Labeling and selective recovery of newly synthesized viral DNA from simian virus 40-infected cells incubated with inorganic thiophosphate

(DNA replication/thiophosphate affinity probe/Hg-affinity chromatography)

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ABSTRACT African green monkey CV-1 cells infected with simian virus 40 (SV40) were incubated in the presence of sodium [35S]thiophosphate. The viral DNA was prepared and the newly synthesized SV40 DNA molecules containing phosphorothioate groups were selectively recovered by affinity chromatography on organomercurial-Sepharose columns. The thio-derivatized viral DNA occurred in both superhelical (form I) and relaxed circular (form II) configurations, and it gave the expected cleavage products upon digestion with the restriction endonucleases Kpn I and HindIII. Enzymatic digestion of [35S]labeled SV40 DNA to its component nucleotides and chromatographic separation of the resultant 5' labeled phosphorothioate esters established the presence of the label in all four major nucleotides, but mainly in thymidine acid. The ability to label and recover selectively the newly synthesized polynucleotides by incubating cells with inorganic thiophosphate complements earlier findings [Sun, I. Y.-C. & Allfrey, V. G. (1982) J. Biol. Chem. 257, 1347-1353] that thiophosphate enters intracellular nucleotide pools and serves as an effective precursor for the phosphorylation of nuclear proteins in vivo.

There are now numerous examples of the use of phosphorothioate analogues of nucleoside triphosphates for the synthesis of polynucleotides in vitro. Eckstein and Gindi (1) showed that the ATP analogue adenosine 5'-O-(1-thiotriphosphate) (ATP[a-S]) was utilized as a substrate by Escherichia coli RNA polymerase. Adenosine 5'-O-(3-thiotriphosphate) (ATP[γ-S]) (2) is effectively utilized for RNA chain initiation as catalyzed by both prokaryotic (3, 4) and eukaryotic RNA polymerases (5-8). A great advantage of phosphorothioate incorporation is that transcripts initiated with ATP[γ-S] can be recovered selectively by affinity chromatography on organomercurial-Sepharose columns. The method has been employed to study the initiation of 5S RNA in myeloma nuclei (5), of preribosomal RNAs in Physoorum polycephalum (6) and Xenopus laevis oocyte nuclei (7), and of pre-tRNAs in yeast nuclei (8).

Thio-derivatized deoxyribonucleoside triphosphates substituted in the α-position have been employed as precursors for the in vitro synthesis of viral DNAs (9, 10), and they permit an analysis of the stereochemistry of the DNA polymerase reaction (11). Some interesting recent applications include the synthesis of a [35S]labeled cDNA of rabbit globin mRNA (12) and the use of dATP[α-35S] for the nick-translation of plasmid DNA (12).

While it is clear that the phosphorothioate analogues of nucleoside and deoxyribonucleoside triphosphates have many important applications, such compounds are not effective substrates for the in vivo synthesis of polynucleotides, due to restricted permeation through the cell membrane. What is needed is a thio-derivatized precursor that can freely enter the nucleotide pools of living cells. We have recently shown that [35S]thiophosphate fulfills this requirement and effectively labels ATP, GTP, and other nucleotides of HeLa cells (13). The subsequent transfer of thiophosphate to nuclear proteins permits their purification by Hg-affinity chromatography and facilitates the analysis of different sites of modification within the polypeptide chain. Given the evidence that inorganic thiophosphate readily enters the ribonucleotide pools of cultured cells, we decided to test whether deoxyribonucleotide pools were also labeled to a degree permitting the isolation of thio-derivatized DNA. The experiments to be described indicate that [35S]thiophosphate can be used to label the DNA of simian virus 40 (SV40) in infected CV-1 cells and that the substitution permits purification of new SV40 molecules by Hg-affinity chromatography.

MATERIALS AND METHODS

The African green monkey cell line CV-1 and SV40 were the generous gifts of M. T. Hsu of the Rockefeller University. The growth and maintenance of the cell line and propagation of SV40 were carried out as described by Kaufmann et al. (14). Sodium [35S]thiophosphate (Na3[35S]PO3; specific activity 600-800 mCi per mmol; 1 Ci = 3.7 x 10^10 becquerels), carrier-free [35P]orthophosphate, and [methy1-3H]thymidine (specific activity 78 Ci/mmol) were purchased from New England Nuclear. Restriction enzymes Kpn I and HindIII were from Bethesda Research Laboratories. Affi-Gel 501 was a product of Bio-Rad. RNase A, DNase I, and snake venom diesterase were obtained from Worthington.

Incorporation of [35S]Thiophosphate by SV40-Infected CV-1 Cells. Two 150-mm plates, each containing 5 x 10^-6 cells grown to confluence, were treated with 3 ml of a SV40-infected cell lysate at a multiplicity of 10 plaque-forming units per cell. One hour after virus adsorption, the lysate was removed and replaced with 5 ml of fresh Dulbecco's modified Eagle's medium (GIBCO) containing 2% dialyzed fetal calf serum. At 24 hr after infection, the medium was replaced with 5 ml of Joklik's modified phosphate-free Eagle's medium containing 2% dialyzed fetal calf serum. Three hours later, the medium was removed and replaced with 3 ml of the same medium containing 1.5 mCi per plate of 2 mM Na3[35S]PO3 (250 mCi/mmol). Uptake was allowed to proceed for 3 hr and then was terminated by washing the cells in 10 ml of ice-cold isotonic saline before the viral DNA was extracted according to the method of Hirt (15) as modified below. In 35P-labeling experiments, the [35S]thiophosphate was replaced by 1 mCi per plate of carrier-free [35P]orthophosphate. Other incubations combined 2 mM non-radioactive Na3PO4 with 1 mCi per plate of [3H]thymidine (78 Ci/mmol) and employed 10 plates per experiment.

Abbreviations: SV40, simian virus 40; ATP[α-S], adenosine 5'-O-(1-thiotriphosphate); ATP[γ-S], adenosine 5'-O-(3-thiotriphosphate); other thio-substituted nucleotides are abbreviated similarly.

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Preparation of SV40 Viral DNA. The washed cells were scraped into 5 ml of TD buffer (10 mM Tris-HCl, pH 7.4/10 mM EDTA), centrifuged, resuspended in 1 ml of the buffer, and mixed prior to addition of 1 ml of 1.2% Sarkosyl (Sigma) dissolved in TD buffer. The suspension was mixed very gently and immediately layered over 10 ml of a 5–20% sucrose gradient in TD buffer containing 0.6% Sarkosyl. After centrifugation at 37,000 rpm for 2 hr at 4°C in a Beckman SW 40 rotor, the gradient was collected from the top in 0.5-ml aliquots. Fifty microliters of each aliquot was taken for measurement of radioactivity, adding 50 μg of nonradioactive yeast tRNA as a carrier and precipitating with 10% trichloroacetic acid. The precipitates were collected on 0.45-μm-pore HA type Millipore filters, washed extensively with cold 10% trichloroacetic acid, and dried, and radioactivity was measured by scintillation spectrometry. Gradient fractions containing radioactivity were pooled, adjusted to 50 mM Tris-HCl/0.2 M NaCl, pH 7.4, and extracted twice with phenol and twice with chloroform/isoamyl alcohol, 24:1 (vol/vol), before the DNA was precipitated in 2.5 vol of ethanol at −20°C. The precipitates were redissolved in 1 ml of 50 mM Tris-HCl, pH 7.4, and treated with pancreatic RNase (75 μg/ml for 30 min at 37°C) and proteinase K (Boehringer Mannheim) (0.8 mg/ml) for 1 hr at 37°C. The enzymes were removed by repeated phenol extractions and the DNA was precipitated in ethanol as before.

Hg-Affinity Chromatography of Thio-Derivatized DNA. SV40 DNA (10–15 μg) was dissolved in 1 ml of buffer A (10 mM Tris-HCl, pH 7.9/20 mM NaCl/5 mM EDTA/0.05% NaDodSO₄) and applied to an Affi-Gel 501 column (0.7 × 10 cm) that had been equilibrated in the buffer. Elution of unbound DNA was carried out in buffer A, collecting 2-ml fractions. DNA bound to the organomercurial column was then eluted in buffer A containing 10 mM dithiothreitol. The eluent was monitored by measuring the radioactivity of 50-μl aliquots of successive 2-ml fractions, precipitating the DNA with 10% trichloroacetic acid in the presence of 50 μg of unlabeled tRNA. Experiments using 32P-labeled or [3H]thymidine-labeled SV40 DNA showed that only 0.1–0.6% of the applied DNA bound to the column and eluted with dithiothreitol under these conditions.

Separation of SV40 DNA Forms I and II. The [32P]thio-derivated SV40 DNA molecules recovered by Hg-affinity chromatography are a mixture of superhelical (form I) and relaxed circular (form II) viral genomes. These were separated by density gradient centrifugation (16). The DNA solution (1–2 ml) was layered over 10 ml of CsCl solution (0.735 g/ml) containing ethidium bromide at 400 μg/ml. After centrifugation at 30,000 rpm for 48 hr in a Beckman SW 41 rotor, the two SV40 DNA bands were visualized under UV light. The gradient was collected in 0.5-ml fractions, monitoring radioactivity in 25-μl aliquots of each fraction. Fractions containing form I DNA (lower band) and form II DNA (upper band) were pooled, extracted with 1-butanol to remove the dye, dialyzed against 10 mM Tris-HCl, pH 7.4/10 mM EDTA/10 mM NaCl, and precipitated in ethanol, using 50 μg of tRNA as a carrier.

Digestion of SV40 DNA with Restriction Endonucleases. Aliquots of purified SV40 DNA were digested with 10–20 units/μg of DNA of Kpn I in 30 μl of 6 mM Tris-HCl, pH 7.5/6 mM NaCl/6 mM MgCl₂/6 mM 2-mercaptoethanol. After 40 min at 37°C, 10–20 units/μg of DNA of HindIII was added and the reaction mixture was adjusted to 20 mM Tris-HCl and 60 mM NaCl. Incubation was continued for an additional 40 min. The reaction was terminated either by freezing and lyophilization or by addition of 10 mM EDTA prior to electrophoretic analysis of the restriction fragments.

Agarose Gel Electrophoresis of SV40 DNA and Restriction Fragments. Purified SV40 DNA and its restriction fragments were separated by electrophoresis in 2% agarose gels in buffer B (40 mM Tris-HCl, pH 8.3/20 mM sodium acetate/10 mM EDTA), using 0.01% bromophenol blue as a tracking dye. DNA bands were stained with ethidium bromide and localized in UV light. Isotopically labeled bands were excised and digested with 2 ml of Protosol (New England Nuclear), and their radioactivities were measured.

SV40 DNA Restriction Fragments with ATP[γ-32P]. The reaction was carried out essentially as described by Beltz and O'Brien (17). Briefly, 1–2 μg of endonuclease-digested SV40 DNA was incubated with bacterial alkaline phosphatase (1.5 units/μg of DNA) in 50 mM Tris-HCl, pH 8.0/10 mM MgCl₂/10 mM dithiothreitol for 30 min at 37°C. The DNA may be either separated from the phosphatase by phenol extraction or subjected directly to the polynucleotide kinase reaction. The reaction mixture contained 66 μM ATP[γ-32P] of specific activity 20 Ci/mmol and T4 polynucleotide kinase at 100 units/ml. After 1 hr at 37°C the reaction was terminated by addition of 0.3 M sodium acetate/0.1 M EDTA, pH 6.0, and the labeled DNA was precipitated with ethanol.

Dissociation of 35S-Labeled DNA to Component 5'-Deoxyribonucleotides. Bands of electrophoretically purified 35S-labeled SV40 DNA (form I) were excised from the gel and crushed into fine particles. The DNA was extracted twice with 400 μl each of 10 mM Tris-HCl, pH 7.25/5 mM NaCl/1 mM EDTA over a period of 16 hr at 4°C. The extract was adjusted to contain 10 mM MgCl₂ and DNase I at 10 μg/ml. After incubation at 37°C for 2 hr, 200 μg of snake venom phosphodiesterase was added and incubation was continued for 2 hr. The reaction was terminated with 0.1 M ammonium formate at pH 3.2. A similar degradation was carried out with 32P-labeled SV40 DNA.

Ion-Exchange Chromatography of 32P- and 35S-Labeled Nucleotides. The mixture of 5'-deoxyribonucleotides produced by DNase I and phosphodiesterase digestion was applied to a Dowex AG50-X8 column (NH₄⁺ form; 0.8 × 30 cm) that had been equilibrated in 0.1 M ammonium formate at pH 3.2. Approximately 6,000 cpm of 35S-labeled 5'-deoxyribonucleotides was applied to the column together with unlabeled dTMP, dGMP, dCMP, and dAMP as markers. The nucleotides were eluted in 0.1 M formate at pH 3.2 (18), collecting 1.3-ml fractions and monitoring UV absorbance at 254 nm and 35S (or 32P) activity.

RESULTS

35S-Phosphate Incorporation into SV40 DNA by Virus-Infected CV-1 Cells. We have shown previously that inorganic thiotophosphate enters the nucleotide pools of HeLa cells to form the thioanalogues of AMP, ADP, ATP, and GTP (13). The formation of ATP[γ-35S] and GTP[γ-35S] was of particular interest because subsequent kinase-catalyzed transfers of their thiotophosphate groups to proteins allow the recovery of the newly phosphorylated proteins by Hg-affinity chromatography.

Thiophosphate substitution at the γ position of ATP would also be expected to result in the enzymatic transfer of thiotophosphate groups to other nucleotides [e.g., by the thymidine kinase reaction (19–21)], whereas direct reduction of the thio analogues of ADP and GDP by ribonucleotide reductases (22) would give rise to the corresponding thioddeoxygenucleotides as potential precursors for DNA synthesis.

To test whether DNA could be labeled in intact cells by adding inorganic thiotophosphate, we selected the well-characterized system of CV-1 cells infected with SV40. The cells were first placed in a phosphate-free medium for 3 hr to deplete their orthophosphate reserves. This medium was replaced with one containing 2 mM sodium [35S]thiotophosphate, and incubation was continued. After 3 hr the cells were lysed in Sarkosyl and the viral DNA was prepared. The DNA was found to be radioactively labeled—with specific activities of 500–2,000 cpm/
μg—and it retained its 35S activity after treatment with RNAse A and proteinase K. This degree of 35S incorporation corresponds to 3–12 35S-labeled phosphorothioate groups per viral DNA molecule [5, 226 base pairs (23)].

In parallel experiments the cells were incubated with [32P]orthophosphate, with [3H]thymidine, or with [3H]thymidine plus nonradioactive thiol phosphate, and the labeled SV40 DNAs were prepared. These DNAs were employed to test the method of Hg-affinity purification of thio-derivatized DNA molecules.

Hg-Affinity Purification of Thiophosphorylated DNA Molecules. All SV40 DNA preparations were fractionated by chromatography on organomercurial-Sepharose columns as described in Materials and Methods. Unbound DNA was washed from the column in pH 7.9 Tris-HCl buffer containing 5 mM EDTA and 0.05% NaDodSO4, and the bound DNA was displaced subsequently by adding 10 mM dithiothreitol to the buffer. Fig. 1 compares the binding and elution profiles of viral DNAs labeled with [35S]thiophosphate and [32P]orthophosphate. It is clear that a substantial proportion (about 30%) of the 35S activity is retained by the column, whereas virtually all (more than 99%) of the 32P-labeled DNA passes through in the run-off peak. In experiments with [3H]thymidine-labeled viral DNA only 0.1–0.6% of the [3H]DNA applied to the column was retained and eluted with dithiothreitol under these conditions.

An alternative labeling procedure, designed to increase the specific activity of the affinity-purified DNA, combined high specific activity [3H]thymidine (78 Ci/mmol) with nonradioactive thiol phosphate in the culture medium. The viral DNA was prepared and subjected to Hg-affinity chromatography. The specific activity of the DNA eluted with dithiothreitol was about 100 times greater than that obtained with [35S]thiophosphate as the precursor (Fig. 2).

Characterization of Thio-Derivatized SV40 DNA. The affinity-purified SV40 DNA was centrifuged in a CsCl gradient to separate its superhelical form (I) from its relaxed circular form (II) (16). Fig. 2A illustrates the separations achieved and the distribution of 32P-labeled DNA along the gradient. Fig. 2B shows the corresponding distribution of SV40 DNA molecules that had been labeled with [3H]thymidine plus nonradioactive thio phosphate. In both cases, the isotopic label is distributed in two peaks that coincide with the positions of viral forms I and II as visualized by ethidium bromide fluorescence staining.

The two viral DNA forms are also separable by electrophoresis. Fig. 3 shows the separation of SV40 DNAs on 1% agarose gels after labeling with [3H]thymidine plus nonradioactive thiophosphate. A comparison of the radioactivity profile of DNA recovered from the sucrose gradient (Fig. 3A) with that of DNA purified by Hg-affinity chromatography (Fig. 3B) indicates that form I DNA is the predominant configuration in the cell lysate, but that form II predominates in the affinity-purified DNA. This is probably due to the diminished accessibility of the phosphorothioate groups in the superhelical form of the viral DNA, thus reducing its Hg affinity relative to that of the more extended relaxed circular form II DNA.

Endonucleolytic cleavage of SV40 DNA by restriction nucleases Kpn I and HindIII produces seven fragments of known size and nucleotide sequence. Electrophoretic analysis of the fragments derived from phosphorothioate-containing SV40 DNA shows that the same cutting pattern is observed as in the restriction digest of unmodified viral DNA (Fig. 3 D–F).

Finally, the 35S-labeled viral DNA was hydrolyzed to its com-

Fig. 1. Fractionation by Hg-affinity chromatography of SV40 DNAs from virus-infected CV-1 cells incubated with [35S]thiophosphate (A) and [32P]orthophosphate (B). The viral DNAs, purified by sucrose density gradient centrifugation, phenol extraction, and treatment with RNAse A and proteinase K, were applied to the Affi-Gel 501 column and eluted, first collecting the run-off peak and then displacing the bound DNA with 10 mM dithiothreitol (arrow). Note that a substantial fraction of the 35S-labeled viral DNA is retained by the column while virtually all of the 32P-labeled DNA (>99%) passes through.

Fig. 2. Separation of SV40 viral forms I and II from CV-1 cells incubated with [35S]thiophosphate (A) or with [3H]thymidine plus nonradioactive thiol phosphate (B). The purified viral DNAs were centrifuged in a CsCl gradient containing ethidium bromide at 400 μg/ml to separate the superhelical form I DNA from the relaxed circular form II DNA, as shown in the bottom photograph. The distributions of the 35S-labeled DNA (A) and affinity-purified 3H-labeled DNA molecules (B) coincide with the band positions visualized by fluorescence.
FIG. 3. Electrophoretic analysis of SV40 viral DNAs and their restriction fragments. (A) Separation in 1% agarose gels of $^{35}$S-labeled viral DNA purified by sucrose density gradient centrifugation but not applied to the Affi-Gel 501 column. The histogram above the fluorescent DNA bands indicates the proportions of the total $^{35}$S activity in forms I and II of the viral DNA. Note the predominance of form I DNA. (B) Electrophoretic separation of $^{35}$S-labeled SV40 DNA forms after Hg-affinity chromatography. The distribution of $^{35}$S activity (histogram) indicates an enrichment of the relaxed circular form II in the DNA bound to the column. (C-F) Electrophoretic analysis on 2% agarose gels of control and phosphorothioate-containing SV40 DNAs and their restriction fragments. The phosphorothioate-containing DNA was doubly labeled with $[3H]$thymidine and nonradioactive thiosphosphate and purified by Hg-affinity chromatography before digestion with Kpn I and HindIII. The electrophoretic banding patterns are shown for intact (unlabeled) DNA standard (C); restriction fragments of SV40 DNA in C (D); restriction fragments of phosphorothioate-containing SV40 DNA passing through the Affi-Gel 501 column (E); restriction fragments of organomercurial-bound SV40 DNA (F).

ponent 5'-deoxyribonucleotides by treatment with DNase I and snake venom phosphodiesterase (see Discussion). The nucleotides were separated by ion-exchange chromatography, as shown in Fig. 4, and the radioactivity in each peak was measured. For purposes of comparison, a similar analysis was carried out on SV40 DNA that had been labeled with $^{32}$P orthophosphate. The distribution of phosphorothioate $^{35}$S-labeled shows an overwhelming predominance in thymidylic acid, which has 86% of the total $^{35}$S, as compared to 3% in dGMP, 3.7% in dCMP, and 7.3% in dAMP. The corresponding figures for $^{32}$P orthophosphate incorporation are 56% in dTMP, 22.4% in dGMP, 12.5% in dCMP, and 9.1% in dAMP. It follows that most of the $^{35}$S incorporation into viral DNA represents the thiophosphorylation of thymidine in the thymidine kinase reaction (19-21) in which ATP$^{[\gamma-S]}$ would serve as the thiophosphate group donor. This is in accord with earlier evidence that inorganic thiophosphate readily enters the $\gamma$ position of ATP in cultured cells (13).

DISCUSSION

The main point of this paper is that it is possible to label DNA molecules in vivo, using a probe ($^{35}$S thiophosphate) that not only labels the newly synthesized molecules but also permits their selective recovery by Hg-affinity chromatography. We anticipate that this technique will find many applications in the study of the mechanism of DNA synthesis (e.g., recovery of Okazaki fragments) and in analyzing temporal aspects of the replication of different DNA sequences. The method has been shown to permit the selective recovery of newly synthesized SV40 viral DNA molecules, and evidence is presented that the thio-derivatized DNAs have been synthesized with fidelity, as judged by their patterns of cleavage with Kpn I and HindIII.

There are, however, certain caveats that require discussion. First among these is the problem of incomplete recovery of $^{35}$S-labeled DNAs from Affi-Gel 501 columns. The retention of DNA molecules on the organomercurial column will depend on the number of phosphorothioate groups in the polynucleotide chain, on the size of the DNA, and on its configuration. The recovery of newly synthesized DNA molecules is skewed in favor of those containing more sulfur-derivatized nucleotides. In our experience with SV40 DNAs, the molecules that bind to Affi-Gel 501 have about 2.5 times more thio-derivatized than the viral DNA fraction that is not retained. We have examined the relationship between the size of the modified DNA and its chromatographic behavior in SV40 restriction fragments that were 5'-end labeled with polynucleotide kinase and ATP$^{[\gamma-S]}$ (17, 24, 25). The distribution of $^{35}$S-labeled DNA molecules was measured in the run-off peak and in the fraction retained on Affi-Gel 501 and subsequently eluted with dithiothreitol. The proportion of DNA molecules retained by the affinity column was found to diminish with increasing size of the restriction fragment: from 33% bound at 215 base pairs to 12% bound at 1,101 base pairs and only 5% bound at 1,768 base pairs. This is in accord with earlier evidence that Affi-Gel 501 retained only 15-25% of the 550-nucleotide-long transcripts initiated by E. coli RNA polymerase with ATP$^{[\gamma-S]}$ (3). Reeve et al. (3) suggested that the low retention of thio-derivatized RNA molecules may be due to their exclusion by the affinity gel, and they recommended that crosslinking of the gel be reduced by the use of lower concentrations of cyanoine bromide during the activation of the agarose. We have prepared the organomercurial-Sepharose after activation with cyanoine bromide at only 2.5 mg/ml, as recommended (3), but there was no significant improvement in the binding of full-length $^{35}$S-labeled SV40 DNA.

Under the labeling conditions described for SV40-infected CV-1 cells, the viral DNA incorporates 3-12 $^{35}$S thiophosphate groups per viral genome (5,226 base pairs). This degree of substitution allows recovery of over 30% of the newly synthesized viral DNA on Affi-Gel 501 columns, but it should be noted that
some selection occurs against the superhelical form of the viral DNA (see Fig. 3).

What are the consequences of thio-phosphate incorporation for DNA susceptibility to restriction nucleases? Although the restriction fragments produced by Kpn I and HindIII digestion of thio-derivatized SV40 DNA are apparently normal, one cannot conclude that all restriction nucleases will cleave phosphothioate-containing DNAs at the appropriate sites. In fact, there is evidence to the contrary. A recent report by Vosberg and Eckstein (26) indicates that phosphothioate-containing DNAs of phages dX174 or fd (synthesized in vitro by E. coli DNA polymerase I) are not cleaved normally, and that various restriction nucleases are inhibited to different degrees, but especially when the DNA contains a phosphothioate exactly at the cleavage site. However, it has also been observed that the presence of dAMP(α-S) within the restriction site for BatIII does not affect DNA cleavage (27).

What are the consequences for the fidelity of DNA synthesis when phosphothioate enters the nucleotide pools? The answer depends, in part, on the source of the DNA polymerase. The deoxyribonucleoside (1-thio)triphosphates have normal base-pairing properties, but if they are once misinserted into the DNA chain they cannot be excised by the proofreading functions of E. coli or bacteriophage T4 DNA polymerases (10). With DNA polymerase β from rat Novikoff hepatoma the substitution of dCTP(α-S) for dCTP did not alter fidelity (10).

The predominant labeling of dTMP by [35S]thiophosphate analogs with the most convincing preferential incorporation of [32P]thiophosphate into the thymidic acid of SV40 DNA, but it should be noted that the quantitation of thiо-derivatized deoxyribonucleotides by enzymatic digestion of [35S]labeled DNAs is complicated by several factors. There are clear indications that phosphothioate diester linkages in polyribonucleotides are hydrolyzed much more readily by exonucleases such as snake venom phosphodiesterase than are the corresponding phosphodiester bonds (1, 11, 28, 29). The deoxyribonucleoside (1-thio)triphosphates have been shown to be effective precursors for DNA synthesis in vitro (10, 26, 30), but the phosphothioate diester bonds resist the proofreading exonuclease functions of E. coli DNA polymerase I (10). Similarly, the introduction of thio-dAMP near the 3′ ends of DNA restriction fragments yields “capped” molecules that resist degradation by exonuclease III (27). Steric factors also influence greatly the rate of enzymatic hydrolysis of phosphothioate diester linkages, and in a complex way. For example, snake venom phosphodiesterase hydrolyzes the R₄ diastereomer of 5′'-adenosyl-3′-O-uridylyl phosphothioate approximately 500 times faster than the S₄ diastereomer (31), whereas the R₄ configuration of adenosine 5′-O-phosphothioate O-p-nitrophenyl ester is hydrolyzed 13,000 times faster than the S₄ diastereoisomer (32). An additional complication is the desulfurization of the nucleotide 5′-phosphothioates produced by exonuclease digestion (11, 31, 33), which would lead to a loss of 35S activity and an underestimation of the extent of thiо-derivatization. In our studies of [35S]thiophosphate incorporation into DNA in vitro, relatively few phosphothioate diester linkages were formed (3–12 per SV40 genome), and this probably accounts for the comparable extents of digestion observed with [35S]-labeled and [32P]-labeled DNA molecules on treatment with DNase I and venom phosphodiesterase. The recovery of the [35S] label in mononucleotide form (particularly as dTMP(α-S) and dAMP(α-S)) indicates that the venom exonuclease was effective in the hydrolysis of the few phosphothioate diester linkages in the newly made viral DNA.

Despite the limitations mentioned, we feel that the unique advantages of the in vitro labeling procedures with [35S]thiophosphate, in particular the ability to recover a large fraction of the most recently synthesized polynucleotide sequences, will recommend its use for many purposes. Moreover, the facts that introduction of as few as three phosphorothioate linkages in the entire SV40 viral genome permits its recovery in appreciable yield and that its restriction patterns by Kpn I and HindIII are normal argue that most DNAs prepared with a similar low level of substitution will yield the expected restriction fragments with other endonucleases. Moreover, recent studies with dATP(α-S) insertions at the 5′ ends of DNA restriction fragments indicate that the phosphothioate allows subsequent ligation of the DNA, restriction of ligated junctions, and in vitro replication of plasmids containing phosphothioate linkages (27).

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