sma I	dGTPαS	30	6	-	6	20	6

a) The dNTPαS indicated was used in the polymerisation reaction in place of the corresponding natural dNTP to produce the phosphorothioate-containing DNA examined.
b) All restriction enzyme digests were performed at 37°C using 1µg double-stranded DNA as substrate. Units as defined by commercial suppliers. Reactions were sampled after 120 min (except for Pvu II which was terminated after 60 min).

DTT in a total volume of 30 µl at 37°C for 15 min. The reaction was halted by heat inactivation at 70°C for 10 min.

Annealing: The appropriate M13 single-stranded DNA, mp2EM3 or mp18EM3 (10 µg) was annealed with the 5′-phosphorylated mismatch primer (mp2EM3 or mp18EM3, 5 µl of the phosphorylation reaction) by heating at 70°C for 5 min in 125 mM Tris·HCl, pH 8, and NaCl 125 mM in a volume of 40 µl. The solution was then transferred immediately to a heating block at 37°C for 20 min and then kept on ice.

The polymerisation reaction and the nitrocellulose filtration step were performed as in the preceding paper (6). After filtration the DNA was precipitated with ethanol at -78°C (11) and pelleted by centrifugation. The DNA pellet was washed with cold 70% ethanol and resuspended in the appropriate nicking buffer to a final concentration of 40-50 µg double stranded DNA/ml.

Nicking Reaction: The ethidium bromide mediated nicking reaction was then performed as outlined in Table II. The enzymatic reactions with Pvu II, Hpa II and Bgl I were terminated by heat inactivation of the enzymes at 70°C for 10 min. The Hind III reaction was terminated by

Table II Conditions Used to Produce Nicked DNA in the Mutagenesis Method

Restriction Enzyme	Reaction Time (min)	Ethidium bromide Concentration (μg/ml)	DNA Concentration (μg/ml)	Enzyme Units[a] (/μg DNA)
Bgl I	180	13	50	4.5
Hind III	180	25	40	11
Hpa II	120	20	50	7
Pvu II	180	10	40	13

a) Units of restriction endonuclease as defined by the commercial supplier.

extracting the nicking reaction solution twice with half its volume of phenol as described (12).

Extraction of Ethidium bromide: The DNA was purified by the Geneclean procedure. The purified DNA was eluted in water and converted to the appropriate buffer for the gapping reaction.

Gapping Reactions: DNA nicked by Pvu II or Hpa II was gapped with T7 gene 6 exonuclease as described (6). Exonuclease III was used to gap the Hind III and Bgl I nicked DNAs under the conditions described in (6).

Repolymerisation: After heat inactivation of the exonuclease the gapped DNA was repolymerised. The solution was adjusted to 800 μM ATP and 200 μM of each of the four natural dNTPs. DNA polymerase I and T4 DNA ligase were added (approx. 1 unit of ligase and 0.5 units of polymerase per μg DNA). The reaction was performed at 37°C (120 min) or at 16°C (overnight).

Transfections: The repolymerisation mixtures were used to transfect competent cells exactly as described in (4). The SMH50 strain of E. coli was used exclusively in this work (13).

RESULTS

Effect of Ethidium Bromide on Restriction Enzyme DNA Cleavage

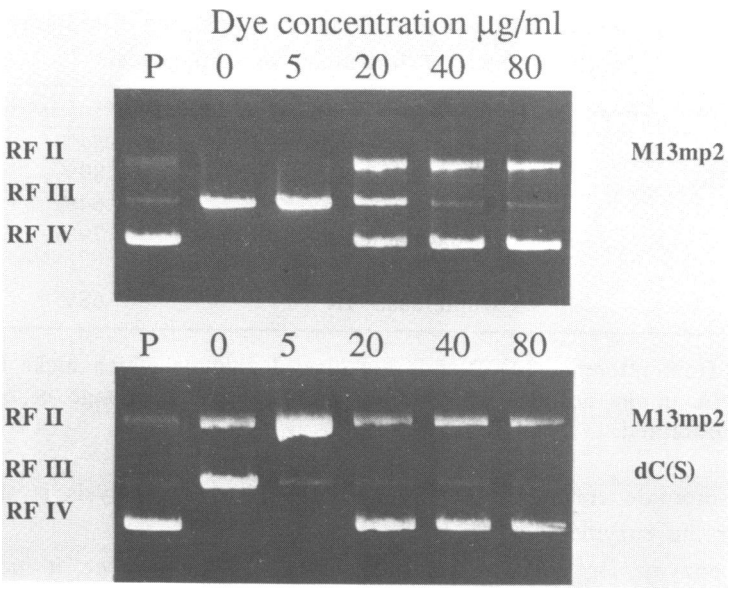
The effects of ethidium bromide on the restriction enzyme cleavage of phosphorothioate-containing, RF IV dN(S), and unmodified DNA are presented in Table III. It can be seen that the concentration of ethidium bromide determines the products obtained in the restriction reactions. Considering first the reactions performed with unmodified DNA it is apparent that all the enzymes linearise the substrate at the lowest

Table III Major Reaction Product(s) Obtained by Restriction Enzyme Digestion of RF IV DNAs in the Presence of Ethidium Bromide

Restriction Enzyme	M13[a] DNA	Ethidium Bromide Concentration (μg/ml) 5	20	40	80
Bam HI	mp18 dG(S)	L	N	C	C
	mp18	L	L	L	L/N
Bgl I	mp18 dA(S)	N/L	N	N	N
	mp18	L	L	L	L
Eco RI	mp2 dA(S)	N	N	N	N
	mp2	L	L	L	L
Hind III	mp18 dA(S)	N	N	C	C
	mp18	L	L	L	N/L
Hpa II	mp18 dC(S)	L	L	N/L	N
	mp18	L	L	L	L
Pvu II	mp2 dC(S)	N	N/C	C	C
	mp2	L	N/L/C	C	C
Sac I	mp18 dC(S)	L	N/L	N	C
	mp18	L	L	N/L	C
Sma I	mp18 dG(S)	L	N/L	N	C
	mp18	L	L/N	N/L	C

a) M13 RF IV DNA or the appropriate phosphorothioate containing RF IV DNA was used as indicated. The reactions were performed as detailed in the Materials and Methods section.
The products obtained are represented as follows: N, nicked DNA (RF II); C, double stranded closed circular DNA (RF IV); L, linearised DNA (RF III).

ethidium bromide concentrations employed. The enzymes Bgl I, Eco RI and Hpa II linearise the substrate even at the highest dye concentration of 80 μg/ml. At this concentration the enzymes Bam HI and Hind III begin to be inhibited in that they produce a mixture of nicked and linear DNA. These



Effect of Ethidium Bromide on the Cleavage of Natural and Phosphorothioate-Containing DNA by Pvu II: Lane P shows RF IV DNA before addition of the enzyme. The remaining lanes show the effect of various concentrations of dye (0-80 µg/ml) on the Pvu II cleavage reaction. The results for unmodified DNA (upper gel) and DNA containing phosphorothioate groups (lower gel) are shown.

products are seen at lower dye concentrations for the enzymes Pvu II, Sac I and Sma I for which the 80 µg/ml dye concentration is completely inhibitory.

The enzymes also differ in their reactivity towards phosphorothioate-containing DNA. Again Bgl I and Eco RI exhibit similar behaviour in that nicked DNA is produced by both enzymes over the dye concentration range of 20-80 µg/ml. The only difference being that at a dye concentration of 5 µg/ml, Eco RI nicks phosphorothioate-containing DNA while Bgl I is capable of producing appreciable amounts of linearised DNA.

Hind III and Pvu II also produce nicked DNA at the lowest dye concentration but are inactivated by increasing the dye concentration, leaving the RF IV DNA intact. For the remaining enzymes nicking sets in at 20 or 40 µg/ml of dye with most of them being inactivated at the highest

Table IV Mutational Efficiencies Obtained Employing Ethidium Bromide Mediated Nicking Reactions in Mutagenesis

Restriction Enzyme	Gapping Enzyme	Mutational Efficiency[b]
Bgl I	Exonuclease III	84%
Hind III	Exonuclease III	89%
Hpa II	T-7 exonuclease	64%
Pvu II	T-7 exonuclease	79%
Nci I[a]	Exonuclease III	68%

a) Taken from reference (4). Nci I is a class I enzyme which nicks RF IV dC(S) DNA in the absence of ethidium bromide. b) Percentage of blue plaques obtained.

ethidium bromide concentration. An example of a gel analysis is shown in Fig. 1 for the enzyme Pvu II.

The enzyme Hgi AI was not fully investigated. However, it too produced nicked phosphorothioate DNA under the conditions outlined in Table I together with 20 μg/ml ethidium bromide.

Strand Selectivity of the Ethidium Bromide Mediated Nicking Reactions

The phosphorothioate-based oligonucleotide-directed mutagenesis method was used as an assay sytem to determine whether or not the nicking reactions showed any strand selectivity. The nicking reactions with either Bgl I, Hind III, Hpa II or Pvu II were performed on RF IV dN(S) DNA containing a mismatch coding for blue plaque formation in the phosphorothioate substituted (-)strand. The RF II DNA so produced was gapped with an exonuclease and repolymerised. The mutational efficiencies obtained upon transfection are shown in Table IV.

DISCUSSION

The restriction endonucleases tested here cleave double stranded DNA under optimal conditions in both strands. This cleavage is thought to proceed via two separate strand cleavage reactions thus generating a nicked intermediate DNA molecule (14). This nicked species has been detected in the cleavage reaction of covalently closed circular DNA by the enzymes Eco RI, Bam HI and Hind III (15) and Hpa II (16). It has been suggested that Eco RI can dissociate freely from this nicked intermediate (15).

In the presence of the intercalating dye ethidium bromide significant

amounts of nicked DNA may occur. This phenomenon has been reported for a few enzymes such as Eco RI, Hind III, Bgl I, Pst I, Hinc II and Pvu II (7,8) and even for DNAse I (17) in the cleavage of covalently closed circular DNA. The mechanism by which the presence of ethidium bromide inhibits the second strand cleavage reaction is not clear. However, it has been demonstrated that nicked DNA has a higher affinity for the dye than covalently closed double stranded DNA (18). Thus, once the enzyme has performed the initial strand cleavage reaction increased ethidium bromide binding may prevent cleavage of the second strand.

We have shown earlier that a few restriction enzymes do not hydrolyse phosphorothioate internucleotidic linkages whereas others do so but slowly (3). When RF IV DNA containing phosphorothioate groups, incorporated into one of the two strands, is employed as substrate for some restriction enzymes nicked DNA (RF II) can be detected either as the final product (class I enzymes) or as a distinct intermediate (class II enzymes). Members of the class I enzymes have been exploited in the phosphorothioate-based mutagenesis method (3,4). There is also a third class of enzymes (class III) where no rate difference between cleavage of phosphate and phosphorothioate internucleotidic linkages has been observed. In these cases nicked intermediate and linearised final product are seen together even at the initial phase of the reaction. Most of the 30 enzymes investigated belong to this latter class.

The data presented in Table III show that for restriction enzymes of class II (Bam HI, Bgl I, Eco RI, Hind III, Hpa II) as well as class III (Pvu II, Sac I, Sma I) ethidium bromide concentrations can be found where nicked DNA is the sole product when RF IV DNA with phosphorothioate groups in only one of the strands is employed. The dye concentrations required are much lower than for nicking of all-phosphate-containing DNA. The difference in concentration is particularly pronounced for the enzymes Bgl I, Eco RI and Hpa II which linearize such DNA even at the highest ethidium bromide concentration. The difference is somewhat less pronounced for Bam HI and Hind III and is quite small for the remaining enzymes.

The interesting question is whether this nicking is strand specific or not. It might be argued that it should be at least so for the class II enzymes as the reaction even in the absence of ethidium bromide is clearly biphasic. For one representative of these enzymes, Eco RI, it has been shown using an oligonucleotide containing a phosphorothioate group

at the cleavage site as substrate that the rate difference between the cleavage of a phosphate and a phosphorothioate internucleotidic linkage is approximately 15 fold (19). This would suggest that when both these groups are present the phosphate linkage should be hydrolysed first. For enzymes of class III such an argument does not hold.

We decided to answer this question by employing some of these enzymes listed in Table III for the phosphorothioate-based mutagenesis procedure (3,4). In this method 70-90% mutational frequency, close to the theoretical maximum of 100%, can be achieved employing enzymes of class I. As mentioned these enzymes nick the (+)strand of RF IV DNA when phosphorothioate groups are present in the (-)strand. This nick forms the starting point for an exonuclease reaction which destroys the (+)strand past a mismatch introduced by an oligonucleotide annealed to the wild type (+)strand and extended with the use of a dNTPαS. If the ethidium bromide mediated nicking reactions of class II and III enzymes are selective for the (+)strand then similarly high mutational efficiencies should result when such reactions are employed in the mutagenesis method. Nicking in the (-)strand is expected to produce a mutational frequency of at most 10% in the system employed here.

The results presented in Table IV do indeed show that high mutational frequencies are obtained which allow the conclusion that the nicking was at least strand selective for the unmodified strand. It is difficult to unambiguously explain the difference between the values obtained and those expected theoretically as both incomplete as well as strand-unspecific nicking could account for this.

This strand specific cleavage of the phosphodiester by class III enzymes raises the question as to what determines this specificity. One of the possible explanations could be that the phosphodiester is indeed cleaved faster than the phosphorothioate diester even by these enzymes but that in the absence of ethidium bromide the nicked intermediate cannot dissociate freely. In the presence of the dye, however, the intermediate dissociates upon additional binding of ethidium bromide to the nicked DNA. On the other hand it is also quite possible that rate of cleavage of both groups is similar in the absence, but different in the presence of ethidium bromide.

The results presented clearly show that strand specific cleavage by restriction enzymes can be achieved on RF IV DNA in the presence of ethidium bromide as long as one strand contains phosphorothioate groups

at the potential site of cleavage. Although only a very limited number of enzymes have been investigated we assume that this observation holds true for most restriction enzymes.

This strand specific cleavage is not only of interest in site directed mutagenesis as documented here but might also be extended to "local" mutagenesis (20) and "gap misrepair" mutagenesis (21) if the gene or gene fragment was cloned into a single stranded phage DNA or any vector containing a single stranded origin of replication e.g. pEMBL plasmids (22).

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