M13 RF DNA was synthesized in vitro in the presence of various single deoxynucleoside 5'-O-(1-thiotriphosphate) phosphorothioate analogues, and the three other appropriate deoxynucleoside triphosphates using a M13 (+)-single-stranded template, *Escherichia coli* DNA polymerase I and T4 DNA ligase. The resulting DNAs contained various restriction endonuclease recognition sequences which had been modified at their cleavage points in the (−)-strand by phosphorothioate substitution. The behavior of the restriction enzymes *AvaI*, *BamHI*, *EcoRI*, *HindIII*, and *SalI* towards these substituted DNAs was investigated. *EcoRI*, *BamHI*, and *HindIII* were found to cleave appropriate phosphorothioate-substituted DNA at a reduced rate compared to normal M13 RF DNA, and by a two-step process in which all of the DNA is converted to an isolable intermediate nicked molecule containing a specific discontinuity at the respective recognition site presumably in the (+)-strand. By contrast, *SalI* cleaved substituted DNA effectively without the intermediacy of a nicked form. *AvaI*, however, is only capable of cleaving the unsubstituted (+)-strand in appropriately modified DNA.

Phosphorothioate analogues of nucleotides have in recent years found extensive applications in enzymology, especially in the determination of the stereochemical courses of enzymatically catalyzed phosphoryl transfer processes, and in the determination of metal binding sites in nucleotides (1). More recently, applications of these analogues in molecular biology have become apparent, such uses being generally based on the higher stability of phosphorothioates with respect to cleavage by enzymes (1).

Phosphorothioates can be incorporated into DNA by enzymatic polymerization of dNTPαS* on a suitably primed template, for example, by *Escherichia coli* DNA polymerase I (2, 3). Only the αS diastereomer of dNTPαS is accepted as a substrate. As inversion of configuration at phosphorus is observed in the polymerization reaction, a phosphorothioate internucleotidic linkage of the R configuration is produced in the new DNA chain. The polymerization rates of these analogues are comparable with those observed using normal substrates (3, 4). In this way double-stranded circular fd RF DNA was prepared in which only the (−)-strand had been modified by the incorporation of dAMPS residues instead of dAMP (2), thus demonstrating the feasibility of modifying DNA using these analogues. Drastic changes in the physico-chemical properties of this modified DNA were not observed, and the DNA remained infective. A later report (5) showed in a semiquantitative fashion that such DNA is often cleaved considerably more slowly by restriction endonucleases than unsubstituted DNA. However, from the data presented it was not possible to ascertain whether the eventual cleavage of this hybrid DNA proceeded, as might be expected, first by attack on the unsubstituted template strand to give a nicked intermediate. If this were the case, as might be predicted from the general properties of phosphorothioate analogues, then phosphorothioate substitution might form a general basis for the generation of a specific nick at any desired restriction site in one strand of a duplex DNA molecule, and permit mechanistic information on the mode of cleavage of DNA by restriction endonucleases to be obtained.

We wish to report here that phosphorothioate substitution, by altering relative cleavage rates, can be an effective probe of restriction enzyme cleavage mechanisms under optimum conditions for cleavage.

**MATERIALS AND METHODS**

M13mp9 (+)-single-stranded DNA and ϕX174 RF DNA were obtained from BRL GmbH, West Germany. M13mp8 (+)-single-stranded DNA was a gift of Dr. M. Darby, Max Planck Institut für Medizinische Forschung, Heidelberg. Primer oligonucleotides were a gift of Dr. H.-P. Vosberg, Max Planck Institut für Medizinische Forschung, Heidelberg and were obtained from denatured calf thymus DNA by limited digestion with pancreatic DNase I, and subsequent fractionation on DEAE-Sephadex G-100. The primer molecules had an average length of 15–20 nucleotides as judged by polyacrylamide gel electrophoresis. On the average, three primer molecules were present per DNA template during in vitro synthesis. dCTP, dGTP, dTTP, dATP, ethidium bromide were obtained from Boehringer Mannheim GmbH. E. coli acetate kinase (200 units/mg, 5 mg/ml), E. coli DNA polymerase I (endonuclease free, 9 units/ml), *BamHI* (11 units/ml), *HaeIII* (5 units/ml), *HindIII* (10 units/ml), and *SalI* (9 units/ml) were purchased from Boehringer Mannheim GmbH. *AvaI* (6 units/ml) was from Amersham Buchler GmbH, Braunschweig. T4 DNA ligase (5–5 units/ml), isolated from the overproducing strain *E. coli* NM 268 was a gift of Dr. F. Winkler, European Molecular Biology Laboratory, Heidelberg. *EcoRI* was a product of Regen GmbH, Heidelberg. Microfiltration apparatus and appropriate nitrocellulose filters (pore size 0.45 µm, diameter 9 mm) used for DNA purification were obtained from Schleicher and Schüll GmbH, Dassel, W. Germany.

Buffers used for reactions involving restriction enzymes were as follows: for *EcoRI*, 100 mM Tris·HCl, 50 mM NaCl, 10 mM in MgCl₂.
RESULTS

Covalently closed circular relaxed M13 RF IV DNAs containing various single phosphorothioate substitutions in the \((-\)-strand were synthesized in vitro by polymerization of a dNTPoS phosphorothioate analogue and the three appropriate normal deoxynucleoside triphosphates on a M13 (\(+\)) single-stranded template using E. coli DNA polymerase I. Closure of the newly synthesized circles was effected by T4 DNA ligase. Internucleotidic phosphorothioate linkages were thus introduced base specifically in the \((-\)-strand and substituted cleavage sites for several DNA restriction endonucleases. The effects of these substitutions on DNA cleavage by these enzymes were studied.

EcoRI—The enzyme EcoRI recognizes the palindromic duplex sequence GAATTC (10) and cleaves between the dA and dG residues. After \((-\)-strand synthesis in the presence of dATP, dAMP, dGMP, and dTMP residues were incorporated into this sequence in the \((-\)-strand, and there is consequently an internucleotidic phosphorothioate bridge of the \(R\) configuration (3) at the normal cleavage point in this strand.

The synthesis of M13mp9 (S)N RF IV DNA duplex material produced \(~90\%\) covalently closed circles when employing the T4 DNA ligase provided by F. Winkler. Commercial preparations gave much poorer yields of ligated material.

M13mp9 RF IV DNA and M13mp9 (S)A RF IV DNA were treated with EcoRI under identical conditions for 30 min at 37 °C and the products of the reactions analyzed by agarose gel electrophoresis (Fig. 1). After this time, M13mp9 RF IV DNA was completely cleaved at the unique EcoRI site to full length M13mp9 RF III linear DNA. M13mp9 (S)A RF IV DNA, however, was transformed under these conditions to M13mp9 (S)A RF II nicked linear DNA by cleavage presumably only in the unsubstituted viral \((+\))-template strand. Reaction with excess enzyme over a longer time period (Fig. 2) demonstrated that eventual cleavage of M13mp9 (S)A RF DNA was not obtained.

**Fig. 1.** Nicking of M13mp9 (S)A RF IV DNA by EcoRI. Samples of M13mp9 RF IV and M13mp9 (S)A RF IV (0.25 \(\mu\)g) were incubated with EcoRI (0.8 unit) in reaction buffer (10 \(\mu\)l) at 37 °C for 30 min, quenched with excess EDTA, and subjected to agarose gel electrophoresis. Lane 1, DNA cleaved with EcoRI under identical conditions; lane 2, M13mp9 (S)A RF IV DNA after incubation with EcoRI; lane 3, M13mp9 (S)A RF IV DNA; lane 4, M13mp9 (S)A RF IV DNA after incubation with EcoRI; lane 5, M13mp9 RF IV DNA.
Phosphorothioate-substituted DNA

Recognition Sequence: GpApApTpTpC

Recognition Sequence: GpGpApTpCpC

Recognition Sequence: CpTpTpApGp

Recognition Sequence: CpGpApTpCpC

Recognition Sequence: CpCpTpGpCpGp

FIG. 2. Cleavage of M13mp9 (S)A RF IV DNA by EcoRI. Samples of M13mp9 (S)A RF IV DNA (0.28 μg) were digested by EcoRI (11 units) in reaction buffer (20 μl) at 37 °C. Samples were quenched at indicated times by addition of excess EDTA and subjected to agarose gel electrophoresis.

FIG. 3. Cleavage of M13mp9 (S)G RF IV DNA by BamHI. M13mp9 (S)G RF IV DNA (0.4 μg) was digested by BamHI (55 units) in reaction buffer (204 μl) at 37 °C. Aliquots of 20 μl were removed at various time intervals, quenched with excess EDTA, and subjected to agarose gel electrophoresis.

FIG. 4. Cleavage of M13mp2 (S)C RF IV DNA by AvaI. Top, Nicking of M13mp2 (S)C RF IV DNA by AvaI. M13mp2 (S)C RF IV DNA (0.2 μg) was treated with AvaI (60 units) in reaction buffer (110 μl) at 37 °C. Aliquots of 10 μl were removed at various time intervals, quenched with excess EDTA, and subjected to gel electrophoresis. Bottom, M13mp2 (S)C RF IV DNA (0.1 μg) was nicked by AvaI (30 units) in a total volume of 55 μl reaction buffer. After 15 min, HaeIII (10 μl) was added and the incubation continued. Aliquots of 10 μl were withdrawn at various time intervals, quenched by addition of excess EDTA, and subjected to agarose gel electrophoresis.
Phosphorothioate-substituted DNA

AuaI under these conditions in approximately 2.5 min. AuaI introduced no nicks into SV40 DNA, which has no AuaI site, under the same conditions. That the enzyme is not rapidly deactivated under the conditions used was tested by incubation of the enzyme alone in reaction buffer for 1 h. DNA was then added and incubation continued. The characteristic cleavage pattern of AuaI was observed, confirming the presence of active enzyme (results not shown). That this DNA itself possessed no other irregularity other than the desired phosphorothioate substitution was tested as shown in Fig. 4, bottom. The DNA was first converted fully to the nicked form by AuaI, and then HaeIII was added. As can be seen from Fig. 4, bottom, the DNA was rapidly cleaved at the phosphorothioate to give HaeIII fragments.

SalI—The enzyme SalI recognizes the DNA sequence GpTpCpGpApC and cleaves between the dG and dT residues (10). M13mp9 (S)T RF IV DNA was prepared using dTTPoS and tested as a substrate for SalI. This DNA contains a unique restriction site for SalI in the multiple cloning region. The results are shown in Fig. 5. It can be seen that in contrast to EcoRI, BamHI, and AuaI essentially no open circle intermediate is observed, and cleavage proceeds directly from the RF IV to the RF III form with only minimal formation of a nicked RF II intermediate. The cleavage reaction was slower than for unmodified DNA which under the condition used was linearized in less than 1 min. SalI introduced no nicks into eX174 DNA, which has no SalI site, under the same conditions.

**DISCUSSION**

Phosphorothioate-containing polynucleotides are generally more slowly hydrolysed by enzymes than those containing phosphate groups (1). Thus, snake venom phosphodiesterase cleaves polynucleotides with phosphorothioate linkages of the $P_2$ configuration approximately 10 times more slowly than normal polynucleotides (3, 11), and exonuclease III (12, 13) as well as the 5' → 3' exonuclease activity of E. coli DNA polymerase I (3, 14, 15) cleave them so slowly that hydrolysis can normally not be detected. Also, the 3' → 5' exonuclease activity of the latter hydrolyses these groups extremely slowly (4, 16). It is this aspect which justifies a more detailed investigation of the cleavage of phosphorothioate groups by DNA restriction endonucleases as it might open a way of achieving cleavage of a particular restriction enzyme site only in one strand by protecting it by phosphorothioate substitution in the other. The earlier report on this subject described the inhibition of cleavage of hybrid phosphorothioate-substituted DNA by six restriction enzymes (5). It established that inhibition was most pronounced when an intramolecular phosphorothioate linkage was introduced exactly at the cleavage point in the appropriate recognition sequence. In this work, electrophoresis was performed on agarose gels in the absence of ethidium bromide and it was not possible to distinguish between RF II DNA containing one or more nicks and covalently closed circular RF IV DNA. Consequently, it was not possible to examine how cleavage of the hybrid DNA molecules thus prepared had been effected.

The reaction catalyzed by Type II restriction enzymes requires subsequent introduction of two single-stranded breaks within a recognition sequence contained in a DNA. The two cleavage events can occur simultaneously or be separated in time. In the latter case, the enzyme will either stay bound or will dissociate between the cuts, and an open circle form II intermediate will be formed. The observation of this intermediate will be indicative of a mechanism where the two cleavages are distinct. When no intermediate is observed the two cleavages can be said to occur simultaneously.

It is thought that sequential cleavage of DNA strands may be a general property of restriction endonucleases. Evidence in the case of the most studied endonuclease, EcoRI, is rather well-founded (17-20). In several other cases the intermediate possessing a single-strand scission at the recognition sequence is isolable under suboptimal reaction conditions, i.e. with limiting amounts of enzyme (18), at low temperatures (21, 22), or in the presence of ethidium bromide (17).

There are several reports concerning the isolation of RF II DNA with single-strand nicks generated by restriction endonucleases in the presence of ethidium bromide (23-26). One of the main uses of such DNA has been its application to site-directed mutagenesis using nick translation in the presence of base-modified nucleotides (27), by the bisulfite method (25) and by gap misrepair mutagenesis including the use of dNTPaS (29). A common limitation of these methods, however, is the great variability in the behavior of restriction enzymes towards DNA in the presence of ethidium bromide (26). Thus, it is sometimes only possible to nick between 50 and 90% of the input DNA, and some linearization and unchanged starting material is almost always observed. The exact conditions must be determined by careful titration for each enzyme. Many enzymes do not exhibit this effect at all, and some only very poorly. Thus it is often difficult to generate reasonable quantities of nicked DNA and many potential mutagenesis sites on a given genome are not available for this technique. In addition, of course, the partial cleavage by restriction enzymes is not strand specific so that the gaps are presumably equally distributed between the (+) and the (−)-strand. Other methods for generating specific nicks would clearly be most useful.

We suspected that cleavage of a hybrid DNA molecule possessing phosphorothioate substitutions at a restriction cleavage site in the (−)-strand, should proceed by an enhanced two-step process for restriction enzymes which are supposed to cleave only by a two-step mechanism, since cleavage of the unsubstituted viral (+)-template strand should be favored, followed by a considerably slower cleavage of the phosphorothioate-containing DNA.
thioate internucleotidic linkage in the (-)-strand. This should permit isolation of a DNA RF II form with a nick only in the (+)-strand.

To this end we decided to reinvestigate the previously reported inhibitory effects of phosphorothioate-substituted DNA on restriction endonucleases (5) using first the enzyme EcoRI, since more mechanistic data is available for this enzyme than for others (17) and also because these data indicate a two-step mechanism for this enzyme.

In this reinvestigation it was important to monitor cleavage products on agarose gels containing ethidium bromide (23), which facilitate the separation of open circle and covalently closed circular forms of DNA. We chose the single-stranded DNA of bacteriophage M13. Since this is currently one of the most popular cloning vehicles (30), it has frequently been employed for site-specific mutagenesis experiments (31) and M13mp9 possesses many unique restriction enzyme cleavage sites in the multiple cloning region.

The mechanism of cleavage by EcoRI has been extensively studied (17-22). Evidence is strongly in favor of the enzyme acting in a two-step fashion with the participation of an open-circle intermediate. Second-strand cleavage is slower than first-strand cleavage by a factor of about two, although this may be accounted for by a statistical factor (19, 20), and the enzyme may dissociate from the DNA between first- and second-strand cleavages, although at 37 °C the cleavage process appears to be coupled. However, slightly different behavior has been observed with different DNA.

When M13mp9 RF IV DNA and M13mp9 (S)A RF IV DNA were compared as substrates for EcoRI under identical conditions, the former DNA was completely cleaved to the linear form, whereas the latter phosphorothioate-substituted DNA was at first only converted to the RF II nicked form (Fig. 1). This result can be explained on the basis of the generally slower rate of enzymatic hydrolysis of phosphorothioates in comparison to phosphates as mentioned above. Indeed, for the synthetic octamer d(GGsAATTCC) containing the EcoRI recognition sequence and a phosphorothioate group at the cleavage site in both strands a decrease in rate of approximately 20 in comparison to the unmodified octamer could be observed (32). The conclusion that EcoRI has placed a nick in its recognition sequence in the unsubstituted viral (+)-strand is therefore most reasonable. The observed rate difference is sufficient to obtain the nicked material as the sole product in the first phase of the reaction.

Incubation with larger amounts of enzyme, when monitored over a period of time, demonstrated that after nicking to form II DNA has taken place, subsequent second-strand cleavage of the phosphorothioate-containing strand occurs resulting in a linear RF III product (Fig. 2). This result is thus in agreement with what has been found with the phosphorothioate octamer d(GGsAATTCC), namely that a phosphorothioate linkage of the Rs configuration can be hydrolyzed by EcoRI, albeit slowly. Moreover, the fact that in this small substrate analogue cleave by EcoRI occurred at the correct position in the recognition sequence provides reassurance that the second strand of M13mp9 (S)A RF IV DNA is indeed also cleaved at the correct site. The overall result demonstrates that even at 37 °C double-strand cleavage by EcoRI is not an obligatory coupled process.

The cleavage of this DNA by HindIII also leads to formation of a nicked intermediate. As this enzyme can nick DNA, like EcoRI, in the presence of ethidium bromide (28) this is probably not surprising; and indeed a two-step cleavage mechanism has been proposed (19).

Evidence in favor of a two-step mechanism for the endonuclease BanHI has been provided by Smith and Chirikjian (33) who showed that form II DNA accumulates during the digestion of plasmid pMB9 with the enzyme at 1 °C with limiting amounts of nucleases. Also Shortle and Botstein (28) have demonstrated that BanHI displays an efficient nicking reaction in the presence of ethidium bromide. On the other hand, Halford et al. (19) have shown that under optimal conditions BanHI displays a higher reactivity towards second-strand cleavage once the first strand has been cut than towards first-strand cleavage. Recent support for this has been provided by examination of cleavage of a supercoiled DNA substrate (34). Thus, normally no nicked intermediate is observable. On cleavage of M13mp9 (S)G RF IV DNA by BanHI the presence of a nicked intermediate can clearly been observed (Fig. 3). Thus, cleavage at the second, the phosphorothioate-containing strand is now slowed down and an isolable form II intermediate is observed suggesting a two-step mechanism at least for this artificial DNA.

A simultaneous strand cleavage mechanism similar to that for BanHI has also been proposed for the enzyme AuaI (35). If this were correct, one would not expect to observe a nicked intermediate, even in the presence of a phosphorothioate group in one strand. The cleavage of M13mp2 (S)C RF IV DNA by this enzyme, however, shows not only this unexpected intermediate but also that it is in this case the end-product of the reaction. This preparation of DNA could be cleaved by HaeIII, an enzyme which attacks M13mp2 DNA at multiple sites cleaving between dG and dC in the sequence GGCC, and is known to cleave M13mp2 (S)C RF IV DNA. As can be seen from Fig. 4, bottom, the DNA was rapidly cleaved to give HaeIII fragments indicating that it could be cleaved by another restriction enzyme sensitive to dCMPM substitution.

Several explanations for this behavior of AuaI are possible. First, it is important to note that the two possible configurations of a phosphorothioate diastereomer in general, normally only one will be cleaved by an enzyme at a reasonable rate. It is normally not possible to predict in advance which one this may be. Thus, among the nonspecific DNA endonucleases, there are enzymes which cleave Rs linkages and those which cleave Ss linkages (1). It is thus quite possible that AuaI might be one of the enzymes cleaving Rs rather than Rs phosphorothioate linkages. As the phosphorothioate groups introduced into DNA by polymerization with DNA polymerases have only the Rs configuration, this would explain the inability of the enzyme to linearize this DNA. At present, only data on the stereospecificity of restriction enzymes are available for EcoRI and this enzyme has been shown to recognize a Rs internucleotidic linkage (32). However, this does not exclude the possibility that other restriction enzymes may show a different stereospecificity. A second explanation could be that the row of three dCMPS residues, in the middle of which the cleavage site resides, in the recognition sequence of the (-)-strand of this particular DNA causes an inhibition in an as yet unexplained way. That in a recognition sequence other residues substituted with phosphorothioate but not directly at the cleavage point can have an inhibitory effect on cleavage was noted by Vosberg and Eckstein (5). Third, the possibility exists that DNA nicked in one strand is not a substrate for AuaI. However, it is not yet known whether AuaI exhibits a good nicking reaction in the presence of ethidium bromide so this cannot at present straightforwardly be tested. That AuaI does, however, nick one strand of a DNA although not natural does seem to rule out a mechanism whereby it is

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obligatory for the enzyme to cut both strands simultaneously, and makes a two-step mechanism of action more likely for this enzyme. Clarification of this aspect, however, must await further work.

A particularly challenging enzyme to investigate with hybrid phosphorothioate-phosphate DNA with the view of isolating nicked intermediates is the restriction endonuclease SalI. On cleavage of normal DNA by this enzyme under optimal conditions no nicked intermediate can be observed (19, 20, 35). It is only under suboptimal conditions at low pH or low MgCl₂ concentration that some nicked DNA can be seen but this DNA is cleaved at a slower rate than the intact duplex and is therefore not an obligatory intermediate. It is therefore suggested that the preferred pathway for this enzyme is a concerted reaction to cleave both strands of the DNA within one enzyme-DNA complex but some nicked DNA can dissociate before cutting of the second strand. To be able to isolate nicked DNA in the cleavage of the phosphorothioate-phosphate DNA would then require that this leakage would have to be increased by virtue of the slow rate of cleavage of the second strand. However, cleavage of M13mp9 (S)T RF IV DNA by SalI gave no indication of such a process. Except for a time point taken at 3 min incubation, either only starting material or linearized DNA as product can be detected. Thus, the observation that even in a DNA where cleavage of one strand is most likely more difficult than the other, the reaction still proceeds without dissociation of the enzyme-substrate complex and provides further evidence for the assumption that the SalI-catalyzed reaction proceeds in a concerted fashion.

These studies demonstrate that for restriction enzymes which cleave both strands of DNA in a stepwise fashion, nicked DNA can in many cases be isolated by employing phosphorothioate-phosphate DNA as substrate when the proper choice of dNTP₆S has been made for the synthesis of the second strand. As the phosphorothioate groups can be cleaved eventually by most enzymes, Avel being at present the exception, the amount of enzyme and the time of incubation have to be determined so as to obtain this nicked DNA as the sole product. Exceptions for the preparation of such nicked DNA by this method seem to be enzymes which cleave the DNA in a concerted fashion as exemplified in this respect by SalI.

This method of protecting restriction sites against cleavage in one strand of double-stranded DNA might prove to be an interesting method for the creation of gaps for the performance of single-site mutagenesis.

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