
Separation of the complementary strands of DNA fragments on polyacrylamide gels*

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ABSTRACT

³²P-labeled (*in vivo*) ØX174 RFI DNA was restricted by Hinc II. Three aliquots of the same digest: a) non-denatured, b) heat denatured, and c) denatured by 5 mM Me-HgOH were analyzed on 3-15% acrylamide gel gradients or on 3% gels with reduced N,N'-methylene-bis-acrylamide. The autoradiography of the gels showed that the non-denatured sample migrates two times faster than the denatured samples. After denaturation each original fragment appeared as a doublet. Using *in vitro* synthesized RFI DNA labeled only in negative strand with ³²P we could identify the position of the negative strand in each denatured doublet. The single strand DNA fragments could be recovered from the gel slices on a semi-preparative scale by electrophoresis into dialysis tubing.

INTRODUCTION

Separation of the complementary strands of DNA fragments generated by restriction enzymes could be very useful for hybridization and sequencing. Differences in base composition have enabled a partial fractionation of the complementary strands of several phage DNAs in alkaline CsCl. Szybalski *et al.*¹ introduced the use of poly U,G in CsCl density gradients and showed separation of the two intact strands of λ DNA. An analytical method for strand separation of different phage DNAs, using alkali denaturation followed by agarose gel electrophoresis, was reported by Hayward².

A combination of these methods has been used by Tibbetts *et al.*³ and Flint *et al.*⁴ to separate the complementary strands of adenovirus (Ad2) DNA and the complementary strands of DNA fragments generated by Eco RI.

We have investigated the separation of the complementary strands of ØX174 DNA fragments. We took advantage of the high resolving power of polyacrylamide gel gradients⁵ to develop a method for strand separation. In this paper we show that DNA fragments ranging from 75-2000 base pairs can be resolved into their complementary strands in one electrophoretic run.

MATERIALS AND METHODS

The chemicals and enzymes were obtained from the following sources: acrylamide, N,N'-methylene-bis-acrylamide, N,N,N',N'-tetra methylethylene diamine, ammonium

persulfate, Dowex 50 W-X2 from Bio-Rad Laboratories, Richmond, California. Sephadex G-50, Lysozyme, RNase A, calf thymus DNA from Sigma Chemical Corp., St. Louis, Missouri. Chloramphenicol B grade, ethidium bromide B grade, propidium diiodide A grade, dATP, dGTP, dCTP, TTP, and NAD⁺ from Calbiochem, San Diego, California. All four α -³²P-dXTP's from New England Nuclear. Dichlorodimethylsilane 99% from Aldrich Chemical Co., Milwaukee, Wisconsin. 1 M solution of methylmercury II hydroxide from Alfa Products, Beverly, Massachusetts. DNA ligase (*E. coli* NAD⁺), Hinc II isolated from *Haemophilus influenzae* serotype C, Hae III from *Haemophilus aegyptius* and Pst I from *Providencia stuartii* were obtained from New England Biolabs. DNA-polymerase I was a gift from Dr. A. Kornberg, Stanford University.

Preparation of ϕ X174 ³²P-RFI DNA. Cells of *E. coli* (strain H514 arg⁻, thy⁻, DNase I⁻, uvrA⁻) were grown at 37° in TPG⁶ low phosphate medium. A one liter culture at cell density of 2×10^8 cells/ml was infected with ϕ X174 am3 phage at a multiplicity of 10; one min later carrier-free ³²P (New England Nuclear) was added to final concentration of 20 μ Ci/ml. At 8 min after infection, chloramphenicol (30 μ g/ml) was added to inhibit single strand DNA formation.

The cells were aerated for 90 min and harvested in a Sorvall rotor GSA at 6000 rpm for 10 min at 0°. The cells were washed, repelleted, and frozen quickly in liquid nitrogen. After slow thawing the pellet was resuspended in 20 ml buffer containing 50 mM Tris-HCl pH 8.0, 15 mM EDTA at 4° and 100 μ g lysozyme was added to the suspension. To achieve complete lysis 10% SDS was added dropwise to a final concentration of 1.25% and the suspension held for 5 min at room temperature.

The majority of the host DNA could be removed by centrifugation of the lysate in a Spinco Type 30 rotor at 30,000 rpm for 30 min at 0°. The proteins were extracted by repeated treatment with phenol, the RNA was digested with heat treated RNase A (25 μ g/ml), and the RNase then removed by repeated phenol treatment. DNA was then precipitated with ethanol, overnight at -20°. The DNA pellet was resuspended in 6 ml of 10 mM Tris-HCl pH 7.4, 1 mM EDTA. 7 M CsCl was added to a final concentration of 1 M. The remaining high molecular weight host DNA was precipitated according to Hirt⁷ by adding 1 ml of 10% SDS (1%) for 1 hr at 0°. This host DNA was pelleted in Sorvall SS34 rotor 20 min at 15,000 rpm.

ϕ X174 RFI DNA was then purified to homogeneity by repeated buoyant density centrifugation in CsCl as described by Radloff *et al.*⁸. Propidium diiodide was removed from RFI DNA by running it through on a 3 x 1 cm Dowex 50 column. The purified RFI had a specific activity 5×10^6 cpm/ μ g DNA.

Isolation of Single Strand (ss) DNA. *E. coli* H514 was grown in 2 liters TPG medium to a titer of 5×10^8 cells/ml. Then the culture was infected with ϕ X174 am3 phage at multiplicity of 10 and aerated for 3 hr at 37°. The cells were collected by

centrifugation and washed with 50 mM sodium tetraborate, 5 mM EDTA pH 9.3. The pellet was resuspended in 9 ml of borate buffer and 1.0 ml of lysozyme (3 mg/ml) was added. The cells were lysed for 20 min at 37°. The lysate was treated with DNase (10 µg/ml) 20 min at 37°. The phage particules were purified on a glass bead column 1 x 75 cm (pore size 240°), followed by CsCl buoyant density centrifugation.

The ssDNA was isolated from the purified phage by the phenol procedure according to Guthrie and Sinsheimer⁹. The purity of the DNA was assayed on 1.2% agarose gels or agarose Me-HgOH gels described by Bailey and Davidson¹⁰. The purified DNA fraction contained 80% single strand circles, 15% single strand linear DNA, 5% host impurities. The purified DNA was stored at -20°.

In Vitro Synthesis of ØX174 RFI Labeled Only in the Negative Strand. The procedure is similar to that described by Dumas *et al.*¹¹ with minor modification. The reaction was carried out in 100 µl volume in 0.1 M Tris-HCl pH 7.4, 15 mM KCl, 9 hr at 12°. The reaction mixture contained: 1.0 µg ØX174 ssDNA, 0.5 µg degraded calf thymus DNA as primer (8-12 nucleotides in length), 1 unit of DNA polymerase I, 60 µM all four dXTP's, 5 mM MgCl₂, 0.3 mM NAD⁺, 10 µCi of all four α-³²P dXTP's and two units of *E. coli* ligase. After incubation the proteins were removed by phenol treatment and the DNA was purified on a Sephadex G-50 column followed by precipitation. The purity of the *in vitro* synthesized RFI DNA was assayed on agarose gels.

Cleavage of the RFI DNA's by Restriction Enzyme. 1 µg of RFI DNA was digested with five units of Hinc II or Hae III for 4 hr at 37° in 6.6 mM Tris-HCl pH 7.4, 6.6 mM MgCl₂, 6.6 mM NaCl and 6.6 mM ME. After complete digestion the samples were treated with chloroform-isoamylalcohol (24:1) to remove the enzyme proteins and the DNA was precipitated with ethanol.

Gel Electrophoresis of the ³²P-labeled Fragments. The separation of DNA fragments obtained by restriction cleavage was carried out on polyacrylamide gels in glycine-SDS-EDTA buffer as described by Studier⁵. 3-15% gradient gels were prepared by using a sucrose gradient mixer connected to two metal needles. The light solution was prepared from a 30% acrylamide stock (acrylamide: bis-acrylamide 30:1.5) the heavy solution from a 60% acrylamide stock (acrylamide: bis-acrylamide 60:0.8). If uniform gels were used for strand separation the 60% stock solution was taken.

Denaturation of DNA Fragments. The purified DNA fragments have been denatured in 50 µl volume:

- a) by heat; 2.5 min in boiling water in cracking buffer, described by Studier⁵,
- b) by treatment with 5 mM Me-HgOH 10 min at room temperature in borate buffer described by Bailey and Davidson¹¹,
- c) by treatment with alkali (0.2 M NaOH, 10 min at room temperature). After denaturation 10 µl of 0.2% brom-phenol-blue and 50% glycerol were added and the samples

were immediately applied to the slab gel. The electrophoresis was carried out at 150 Volts for 4-5 hr at room temperature. Kodak medical X-ray film RP-R54 was used for autoradiography.

Recovery and Purification of Separated DNA Bands and Separated Complementary Strands. Gel slices containing one DNA band were pressed through a plastic syringe into a plastic pipette with an agar plug at the bottom. The DNA fragments were recovered from the gel by electrophoresis into dialysis tubing and purified further by phenol extraction.

RESULTS

Purification of RFI DNA. The goal of these studies was the separation of complementary strands of DNA fragments generated by restriction enzyme cleavage. It was therefore necessary to insure the homogeneity of the ϕ X174 RFI DNA for enzyme cleavage. The ^{32}P -labeled RFI DNA was purified on CsCl-propidium diiodide density gradients as described in Materials and Methods. The two bands shown in Figure 1a correspond to RFI and RFII. Although these bands are very well resolved we found by electrophoresis on agarose gel that they are still cross-contaminated. After two further centrifugations RFI was obtained essentially free of any impurities as shown in Figure 1b. Figure 1c is the rerun of RFII showing the contamination by RFI.

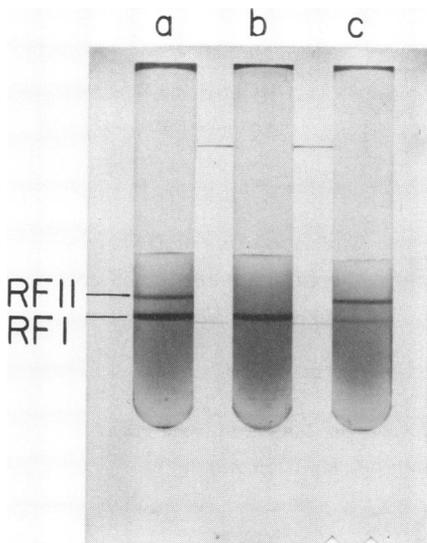


Figure 1: CsCl, propidium diiodide density gradient centrifugation of ϕ X174 RF DNA. a) RFI + RFII, b) RFI, c) RFII. Buffer: 10 mM Tris-HCl pH 7.4, 2 mM EDTA, 500 $\mu\text{g}/\text{ml}$ PDI. Centrifugation: SW41 35,000 rpm, 48 hr, 20 $^{\circ}$.

Cleavage of RFI DNA with Restriction Endonuclease Hinc II and Separation of the Fragments on Polyacrylamide Gels. In vivo ^{32}P -RFI DNA was cleaved by Hinc II

enzyme. The fragments were separated on polyacrylamide gel. Figure 2a shows the 10 separated bands (6 is a triplet, 7 is a doublet) on a 3-15% gradient gel. When the DNA fragments were heated to 100° prior to loading onto the gel, the pattern shown in Figure 2b was obtained.

Two features of these data are noteworthy:

- a) The undenatured fragments migrate faster than the denatured ones.
- b) After denaturation, two bands correspond to each DNA fragment.

Exactly the same results were obtained after denaturation of the fragments with Me-HgOH; the denaturation with alkali did not give clean separation of the doublet.

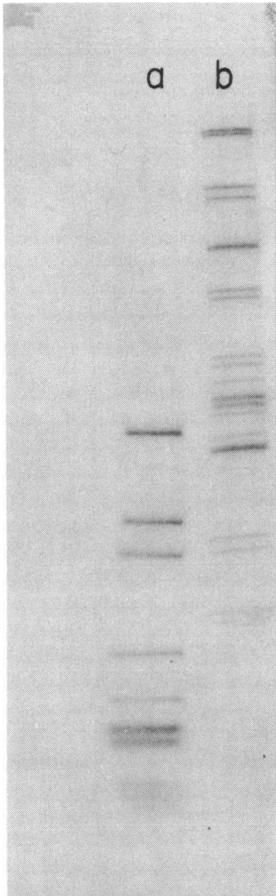


Figure 2: Polyacrylamide gradient gel electrophoresis of Hinc II fragments (ranging in size from 1000 to 80 base pairs)¹² from RFI DNA. a) Nondenatured, b) heat denatured 2.5 min at 100°. Gradient gel 3-15% acrylamide run at 150 volts for 5 hr at room temperature.

In Vitro Synthesis of ^{32}P -labeled RF DNA and Strand Separation of the Fragments Obtained by Cleavage with Hinc II. To synthesize ^{32}P -labeled RF DNA, cold ssDNA was used as a template for DNA-polymerase I. The reaction was primed with oligonucleotides of chain length of 8-12. These were obtained by DNase I digestion of calf thymus DNA and isolated on DEAE cellulose. The reaction was carried out in the presence of *E. coli* DNA ligase. The synthesized DNA was analyzed on a 1.2% agarose gel. Figure 3a shows the pattern of the in vitro synthesized DNA. This DNA contains a mixture of molecules differing in the number of superhelical turns¹³. A comparison with the in vivo labeled RF DNA Figure 3b demonstrates that the in vitro product is indeed full length, double-stranded DNA.

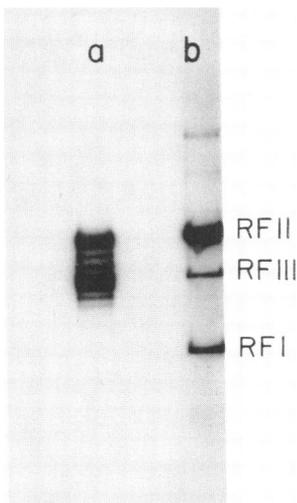


Figure 3: Agarose gel electrophoresis of a) in vitro synthesized RF DNA, b) in vivo labeled RFI, RFII and RFIII DNA as markers. 1.2% agarose gel run in Tris-acetate-buffer (40 mM Tris, 20 mM sodium acetate, 2 mM EDTA pH 8.3).

The in vitro synthesized RF was used as a substrate for restriction endonuclease Hinc II. The fragments separated on polyacrylamide gel gradients (Figure 4a,b) demonstrate the identical mobilities of DNA fragments obtained from in vivo and in vitro synthesized RF DNA. When the digested fragments were heat denatured prior to electrophoresis the pattern shown in Figure 4d is obtained. The most striking feature is the absence of the doublets as seen in the pattern of in vivo synthesized ^{32}P DNA shown in Figure 4c.

Since only the negative strand is labeled in vitro the positive strand would not be visible on the radioautograph. The band missing in the in vitro pattern but present in the in vivo is therefore the positive strand.

The difference in the mobilities of the complementary strands of various fragments is not always the same, e.g. in Figure 4c fragment 2 is well separated while the strands of fragment 3 overlap one another. These results suggest that these two fragments have a difference in the base composition. This is also evidenced by the mobility of the negative strand of fragment 5. In most of the fragments the negative strand migrates faster, but in fragment number 5 the mobility is reversed.

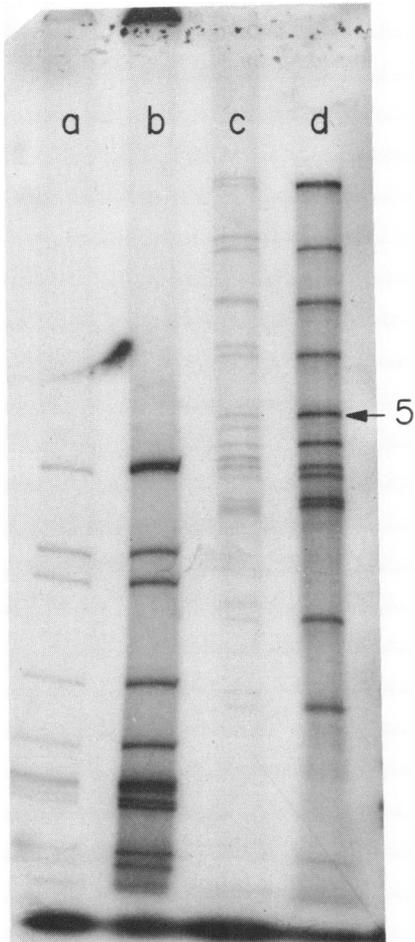


Figure 4: Radioautograph showing the separation of Hinc II cleavage products of ØX174 RF DNA synthesized *in vivo* and *in vitro*. Nondenatured: a) *in vivo*, b) *in vitro* non-denatured, c) *in vivo*, d) *in vitro* heat denatured 2.5 min at 100°. Gel gradient 3-15% acrylamide, run at 150 volts, 5 hr at room temperature.

Preparative Scale Separation of Complementary Strands of DNA Fragments.

For biochemical use of the separated strands it is necessary to isolate them in a large

scale. For this purpose we use 3 or 5% polyacrylamide gels containing acrylamide to bis-acrylamide in a ratio of 60:1. The 3% gels were suitable for fragments ranging in size from 600-2000 base pairs. Smaller fragments were better separated on the higher acrylamide concentration. Figure 5 shows the strand separation of a 50 μ g sample of in vivo RFI DNA after cleavage by Hinc II. The first four doublets were cut out and eluted by electrophoresis into dialysis bags. The positive and negative strands of the DNA fragments were digested with DNases to mononucleotides for base composition analysis. Preliminary results indicate that the single strands containing a higher content of T migrate slower than the corresponding complementary strand.

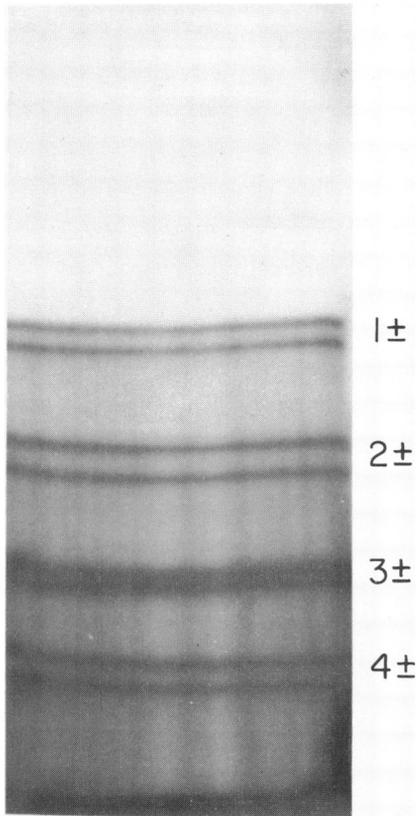


Figure 5: Strand separation on semipreparative scale: 50 μ g of RFI DNA was digested by 200 units of Hinc II for 4 hr at 37°. The digest was fractionated on 3% acrylamide gel containing acrylamide and bis-acrylamide in a ratio 60:1 run at 150 volts, 3.5 hr at room temperature.

DISCUSSION

The results presented here demonstrate that complementary strands of DNA can

be resolved on polyacrylamide gels. This separation does not require electrophoresis in denaturing conditions, it only requires denaturation of DNA prior to electrophoresis. Treatment of the DNA fragments with 5 mM Me-HgOH or heating in boiling water for 2-3 min gave very good separation of the complementary strands. The separation is independent of temperature (4°-22°) and pH (7.0-9.0).

The ratio of acrylamide to N,N'-bis-acrylamide with gel was found to play a very important role in the strand separation. Best results were achieved when the ratio was 60:1. By choosing the appropriate concentration of acrylamide it is possible to separate complementary strands of fragments containing 75-5500 base pairs. Full length double stranded ØX174 RFIII DNA (5500 b.p.) obtained by cleaving RFI with Providencia stuartii endonuclease could be resolved as two sharp bands on 2% polyacrylamide gel. For preparative isolation of strands containing 600-2000 nucleotide 3% acrylamide gels were most suitable.

For analytical purpose gradient gels have good resolution in the range of 75 to 2000 nucleotide long fragments.

The basis of strand separation is not at present clear but it appears that the base composition plays an important role. Our results show that the slow moving strand of the doublets is the positive (+) strand in most of the ØX DNA fragments. Preliminary experiments on base composition of separated strands suggest that the slow moving strands are rich in T (30-40%). Available nucleoside sequence analysis on both strands of fragment numbers 9 and 10 generated by Hinc II¹⁴ show that the T rich strands are the + strands which migrate on our gels slower than the in vitro labeled minus (-) strands.

In the fragment number 5 produced by Hinc II the negative strand migrates slower. This is not an artifact of Hinc II digestion. Fragment number 3 produced by Hae III from the same region of the ØX genome also shows the same results. Fragments of HeLa mitochondrial DNA and of SV40 DNA obtained by Hpa II and Hind III restriction endonucleases could also be resolved into complementary strands suggesting the general applicability of the method. A combination of this technique with the transfer-method developed by Southern¹⁵ could be useful for identification of the DNA strand with its transcription product.

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