DNA and RNA Sequence Determination Based on Phosphorothioate Chemistry

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The difference in reactivity between phosphate and phosphorothioate diesters is the basis of a chemical degradation scheme for the sequencing of DNA and RNA. The phosphorothioate groups are incorporated into the nucleic acid in four separate enzymatic reactions, with three of the natural nucleoside triphosphates and one α-thiotriphosphate in each reaction. Selective strand cleavage is achieved through alkylation to form the hydrolytically labile phosphorothioate triester. As an example, the sequence analysis is presented of M13 phage DNA and of RNA prepared by transcription with SP6 RNA polymerase.

RECENT ADVANCES IN DNA TECHnology have relied heavily on the methods of Sanger (*1*) and Maxam and Gilbert (*2*) for the sequencing of DNA. Although much work has been directed toward the automation of the existing sequencing methods (*3, 4*) and the use of fluorescent labels for detection (*5, 6*), little progress has been reported on the development of alternative methods for performing the sequencing reactions. We describe a novel method based on the enzymatic incorporation of phosphorothioate groups into DNA with a subsequent chemical degradation procedure that takes advantage of the difference in reactivity between a phosphate and phosphorothioate diester to achieve the cleavage required for sequencing. An analogous procedure can be used to sequence RNA with the use of the SP6 RNA polymerase transcription system.

The *Sp*-diastereomers of deoxyribonucleoside and ribonucleoside 5′-*O*-(1-thiotriphosphates) are analogs of the naturally occurring nucleotides and are incorporated readily into nucleic acids by DNA or RNA polymerases [(*7*); for abbreviations, see (*8*)]. A method for the selective chemical degradation of this nucleic acid at the phosphorothioate residues was suggested from the observation that the sulfur atom of the phosphorothioate functional group of ATPαS is readily alkylated by methyl iodide (*9*). In DNA and RNA this alkylation would form the phosphorothioate triesters, which would be more labile to hydrolysis than the phosphate diesters (*10*). We believed that by controlling the extent of hydrolysis, random strand breaks suitable for sequencing could be generated. As a test system, we studied the hydrolysis of dinucleoside monophosphorothioates and monophosphates in the presence of alkylating reagents.

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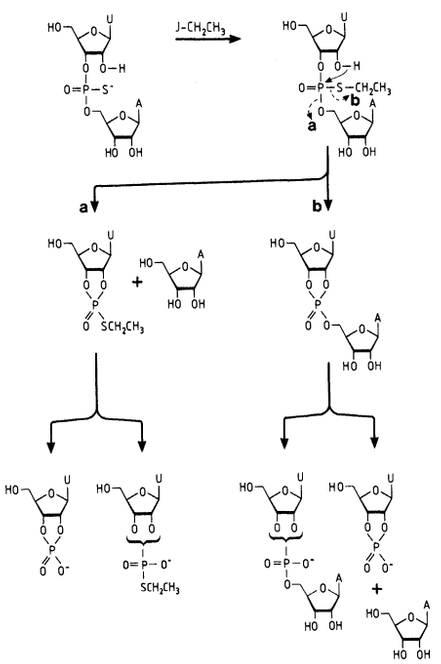
Reaction of an aqueous ethanol solution of the diribonucleoside monophosphorothioate *Rp*-[Up(S)A] (*11*) with an excess of iodoethane at 56°C caused decomposition of the dinucleotide and the formation of adenosine, 2′,3′-cUMP, 2′-UMP, 3′-UMP, and UpA, as monitored by reversed-phase HPLC after 2.5 hours. In contrast, the diribonucleoside monophosphate UpA remained unchanged when treated under these conditions. These results suggest that alkylation of the phosphorothioate occurs to form the triester. Subsequent nucleophilic addition of the 2′-hydroxyl of the uridine ribose ring to the phosphorus atom leads to displacement of either adenosine (path a) or ethanethiol (path b). The resulting 2′,3′-cyclic triesters are then hydrolyzed.



Treatment of the dinucleoside phosphorothioate, d[Cp(S)T] (*12*), with iodoethane gave quantitatively the phosphorothioate triester, which was stable under the conditions of the alkylation. With the addition of dilute sodium hydroxide, the triester was hydrolyzed to d[CpT]. When d[Cp(S)T] was treated with 2-iodoethanol [a reagent, which upon alkylation, places a hydroxyl group in a position equivalent to the 2′-hydroxyl of the uridine in Up(S)A], direct conversion to d[CpT] was observed. This desulfurization reaction, which would not lead to breakage of a phosphorothioate-DNA strand, is probably analogous to that observed for the reaction of oxirane with phosphorothioate compounds (*13, 14*). We did observe, however, that DNA strand cleavage does occur to a small extent as M13 RFIV DNA with phosphorothioate nucleotides incorporated into the (−)strand was nicked after treatment with 2-iodoethanol (*15, 16*).

From these chemical studies we developed a simple procedure for DNA sequencing involving treatment of phosphorothioate DNA with 2-iodoethanol (Fig. 1). The level of chemical degradation of the DNA is not excessive and more than 100 bases can be read easily. Longer fragments could be read if different electrophoresis conditions were used. The chemical reaction can produce a mixture of fragments with either 3′-hydroxyl or 3′-phosphate ends, depending on whether the nucleoside 3′- or 5′-oxygen is the leaving group. Such fragments should have different mobilities and be resolved during electrophoresis. Indeed, on close inspection of the smaller DNA fragments, one can observe two bands that are of very different intensity. By reading only the more intense band, the correct sequence can easily be determined. For DNA fragments greater than 50 bases (included in this value is the length of the primer), only one band is observed (*17–19*).

Operationally this method is similar to and is as convenient as the Sanger approach (*1*). It differs in the respect that the dNTPαS required in a particular reaction is used solely and not in a mixture with the natural nucleotide. As it does not rely on chain termination, it has the advantage that the level of polymerization can be monitored independently of the sequencing reaction. This is important, as certain difficulties encountered with the Sanger method arise because of poor polymerization caused by contaminants in the DNA preparation or anomalies in the template sequence. With the use of this method new polymerization conditions can be tested, analyzed quickly by agarose gel electrophoresis for the conversion of single-stranded DNA to RFII DNA, and then used directly in the sequencing protocol. The incorporation of phosphorothioate nucleotides avoids a problem that arises in the Sanger method due to inhibition of the enzyme in the polymerization reaction by the 2′,3′-dideoxyribonu-

cleotides. As demonstrated by Atkinson *et al.* (*20*) and Webb *et al.* (*21*) this inhibition results from trapping of the DNA polymerase in a tight association with the chain-terminated DNA. In contrast, the phosphorothioate analogs display kinetic properties with *Escherichia coli* DNA polymerase I (*22*) and chemically modified T7 DNA polymerase (*23*) that are essentially identical to the natural nucleotides. This greater flexibility allowed by the better substrate properties of the phosphorothioate analogs and the ability to monitor the polymerization reaction in the sequencing procedure should make this approach to DNA sequencing preferable to the Sanger method for automation.

The sequence of phosphorothioate-RNA, prepared with the SP6 (*24*) or T7 (*25*) RNA polymerase transcription systems, can be determined with an analogous procedure (Fig. 2). The "run-off" RNA transcripts, from SP6 RNA polymerase transcription of Bst N1 linearized pGEM1, were labeled at the 5′ end by including [γ-^{32}P]GTP, the first nucleotide incorporated by the polymerase, in the reaction mixture. As measured with a Geiger counter, the gel-purified full-length RNA typically contained approximately 6000 cpm of radioactivity. For the transcripts tested in this study, and for others we have tried that range in size from 100 to 350 bases long, this level of incorporation of radioactivity was sufficient for sequencing. For the degradation reaction, we found that 2-iodoethanol was a better reagent than iodoethane, which was used in the preliminary study, because iodoethanol is more soluble in water.

The RNA sequence determined by this method is accurate when compared with that expected from the corresponding DNA sequence of pGEM1. There is a greater variation in band intensity when compared with the DNA sequencing results and may be due to secondary structure present in the single-stranded RNA that is not encountered in double-stranded DNA. In sequences that have a series of the same nucleotide in a row, the band intensities varied. Generally the smaller, faster migrating RNA fragments in the series gave the more intense bands.

These experiments demonstrate that the chemical sequencing of DNA and RNA can be achieved through the incorporation of phosphorothioate groups into nucleic acids followed by hydrolysis in a statistically random manner at the phosphorothioate positions. As these reactions are sufficiently mild as to not interrupt nucleic acid–protein complexes, it may be possible to use this sequencing reaction in the presence and absence of the protein to probe the interactions within such a complex.

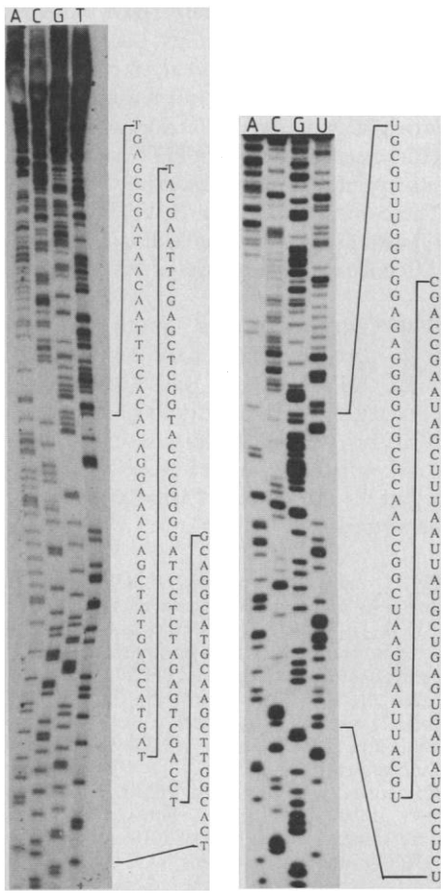


Fig. 1 (left). DNA sequencing result with the use of M13mp18 as template. A ^{32}P-labeled primer (0.5 pmol, 1.5 μl of a stock primer solution prepared as described below) was annealed to 1 μg of single-stranded DNA template in a 9-μl reaction volume containing 83 m*M* tris-HCl, *p*H 8.3, and 33 m*M* $MgCl_2$, by incubating at 70°C for 5 min and then at 37°C for 30 min. Portions (2 μl) were transferred to four marked 1.5-ml Eppendorf tubes containing 3 μl of the appropriate dNTP mix [each mix contained 0.5 m*M* dNTPαS (*15*) and 0.5 m*M* of each of the other three dNTPs (Boehringer Mannheim), 8 m*M* tris-HCl, *p*H 8.0, 80 μ*M* Na_2EDTA]. DNA polymerization was initiated by adding 2 μl of an enzyme solution composed of 10 m*M* dithiothreitol (DTT) and Klenow fragment (1 U/μl) (*26*). After a 40-min incubation at 37°C, the reactions were stopped (by addition of 2 μl of 10 m*M* Na_2EDTA, 95% (v/v) formamide, 0.1% (w/v) xylene cyanol FF, 0.1% (w/v) bromophenol blue, and 7% (v/v) iodoethanol), heated at 95°C for 3 min, and then placed on ice. Approximately 7 μl of sample was applied to a 6% polyacrylamide buffer gradient gel (*27*) of dimensions 40 by 20 by 0.4 cm, and the electrophoresis was performed until the bromophenol blue dye migrated off the gel. The gel was transferred to Whatman 3MMChr filter paper and dried under vacuum at 80°C. Autoradiography was performed at −78°C with Kodak X-Omat XAR-5 film for 48 hours. To label the sequencing primer, 34 pmol (0.01 A_{260} absorbance units) of M13 primer [sequence d(AGGGTTTTCCCAGTCACG) prepared with an Applied Biosystems Model 308A DNA synthesizer), 8 units of T4 polynucleotide kinase (Boehringer Mannheim), and 40 μCi of [γ-^{32}P]ATP (5000 Ci/mmol, Amersham] were combined in 30 μl with 100 m*M* tris-HCl, *p*H 8.0, 10 m*M* 2-mercaptoethanol, and 10 m*M* $MgCl_2$, and then incubated for 30 min at 37°C. The enzyme was denatured by heating at 70°C for 15 min. A SEP-PAK C_{18} cartridge (Waters Associates) was used to purify the phosphorylated primer (*28*). The residue was dissolved in 100 μl of water to give a stock 340 n*M* (0.1 A_{260} absorbance units per milliliter) radioactive primer solution. **Fig. 2 (right).** Sequence of RNA obtained from SP6 RNA polymerase transcription of Bst NI linearized pGEM1. Into each of four 1.5-ml Eppendorf tubes, 50 μCi of [γ-^{32}P]GTP (30 Ci/mmol, Amersham) was added and then dried with a Speed Vac concentrator. To each tube was added (15 μl) 40 m*M* tris-HCl, *p*H 7.5, 8 m*M* $MgCl_2$, 2 m*M* spermidine-3HCl, 13 m*M* DTT, 31.5 units ribonuclease inhibitor (Boehringer Mannheim), 0.4 m*M* of one NTPαS (*15*) and 0.4 m*M* of each of the three other NTPs (Boehringer Mannheim), 2 μg of Bst N1 linearized pGEM1 DNA, and 20 units of SP6 RNA polymerase (Promega Biotec) (*24*). The reactions were incubated at 37°C for 2.5 hours and then stopped by addition of 2 μl of 95% (v/v) formamide, 10 m*M* Na_2EDTA, 0.1% (w/v) xylene cyanol FF, and 0.1% (w/v) bromophenol blue. The Bst NI (New England Biolabs) reaction was performed as specified by the supplier and, after phenol extraction and ethanol precipitation, the DNA was dissolved in water to give a 1 μg/μl solution. The RNA was purified by denaturing 8% polyacrylamide gel electrophoresis. The appropriate section of the gel, with reference to a 1-hour autoradiographic exposure of the gel with Kodak X-Omat XAR-5 film, was cut out with a sterile scalpel and transferred to an 1.5-ml Eppendorf tube. The procedure of Rubin (*29*) was used to isolate the RNA, with the modification that the gel piece was first ground against the sides of the Eppendorf tube with the end of an Eppendorf 0.5-ml combitip until it was the consistency of a gummy paste. The level of radioactivity resulting from each of the four reactions was estimated with a Geiger counter. The RNA was dissolved in a sufficient amount of water to give a solution of approximately 10 cps/μl. To 4 μl (40 cps) of RNA was added 2 μl of 10 m*M* Na_2EDTA, 95% (v/v) formamide, 0.1% (w/v) xylene cyanol FF, 0.1% (w/v) bromophenol blue, and 7% (v/v) iodoethanol, which was then heated at 95°C for 3 min and then placed on ice. Approximately 5 μl of each reaction mixture was applied to a 8% polyacrylamide buffer gradient gel (*27*) as described in Fig. 1. Autoradiography was performed at −78°C with Kodak X-Omat XAR-5 film for 72 hours.

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8. The abbreviations used in the text are: ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); *Rp*-[Up(S)A], the *Rp* isomer of 5′-*O*-adenosyl 3′-*O*-uridyl phosphorothioate (*Sp*, *Rp* designates the chirality of

phosphorus); UpA, 5′-*O*-adenosyl 3′-*O*-uridyl phosphate; d[Cp(S)T], 5′-*O*-thymidyl 3′-*O*-cytidyl phosphorothioate; d[CpT], 5′-*O*-thymidyl 3′-*O*-cytidyl phosphate; d[Cp(S)G], 5′-*O*-guanosyl 3′-*O*-cytidyl phosphorothioate; 2′,3′-cUMP, uridine 2′,3′-cyclic monophosphate; 2′-UMP, uridine 2′-monophosphate; 3′-UMP, uridine 3′-monophosphate; HPLC, high-performance liquid chromatography; dNTPαS, 2′-deoxyribonucleoside 5′-*O*-(1-thiotriphosphate); and NTPαS, ribonucleoside 5′-*O*-(1-thiotriphosphate).

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16. Agarose gel electrophoresis run in the presence of ethidium bromide, as described in T. Maniatis, E. F. Fritisch, and J. Sambrook [*Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982), pp. 151–152], was used to assay the reaction.
17. When the electrophoresis period was short and the primer had not been run off the gel, a series of bands across all four lanes was observed. The uppermost band was unextended primer, whereas the lower bands presumably arose from primer that had been degraded by the 3′ to 5′ exonuclease activity of the Klenow fragment, but not then extended. As a test, we prepared a sequencing primer that contained a phosphorothioate group at the first internucleotide linkage from the 3′ end. Our expectations, based on precedent (*18, 19*) that this primer would be resistant to the exonuclease activity of the Klenow fragment, were confirmed, and only the unreacted primer was observed in all four lanes. However, this partial degradation of the primer does not interfere with reading the sequence.
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30. We thank I.-M. Ehbrecht, H.-H. Förster, K. Nakamaye, J. Ott, J. Sayers, and W. Schmidt for helpful discussions and D. Brunsing, A. Fahrenholz, and A. Wendler for excellent technical assistance. G.G. was the recipient of a postdoctoral research fellowship from the Alexander von Humboldt Stiftung.

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Characterization of a Noncytopathic HIV-2 Strain with Unusual Effects on CD4 Expression

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A new isolate of the human immunodeficiency virus type 2, designated HIV-2_{UC1}, was recovered from an Ivory Coast patient with normal lymphocyte numbers who died with neurologic symptoms. Like some HIV-1 isolates, HIV-2_{UC1} grows rapidly to high titers in human peripheral blood lymphocytes and macrophages and has a differential ability to productively infect established human cell lines of lymphocytic and monocytic origin. Moreover, infection with this isolate also appears to involve the CD4 antigen. However, unlike other HIV isolates, HIV-2_{UC1} does not cause cytopathic effects in susceptible T cells nor does it lead to loss of CD4 antigen expression on the cell surface. These results indicate that HIV-2 may be found in individuals with neurologic symptoms and that the biological characteristics of this heterogeneous subgroup can differ from those typical of HIV-1.

TWO PREVIOUSLY DESCRIBED ISOlates of HIV-2, LAV-2 [now called HIV-2_{ROD} (*1*)] and SBL6669 (*2*) obtained from West African patients with AIDS or AIDS-related disease induce cytopathic effects in cultured cells. Here we report the isolation and characterization of a noncytopathic HIV-2–like virus from a patient in the Ivory Coast. Its biologic properties in vitro appear distinct from many iso-

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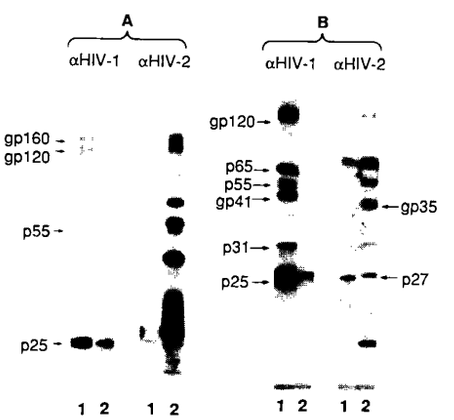

Fig. 1. Immunoblot analysis of HIV viral antigens. (**A**) Sera of the African patients from whom HIV-1_{SF471} (αHIV-1) and HIV-2_{UC1} (αHIV-2) were isolated were tested by immunoblot with electrophoretically separated HIV-1_{SF2}– [also called AIDS-associated retrovirus, ARV-2 (*15*)] (lane 1) and SIV_{mac}– (lane 2) infected HUT-78 cell lysates (*6*). (**B**) Isolates of HIV-1 and HIV-2 were grown to high titer (>1 × 10^6 cpm/ml of RT activity) and centrifuged (20,000*g*) through a layer of 20% glycerol for 2 hours. Viral pellets were suspended in 0.1 ml of PBS and stored at −70°C. Individual proteins were resolved by polyacrylamide gel electrophoresis and transferred to nitrocellulose for immunoblot analysis (*5*). Viral antigens present in the pellets from HIV-1_{SF471}– (lane 1) or HIV-2_{UC1}– (lane 2) infected cultures were reacted with serum from the patient infected with HIV-1_{SF471} (αHIV-1) or from the patient infected with HIV-2_{UC1} (αHIV-2). A similar result to that obtained with HIV-2 serum in lane 2 was observed with an antiserum to SIV_{mac}.

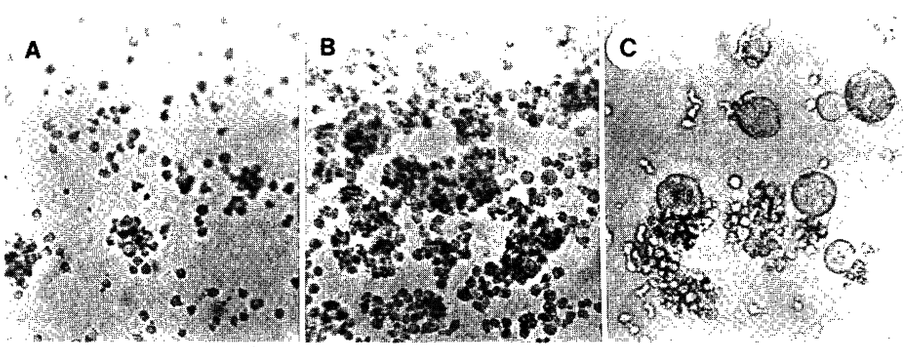

Fig. 2. Induction of cytopathology by cocultivation with uninfected SupT1 cells. (**A**) Uninfected; (**B**) HIV-2_{UC1}–infected (100% HIV antigen-positive); and (**C**) HIV-1_{SF471}–infected (35% HIV antigen-positive) SupT1 cells were mixed with uninfected SupT1 cells (99% $CD4^+$) at a ratio of 1:10 and observed for the formation of multinucleated giant cells and balloon degenerative forms. Note the CPE only in (C) (×400).