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A DNA fragment with an α-phosphorothioate nucleotide at one end is asymmetrically blocked from digestion by exonuclease III and can be replicated *in vivo*

(subcloning/DNA endwise digestion/nuclease inhibition/plasmid reconstruction)

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ABSTRACT **2′-Deoxyadenosine 5′-*O*-(1-thiotriphosphate) (dATP[αS]) was introduced into the 3′ ends of DNA restriction fragments with *Escherichia coli* DNA polymerase I to give phosphorothioate internucleotide linkages. Such "capped" 3′ ends were found to be resistant to exonuclease III digestion. Moreover, the resistance to digestion is great enough that, under conditions used by us, just one strand of a double helix is digested by exonuclease III when a cap is placed at only one end; when digestion is carried to completion, this results in production of intact single strands. When digestion with exonuclease III is limited and is followed by S1 nuclease treatment, double-stranded DNA fragments asymmetrically shortened from just one side are produced. In this way thousands of nucleotides can be selectively removed from one end of a restriction fragment. *In vitro* introduction of phosphorothioate linkages into one end of a linearized replicative plasmid, followed by exonuclease III and S1 nuclease treatments, gives rise to truncated forms that, upon circularization by blunt-end ligation, transform *E. coli* and replicate *in vivo*.**

It is often useful to decrease the length of a given DNA fragment in a progressive, controlled manner (1–7). Two different schemes are currently employed for progressively varying the length of double-stranded DNA fragments. One is the use of exonuclease III followed by single-strand-specific S1 nuclease (1, 2, 4, 5, 7, 8). Another scheme uses Bal 31 nuclease (3, 6), which simultaneously digests both 5′ and 3′ ends of a double-stranded DNA fragment (9). The primary disadvantage of these approaches is that the nucleases act simultaneously at both ends of the fragment so that desirable sequences at one end are not preserved.

The S_P diastereomer of the nucleoside α-thiotriphosphates (which have a sulfur substituted for an oxygen at the α-phosphate) act as substrates for *Escherichia coli* DNA polymerase I in DNA synthesis (10). These compounds, although incorporated at an efficiency similar to that of the natural substrates, effectively block the 3′ exonuclease activity of DNA polymerase I at the phosphorothioate nucleotide linkage (unpublished results). In work reported here we found that placement of an α-thionucleotide at only one of the 3′ ends of a DNA fragment effectively blocks that end from digestion with exonuclease III. Digestion proceeds progressively from the opposite end and, after S1 nuclease treatment, the fragment is shortened with the integrity of the nucleotide sequence at the "capped" end preserved. Alternatively, if exonuclease III digestion is allowed to proceed to completion, a full-length single strand is generated from the original double strand.

This work also demonstrates that, when 2′-deoxyadenosine 5′-*O*-(1-thiotriphosphate) (dATP[αS]) is inserted into 3′ recessed ends of restriction fragments, the phosphorothioate linkage allows subsequent ligation of the DNA, restriction of ligated junctions, and *in vivo* replication of plasmids containing phosphorothioate nucleotide linkages.

EXPERIMENTAL PROCEDURES

Preparation of dATP[αS]. The dATP[αS] S_P diastereomer was prepared by using methods discussed in ref. 11.

Purification of DNA. Plasmids pBR322 and pSP201 (12) were replicated, separately, in *E. coli* host KL386. Isolation and purification were performed by preparation of a cleared lysate followed by cesium chloride centrifugation (13). The 5.3-kilobase (kb) (with *Bst*EII ends) and 302-base pair (bp) (with a *Bst*EII and a *Kpn* I end) fragments, which are both part of pSP201, were obtained by digesting the plasmid with the appropriate enzymes and electrophoresing the products through a 5% polyacrylamide gel (14). The DNA was visualized by UV shadowing and isolated from the gel (15).

RESULTS

Protection of a DNA Strand Containing dAMP[αS] from Exonuclease III Digestion. To show that the presence of dAMP[αS] renders that strand resistant to exonuclease III digestion, a 5.3-kb fragment, with *Bst*EII ends at both sides, was treated with DNA polymerase I, dCTP, dTTP, [α-^{32}P]dGTP, and either dATP[αS] or dATP (Fig. 1 *Left*). Because [α-^{32}P]dGTP was included as a substrate, the fragments were labeled at both ends and dAMP[αS] was positioned between the end of the fragment and the labeled dGMP. The result of this treatment was, therefore, a blunt-ended fragment with dAMP[αS] located one nucleotide from one end.

These fragments were incubated with excess exonuclease III and the release of label was monitored by measuring trichloroacetic acid-precipitable radioactivity. Because dAMP[αS] lies between the end of the fragment and the labeled dGMP (Fig. 1 *Left*), the fragment will remain labeled only if dAMP[αS] prevents removal of the label by blocking exonuclease III digestion from the ends of the molecule. On the other hand, loss of label should be rapid if dAMP[αS] does not inhibit exonuclease III digestion. The results (Fig. 1 *Right*) show that, at both 22°C and 37°C, the amount of radioactivity in the capped fragment is almost unchanged during the duration of the reaction (60 min), while the label is rapidly lost (1 min) from the uncapped frag-

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Abbreviations: dATP[αS], the S_P diastereomer of 2′-deoxyadenosine 5′-*O*-(1-thiotriphosphate); dAMP[αS] and dNTP[αS] are corresponding α-thio analogs; kb, kilobase(s); bp, base pair(s).

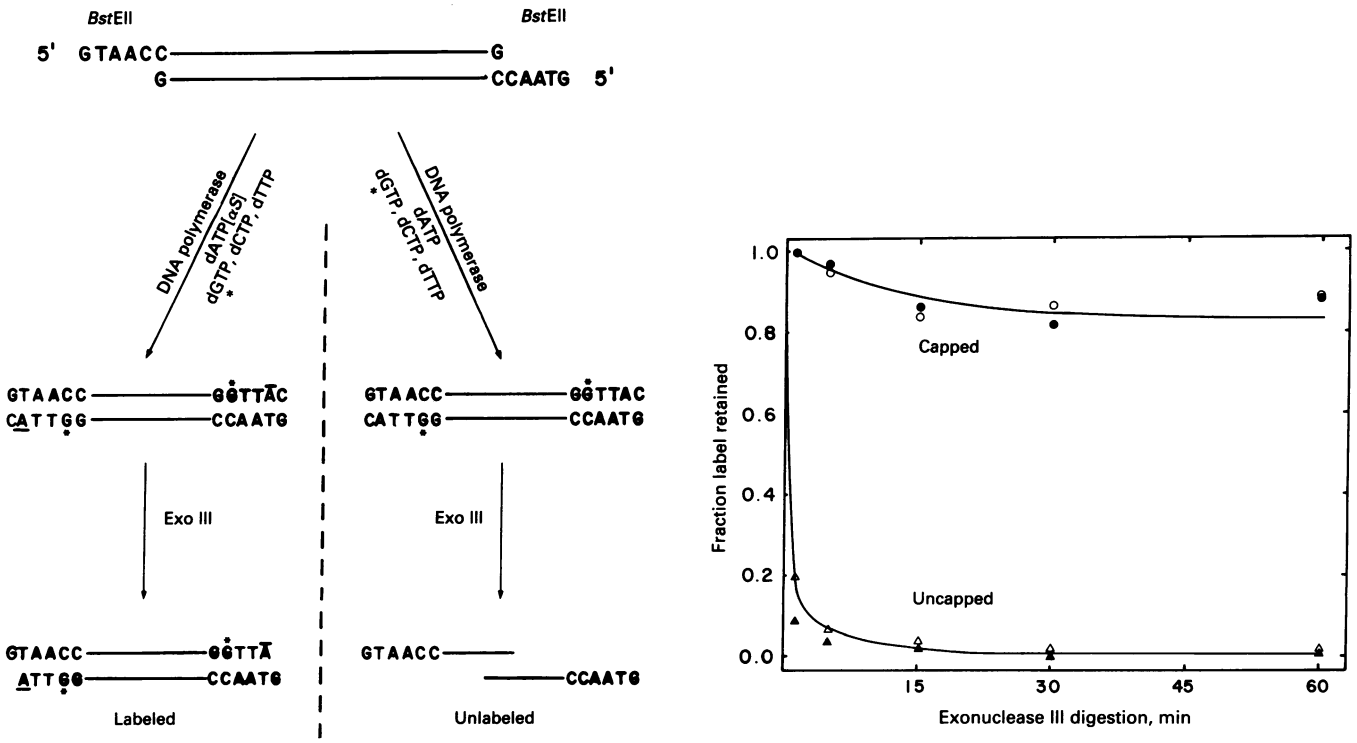

FIG. 1. (*Left*) Sequential action of DNA polymerase and exonuclease III (Exo III) on the 5.3-kb *Bst*EII fragment. The phosphorus of the dGTP was labeled (indicated by an asterisk) and the dAMP[αS] (indicated by an over- or underbar) is positioned between the label and the end of the fragment. The filling-in reaction was done by incubating 20 μg of DNA for 30 min at 18°C with 50 mM Tris·HCl (pH 8.0)/5 mM $MgCl_2$/10 mM 2-mercaptoethanol/50 μM dTTP and dCTP/10 μM [α-^{32}P]dGTP/50 μM dATP[αS] or dATP/4 units of the large fragment of DNA polymerase (Bethesda Research Laboratories). The products were purified by polyacrylamide electrophoresis (14, 15). To perform the exonuclease III reactions, 0.24 μg of DNA was incubated in 6.6 μM Tris·HCl (pH 7.5)/6.6 μM $MgCl_2$/6.6 μM 2-mercaptoethanol/50 μM NaCl/2.5 units of exonuclease III (Bethesda Research Laboratories) at 22°C for the indicated times. (*Right*) Release of label from capped and uncapped 5.3-kb *Bst*EII fragments during exonuclease III digestion. DNA was treated with a 1:8 molar ratio of DNA to exonuclease III at either 22°C or 37°C and the amount of acid-precipitable radioactivity was determined. Label was retained in the capped fragment at both temperatures (○, 22°C; ●, 37°C), while label was rapidly lost from uncapped fragment (△, 22°C; ▲, 37°C). [The amount of label retained in the capped fragment initially (≈1 min) drops approximately 20%, and this is attributed to the release of label from uncapped fragments that were incompletely filled in.]

ment. Thus, a single dAMP[αS] protects the end of a DNA fragment from prolonged exonuclease III digestion.

Experiments were performed to explore the effect of Bal 31 nuclease on capped and uncapped flush-ended fragments. In addition to being a single-strand-specific nuclease, Bal 31 possesses an exonuclease activity whereby both the 3′ and 5′ ends of a double-stranded DNA fragment are digested synchronously (9). Treatment with Bal 31 resulted in loss of label from the dAMP[αS] fragment and from the control fragment at identical rates and at identical Bal 31 concentrations (results not shown). Hence, the presence of dAMP[αS] does not affect digestion with Bal 31.

Generation of Full-Length Single-Stranded DNA from a Double-Stranded Template. A 130-bp fragment, produced by *Dde* I (5′ overhang) and *Pvu* I (3′ overhang) cleavages of pBR322, was filled in at the *Dde* I end with dAMP[αS]. Fig. 2 shows untreated fragment (lane 1) electrophoresed in parallel with exonuclease III-digested fragment (lane 2). The uncapped strand was digested and the undigested single strands migrate above the double stranded form. In an identical experiment using uncapped fragment, no DNA was visible after exonuclease III digestion (data not shown).

Ligation and Restriction of Fragments Containing dAMP[αS]. The dAMP[αS]-containing *Bst*EII/*Kpn* I fragment and the uncapped fragment were incubated separately with phage T4 DNA ligase under conditions that promote intramolecular flush-end ligation. As explained in Fig. 3 *Left*, such a ligation joins the *Bst*EII and *Kpn* I ends. To assay the extent of ligation, the products were digested with *Msp* I, which cleaves the fragment 38 bp from the *Kpn* I end. After digestion and electrophoresis through a denaturing gel, two *labeled* bands are observed for both the capped and uncapped fragment (Fig. 3 *Right*, lanes 1 and 3). One, 302 nucleotides in length, results from fragments that underwent ligation. The other, 264 nucleotides, is unligated fragment. This experiment demonstrates that dAMP[αS], located only one nucleotide from the site of joining, has no significant effect on the efficiency of this ligation reaction.

Fortuitously, when the fragments are circularized, the *Bst*EII restriction site is regenerated (see Fig. 3 *Left*). This offers a test of whether dAMP[αS] interferes with the recognition of a restriction endonuclease. When the *Msp* I-treated ligation products were restricted with *Bst*EII and electrophoresed (Fig. 3 *Right*, lanes 2 and 4), the 302-bp fragment disappeared and a new fragment appeared at 38 bp. Hence, the presence of dAMP[αS] within the recognition site for this endonuclease does not affect cleavage.

Use of dATP[αS] to Construct a Truncated Form of pBR322. The design of the experiment is outlined in Fig. 4 *Left*. pBR322 was opened at the unique *Eco*RI site and the recessed 3′ ends were rendered flush with DNA polymerase in the presence of dATP[αS] and dTTP. Restriction was then performed with *Hin*dIII to generate a fragment with dAMP[αS] at one end. Limited exonuclease III treatment followed by S1 nuclease (to produce flush ends) resulted in forms of pBR322 that were shortened from the end that lacked dAMP[αS]. This treatment

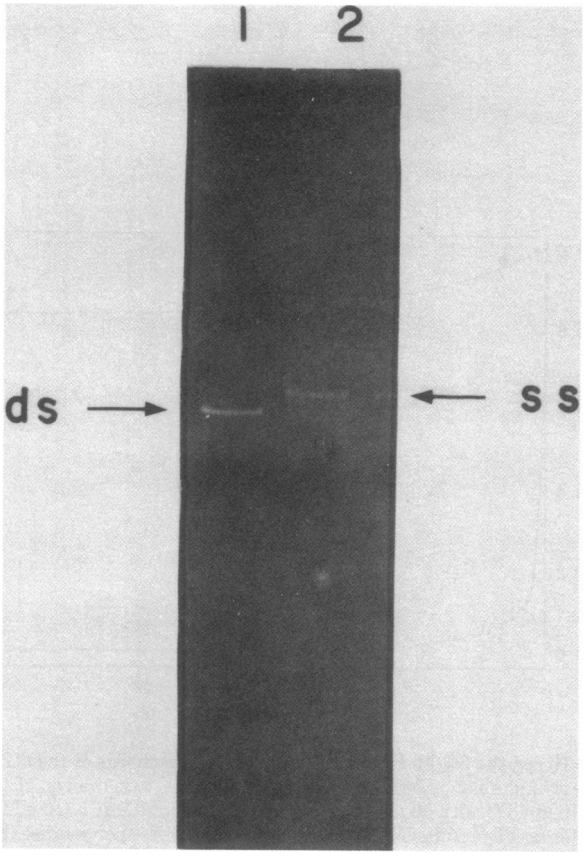

FIG. 2. Exonuclease III digestion of a 130-bp *Dde* I/*Pvu* I fragment containing dAMP[αS] at one end. Lane 1 shows 0.016 μg of undigested fragment and lane 2 contains 0.040 μg of fragment treated with 200 units of exonuclease III per μg. Exonuclease III digestion produces single-stranded DNA (ss) which migrates above the double-stranded DNA (ds). Electrophoresis was through a 7.5% polyacrylamide gel (14) and the DNA was visualized with ethidium bromide staining.

destroyed the region conferring tetracycline resistance. The fragments were then ligated under conditions that promote recircularization, and an ampicillin/tetracycline-sensitive host was transformed.

Selection was made either for ampicillin resistance or for tetracycline resistance. No clones exhibiting tetracycline resistance were found, but many were isolated as ampicillin resistant. (A control experiment, in which no exonuclease III treatment was performed, revealed that clones carrying pBR322 missing only the region between the *Eco*RI and *Hin*dIII sites are tetracycline resistant.) This suggests that, due to dAMP[αS] incorporation, exonuclease III digestion proceeded from the *Hin*dIII end and not from the *Eco*RI end.

Plasmid DNA was isolated from one ampicillin-resistant tetracycline-sensitive clone to assess the nature and extent of exonuclease III digestion. Upon digestion with *Pst* I (which cuts pBR322 once within the region coding for ampicillin resistance), it was found that the resulting plasmid was about 2.0 kb in length and therefore 2.4 kb had been removed by exonuclease III (results not shown). Determination of the precise region removed by exonuclease III was made by digesting the truncated plasmid with *Hae* III followed by subsequent electrophoresis of the products. Because *Hae* III cleaves pBR322 22 times (18), identification of the *Hae* III sites present in the modified plasmid reveals the region missing from the original pBR322.

The results of *Hae* III digestion of intact pBR322, in parallel with those of the shortened plasmid, are shown in Fig. 4 *Right*, lanes 1 and 2. All fragments produced from *Hae* III cleavages within the region from nucleotides 174 to 1949 in pBR322 are missing from the truncated plasmid, whereas all other fragments are present. *Hae* III cleavage of the truncated plasmid generates a fragment of approximate length 250 nucleotides, which is not present in the pBR322 digest. On the basis of the lengths of the fragments produced, we estimate that the plasmid has a length of 2120 nucleotides, which means that the exonuclease III digestion proceeded to approximately 160 nucleotides from the origin of replication (19). Particularly important is the presence in the truncated plasmid of the *Hae* III site at position 4344, which is only 18 nucleotides from the original *Eco*RI site (18). Another point is that the *in vitro* ligation proceeded even though the dAMP[αS] was at the very end of the fragment. This confirms data in Fig. 3 *Right* and shows that ligation is not affected by the thio analog. Finally, this demonstrates that a plasmid containing the phosphorothioate internucleotide linkage is viable for *in vivo* replication.

DISCUSSION

The NTP thio analogs have been used extensively as tools for analysis of the stereochemistry of enzyme mechanisms. For example, the mechanisms of polymerases (10, 20), kinases (21), exonucleases (11, 22), and of nucleotidyltransferases (23) have been explored with these compounds. Their significance for such studies is that the stereochemistry of the reaction about the chiral phosphorus atom can easily be determined. We have investigated here the utility of the NTP[αS]s for certain aspects of *in vitro* modification of DNA, with emphasis on their potential as aids to recombinant DNA manipulations. Because the sulfur at the α phosphate interferes with some enzymatic functions but not others, modification of one end of a DNA molecule with the analog makes that end inert to specific processes such as exonuclease III digestion.

The dideoxynucleotides, when incorporated into DNA fragments, also inhibit the 3′-5′ exonuclease activity of DNA polymerase (24). Unlike the dNTP[αS]s, the dideoxynucleotides are unattractive for use in asymmetrically blocking digestion of DNA fragments. Because they lack the 3′-hydroxyl group, fragments with terminal dideoxynucleotides are inert to ligation. Consequently, although double-stranded fragments containing dideoxynucleotides can be asymmetrically digested, they cannot be made viable for *in vivo* functions.

The interactions between dAMP[αS] (inserted into the end of a DNA fragment) and DNA polymerase, exonuclease III, T4 DNA ligase, restriction enzyme *Bst*EII, and nuclease Bal 31 were investigated here. DNA ends containing dAMP[αS] act normally for the polymerization activity of DNA polymerase and for T4 DNA ligase but are not susceptible to digestion by the 3′ exonuclease activities of DNA polymerase and exonuclease III. In the case of Bal 31 nuclease, endwise digestion occurs with the capped fragment, but this does not necessarily mean that the nuclease successfully splits the phosphorothioate internucleotide linkage. Even though the nuclease carries out endwise digestion of both strands (25), current data do not rule out the possibility that, as both 5′ and 3′ ends are degraded, the nuclease can "hop" past the phosphorothioate linkage and cut the next internucleotide linkage. In the case of restriction with *Bst*EII, our experiments show that the presence of dAMP[αS] in the recognition sequence does not block cleavage. But the results do not determine whether cleavage can occur at the phosphorothioate linkage itself. Thus, all results could fit with the viewpoint that, for many nucleolytic activities, cleavages are inhibited at phosphorothioate linkages and that the resistance of such linkages to exonuclease III digestion is but one specific example of this phenomenon.

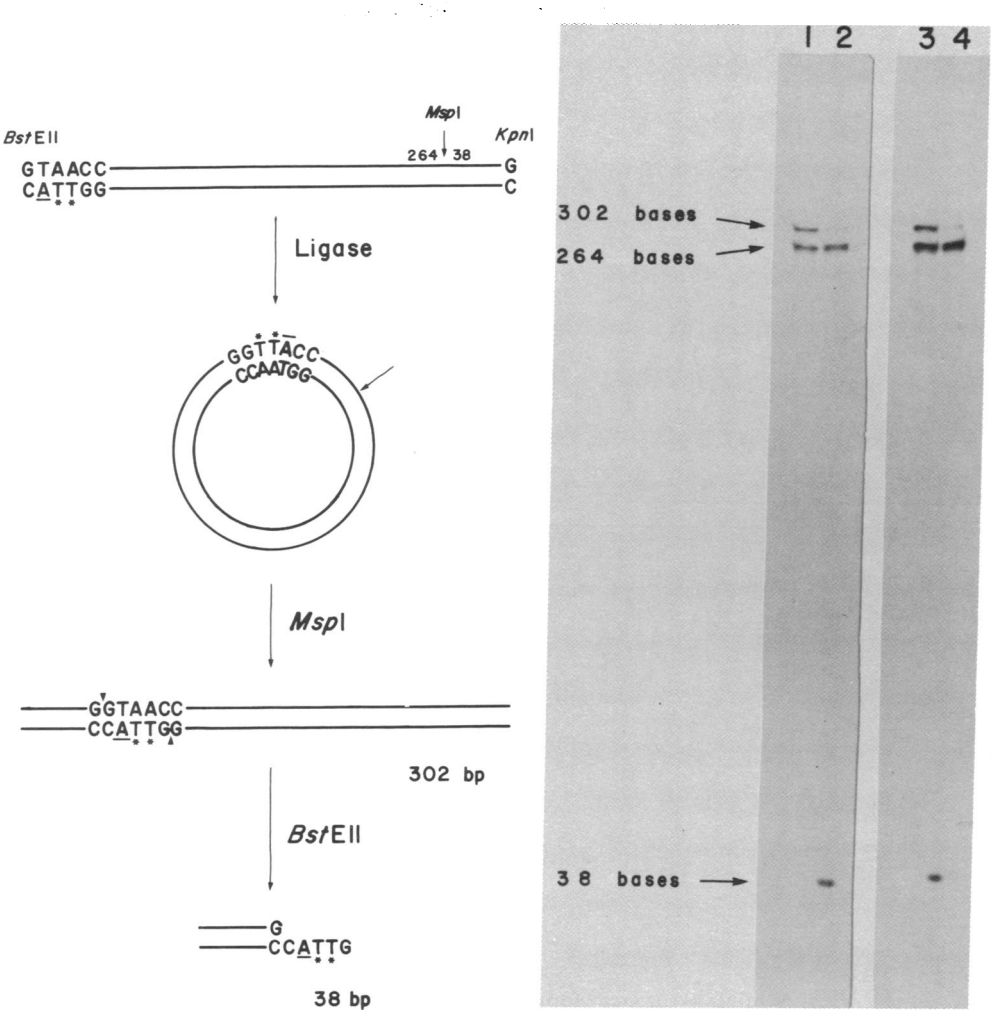

FIG. 3. (*Left*) Ligation and restriction of capped fragment. The label is indicated by an asterisk and the dAMP[αS] is underlined. The labeled fragment was prepared by incubating 1.5 μg of DNA with [α-^{32}P]dTTP, dCTP, dGTP, dATP[αS], or dATP, and 0.75 unit of the large fragment of DNA polymerase I under conditions described for Fig. 1 *Left*. The 3'-exonuclease activity of DNA polymerase I makes flush the *Kpn* I end. The fragment was purified by electrophoresis through a 5.0% polyacrylamide gel (14, 15). Ligation was performed with 0.006 μg of DNA (at a concentration of 0.075 μg/ml) in 50 mM Tris·HCl (pH 7.8)/8.7 mM $MgCl_2$/1.0 mM ATP with 1.5 units of T4 DNA ligase (Bethesda Research Laboratories) for 16 hr at 22°C. Products were analyzed on a 6% polyacrylamide gel with 7 M urea. (*Right*) Restriction with *Msp* I and *Bst*EII of ligated fragments followed by denaturing gel electrophoresis. Lanes 1 and 3 are, respectively, the ligated capped and uncapped fragments cleaved with *Msp* I and lanes 2 and 4 show the *Bst*EII cleavage products of the *Msp* I-treated capped and uncapped fragments, respectively.

A major use of the thionucleotides is the ability to generate single-stranded DNA from a double-stranded fragment. Single-stranded DNA of fixed length is useful for several purposes, including DNA sequence analysis by the chain termination technique (26), S1 nuclease mapping of RNA transcripts (27), and site-directed mutagenesis (28, 29). When an α-thionucleotide is inserted into only one end of a fragment, limit digestion with exonuclease III destroys only the complementary strand. Such treatment provides a full-length single strand and, if the complementary strand is desired, it can be obtained by appropriate choice of a restriction site at the other end of the fragment and of the α-thionucleotide used for the filling-in reaction. Unlike other methods currently used for generating single strands [e.g., gel electrophoretic strand separation (15, 30)], the thionucleotide procedure creates intact single strands regardless of length or sequence.

Most importantly, DNA containing the thio analog is replicated *in vivo* and, therefore, plasmids modified with this analog are competent for cellular transformation. Although only the dATP[αS] analog was used for the present study, there is no reason to believe that the other dNTP[αS]s would not have similar properties.

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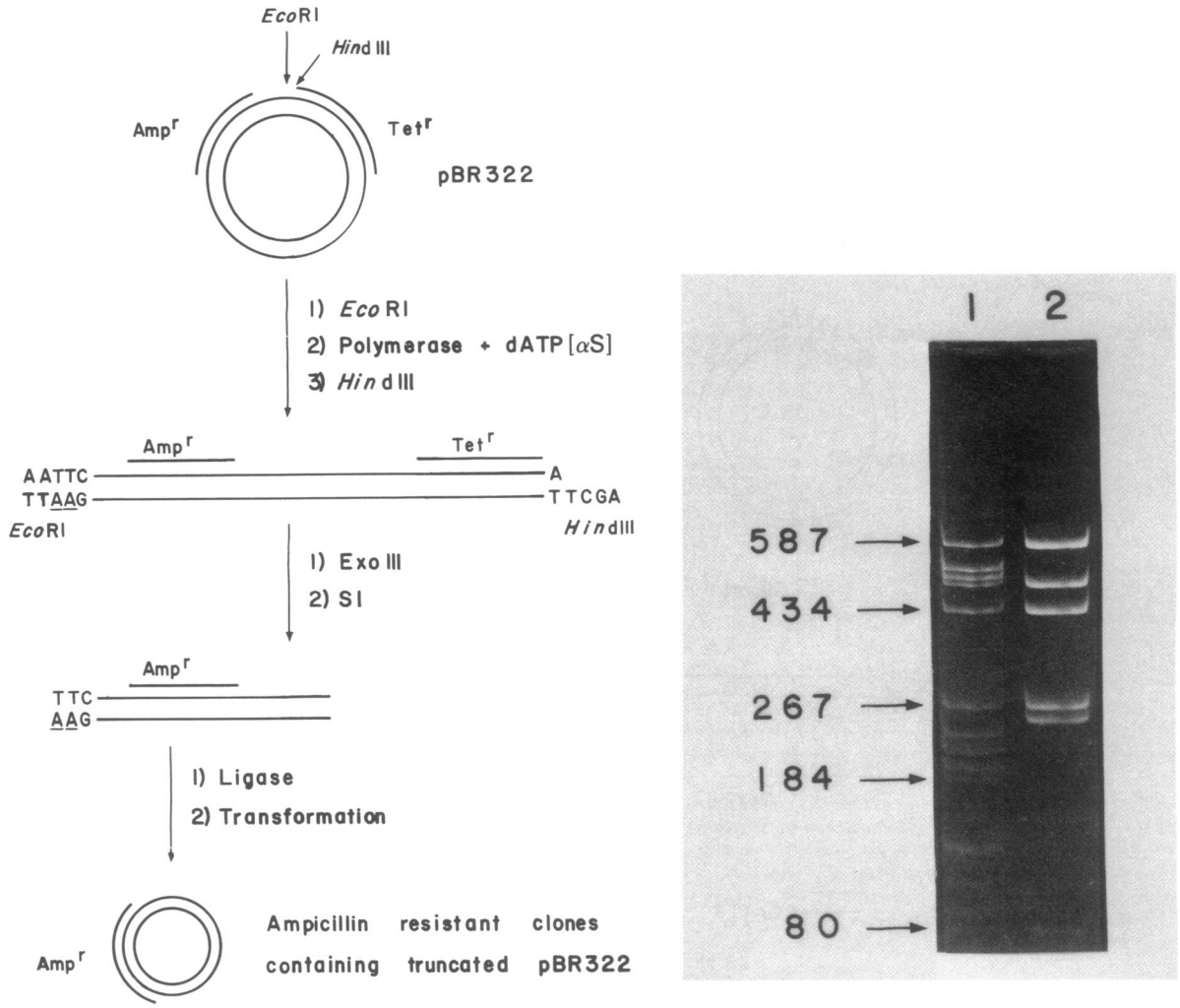

FIG. 4. (*Left*) Use of dATP[αS] to construct a truncated form of pBR322. To generate the truncated plasmid, 2.0 μg of pBR322 was digested with *Eco*RI and the fragment was incubated with dATP[αS], dTTP, and DNA polymerase. After digestion with *Hin*dIII, the fragments were incubated with 20 units of exonuclease III for 15 min at 37°C as in Fig. 1 *Left*. Subsequent S1 nuclease treatment and ligation were performed essentially as in ref. 16. The DNA was then used to transform cell strain KL386 (17). Selection was made for either ampicillin resistance (Amp^r) or tetracycline resistance (Tet^r). While no colonies grew on tetracycline, 40 were present on ampicillin. From one of these, plasmid was isolated and 0.3 μg was digested with *Hae* III and electrophoresed in parallel with *Hae* III-digested pBR322 on a 7.5% polyacrylamide gel (14). (*Right*) Electrophoresis of *Hae* III digestion products of pBR322 (lane 1) and truncated pBR322 (lane 2). Fragment lengths are indicated in bp.

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