Protection of Oligonucleotide Primers against Degradation by DNA Polymerase I

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ABSTRACT: By use of a mutational assay employing an octadecamer with a mismatch in the center, it is shown that the introduction of phosphorothioate groups near the 5′-end can protect the mismatch against degradation by the 5′–3′-exonuclease activity of *Escherichia coli* DNA polymerase I. An optimal level of protection is achieved when the phosphorothioate groups are incorporated in at least the second and third internucleotidic linkages from the 5′-end. However, gel electrophoretic analysis as well as the use of an octadecamer with a mismatch closer to the 5′-end in the mutational assay reveals that degradation of the oligonucleotide is not completely blocked but only slowed down.

Oligonucleotide-directed mutagenesis requires primers containing one or more mismatches for the synthesis of the circular covalently closed double-stranded viral DNA (RF IV DNA)[1] [for reviews, see Zoller and Smith (1983), Smith (1985), and Carter (1986)]. *Escherichia coli* DNA dependent DNA polymerase I is not suitable for this reaction because it has a 5′–3′-exonuclease activity that will degrade the primer from the 5′-end and leads to loss of the mismatch (Goulian, 1968a,b). Although the Klenow fragment of polymerase I (Klenow & Henningsen, 1970) can be used, it produces both RF IV and RF II (nicked) DNA in approximately a 1:1 mixture (Nakamaye & Eckstein, 1986).

Phosphorothioate linkages are not hydrolyzed by the 5′–3′-exonuclease activity of polymerase I (Eckstein, 1985). It therefore occurred to us that the use of oligonucleotides that contain one or more phosphorothioate internucleotidic linkages at or near the 5′-end of the mismatch primer might allow the use of polymerase I for the synthesis of RF IV DNA as the main product. To determine whether protection against this exonuclease could be achieved by the presence of these groups, we have altered the number and position of phosphorothioate groups in the mismatch primer and employed them for mutagenesis of M13mp2 ochre DNA (Taylor et al., 1985), comparing the mutational efficiency for DNA synthesized with either polymerase I or Klenow fragment.

Materials and Methods

E. coli DNA polymerase I (endonuclease free, 5 units/μL) and T_4 polynucleotide kinase (10 units/μL) were purchased from Boehringer Mannheim (Mannheim, FRG); DNA polymerase large fragment [Klenow fragment, obtained from a cloned gene according to Joyce and Grindley (1983), 15 units/μL] was from New England Nuclear (Dreieich, FRG); [γ-^{32}P]ATP was from Amersham-Buchler (Braunschweig, FRG). T_4 DNA ligase was prepared as described (Taylor et al., 1985). Nitrocellulose filters (pore size 0.45 μm) used for DNA purification were obtained from Schleicher & Schüll (9-mm diameter, BA 85) and were used in appropriate filter units from Schleicher & Schüll. The following oligonucleotides (mismatch position underlined) were synthesized on an Applied Biosystems 380B DNA synthesizer on 0.2-μmol columns employing nucleoside methoxyphosphoroamidites purchased either from Applied Biosystems (Foster City, CA) or Merck (Darmstadt, FRG): EM3 [d(CGG CCA GTT GAT TCG TAA)], EM3S1 [d(C_SGG CCA GTT GAT TCG TAA)], EM3S2 [d(CG$_S$G CCA GTT GAT TCG TAA)], EM3S12 [d(C_SG$_S$G CCA GTT GAT TCG TAA)], EM3S23 [d(CG$_S$G $_S$CCA GTT GAT TCG TAA)], EM3S123 [d(C_SG$_S$G $_S$CCA GTT GAT TCG TAA)], EM3S234 [d(CG$_S$G $_S$C$_S$CA GTT GAT TCG TAA)], EM3N [d(AGT TGA TTC GTA ATC ATG)], and EM3NS123 [d(A_SG$_S$T $_S$TGA TTC GTA ATC ATG)]. The phosphorothioates were introduced by replacing the normal phosphate I_2/H_2O oxidation step with a treatment using a solution of 5% elemental sulfur in CS_2/pyridine (1:1 v/v) for time periods of 4 × 450 s. All oligonucleotides were purified by preparative reverse-phase HPLC before and after removal of the dimethoxytrityl group under conditions described previously (Ott & Eckstein, 1984). The oligonucleotides were digested with snake venom phosphodiesterase or nuclease P_1 followed by dephosphorylation with alkaline phosphatase and HPLC analysis of the reaction products (Connolly et al., 1984).

Agarose gel electrophoresis was performed as described previously (Taylor et al., 1985), on 1% agarose slab gels containing ethidium bromide (0.5 μg/mL). Samples were diluted to 10–20 μL with H_2O and mixed with 6 μL of 0.05% (w/v) bromophenol blue in 100 mM EDTA and 50% (v/v) glycerol, pH 7.0, before electrophoresis. DNA bands were visualized by exposure of the gels to UV light (254 nm).

Oligonucleotides were labeled with ^{32}P according to the published procedure (Taylor et al., 1985) with polynucleotide kinase. In order to purify them from mononucleotides, they were applied to primed SEP-PAK cartridges (Waters Associates, Milford, MA). The cartridges were primed by being washed with 5 mL of acetonitrile, 5 mL of 100 mM TEAB containing 40% acetonitrile (solvent B), and 5 mL of 100 mM TEAB containing 1% acetonitrile (solvent A). After loading of the samples, the cartridges were washed with 30 mL of solvent A and eluted by 600 μL of solvent B. The samples were evaporated to dryness with a Speed Vac concentrator (Savant Instruments, Hicksville, NY) and redissolved in 50 μL of 50 mM NaCl.

M13mp2 ochre ssDNA (Taylor et al., 1985; 5 μg) was annealed with the 5′-phosphorylated oligonucleotides (23 ng; molar ratio primer:DNA ≈ 1.5:1) and the polymerization reaction carried out in the presence of 250 μM of each dNTP

[1] Abbreviations: cccDNA, circular covalently closed DNA; d(S)N, phosphorothioate nucleotide; dNTP, 2′-deoxynucleoside triphosphate; dsDNA, double-stranded DNA; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; RF, replicative form; RF II, nicked circular double-stranded DNA; RF III, linear double-stranded DNA; RF IV, circular covalently closed double-stranded DNA; ssDNA, single-stranded DNA; TEAB, triethylammonium bicarbonate. R_P and S_P denote the configuration of the phosphorothioate internucleotidic linkage (Eckstein, 1985). For abbreviations of primers, see Materials and Methods.

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employing either large fragment (7 units) or DNA polymerase I (2.5 units) and T_4 DNA ligase (10 units) in 50-μL reaction solution at 16 °C overnight following the published protocol (Taylor et al., 1985; Nakamaye & Eckstein, 1986). In order to remove ssDNA, the polymerization reaction mixture was filtered after addition of 5 μL of 5 M NaCl through two nitrocellulose filters, precipitated with EtOH, redissolved in 50 μL of buffer, and used to transform competent JM107 or SMH-50 cells as described (Taylor et al., 1985; Nakamaye & Eckstein, 1986). Mutants were indicated by a color change of the plaques from white to blue.

Phosphorothioate-based mutagenesis was carried out according to Nakamaye and Eckstein (1986) employing *Nci*I for the nicking reaction.

In order to determine the amount of label released during the enzymatic synthesis of the complementary strand, 12 polymerization reactions were performed employing the three radioactively labeled primers EM3, EM3S1, and EM3S123. The reactions were carried out in the presence or absence of ligase and either with Klenow fragment or DNA polymerase I. Products were analyzed by three methods: gel electrophoresis; precipitation with 1 M CCl_3COOH followed by measurement of the ^{32}P label in the supernatant and in the pellet; gel permeation chromatography with determination of the label in the flow through. In these experiments the polymerization reactions were analyzed without prior precipitation or filtering.

Analysis by electrophoresis was performed on a 20-cm 20% polyacrylamide gel. Equivalents of the polymerization assays were mixed with 8 M urea, heated to 85 °C, and applied onto the gel. Untreated labeled primer and also oligonucleotide that had been completely digested with snake venom phosphodiesterase (yielding [^{32}P]dCMP) were used as length standards (Jay et al., 1974). After about 30 min of electrophoresis with a power of 17 W, the gel was autoradiographed overnight with Kodak XAR-5 film.

For the precipitation with trichloroacetic acid, aliquots of the polymerization assays and of the labeled primers were mixed with 50 μg of calf thymus carrier DNA and 200 μL of aqueous 1 M CCl_3COOH, left for 20 min on ice, and then centrifuged for 20 min at 16000*g*. The supernatant and the pellet, after the pellet was dissolved in 300 μL of 50 mM NaCl, were mixed separately with scintillation fluid and counted.

Gel filtration was performed on spun columns packed with 1 mL of Sephadex G-50 (Maniatis et al., 1982). Each polymerization mixture was divided in two equal volumes. One of them was mixed immediately with scintillation fluid and counted; the other one was first passed through a spun column after dilution to 100 μL with 50 mM NaCl.

RESULTS

Synthesis of Phosphorothioate Oligonucleotides. Phosphorothioate-containing oligonucleotides were synthesized by oxidation of the phosphite triesters with a solution of sulfur in carbon disulfide/pyridine instead of iodine/water. The cycle times for the reaction have not been optimized and can certainly be reduced. During purification of the oligonucleotides on HPLC, the main product was obtained in a varying number of fractions (Table I). These fractions contained the various diastereoisomers of the phosphorothioate oligonucleotide. Complete separation of the diastereoisomers could be achieved only for the oligomer EM3S1. The other diastereoisomeric oligonucleotides were only resolved partially. The introduction of the phosphorothioates was checked by cleavage of the oligonucleotides with snake venom phosphodiesterase or nuclease P_1 followed by dephosphorylation with alkaline phosphatase.

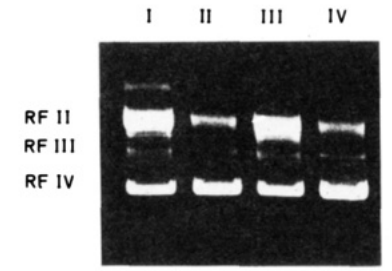

FIGURE 1: Polymerization reactions using mismatch primers and Klenow fragment (I + III) or DNA polymerase I (II + IV) in the presence of T_4 DNA ligase. M13mp2 ochre DNA as template is primed with EM3 (I + II) or EM3S123 (III + IV).

Table I: Average Mutation Rates Using M13mp2 Ochre dsDNA from Polymerizations Employing Klenow Fragment or DNA Polymerase I and T_4 Ligase with Regular or Phosphorothioate-Containing Mismatch Primers

primer	mismatch position[a]	sulfur position[b]	fractions[c]	mutation rate (%) Klenow fragment	mutation rate (%) Pol I
EM3	10		1	17.9	1.1
EM3S1		1	2	20.0	1.0
EM3S2		2	1	15.1	6.7
EM3S12		1, 2	2	13.0	5.4
EM3S23		2, 3	1	12.5	14.5
EM3S123		1, 2, 3	5	12.3	16.2
EM3S234		2, 3, 4	3	16.4	16.5
EM3N	5		1	15.8	0.3
EM3NS123		1, 2, 3	5	15.3	0.9

[a] Counted from 5′-end of the primer. [b] Number of internucleotide phosphate position from 5′-end. [c] From HPLC.

The digestion products were analyzed by HPLC (Connolly et al., 1984).

Mutagenesis Study. Mutation experiments were performed with DNA samples prepared with polymerase I and Klenow fragment in parallel. The reaction with Klenow fragment served as the standard to indicate the mutational frequency to be expected if the mismatch had not been removed by degradation of the primer oligonucleotide.

Polymerization and ligation reactions carried out with phosphorothioate-containing primers produced the same distribution of RF II and RF IV DNA as those carried out with unmodified primers (Figure 1). As noted before (Nakamaye & Eckstein, 1986), the difference between reactions employing DNA polymerase I and large fragment is striking. The former produces almost completely RF IV DNA whereas the latter produces an approximate 1:1 mixture of RF IV and RF II DNA. Figure 1 shows an example for this. Lanes I and III are from a polymerization with Klenow fragment whereas in lanes II and IV polymerase I was employed for the synthesis of the DNA. Additionally, there is almost no difference between normal (lanes I and II) and phosphorothioate-containing oligonucleotides (lanes III and IV). Longer incubation times, up to 48 h, increase the amount of RF IV even further (data not shown).

The results of the mutagenesis experiments are summarized in Table I. The difference in mutation rates between fractions containing different diastereoisomers is only marginal and therefore shown as average values. As expected, essentially no mutants are produced when an oligonucleotide without a phosphorothioate group is used in the polymerization with DNA polymerase I. Introduction of a phosphorothioate group of either configuration at the first internucleotidic linkage at the 5′-end produces the same result. An increase in mutational frequency is observed when phosphorothioate groups are in

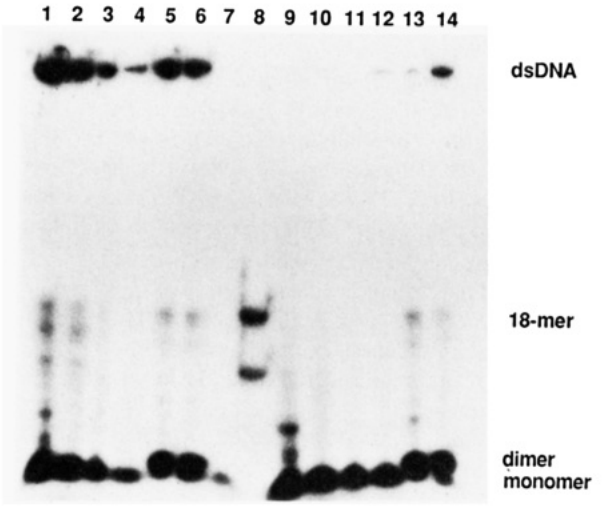

FIGURE 2: Gel analysis for cleavage of 5′-^{32}P-labeled primers by the 5′–3′-exonuclease of DNA polymerase I. EM3 (lanes 1, 2, 9, and 10), EM3S1 (lanes 3, 4, 11, and 12), or EM3S123 (lanes 5, 6, 13, and 14) was annealed to M13mp2 ochre ssDNA as template and elongated with Klenow fragment (lanes 1–6) or DNA polymerase I (lanes 9–14) in the absence (lanes 1, 3, 5, 9, 11, and 13) or presence (lanes 2, 4, 6, 10, 12, and 14) of T_4 ligase. Length standards are dCMP (lane 7) and EM3 (lane 8, somewhat degraded).

the second position as well as in the first plus second positions from the 5′-end of the oligonucleotide.

Mutational frequencies equivalent to those observed with the Klenow fragment are achieved when the phosphorothioate groups are located in the first, second, and third positions of the primer. Similar values are observed when phosphorothioate linkages are at the second and the third positions only. A third phosphorothioate in position 4 does not lead to further improvement in the mutation rates.

When the mismatch is located closer to the 5′-end of the oligomer, at position 5 rather than at position 10, almost no mutations are observed with both phosphate and phosphorothioate oligonucleotides after polymerization with DNA polymerase I.

The primer EM3S123 was also employed in the phosphorothioate-based mutagenesis method (Taylor et al., 1985; Nakamaye & Eckstein, 1986) where mutational frequencies of 82% were obtained when Klenow fragment and 74% when polymerase I was used for RF IV synthesis.

Release of Oligonucleotides by the 5′–3′-Exonuclease. The polymerization reactions were also analyzed by gel electrophoreses on 20% polyacrylamide gels to determine whether any 5′-labeled mono- or oligonucleotides had been released during the reactions. Figure 2 shows a typical autoradiograph of such gels. Release of predominantly mono- and dinucleotides can be observed with the normal primer (EM3, lanes 1, 2, 9, and 10) as well as with the phosphorothioate-containing primers (EM3S1, lanes 3, 4, 11, and 12; EM3S123, lanes 5, 6, 13, and 14). The latter shows a difference in the digestion products. Whereas mononucleotides are the main cleavage products of EM3S1, the primer EM3S123 gives predominantly dinucleotides. In polymerization reactions in the presence of ligase (lanes 2, 4, 6, 10, 12, and 14) the amount of cleavage products, particularly oligonucleotides longer than dimers, is somewhat lower than that without ligase (lanes 1, 3, 5, 9, 11, and 13). As expected, the amount of radioactive label residing in the fully polymerized DNA is much higher when Klenow fragment (lanes 1–6) is employed for polymerization instead of polymerase I (lanes 9–14), where essentially none is detected except when EM3S123 is used together with ligase (lane 14).

The results of the acidic precipitation and the gel filtration are not clear as the released oligonucleotides are neither completely retained in the precipitate or the gel nor do they stay completely in solution. Thus, no quantitative evaluation of the results was possible. Qualitatively, these experiments confirmed the results of the gel electrophoreses.

Discussion

Several studies have demonstrated that DNA containing phosphorothioate in place of phosphate is resistant to hydrolysis by the 5′–3′-exonuclease of polymerase I. In particular, the phosphorothioate analogue of poly(dA), poly[dp(S)A] of the R_P configuration, as the complementary strand to poly(dT) is stable against this enzyme activity (Burgers & Eckstein, 1979). Also poly[dTp(S)dA], the poly(dA-dT) analogue where the internucleotidic linkage between dT and dA is a phosphorothioate group, is degraded by hydrolysis of the phosphate internucleotidic linkage to yield d[pTp(S)A] (Brody & Frey, 1981; Romaniuk & Eckstein, 1982). This result suggests that the enzyme also has an endonucleolytic activity. Indeed, earlier investigators (Kornberg & Kornberg, 1974) had reported this endonucleolytic activity after they observed both mono- and oligonucleotide products while monitoring the exonuclease reaction. This was confirmed in nick translation experiments (Lundquist & Olivera, 1982), which suggestd that 5′–3′-exonuclease activity of polymerase I acts by hydrolyzing overhangs produced by strand displacement during the polymerization. The longest oligonucleotides identified were decamers while the monomer, dimer, and trimer products accounted for more than 80%. This combination of endo- and exonuclease activity of polymerase I complicates any strategy aimed at protecting mismatch primers by incorporating phosphorothioate groups into the DNA. In order to determine the requirements for optimal protection of mismatch primers, we synthesized two series of octadecamer mismatch primers with between one and three phosphorothioates located in different positions from the 5′-end. In one set of primers the mismatch was positioned 10 nucleotides from the end and in the other 5.

Synthesis of Phosphorothioate Oligonucleotides. The synthesis of phosphorothioate-containing oligonucleotides in an automated synthesizer requires that the elemental sulfur for the oxidation reaction is in solution to avoid blockage of valves. A solution of sulfur in 2,6-lutidine warmed to approximately 55 °C has been employed by other authors previously (Stec et al., 1984; Stec & Zon, 1984). We found that a solution of sulfur in CS_2/pyridine (Connolly et al., 1984) can also be successfully applied in automated synthesis.

As the phosphorus in internucleotidic phosphorothioate groups is chiral (Eckstein, 1985), a number of diastereoisomers are produced in such a synthesis. The number of diastereoisomers increases by 2^n so that an oligonucleotide having three of these groups should possess eight diastereoisomers. Although separation of diastereoisomers of phosphorothioate-containing oligonucleotides by reverse-phase HPLC has been reported for some cases (Stec et al., 1984; Stec & Zon, 1984; Connolly et al., 1984), it is by no means a foregone conclusion that this can be achieved in every case. We have indeed been able to separate by preparative HPLC the two diastereoisomeric oligonucleotides that have one phosphorothioate internucleotidic linkage at the 5′-terminal position, but our attempts to separate diastereoisomers with the phosphorothioate located in position 2 or 3 failed. For all oligonucleotides the number of fractions obtained is listed in Table I. These results confirm the observation that the diastereoisomers seem to separate more easily when the position of the phosphorothioate linkages

is closer to the 5′-end (Stec et al., 1984). However, we have not undertaken a configurational analysis of the various fractions so there is no information on their diastereoisomeric composition.

Mutagenesis Study. A mutagenesis assay is a sensitive probe for the efficiency of oligonucleotide protection from degradation by polymerases. If the oligonucleotide is degraded past the mismatch, the mutational frequency will decrease. We employed an M13 system with M13mp2 ochre DNA, in which the wild-type TCA Ser triplet in position 6235–6237 has been mutated to the stop codon TAA (Taylor et al., 1985) and thus produces colorless plaques upon transformation in the α-complementation β-galactosidase assay. However, when oligonucleotides EM3 and EM3N are used as primers for the synthesis of the complementary strand, this triplet is mutated to a CAA Gln triplet, and blue plaques are produced (Taylor et al., 1985). Thus, the comparison of the number of blue to white plaques gives the mutational efficiency of the system, which reflects the stability of the mismatch primer. It is necessary to have parallel experiments with Klenow fragment and polymerase I as different cell preparations can produce differences in mutational frequencies presumably as a result of varying degrees of efficiency of the repair system (Claverys & Lacks, 1986).

One advantage in using polymerase I instead of Klenow fragment can be seen in Figure 1. With polymerase I the desired RF IV DNA is almost the exclusive product from a polymerization/ligation reaction, whereas a considerable amount of RF II DNA remains unligated when Klenow fragment is used. The reason for this difference is undoubtedly the fact that both enzymes carry out strand displacement of the 5′-end at a nick, but polymerase I is able to hydrolyze these overhangs, whereas the large fragment is not. The interpretation is supported by the finding that addition of T_4 DNA ligase to a polymerization reaction after polymerization is complete does not yield RF IV DNA when Klenow fragment but not when polymerase I has been employed for this reaction (Nakamaye & Eckstein, 1986).

The mutagenesis experiments with the non-phosphorothioate-containing primers EM3 and EM3N confirm the well-known fact that the 5′–3′-exonuclease activity of the DNA polymerase I degrades oligonucleotides and as a consequence destroys mismatch primers (table I) (Goulian, 1968a,b). Our results show that the introduction of one phosphorothioate at the 5′-end of an oligonucleotide (EM3S1) cannot prevent the destruction of the primer. This could be explained by earlier observations which showed that the enzyme can jump over at least one phosphorothioate to cleave at the following phosphate (Brody & Frey, 1981; Romaniuk & Eckstein, 1982). Introduction of a phosphorothioate linkage at position 2 of the primer (EM3S2 and EM3S12) improves the mutation rate to about 40% of that obtained with Klenow fragment.

Three phosphorothioates at the 5′-terminus of the primer (EM3S123), however, give sufficient protection as judged by the mutation yields, which are equivalent to those achieved with Klenow fragment. Equivalent protection can also be obtained when the phosphorothioate linkages are located only at positions 2 and 3 of the oligonucleotide (EMS23). On the other hand, an additional phosphorothioate in position 4 (EM3S234) does not lead to further improvement. Thus, positions 2 and 3 are the most susceptible to cleavage by the 5′–3′-exonuclease. This is in agreement with findings by Lundquist and Olivera (1982), who observed mono- and dinucleotides as main digestion products.

Surprisingly, such protection of the mismatch against degradation is only observed if the mismatch is located in the middle of the oligonucleotide primer at position 10 and not close to its 5′-end at position 5. As shown in Table I, almost no mutants are obtained when the primers EM3N and EM3NS123 are elongated by polymerase I. This observation indicates that the 5′–3′-exonuclease activity is not completely blocked by the phosphorothioate internucleotide linkages.

An additional set of experiments was carried out to ascertain that the mismatch had not been excised from the phosphorothioate-containing primer EM3S123. The phosphorothioate-based mutagenesis method described by Taylor et al. (1985) and Nakamaye and Eckstein (1986) produces mutational frequencies of routinely 80%. If the phosphorothioate-containing primers such as EM3S123 had not lost their mismatch during polymerization with DNA polymerase I, similar mutational frequencies should be observed. Indeed, this expected result was obtained with primer EM3S123 elongated either with polymerase I or with Klenow fragment.

Mismatch oligonucleotide 28-mers with either a phosphorothioate group at the 5′-terminal internucleotidic linkage or two phosphorothioate groups at the two terminal positions have been employed for oligonucleotide-directed mutagenesis by H. J. Fritz and co-workers (private communication). These authors also observe a significant increase in mutational frequency for the modified oligomers.

Cleavage of Primers by the 5′–3′-Exonuclease. Although the mutation assay is sensitive to overall degradation of the primer, it does not provide any information as to the nature of degradation. In order to determine the type of degradation, we attempted to isolate the released mono- or oligonucleotide degradation products. For this purpose the primer oligonucleotide was ^{32}P labeled at the 5′-terminal phosphate. After polymerization and ligation, the amount of ^{32}P label present in oligonucleotides as well as in the RF IV DNA was determined. Electrophoresis (Figure 2), precipitation, and gel filtration (data not shown) indicated that the amount of radioactivity present in the RF DNA varied. In polymerizations carried out in the presence of Klenow fragment, a considerable amount of radioactivity resides in the DNA almost independent of whether T_4 ligase was present or not (Figure 2, lanes 1–6). This observation is in agreement with the mutagenesis results where all these primers produce a high mutational frequency. In polymerizations with polymerase I, essentially no radioactivity is seen in the DNA when polymerized in the presence of ligase except for reactions with primer EM3S123. This again is in accordance with the mutagenesis experiments where primers EM3 and EM3S1 yield essentially no mutants whereas primer EM3S123 shows high mutational frequency. The main labeled degradation products are mono or dinucleotides (Figure 2, lanes 1–6 and 9–14). These products are probably not caused by contaminant nuclease or phosphodiesterase activities as they were not observed when oligonucleotide primers were incubated with polymerase, ligase, and the complete set of substrates and buffers but in the absence of DNA (not shown). These digestion products were observed after reactions not only with DNA polymerase I (Figure 2, lanes 9–14) but, rather unexpectedly, also with the Klenow fragment (Figure 2, lanes 1–6). This preparation of Klenow fragment from New England Nuclear is isolated from an overproducer (Joyce & Grindley, 1983) and should thus be devoid of 5′–3′-exonuclease activity. We had previously found it to give the best results in site-directed mutagenesis (Taylor et al., 1985; Nakamaye & Eckstein, 1986). Thus, we have to conclude that there is sufficient 5′–3′-exonuclease

activity even in cloned Klenow fragment to partially destroy primers. It had been reported previously that some commercial preparations of large fragment have enough residual 5′–3′-exonuclease activity to remove the mismatch in the priming oligonucleotide (Baas et al., 1981; Smith, 1985), but it is not clear whether cloned Klenow fragment had been employed in these experiments. To explain the release of dinucleotides from the phosphorothioate-containing primer EM3S123, we have to assume either that the S_P diastereoisomer is cleaved—in previous experiments where no cleavage was observed (Burgers & Eckstein, 1979; Brody & Frey, 1981; Romaniuk & Eckstein, 1982) the polymer contained only the R_P diastereoisomer—or that one or both diastereoisomers are cleaved relatively slowly.

When the polymerization reaction is carried out in the absence of T_4 ligase (Figure 2, lanes 1, 3, 5, 9, 11, and 13), the amount of radioactive label incorporated into the newly synthesized DNA is somewhat lower than that in the presence of ligase (lanes 2, 4, 6, 10, 12, and 14), particularly when polymerase I is used (lanes 9–14). Additionally, more oligonucleotides of different length can then be detected by gel electrophoreses. Thus, there is obviously competition between the ligase and the 5′–3′-exonuclease of polymerase I, as they require the same substrate. Whereas the exonuclease activity starts at nicks with digestion and strand displacement, the ligase will protect nicks against the action of the 5′–3′-exonuclease by ligation. It is well-known that phosphorothioates are not or only slowly hydrolyzed by the 5′–3′-exonuclease and other enzymes (Eckstein, 1985). Thus, when the digestion rate of the exonuclease is reduced by the phosphorothioates, the ligase is able to ligate the nick more efficiently. This difference in rate of hydrolysis of phosphate and phosphorothioate internucleotidic linkages could then explain the protection of the mismatch when in the center of the oligomers EMS123 and EMS23. The somewhat puzzling result remains that the mismatch only five positions removed from the 5′-end is destroyed even in the presence of phosphorothioates. At present we can only assume that the reduction in rate of hydrolysis in the presence of phosphorothioates is sufficient to slow down the degradation of the oligomer up into the center but that the hydrolysis rate is still high enough to reach at least the fifth position from the 5′-end.

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