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Inhibition of restriction endonuclease Nci I cleavage by phosphorothioate groups and its application to oligonucleotide-directed mutagenesis

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ABSTRACT

M13 RF IV DNA where phosphorothioate groups are incorporated at restriction endonuclease Nci I recognition sites in the (-)strand is efficiently nicked by the action of this enzyme. Incubation of such nicked DNA with exonuclease III produces gapped DNA. The gap can be filled by reaction with deoxynucleoside triphosphates and DNA polymerase I. When this sequence of reactions is performed with DNA containing a mismatch oligonucleotide primer in the (-)-strand mutational frequencies of 70 - 90 % can be obtained upon transformation. The general nature of this methodology has been further shown to be applicable to other restriction enzymes such as Hind II, Pst I and Fsp I. The mutational frequency obtained using these enymes is between 40 - 80 % mainly because of less efficient nicking and gapping. Studies on inhibition of Nci I cleavage show that in addition to a phosphorothioate group at the position of cleavage an additional group in the 5'-neighbouring position is necessary for complete inhibition.

INTRODUCTION

We have recently reported on the inhibition of restriction endonuclease cleavage of M13 DNA by incorporation of phosphorothioate groups in the (-)strand at the position of cleavage (1). Three classes of enzymes could be distinguished in the examination of about 30 restriction enzymes: Those where only nicking was observed indicating resistance towards hydrolysis of the phosphorothioate group in the (-)strand (class I); those where complete nicking was followed by linearisation indicating a slow hydrolysis rate of the phosphorothioate group (class II) and those where nicking and linearisation occured with similar rates indicating no or only a small degree of inhibition by the phosphorothioate group (class III). One representative of the class I enzymes, the restriction endonuclease Pvu I, was investigated in more detail and its nicking ability of the (+)strand was used as the basis for the development of an efficient method of oligonucleotide directed mutagenesis (2). Mutation frequencies between 40 - 66 % were achieved by this method. We would like now to report an investigation of other restriction enzymes of class I

and their suitability for oligonucleotide-directed mutagenesis by the phosphorothioate method, with particular emphasis on the use of the restriction enzyme Nci I which yields mutational efficiencies of 70 - 90 %.

EXPERIMENTAL

The majority of the experimental techniques, materials and methods are given in the previous papers (1,2). Only additions and changes are reported here.

MATERIALS AND METHODS

Nci I, Pst I, Hind II and DNA polymerase I were obtained from Boehringer Mannheim; Fsp I, Hpa II and exonuclease III were from New England Biolabs; Taq I was from Bethesda Research Labs, Bgl II from Anglican Biotechnology Ltd., DNA polymerase I large fragment (Klenow enzyme) was from New England Nuclear. Sephadex G-50 used for spun columns was coarse grade from Pharmacia, swelled in water, packed into 1 ml columns and equilibrated with NTE buffer (20 mM NaCl, 10 mM Tris-Cl and 1 mM MEDTA, pH 8.0) as described (3). Phenol was redestilled and used as described (3). The phosphorothioate analogues dNTPαS were either synthesized (2) or obtained from New England Nuclear.

Preparation of Single-Stranded M13 DNA

The procedure previously described (2) was used with the following addition. After the first isolation of the phage precipitate, the phage is resuspended in 1 ml of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) and diluted to 10 ml. The solution is centrifuged (5000 xg for 20 min) and the supernatant transferred to a new centrifuge tube along with 2.5 ml of 20 % PEG-6000 in 2.5 M NaCl for a second phage precipitation of 30 min at 4°C. The remainder of the procedure is identical. A further test for contamination by cellular RNA or pieces of DNA is conducted by performing a "self-primed" polymerization, i.e. in the presence of no added primer. The double precipitation of phage typically yields a cleaner single-stranded DNA than previously found and comparable to CsCl-gradient centrifugation purified DNA.

Oligonucleotide Synthesis

NCI-1 (5'-CATGTCAATCATATGTACCC-3'), NCI-2 (5'-GCATGTCAATCATATGTACC-3'), and NCI-4 (5'-GCATGTCAATCATATGTA-3') oligonucleotides, extension primers for the Nci I site at position 6786 and 6839 in M13mp2 and M13mp18 respectively, were synthesized using the Omnifit Bench Synthesizer according to the published procedure as were the mp2EM2 and mp2 EM3 oligonucleotides (2). The mp18EM2 (5'-GTACCGAGCTTTAATTCGTAATC-3') and mp18EM3 (5'-CCGAGCTTTGATTCGTAATC-

Table 1 Nicking Protocols for Restriction Endonucleases[a]

	Nci I (CCGGG)	Hind II (GTTAAC)	Hind II (GTCGAC)	Pst I (CTGCAG)	Fsp I (TGCGCA)
RF IV DNA[b]	ca. 1.5 µg (-)sC	ca. 1.5 µg (-)sA	ca. 1.5 µg (-)sG	ca. 1.5 µg (-)sG	ca. 1.5 µg (-)sG
NaCl	30 mM	95 mM	95 mM	95 mM	50 mM
Tris-Cl (pH 8.0)	12 mM	12 mM	12 mM	12 mM	6 mM
Endonuclease	9 units	10 units	15 units	25 units	10 units
Time (Nicking)	90 min	90 min	ca. 15 h	90 min	ca. 15 h

[a] All reactions were carried out at 37°C in the presence of 6 mM $MgCl_2$ and 10 mM DTT;

[b] (-)sN indicates the presence of the corresponding dNMPS in the (-)strand.

3') oligonucleotides were synthesized on the Applied Biosystems 380B DNA Synthesizer, as were the mismatch oligonucleotides INNCIB (5'-GGGTAACGCCCGGATTTT CCAG-3'), PELNCI (5'-GAGGATCCCCAGGTACCGAG-3') and ELNCIA2 (5'-GTACCCCGATTGAT-AACT-3'), used for site-specific mutations at various positions on M13mp2 and M13mp18. Purification of the oligonucleotides was conducted as described previously (2).

Mutagenesis Procedure

The exact procedure differs slightly for each different restriction endonuclease in the nicking reaction buffer and post nicking treatment. A general description of these sections is given and the accompanying Table 1 shows the exact quantities for the individual restriction endonucleases. The procedures have been altered somewhat from those described previously as Mutagenesis Method A (2), so they will be completely shown here.

Polymerization: The appropriate M13 single-stranded DNA (10 µg) was annealed with the 5'-phosphorylated oligomer (5 µl of phosphorylation reaction, ca. 2 equivalents) in 50 µl of 120 mM NaCl and 120 mM Tris-Cl, pH 8.0 by heating to 70°C in a water bath for 5 min, immediately transferring to a 37°C heating block for 25 min and then to 0°C. To the solution was added $MgCl_2$ (10 mM), ATP (1 mM), the appropriate dNTPαS (250 µM), the other 3 dNTP (250 µM each), Klenow fragment (15 units), and T4 ligase (15 units) and diluted to 100 µl total volume. Incubation of this reaction mixture overnight at 16°C or normally even better for about 40 h, normally yielded double-stranded DNA that is about 2/3 RF IV and 1/3 RF II with a small amount of RF III by standard agarose gel analysis (2).

Purification: The polymerization reaction mixture was made 300 mM in NaOAc and slowly filtered through 2 nitrocellulose filters (13 mm diameter, SM 11366, Sartorius) that were prewetted with 500 mM NaCl (40 µl). The filters were washed with 500 mM NaCl (2 x 80 µl) and the combined filtrate diluted with 700 µl of absolute ethanol and the DNA precipitated as previously described. The DNA was redissolved in 50 µl of NTE buffer (20 mM NaCl, 10 mM Tris-Cl and 1 mM EDTA, pH 8.0). Approximately 40 - 60 % of the RF IV DNA was recovered as estimated by agarose gel analysis.

Nicking and Postnicking Treatment: Approximately 1.5 µg of RF IV DNA was diluted in the appropriate buffer and the nicking reaction conducted as shown in Table 1. Upon completion of nicking the solution was diluted to 100 µl and appropriately converted if necessary to Exonuclease III buffer (90 mM NaCl, 60 mM Tris-Cl, 6 mM $MgCl_2$, 10 mM DTT, pH 8.0). In the case of Hind II, Pst I, and Fsp I this solution was then extracted with 100 µl of phenol (2 x for Pst I) followed by ethyl ether extraction (4 x 200 µl water saturated ether).

Gapping: The nicked or if necessary postnick treated solution was gapped by the addition of exonuclease III (50 units) and placed in a 37°C heating block for the appropriate time, assuming about 100 bases digested per minute. Gapping was terminated by heating the reaction mixture to 70°C for 15 min.

Repolymerization: The gapped solution was then diluted to about 120 µl with the addition of the 4 dNTP's (250 µM each), ATP (350 µM) and $MgCl_2$ (10 mM), DNA Polymerase I (2.5 units) and T4 ligase (3 units) and incubated at 16°C for 3 - 16 h depending upon the extent of gapping.

Transformation: The repolymerization reaction mixture was used directly to tranform competent cells as previously described (2). The 1.5 min heat-shock at 42°C, however, was found to be superfluous and was omitted. In some studies the JM107 cell line was used but in later work the SMH-50 (4) cell line was preferred. This cell line is indentical with CSH-50 cells used by Kunkel (5). For a higher plaque yield which is not necessary when working with SMH-50 cells, the reaction mixture was passed through a 1 ml Sephadex G50 spun column and then transfected.

Hybridization Assay

The published procedure by Zoller and Smith (6) was used with the appropriate 5'-^{32}P labelled oligonucleotide. The autoradiographs were found to be more quickly exposed by using Kodak XAR-5 X-ray film. The final washing temperatures used ranged from about 4°C below the calculated Wallace temperature to about 5°C above.

RESULTS

Mutational Systems

Model mutational systems were used for most of the mutational efficiency studies which allowed easy scoring of plaques. The model system for the M13mp2 phage was identical to that used in the Pvu I studies (2). The oligomers EM2 and EM3 were designed to be mismatch primed to M13mp2 and M13mp2*och* respectively. The EM2 oligomer converts the seventh codon (TCA) in the ß-galactosidase gene fragment to an ochre (TAA) stop codon, by a single C→A base change. The prevention of expression of the *lacZ*α fragment allows scoring of mutants as colorless plaques on X-Gal plates. Oligonucleotide EM3 converts the colorless plaque producing ochre mutant to a blue plaque producing strain by converting the TAA stop codon to a glutamine (CAA) codon again by a single T→C base change and restoring the α-complementation. Since Pst I and Fsp I sites are not present in M13mp2, an analogous mutation system was prepared for M13mp18 phage. The two new oligomers mp18EM2 and mp18EM3 were synthesized, mp18EM2 converting the wild type M13mp18 codon TCG at the Eco RI site to the ochre stop codon (TAA) with a double mismatch C/T-G/T and thus producing colorless plaques on X-Gal plates. mp18EM3 reverts this M13mp18*och* mutant to a glutamine (CAA) codon as in the M13mp2 system with the single T/G mismatch.

Specific mutations were also created by the reported methodology for other purposes and were scored by standard dot blot hybridization techniques. These are reported and discussed under the individual endonuclease that was utilized for the mutations.

The previous publication (2) reported two methodologies, A and B, which were developed for the use of the enzyme Pvu I. The Method A methodology (Figure 1) involves the polymerization of the mismatch oligonucleotide primer on the template DNA in the presence of the appropriate dNTPαS necessary for the restriction endonuclease to be used. The thus produced double stranded DNA is nicked with the restriction endonuclease, gapped by exonuclease III to a point beyond the mismatch, repolymerized in the presence of the normal dNTP's to the double-stranded DNA, and transfected into *E. coli* competent cells. Because of the excellent results obtained using Method A, particularly with Nci I and with the new host *E. coli* strain SMH-50, Method B was only briefly studied.

General Procedures

Polymerization Ligation

In order to obtain high yields of mutants utilizing any mutagenic oligonucleotide technique, the oligonucleotide priming, polymerization, and liga-

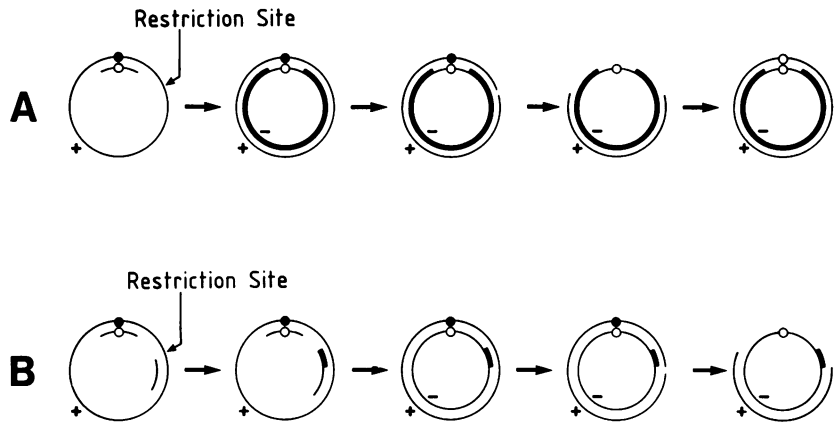

Figure 1.
Schematic Representation of the Mutagenic Methods A and B.

tion to produce the double-stranded DNA are of primary importance (for reviews see 6,7,8). We found that for an oligonucleotide primer containing 1 - 2 base mismatches a ratio of oligonucleotide:DNA of 2:1 is optimal. Klenow fragment catalyzed polymerizations have consistently worked very well if the enzyme is pure. Some commercial preparations of the Klenow enzyme, however, have enough residual exonuclease activity to remove the mismatch in the priming oligonucleotide (7,9). A critical factor in the polymerization/ligation reactions is the ratio of Klenow enzyme to T4 ligase (6). A systematic change in this ratio (fig. 2) revealed that a 1:1.5 ratio of units produces optimal results. At a lower ratio of T4 ligase higher molecular DNA is produced presumably by strand displacement. Consequently, later addition of T4 ligase does not result in an increase of RF IV DNA which is in contrast to the situation where DNA polymerase is used instead of Klenow enzyme (fig. 2). Because of these improvements in RF IV DNA synthesis, the previously reported (2) purification of the DNA by denaturation, filtration through nitrocellulose filter, precipitation of the DNA and passage of the redissolved DNA through Elu-tip D columns has been found to be unnecessary. Only a nitrocellulose filtration is required in order to remove any unpolymerized single-stranded template DNA.

Gapping

The gapping reaction with exonuclease III has been studied at different temperatures and is quite easily controlled at 37°C for much shorter periods of time than reported previously (2) at 25°C. In fact, for gaps longer than

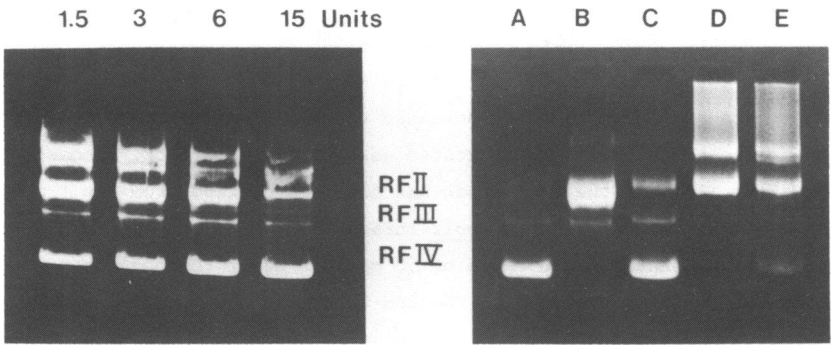

Figure 2.
Determination of Optimal Ratio of Klenow Polymerase to T4 DNA Ligase to Produce RF IV DNA. Left Panel: M13mp2 DNA was primed with a 5'-phosphorylated octadecanucleotide and the polymerisation conducted as described in the Experimental Section using 9 units of Klenow fragment and 1.5 to 15 units of T4 DNA ligase. Right Panel: polymerisation in presence of DNA polymerase I and ligase (A); in absence of ligase (B); subsequent addition of ligase to B (C); polymerisation in the presence of Klenow fragment but absence of T4 DNA ligase (D); subsequent addition of ligase to D (E). Analysis is by agarose gel electrophoresis in the presence of ethidium bromide (2).

about 600 bases, the higher temperature appears to be more reproducible in gap length versus time. The salt concentration for the exonuclease III reaction appears to be quite critical as is an excess of exonuclease III (10) to maintain a relatively processive digestion. The exonuclease III buffer of 90 - 100 mM NaCl and 60 mM Tris-Cl, pH 8.0, (2,10) produces a rate of digestion by exonuclease III from a nick on the order of 100 bases per minute, can create gaps in excess of 4500 bases, and is relatively processive as seen by gel analysis. These results are not directly comparable but are in relative agreement with the work of others (11,12) on double-stranded linear DNA, who report that at 37°C exonuclease III is somewhat processive and an approximation of their data shows approximately 75 bases digested per minute.

Transfection

Considerable experience with the transfection of JM107 competent cells and the repolymerized phosphorothioate-containing double-stranded DNA, shows for unknown reasons a very wide range of competency, appearing in results as highly variable plaque counts. This often resulted in very poor plaque yields in the transfections. Studies conducted to improve plaque yields and reproducibility provided interesting results. In agreement with the findings of Dagert and Ehrlich, the competent cells become most susceptible to transfection about 24 h after preparation (13). Taketo (41) reports the inhibition

of transfection by a number of agents including phosphate, polyphosphate, and ATP. Consistent with these findings, the use of a Sephadex G50 spun column (3) after the repolymerization of the DNA to remove the unreacted dNTP's, ATP, phosphate and pyrophosphate increased the plaque count 4 - 5 fold. Studies on the heat shock at 42°C indicated essentially no effect, also supported by Taketo (14) and the heat shock has now been omitted from the transfection procedure. A different E. coli strain, SMH-50 (4), provided greatly improved plaque counts in the range of 50 plaques/ng of thiophosphate containing double stranded DNA. Thus, the SMH-50 strain has solved the difficulty with variable and sometimes low plaque yield of the JM107 strain and is a highly recommendable cell line for these procedures, although other strains may work as well.

Mutagenesis Efficiency Results

Nci I

The restriction endonuclease Nci I is a Class I endonuclease by the definition given in ref. 1 and recognizes the 5 base sequence CCGGG (i) with cleavage between C-G or CCCGG (ii) with cleavage between C-C. The sequence ii is not present in the (+)strand of M13mp2, and in M13mp18 only in the polylinker region as CCCGGG which is a Sma I site as well as an overlap of the two Nci sites i and ii. Mutagenesis has only been studied from sequence i. The protection of the CCGGG recognition site requires the incorporation of dCTPαS in the oligonucleotide primed polymerization of the (-)strand, but in the cases so far investigated, the presence of at least one sequence i results also in the protection of the other Nci I site ii by this incorporation. This will be discussed in more detail below. The procedure for mutagenesis using Nci I is direct and simple as presented in the Experimental Section. For best results, the nicking reaction with Nci I should be conducted at the lower salt concentration (30 mM NaCl, 12 mM Tris-Cl, pH 8.0) in which Nci I is most active with a subsequent addition of NaCl and Tris-Cl pH 8.0 to the higher concentrations (90 mM NaCl, 60 mM Tris-Cl) used for the exonuclease III reaction. Figure 3 shows an agarose gel of the sequence of reactions used in the mutagenesis. As is observed in this figure, the nicking by Nci I is fast and complete, the exonuclease III gapping reaction is essentially processive, and the repolymerization reaction proceeds very efficiently. Inspection of Table 2 shows the excellent results obtained with the Nci I methodology. The experiments consistently proceed with 70 - 90 % mutational efficiency. The number of Nci I sites is not normally critical as shown in the examples with 1 - 4 Nci I sites present.

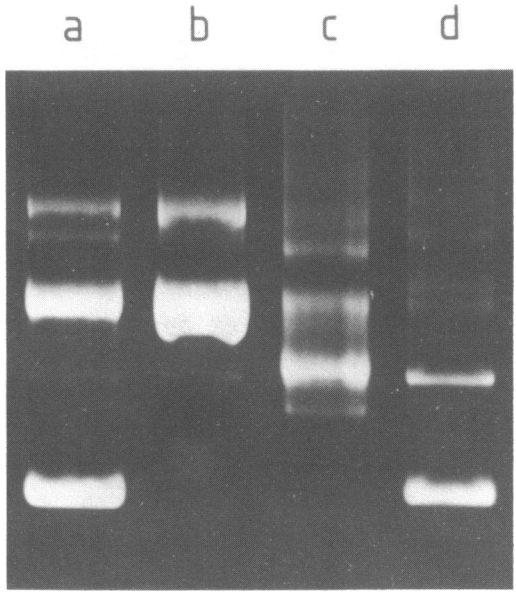

Figure 3.
Agarose Gel Analysis of Sequence of Reactions Used in Mutagenesis (Method A). Polymerisation reaction after filtration (a); Nci-nicking (b); Exo III-gapping (c); Repolymerisation (d). All reaction conditions as in the Experimental Section. Gel analysis as in fig. 2.

Table 2. Efficiency of Mutation

Restriction Enzyme	M13 Phage	Number of Restriction sites	Oligonucleotide used	Distance Nick-Mismatch	Directed Mutation	Total Number of Plaques or Plaques analyzed (% Mutation)	Method of Analysis
Nci I[a]	mp2	2	EM2	550	C→A	551 (82 %)	Plaque Count (Bl→Cl)
Nci I[a]	mp2 ochre	2	EM3	550	T→C	669 (68 %)	Plaque Count (Cl→Bl)
Nci I[a]	mp2ElB[g]	1	EM2	550	C→A	351 (89 %)	Plaque Count (Bl→Cl)
Nci I[a]	mp18	4	EM2	15	C→A G→A	53 (87 %)	Plaque Count (Bl→Cl)
Nci I[a]	mp18	4	INNCIB	600	C→T T→G	37 (86 %)	Dot Hybridisation[f]
			PELNCI		C→T	37 (51 %)	Dot Hybridisation
Nci I[a]	mp18	3	ELNCIB	4320	G→A	44 (68 %)	Dot Hybridisation
Hind II[b]	mp2 ochre	1	EM3	920	T→C	219 (69 %)	Plaque Count (Cl→Bl)
Hind II[c]	mp18 ochre	1	EM3	30	T→C	250 (86 %)	Plaque Count (Cl→Bl)
Hind II[b]	mp2	1	ELNCIA2	410	C→T	22 (50 %)	Dot Hybridisation
Hind II[b]	mp2	1	ELNCIA2	410	C→T	22 (36 %)	Dot Hybridisation
Pst I[d]	mp18 ochre	1	EM3	36	T→C	954 (75 %)	Plaque Count (Cl→Bl)
Pst I[d]	mp18	1	EM2	36	C→A G→A	373 (54 %)	Plaque Count (Bl→Cl)
Fsp I	mp18	1	EM2	200	C→A G→A	170 (22 %)	Plaque Count (Bl→Cl)
Fsp I[e]	mp18 ochre	1	EM3	200	T→C	113 (73 %)	Plaque Count (Cl→Bl)

a, Recognition sequence CC-GGG, dCMPS incorporated into (-)strand; b, recognition sequence GTT-AAC, dAMPS incorporated into (-)strand; c, recognition sequenceGTC-GAC, dGMPS incorporated into (-)strand; d, recognition sequence CTGCA-C, dGMPS incorporated into (-)strand; e, recognition sequence TGC-GCA, dGMPS incorporated into (-)strand; f, represented in fig. 4; g, mp2ELB is mp2 with Nci I-site at position 1926 removed.

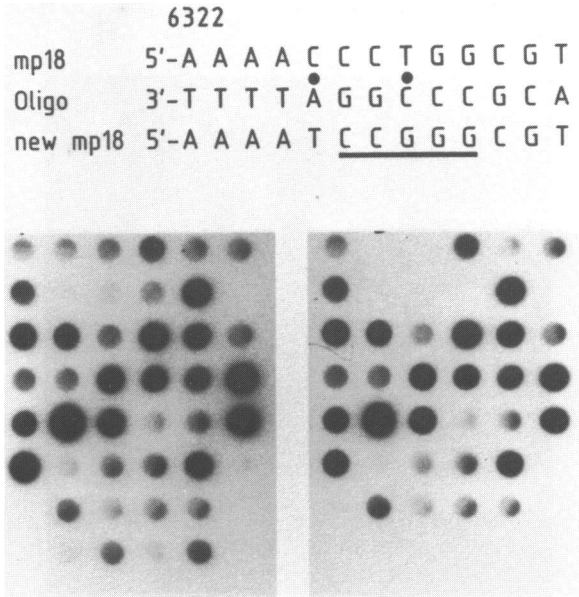

Figure 4.
Dot Blot Hybridisation Analysis of a Mutagenesis to Create a New Nci Site in M13mp18. The experimental conditions were as described in the Experimental Section. Washing at room temperature (left) and at 62° - 63°C (right). The bottom row at the left panel is M13mp18 DNA as a control. (See Table 2 for more details).

As the gapping by exonuclease III is critical for the wide applicability of this mutagenesis procedure a mutagenesis was conducted which required the removal of over 4320 bases in the gapping reaction. This was conducted on M13mp18 using a mismatch oligonucleotide to remove the Nci I site at position 1926 from the Nci I site in the polylinker region at position 6249. The dot hybridization analysis of this mutation showed 30 positive hybridizations out of 44 samples for a 68 % mutational efficiency. This result confirms that exonuclease can produce long gaps. As good mutational frequencies have been obtained from mismatches as close as 15 and as far as 4300 bases from the Nci nicking site, the length of the gap to be created is not a limiting factor in this methodology.

As the mutational efficiency is normally high we also attempted the similtaneous introduction of multiple mutations by employing two mismatch primers. One, INNCIB, was designed to introduce a new Nci site immediately upstream from the mp18 multiple cloning site by changing an Asn triplet AAC

into AAT and a Pro triplet CCT into CCG, thus creating a new CCGGG Nci site. The triplet change from AAC to AAT was necessary to prevent the creation of a double Nci site CCCGGG. The second oligonucleotide, PELNCI, was constructed to eliminate such a double site about 80 bases downstream by changing a CCC Pro triplet to CCT. Fig. 4 shows the dot hybridization analysis with the first oligonucleotide. The initial plaque picking was a sampling of 40 plaques, but 3 plaques failed to produce phage. The figure shows 32 positive dot hybridizations out of the 37 original samples for a 86 % mutational efficiency. Dot hybridization analysis with the second oligonucleotide produced only 19 positives of the 37 samples for a 51 % mutation rate. Interestingly, 100 % of the positive samples of the second mutation also were positive for the first mutation, showing the feasibility of multiple site-directed mutagenesis.

Hind II

The Class I (2) restriction endonuclease Hind II recognizes the 6 base sequence GTPyPuAC with cleavage between the Py-Pu. Since the Pu can be either A or G, depending on the specific sequence of the Hind II site, it can be protected by the incorporation of dAMPS or dGMPS into the (-)strand or perhaps both if both types of sites are simultaneously present. Both types of sequences were studied, GTTAAC in M13mp2 at position 7196 and the GTCGAC sequence in the M13mp18 polylinker region at position 6266. The M13mp2 protected by dAMPS incorporation is nicked quite easily, but the M13mp18 protected by dGMPS requires an overnight digestion with Hind II to complete nicking. The only other basic difference in procedure is the requirement of a phenol extraction after nicking to remove the endonuclease. Without the extraction, much of the nicked DNA is not gapped by the exonuclease III, suggesting that the endonuclease may remain at the nicked site and is not displaced by the exonuclease III. As seen in Table 2, the mutational efficiency of Hind II is lower than that of Nci I, in the range of 50 - 85 % for either type of recognition site.

Pst I

The restriction endonuclease Pst I was also found to be a Class I enzyme (2), recognizing the 6 base sequence CTGCAG and cleaving between A-G. Protection for the nicking reaction is accomplished by the incorporation of dGMPS into the (-)strand. The effectiveness (purity) of Pst I appeared to be variable and some batches did not function well in the mutagenesis procedure. The protocol for mutagenesis with Pst I is essentially identical to that with Hind II although it was found that a second phenol extraction

was helpful in completely removing the Pst I after nicking in order for the exonuclease III reaction to proceed.

As shown in Table 2, Pst I could be studied in the M13mp18 phage with a single recognition site in the polylinker region. It showed a mutational efficiency of 45 - 75 %.

Fsp I

Fsp I is a restriction endonuclease not studied previously. It was characterized as a Class I enzyme (nicking only) and thus studied briefly for mutagenesis efficiency. Fsp I recognizes the 6 base sequence TGCGCA. It cleaves between C-G and is also inhibited by the incorporation of dGMPS into the (-)strand. The procedure is essentially identical to the Hind II procedure with a slightly different buffer for the nicking reaction. Fsp I is available only in rather dilute concentration and nicks very slowly, requiring about 16 h at 30°C to complete nicking.

As shown in Table 2, the mutational efficiency is widely variable with the M13mp18 system, with a range of 25 - 75 %. The wide variability in efficiency appears to be mainly the result of the inefficient nicking ability of this enzyme.

Effect of Phosphorothioate Groups in Recognition Sequence for Cleavage by Nci I

To determine whether a phosphorothioate group in the (-)strand located at the position of cleavage of the Nci I sites is sufficient for inhibition of cleavage, a M13mp2 derivative was first constructed which has only the one Nci site at position 6786 with the sequence i (CCGGG). To guide the number of phosphorothioate groups incorporated, three oligonucleotides, Nci I-1, Nci I-2 and Nci I-4, were synthesized which allowed the control of the phosphorothioate incorporation by providing only dCTPαS, dGTP and dATP for the first 10 min. of polymerization at 50 μM omitting dTTP to prevent elongation past the first dA in the template. To complete DNA synthesis all four dNTP's, and no dCTPαS, were then provided at 250 μM and the procedure followed for RF IV synthesis. This procedure is analogous to that described for mutagenesis method B in ref. 2. The results obtained on incubation with Nci I under standard conditions are reproduced in fig. 5. One can see that incorporation of only the first dCMPS which is then located at the point of normal cleavage by Nci I results first in nicking but is followed by linearization. This linearization reaction, however, is considerably slower than in the absence of dCMPS. Unmodified DNA would be completely linearized in about 2 min. Incorporation of a second dCMPS 5' next to the first essentially prevents linearization and obviously incorporation of four dCMPS which is

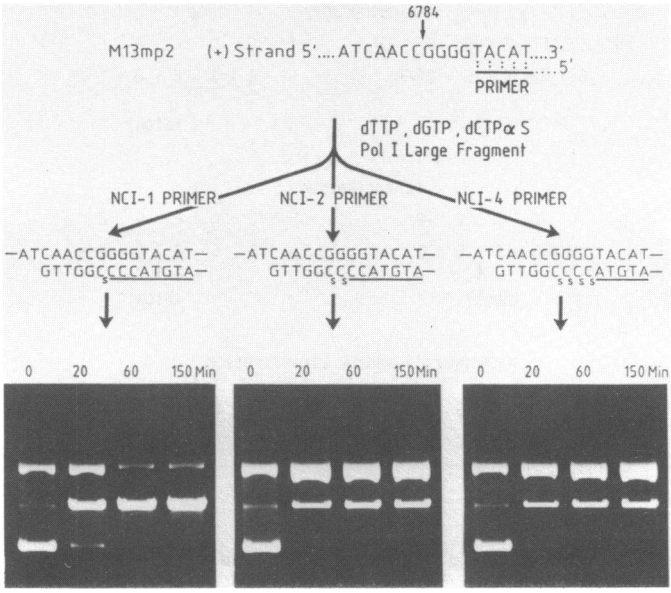

Figure 5.
Effect of Position of Phosphorothioate Groups on Cleavage by Nci. The experimental conditions for the extension of the Nci-primers and the nicking reaction is described in the Results Section. Gel analysis as in fig. 2.

the situation encountered in the normal polymerization reaction in the presence of dCTPαS gives the same result. From previous studies (Table 2, ref. 1) it is known that incorporation of dGMPS 3' to dCMPS has no effect on the normal linearization.

To study the second sequence, ii, a derivative of M13mp2 was made with this site at position 6276 as the only Nci I site as well as one of M13mp18 with this site at position 6328 in addition to the CCCGGG sequence in the polylinker region. After incorporation of dGMPS both of these DNAs were rapidly nicked and then linearized although dGMPS was present at the position of cleavage. However, when the mp2 derivative was tested with dCMPS incorporated linearization was strongly inhibited and no nicked intermediate was detected. Incorporation of both dCMPS and dGMPS resulted in DNA which was nicked slowly. When M13mp2 and M13mp18 derivatives containing both possible Nci sequences were protected only with dCMPS, only nicked DNA was observed with only a slight increase in linear DNA. Thus, complete protection occured. These results are schematically represented in fig. 6.

The conclusion of these studies is therefore that DNA containing both

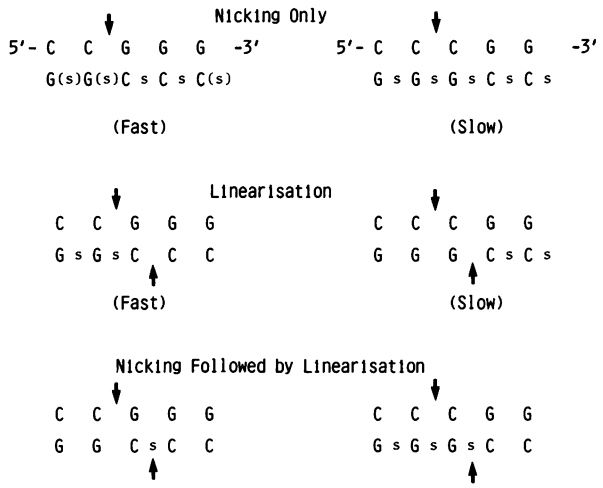

Figure 6.
Influence of Phosphorothioate Groups on Cleavage of the Nci I Sites.

possible sequences can be protected against cleavage of the (-)strand by incorporation of only dCMPS but nicking by Nci I will only occur at the CCGGG sites. High levels of Nci I or prolonged treatment with this enzyme might linearize also the CCCGG site.

DISCUSSION

The most established procedure for site-directed mutagenesis is the construction of an oligonucleotide containing the desired mutant sequence and targeted to the specific base sequence contained in a single-stranded template. Hybridization of this oligonucleotide to the template and *in vitro* extension of the mismatched priming oligonucleotide produces a heteroduplex DNA capable of cell transformation to produce the desired mutant (for reviews see ref. 7,8). A major barrier to high efficiency of mutagenesis is presented by the mismatch repair systems of the *E. coli* host cell which corrects the desired mutation. A further complication is the continued presence of the unmutated template strand. A number of creative strategies have been developed to circumvent these barriers and increase the efficiency of mutation, but a majority of the strategies require special mutant strains or relatively complex manipulations (7,8). A methodology designed to overcome both barriers to efficient mutagenesis was reported in two previous publications (1,2). The strategy is founded on the ability to incorporate phosphorothioate linkages into the *in vitro* polymerized newly formed DNA strand

by substituting the Sp diastereomer of any dNTPαS for the normal dNTP during mismatched oligonucleotide extension by the Klenow fragment. The substitution of a phosphorothioate linkage at the site of cleavage for certain restriction endonucleases, designated as Class I enzymes (1), prevents the hydrolysis of this strand but allows cleavage of the other strand, thus producing a nicked double stranded DNA at a specific site, the nick located in the template strand. The template strand can be digested by exonuclease III beyond the point of mismatch forming a gapped DNA which subsequently can be repolymerized back to the double-stranded DNA. By this strategy the mismatch is not only removed but both strands now contain the mutation. This technique was first developed and reported (2) with the restriction endonuclease Pvu I in two different methodologies (fig. 1) yielding mutational efficiencies of 40 - 60 % and genotypically pure.

Although these efficiencies are acceptable they fall short of the theoretically possible 100 %. Although there probably can be many reasons for not approaching this theoretical value such as incomplete mismatch priming and incomplete gapping, we felt that one of the main reasons was that the nicking reaction by restriction endonuclease Pvu I is rather inefficient. It was for this reason that we tested some of the other restriction enzymes of class I (1) for their efficiency in mutagenesis. The enzymes tested were Nci I, Hind II, Pst I and Fsp I. Inspection of Table 2 shows that the restriction enzyme Nci I is indeed capable of yielding mutational frequencies of 70 - 90 % which comes much closer to the theoretical value than previously observed. The other enzymes Hind II, Pst I and Fsp I give similar results to Pvu I with mutational rates generally in the range of 40 - 85 %.

There are variations in mutagenesis efficiency depending on the restriction enzyme utilized and also among experiments using the same enzyme. We ascribe these variations to the properties of the restriction enzymes in the nicking reaction and the subsequent exonuclease III gapping reaction. As was reported earlier (2) RF IV DNA containing phosphorothioate and the mismatch primer in the (-)strand, as is the case in these systems, produces 1 % or less mutants. Thus, any RF IV DNA where the (+)strand opposite the mismatch is not destroyed will greatly reduce the apparent mutational efficiency. It is within this framework that the superior results of the Nci I can be explained. Gel electrophoretical analysis of these reactions shows that Nci I nicks very cleanly and completely under the given buffer and salt conditions at the recognition sequence CCGGG protected by dCMPS. Pst I is also active and nicks very well. Pvu I is not nearly as active as either

Nci I or Pst I in nicking. Hind II appears to nick the GTTAAC sequence in the M13mp2 quite well, but has difficulty with the GTCGAC sequence in the polylinker of M13mp18. Fsp I is available only in low concentration and shows very weak nicking ability. Concerning the exonuclease III reaction, Nci I and Pvu I can be directly treated with the exonuclease, while Hind II and Fsp I show only partial digestion of the nicked DNA unless a phenol extraction is conducted. Pst I nicked DNA does not react with exonuclease III at all unless a phenol extraction is performed. Nci I thus nicks completely and does not affect the exonuclease III and therefore has the properties that produce consistent and high efficiency mutations that approach the theoretical limit.

It is not clear why there should be these differences with the exonuclease III reactions after the restriction enzyme reaction. As the inhibition can be relieved by phenol extraction, an explanation might be that some of the restriction enzymes remain at the nicked site and are not displaced by the exonuclease.

The exonuclease III digestion has been found to work surprisingly well and reliably up to lengths of $\geq$ 4300 bases in actual mutational studies. Since it was originally thought that the distance of the mutation from the nicking site might be limiting, it was comforting to find that this is probably not so. It has been found in fact that if only a single restriction site is present in the DNA, exonuclease III can digest nearly the entire template strand (W. Schmidt, F. Eckstein, unpublished). The enzyme, however, seems to leave a hybridized double-stranded region of around 800 bases. In the M13mp2 system, therefore, it means that exonuclease III can digest approximately 6300 bases from a single Nci I site. The reason for the incomplete digestion is unknown. However, the problems encountered in one particular experiment which is shown schematically in fig. 7 were probably caused by this property of exonuclease III. The aim was the removal of the Nci I site at position 6839 in M13mp18 by a normal mismatch oligonucleotide mutagenesis from the Nci I site at 1926. However, a new Nci I site had previously been introduced at position 6328, and the double Nci I site (Sma I site) at position 6249 in the polylinker was also present. The standard mutational protocol proceeded apparently in normal fashion as followed by agarose gel analysis before and after nicking, gapping, and repolymerization. When the plaques were sampled for a positive mutation by standard dot hybridization techniques, only 1 out of 22 (2 out of 44 if the similar mutation conducted at the same time is included) or less than 5 % mutational efficiency was ob-

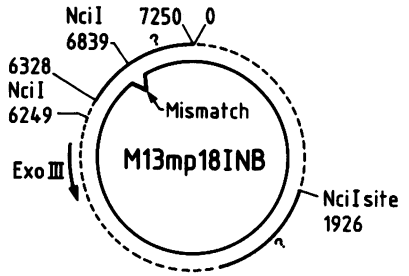

Figure 7.
Schematic Representation of Removal of Nci I site at Position 6839.

served. Since this is at least an order of magnitude lower than normally observed, and the exonuclease digestion required is about 2300 bases and has been shown to proceed at least 4300 bases, the explanation appears to lie in the inability of exonuclease III to digest past this mismatch because either a "stop" region for the exonuclease III exists in this specific area of the M13 DNA or the exonuclease III generally stops about 800 bases before a succeeding nick or gap. This may be one area of caution therefore in utilizing exonuclease III digestion in this procedure.

A crucial step in this mutagenesis method is the nicking reaction and it is, therefore, imperative to understand what requirements must be met to achieve this. The data presented in Table 2 of ref. 1 led us to conclude that the presence of a phosphorothioate group at the position of cleavage is a necessary requirement for this. Whether, however, it is sufficient could rigorously only be shown for the enzyme Ava I as the recognition sites of all other enzymes tested had the nucleotide 5' to which cleavage takes place occuring at least in one other position. By the development of a specific primer for the Pvu I site, necessary for Method B, it could be shown that also this enzyme fails to cleave if a phosphorothioate group is present solely at the position of cleavage (2). In investigating this question for Nci I one faces the problem that the enzyme recognizes two different sites, CCGGG and CCCGG (fig. 6) and therefore must be asymmetric, one binding area recognizing the first sequence and the other the second. Studies of the first sequence employing the oligonucleotide primers Nci-1 and Nci-2 (fig. 5) demonstrated that the presence of a dCMPS at the position of cleavage in the (-)-strand is not sufficient by itself to prevent hydrolysis in this strand as linearization is observed although it is slowed down. An additional dCMPS in the 5'-position next to the first is necessary for complete protection.

Interestingly, incorporation of a dGMPS in the 3'-position next to the dCMPS at the cleavage site of Nci I has no effect on hydrolysis (1).

We have not been able to study the second Nci I recognition sequence CCCGG in the same detail but the following conclusions can be drawn from the studies described in the Results Section. Incorporation of dGMPS which includes one at the position of cleavage and the other two in the positions 3' to it results in nicking and then linearization. Incorporation of dCMPS both of which are 5' to the position of cleavage strongly influences the reaction in that linearization is greatly reduced. Incorporation of both dGMPS and dCMPS results in DNA which is slowly nicked. Thus, the picture is similar though not necessarily identical to that seen with the sequence CCGGG in that a phosphorothioate 5' to the cleavage site exerts a strong influence so that combination with a phosphorothioate at the cleavage site prevents hydrolysis whereas that in the 3'-position is essentially without effect. These studies, in particular those with the sequence CCGGG very dramatically demonstrate the influence of a neighbouring group on the hydrolysis by a restriction enzyme. That such influences exist has already been evident from some of the examples given in Table 2 of ref. 1 where a reduction in rate of linearization caused by one or more phosphorothioate groups at other positions than that of cleavage in the recognition sequence is reported.

Obviously, the influences exerted by phosphorothioate groups on restriction enzymes are more complex than originally anticipated and cannot be generalized at present. Further studies with the phosphorothioates seem to be warranted as they might reveal details of the mechanism by which these enzymes function.

The studies with the enzymes Hind II, Pst I and Fsp I reported here indicate that probably all enzymes of class I (1) can be utilized for mutagenesis although, at present, the frequencies of mutation achieved with them are not competitive with those obtained with Nci I which approach the theoretical maximum.

A complete study of mutagenesis using a Class II enzyme has not been performed, but Hpa II, Taq I and Bgl II have been studied with respect to stopping the reaction after nicking and manipulation of the nicked DNA subsequently. The enzyme Hpa II recognizes CCGG, cleaving between C-C, and can be protected by the incorporation of dCMPS. The Hpa II reaction can be stopped at the nicked DNA stage by passing the reaction mixture through a NENSORB column to remove the protein. Since there are 17 Hpa II sites in M13mp2 it is not possible to determine how many nicks and at what positions they are

present when the reaction is stopped. Nevertheless, exonuclease III can digest from the nicks. Taq I can also be protected by dCMPS and has 10 sites in M13mp2. Taq I could be stopped at the nicked intermediate by the addition of EDTA to the reaction mixture and phenol extraction. The restriction endonuclease Bgl II was not previously studied (1). It recognizes the sequence AGATCT, cleaving between A-G, and is protected by dGMPS incorporation. Bgl II has a single recognition site in M13mp18 which was studied and found to be basically Class II, nicking followed by linearization. The reaction was found to be stopped by phenol extraction but it was difficult to control maximum nicking and minimum linearization. The nicked intermediate is digested by exonuclease III very much like Hind II and Pst I, i.e. the agarose gel analysis indicates some remaining nicked DNA after exonuclease III treatment and some nonprocessively digested DNA. The gapped DNA can be repolymerized.

The results presented demonstrate the potential of blocking restriction endonuclease cleavage in one strand and, thus, allowing a differentiation between the two complementary DNA strands. As one particular application a mutagenic technique is presented which is not affected by in vivo mismatch repair systems, is not dependent upon any special cell line with gene deficiencies, requires no special manipulations, is rapid, and produces high frequencies of mutations that approach the theoretical maximum.

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