Electrophoretic strand separation of long DNAs with poly(U,G) in agarose gels

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ABSTRACT

We have found that binding of poly(U,G) to single-stranded DNA decreases its mobility in 0.3% agarose gels. Differential binding to the complement- ary strands of denatured duplex DNA provides a simple method for strand separation. The method is shown to work with bacteriophage lambda DNA, adenovirus DNA and mtDNA for Tetrahymena pyriformis. In all cases the strand that binds more poly(U,G) in CsCl gradients also binds more in gels. The separated strands can be directly blotted from the gel onto nitrocellulose filters and used for hybridization experiments.

INTRODUCTION

Several methods exist for the separation of the complementary strands of DNA [1]. If strand-specific transcripts are available, these can be used to isolate the complementary strands. If the A/T or G/C ratios of the strands are sufficiently different, they may be separated in alkaline CsCl or Cs2SO4 equilibrium gradients. If pyrimidine clusters are asymmetrically distributed between both strands, the strands may be separated in neutral CsCl after complexation with polyribonucleotides. If the strands differ sufficiently in secondary structure under neutral conditions, they can be separated by electrophoresis in agarose or acrylamide gels [2,3]. Finally, the complementary strands of some DNA fragments can be separated by chromatography on RPC-5 columns at pH 12; the basis for strand separation in this procedure is not known [4].

In the course of experiments on the location of ribosomal RNA (rRNA) genes on the mtDNA of *Tetrahymena pyriformis*, we found that a convenient and reproducible strand separation can be obtained with this DNA by electrophoresis through agarose gels of the denatured DNA complexed with poly(U,G). In this paper we show...
that this method also works with bacteriophage lambda DNA and even with adenovirus DNA, for which strand separation by any other method is difficult. In conjunction with the Southern blotting technique [5], this procedure provides a rapid way of determining the orientation of transcripts on some DNAs.

METHODS AND MATERIALS

Preparation of E.coli phage lambda DNA

E.coli phage lambda DNA was prepared as described by Hershey et al. [6] and was kindly provided by Mrs. F. Fase-Fowler.

Preparation of T.pyriformis mtDNA

T.pyriformis strain ST was originally obtained from Dr. Y. Suyama, Philadelphia, Pa., USA. mtDNA was isolated from mitochondrial preparations as described before [7].

Partial fragmentation of poly(U,G) in alkali

Poly(U,G) (U to G ratio 1.0 : 1.3, purchased from Miles Laboratories, code no. 14-381, lot no. 7A) was fragmented in freshly made 0.05 M Na₂CO₃ at 80° C for 6 min (unless stated otherwise). The solution was neutralized by addition of 1 volume 80 mM Tris-HCl (pH 7.6) and 1 volume 0.05 M HCl.

Binding of poly(U,G) to denatured DNAs

DNA (in 20 mM Tris-HCl, 1 mM EDTA (pH 7.6)) was mixed with partially fragmented poly(U,G) heated 3 min at 95° C and directly chilled in ice. The poly(U,G)/DNA ratios (in μg) used are specified in the legends to the figures.

Strand separation of E.coli phage lambda DNA in a CsCl density gradient

40 μg phage lambda DNA was treated with 80 μg poly(U,G) as described above and mixed with a CsCl solution to give a final volume of 4.3 ml with a density of 1.742 g/ml. The solution was centrifuged for 50 h at 25° C in a Spinco SW-50 rotor at 35 000 rpm. The gradient was collected from the bottom and, after measurement of the refractive index, fractions were diluted 5 times in 20 mM Tris-HCl, 1 mM EDTA (pH 7.6) and absorbance at 260
nm was measured in 0.5-ml quartz cuvettes. r-strand and l-strand-containing fractions were separately pooled and dialysed against 20 mM Tris-HCl, 1 mM EDTA (pH 7.6).

Strand separation of DNAs by means of agarose gel electrophoresis

Poly(U,G)-treated DNA was mixed with 1/4 vol of a 20% ficoll solution and loaded into 5 x 2 mm slots (3 mm deep) in 12 x 12 x 0.4 cm horizontal 0.3% agarose slab gels. Gels were run at 4°C for 16 h at 40 mA using 40 mM Tris-acetate, 1 mM sodium-EDTA (pH 7.7) containing 0.5 µg ethidium bromide per ml [8,9]. Gels were photographed as described [9].

Preparation of strand-specific end-labelled fragments of adenovirus type 5 DNA

End-labelled adenovirus 5 DNA was prepared by repairing E.coli exonuclease III-treated DNA with E.coli DNA polymerase using [α-³²P]dTMP as radioactive precursor [10]. Separation and isolation of the terminal fragments with restriction endonuclease HpaI was done as described in ref. 10.

DNA-RNA and DNA-DNA hybridizations

Southern nitrocellulose strip filters (Sartorius, 0.1 µm pore size) were prepared by the standard procedure [5] except that complete gels were used for transfer from gel to strip. Hybridizations were carried out in 0.3 M NaCl, 0.03 M Na-citrate, 0.1% sodium dodecylsulphate (pH 7.0) at 58°C as described elsewhere [11].

Preparation of T.pyriformis mitochondrial rRNAs

Mitochondrial rRNAs were prepared and purified as described in ref. 11. These RNA preparations were partially degraded with alkali and labelled in vitro using [γ-³²P]ATP and T4 polynucleotide kinase (Boehringer) as described in ref. 11.

Preparation of complementary RNA

Complementary RNA (cRNA) was transcribed from DNA using [α-³²P]ATP (The Radiochemical Centre, Amersham, UK; 250 Ci/m mole) as radioactive substrate [12].
RESULTS

Strand separation of viral DNAs

The effect of poly(U,G) on the electrophoretic mobility of phage lambda DNA is shown in Fig. 1. In the absence of poly(U,G) only one denatured DNA band is found without indication of strand separation (lane b). After complexing to poly(U,G) this band is replaced by two new bands of equal intensity and reduced mobility (lane c). To verify that these two bands represent the complementary strands, they were transferred to nitrocellulose filters by the Southern technique and hybridized to strand-specific cRNA probes. These probes were obtained by transcribing the complementary strands of phage lambda DNA separated with poly(U,G) in CsCl (Fig. 2). The hybridization experiments presented in Fig. 3, show that the transcript of the r-strand (the strand which binds more poly(U,G) in CsCl) hybridizes to the slower-moving band in agarose, the l-strand transcript hybridizes to the other band. This indicates that the binding of poly(U,G) decreases the electrophoretic mobility of DNA in the gel and that the r-strand binds more poly(U,G) than the l-strand in the gel, like in the gradient.

Good strand separation with our procedure was also obtained

Fig. 1. Electrophoresis of phage lambda DNA in a 0.3% agarose gel. A, Undenatured DNA; B, denatured DNA; C, DNA denatured in the presence of poly(U,G). Input DNA in all slots, 1 pg. Poly(U,G)/DNA ratio in lane C is 2. Poly(U,G) was fragmented for 6 min as described in Methods.
Fig. 2. CsCl density gradient with poly(U,G)-treated phage lambda DNA. The DNA (40 μg) was denatured in the presence of 80 μg poly(U,G). The gradient was prepared, run and fractionated as described in Methods. A_{260} = absorbance at 260 nm; ρ = density (g/ml). Both r-strand and l-strand were isolated and used for preparation of strand-specific 32P-labelled cRNAs.

Fig. 3. Characterization of electrophoresed phage lambda DNA denatured in the presence of poly(U,G). The figure shows the original 0.3% agarose gel aligned with autoradiograms of Southern strips after hybridization with: A, 32P-labelled cRNA (specific activity 4 x 10^6 cpm/μg) from unfractionated DNA; B, 32P-labelled cRNA (specific activity 1.8 x 10^6 cpm/μg) from the r-strand; C, 32P-labelled cRNA (specific activity 2.2 x 10^6 cpm/μg) from the l-strand. Inputs of DNA, 1 μg per slot. 6-min degraded poly(U,G) was used at a poly(U,G)/DNA ratio of 2. The input of labelled cRNA was 100,000 cpm per filter.

with adenovirus type 5 DNA, as illustrated in Fig. 4. In this case the two bands observed in the gel with poly(U,G) were identified as the complementary strands by hybridization with the separated terminal HpaI fragments (fragments D and E; ref. 13) of
Fig. 4. Electrophoresis of adenovirus 5 DNA denatured in the presence of poly(U,G). The figure shows the original 0.3% agarose gel aligned with autoradiograms of Southern strips after hybridization with the terminal endonuclease HpaI fragments of 32P-end-labelled adenovirus 5 DNA. Input of DNA, 1 ug per slot. 6-min degraded poly(U,G) at a poly(U,G)/DNA ratio of 2 was used. A, Hybridization of native DNA with HpaI fragment E; B, hybridization of poly(U,G)-treated DNA with HpaI fragment E; C, hybridization of poly(U,G)-treated DNA with HpaI fragment D. The input of 32P-labelled fragment E (specific activity 25 x 10^6 cpm/ug) and fragment D (specific activity 10 x 10^6 cpm/ug) was 100 000 cpm per strip.

32P-end-labelled adenovirus DNA. The labelled strands of fragments D and E represent the strands which bind more (H-strand) and less (L-strand) poly(U,G) in CsCl, respectively [14]. Therefore, the strand that binds more poly(U,G) in CsCl again moves slower in the gel.

An attempt was also made to separate the complementary strands of the plasmid P8G1 linearized by treatment with restriction endonuclease HindIII. This is a 5.6 kb plasmid, constructed by Maniatis et al. [15], which contains a double-stranded DNA copy of rabbit B-globin mRNA. Fig. 5 shows a gel containing this DNA, denatured in the presence of various preparations of poly(U,G). A clear separation of the DNA in two bands of equal intensity is observed in slots H and I. Attempts to improve separation were unsuccessful and no experiments were done to verify whether the two bands are the complementary strands of P8G1 DNA.

Strand separation of Tetrahymena mtDNA

This is illustrated in Fig. 6. We have previously shown [11] that this DNA contains two genes for 21S rRNA (large ribosomal subunit) in inverted position and only one gene for the 14S rRNA (small ribosomal subunit). These three genes are more than 8000 base pairs apart on the DNA. Pure 21S rRNA should hybridize to
Fig. 5. Electrophoresis of HindIII-cleaved plasmid P811 DNA in 0.3% agarose. Poly(U,G)/DNA ratio used was 4. Input per slot, 1 μg DNA. A, Denatured DNA; B-D, DNA denatured in the presence of poly(U,G) pre-incubated in 0.05 M Na₂CO₃ at 80°C for 6 min; E-G, as B-D, but poly(U,G) pre-incubated for 12 min; H and I, as B-D, but poly(U,G) pre-incubated for 24 min.

Fig. 6. Characterization of poly(U,G)-treated T. pyriformis mtDNA in a 0.3% agarose gel. The figure shows the original agarose gel aligned with autoradiograms of Southern strips after hybridization to 32P-labelled mitochondrial rRNA. Input of DNA, 1 μg per slot. Poly(U,G)/DNA ratio 2; 6-min fragmented poly(U,G). A, Hybridization with 14S rRNA (specific activity 35 x 10⁶ cpm/μg; input 100 000 cpm); B, hybridization with 21S rRNA (specific activity 30 x 10⁶ cpm/μg; input 100 000 cpm).
both complementary strands, pure 14S rRNA only to one. Fig. 6 shows that *Tetrahymena* mtDNA separates into two bands in the presence of poly(U,G). The labelled 21S rRNA hybridizes equally to both bands; the 14S rRNA only to the slower-moving band. The hybridization of both bands with 21S rRNA is in full agreement with our previous finding that the 21S rRNA cistron lies within the duplication-inversion of this mtDNA.

In a previous paper from this laboratory Schutgens *et al.* [16] reported that total *Tetrahymena* mitochondrial rRNA hybridized with only one of the complementary strands of *Tetrahymena* mtDNA. This is incompatible with the known arrangement of rRNA genes on this DNA and the results in Fig. 6. We attribute the results of Schutgens *et al.* to extensive self-annealing of the DNA, which may have eliminated hybridization of the 21S rRNA genes located in the inverted repetition. This would leave only the 14S rRNA gene to hybridize (and genes for mRNAs possibly present as contaminants in the rRNA probe). In fact, only hybridization was found with the strand which bound more poly(U,G) in CsCl (the H-strand). This agrees with the hybridization of 14S rRNA with the slower-moving strand in the gel of Fig. 6.

**Optimal conditions for strand separation**

Optimal separation of DNA strands in CsCl gradients containing poly(U,G) is dependent on the size of the polyribonucleotide and the poly(U,G)/DNA ratio. The effect of these parameters on strand separation in gels is illustrated with phage lambda DNA in Fig. 7. Undegraded poly(U,G) gave poor separation and usually only a vague smear without clear bands, but good strand separation was obtained with poly(U,G) partially degraded with alkali. For optimal strand separation higher poly(U,G)/DNA ratios are required than are used in our standard procedure. Too much poly(U,G) leads to renaturation of the DNA (cf. Fig. 7 f and n), however, as we have observed in several experiments.

The electrophoretic strand separation can be easily scaled up and an example of the separation of the complementary strands of 80 µg phage lambda DNA is given in Fig. 8. By using larger and thicker gels it should be possible to separate the strands of mg amounts of DNA.
Fig. 7. Effect of size and poly(U,G)/DNA ratio on the separation of phage lambda DNA strands in 0.3% agarose. Input of DNA, 1 µg per slot. A-E, DNA complexed with poly(U,G) pre-incubated in 0.05 M Na2CO3 at 80°C for 0, 1, 6, 12, and 24 min, respectively. The poly(U,G)/DNA ratio was 4 in all cases. F, Undenatured DNA (0.1 µg); G-N, DNA denatured in the presence of poly(U,G) at a poly(U,G)/DNA ratio of 0 (no poly(U,G) added), 0.25, 1, 2, 4, 8, 16 and 32, respectively.

Fig. 8. Large-scale separation of phage lambda DNA strands. 80 µg DNA was denatured in the presence of 160 µg poly(U,G) and loaded into a 100 mm long x 2 mm broad slot (3 mm deep) in a 0.3% agarose gel and electrophoresed as described in Methods.

DISCUSSION

Our results show that differential binding of poly(U,G) can be used to separate the complementary strands of some DNAs in agarose.
rose gels. The mechanism of strand separation can be inferred from the extensive work on binding of ribopolymers to denatured DNA in CsCl or Cs₂SO₄ gradients [1,17,18]. G-rich polymers (or poly(I)) bind to C-rich clusters in the DNA; U-rich polymers bind to A-rich clusters. Binding apparently occurs via standard Watson-Crick base-pairing. The ribopolymer-DNA complex has a higher equilibrium density in CsCl than free DNA and separation only occurs if one strand contains more ribopolymer-binding clusters than the other. In the high salt concentration used for CsCl gradients additional polymer aggregates onto the free polymer 'tails' of DNA-bound polymer, increasing strand separation [1,19].

When DNA is complexed with ribopolymers, its mass (and hydrodynamic volume) increases and its electrophoretic mobility in agarose should, therefore, decrease. This is what we found. The DNA strand which binds more poly(U,G) in CsCl (the H-strand), is slowed down more by poly(U,G) in agarose, as we have found with phage lambda and adenovirus DNA. We, therefore, expect that any DNA that shows strand separation with any ribopolymer in CsCl gradients, will also show strand separation with the same polymer in agarose gels, if the proper conditions are used.

In our experiments the mixture of DNA and poly(U,G) is kept at low ionic strength and this should minimize aggregation. Nevertheless, the results in Fig. 7 suggest that poly(U,G)-poly(U,G) aggregation occurs at high poly(U,G) concentrations and that this leads to a further decrease in mobility of the DNA-poly(U,G) complex. It is possible that further improvements in separation could be obtained by altering the ionic conditions or by adding Mg²⁺ ions. It should also be possible to separate strands in gels with polymers that cannot be used in CsCl gradients, like poly(C) which does not self-aggregate [1,18] or poly(A), which is insoluble at high Cs⁺ concentrations (see [20,21]). Poly(C), for instance, should preferentially bind to the 1-strand of phage lambda DNA, making it the slower-moving band in agarose and reversing the mobility found with poly(U,G). This would be useful, because the faster-moving strand is usually contaminated with some fragments of the slower-moving strand.

The separation of ribopolymer-bound DNA strands in agarose gels has obvious advantages over other methods of strand separat-
Electrophoretic strand separation in the absence of ribopolymers is often limited or difficult to achieve (cf. Figs 1 and 7, but contrast [2]). Strand separation with ribopolymers in CsCl gradients is time-consuming, expensive and the separations obtained are often strongly dependent on the size and composition of the polymer and the exact experimental conditions [19]. This may explain why we have been unable to reproduce the gradient separation of the complementary strands of Tetrahymena mtDNA (strain ST) reported by Schutgens et al. [16], even though this experiment was done five years ago in the same lab with the same batch of poly(U,G). In contrast, our gel procedure for strand separation has been highly reproducible.

A disadvantage of our procedure is that strand separation of multiple-nicked DNA is impossible, but this gives problems with any other procedure as well. Another potential disadvantage is that separation could be poor for very large DNAs (>100 000 bp) but this remains to be tested. With small DNAs (<2000 bp), however, separation in gradients is inherently difficult because of peak broadening. Resolution in the gel procedure should be good in this size range and, in fact, the gel procedure may also find useful application in the separation of the complementary strands of larger restriction fragments.

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