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HIGH-RESOLUTION CHROMATOGRAPHY OF NUCLEIC ACIDS ON THE GEN-PAK FAX COLUMN

DEBORAH J. STOWERS* and JENNY M. B. KEIM
Nucleic Acid Facility, Iowa State University, Ames, IA 50011-3223 (U.S.A.)
PREM S. PAUL and YOUNG S. LYOO
Veterinary Medical Research Institute, Iowa State University, Ames, IA 50011 (U.S.A.)
MICHAEL MERION
Waters Chromatography Division, Millipore Corporation, Milford, MA 01757 (U.S.A.)
and
ROBERT M. BENBOW
Nucleic Acid Facility and Department of Zoology, Iowa State University, Ames, IA 50011-3223 (U.S.A.)

SUMMARY

High-performance liquid chromatography (HPLC) on a Gen-Pak FAX column has been used to separate and purify microgram amounts of single- and double-stranded DNA and RNA molecules. HPLC of mixtures of DNA restriction fragments showed that fragments within the size range 0.125–23.1 kilobase were easily resolved. Supercoiled (form I) plasmid DNA molecules were readily separated from single-stranded circular DNA of the same length and from various DNA conformational isomers including nicked (form II) and linear (form III) species. Topological isomers generated from supercoiled plasmid DNA molecules by DNA topoisomerase I exhibited different retention times than supercoiled molecules. Supercoiled (form I) DNA molecules were resolved from fully relaxed (form IV) molecules. Synthetic oligonucleotides of 74 and 128 nucleotides in length were separated from failure sequences, as well as from other contaminating synthesis products. Single-stranded circular M13mp18 DNA molecules sufficiently pure for use in automated DNA sequencing systems were prepared by HPLC on a Gen-Pak FAX column. HPLC was also used to fractionate linear double-stranded porcine rotavirus genomic RNA fragments into size classes between 0.3 and 3 kilobase. Finally, HPLC of unfractionated *Escherichia coli* tRNA molecules resolved multiple species. In all cases, HPLC on Gen-Pak FAX was carried out in phosphate or Tris buffers at neutral pH in the presence of sodium chloride. Columns were not damaged by repeated exposure to impure samples, provided they were cleaned frequently with sodium hydroxide and acetic acid. Although procedures for resolution of the various size ranges for each class of DNA and RNA molecules require further optimization, our preliminary data on the separations obtained, the moderate salt concentrations employed, and the durability of the matrix suggest that this column merits further study.

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INTRODUCTION

The use of high-performance liquid chromatography (HPLC) in nucleic acid research has recently been reviewed in exhaustive detail[1–5]. Although the advantages of HPLC are manifest and some significant advances have been made[6–18], no column has yet emerged that is satisfactory for most types and sizes of DNA and RNA molecules[14]. In particular, no columns that are both durable and suitable for relatively large nucleic acids have been perfected. Very recently, Merion[19] has reported that HPLC on Gen-Pak FAX resolved all eight DNA restriction fragments from a Hind III digest of bacteriophage lambda DNA. In addition, Merion[19] reported complete resolution of all 22 fragments of the BRL 1-kilobase (kb) ladder.

In this paper we have examined the ability of this new type of column, the Gen-Pak FAX, to separate and purify a variety of nucleic acids. Our preliminary experiments include: (1) separation of double-stranded DNA restriction fragments; (2) separation of supercoiled (form I) double-stranded plasmid DNA molecules from other single- and double-stranded forms; (3) temporal resolution of topological isomers of covalently closed circular DNA molecules; (4) purification of synthetic single-stranded oligonucleotides; (5) purification of single-stranded DNA molecules for use in automated DNA sequencing systems; (6) separation of linear double-stranded genomic RNA fragments, such as are found in rotaviruses, into discrete size classes; (7) separation of multiple tRNA species; and (8) separation of complex mixtures of nucleic acids.

EXPERIMENTAL

Chemicals

Tris(hydroxymethyl)aminomethane (Tris), acrylamide, bisacrylamide, and Bromophenol Blue were purchased from Sigma, St. Louis, MO, U.S.A. Disodium ethylenediaminetetraacetate (EDTA), sodium phosphate monobasic, sodium chloride, sodium hydroxide, glycerol, acetic acid (glacial), phosphoric acid and hydrochloric acid were obtained from Fisher Scientific, Itasca, IL, U.S.A. Electrophoresis-grade agarose, urea, ammonium persulfate, N,N,N′,N′-tetramethylethylenediamine (TEMED), and N,N′-methylenebisacrylamide were obtained from Bethesda Research Labs., Gaithersburg, MD, U.S.A. Electrophoresis-grade acrylamide was purchased from Bio-Rad, Richmond, CA, U.S.A. [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from DuPont New England Nuclear, Boston, MA, U.S.A.

Buffer A contained 20 m*M* sodium phosphate (pH 6.5). Buffer B was buffer A, containing 1 *M* sodium chloride. Buffer C contained 25 m*M* sodium phosphate (pH 7.0). Buffer D was buffer C, containing 1 *M* sodium chloride. Phosphate buffers were adjusted to the specified pH by adding 10 *M* sodium hydroxide not by titrating with dibasic sodium phosphate (which may be contaminated with polyphosphates). Buffer E was 25 m*M* Tris–HCl (pH 7.5). Buffer F was buffer E containing 1 *M* sodium chloride. All buffers were made with high-purity water (Type 1A water, Culligan, Boone, IA, U.S.A.) and filtered through a GF/F filter (Whatman, Maidstone, U.K.) before use.

Cleaning solutions for the column were 0.2 *M* acetic acid, 0.2 *M* sodium hydroxide, and HPLC-grade water.

Nucleic acids and enzymes

Plasmid DNAs, pXG$\alpha\beta$1 (obtained from R. Patient, Kings College London, London, U.K.), p1934 (pACYC184 with a NARC gene inserted obtained from Moon Youyoun, Iowa State University), MG 001 (obtained from D. Grant, Pioneer Hi-Bred International, Johnston, IA, U.S.A.), SBPSB105 (obtained from H. Zhou, Iowa State University) and pUC19 (purchased from New England Biolabs, Beverly, MA, U.S.A.), were purified according to the procedure of Bayne and Dumas[20]. M13mp18 single-stranded circular DNA was obtained from Pharmacia, Piscataway, NJ, U.S.A. and purified as described by Pharmacia[21]. The 1-kb DNA ladder was purchased from Bethesda Research Laboratories. *Escherichia coli* DNA was purchased from Sigma.

Oligonucleotides were synthesized on a Biosearch 8750 DNA synthesizer with the final DMT (dimethoxytrityl) group attached. After synthesis, the oligonucleotides were cleaved from the controlled pore glass columns and deprotected by incubation at 55°C in fresh ammonium hydroxide for 5 h, then dried in a Savant (Farmingdale, NY, U.S.A.) vacuum centrifuge. The pellet was resuspended in sterile 10 m*M* Tris–HCl (pH 8.0), 1 m*M* EDTA.

Double-stranded genomic RNA molecules from porcine rotavirus, Strain IA86, Serotype 5, according to the classification reported by Hoshino *et al.*[22], were isolated at Iowa State University from pigs with diarrhea. Virions were extracted from infected cells[23] and partially purified by freon extraction[24]. Viral RNA was extracted with phenol–chloroform and dialyzed against 50 m*M* Tris–HCl (pH 8.0), 10 m*M* EDTA. The molecular weights of the 11 genomic double-stranded RNA segments were estimated using lambda DNA cut with Hind III and Eco R1 restriction endonucleases as a standard; they were 3800, 2200, 2200, 1870, 1425, 1300, 1035, 1025, 1020, 770 and 580 base pairs.

E. coli tRNA was obtained from J. Horowitz, Iowa State University. *Xenopus laevis* DNA topoisomerase I was provided by H. Kaiserman, also of Iowa State University. Eco R1 and Sal I restriction endonucleases were purchased from New England Biolabs. Kpn I was purchased from BRL. T4 polynucleotide DNA kinase was purchased from Amersham, Arlington Heights, IL, U.S.A.

Sample preparation for HPLC

Before samples were injected into the column, they were centrifuged at 12000 *g* for 2 min. If the sample was too small for convenient injection, it was diluted with sterile 10 m*M* Tris–HCl (pH 8.0)–1 m*M* EDTA. Samples were collected in sterilized plastic microcentrifuge tubes.

HPLC equipment

Data were collected using two HPLC systems controlled by a Digital Professional 380 computer with 1024 RAM, 10 megabyte hard disk, 400K floppy disk, PDP 11/70 processor, and color monitor. The first system included a 710 WISP with cooler, a 490 programmable multiwavelength detector and 600 multisolvent delivery system equipped with a WAT-062079 column heater. The second system included two 510 HPLC pumps and a Lambda-Max (Waters) Model 481 LC spectrophotometer. All HPLC data were plotted as mV *versus* retention time (min). 1000 mV equals approximately 1 A_{260}.

The parameters of the gradient curves used were defined by Waters Expert software, version 5.1. Curves 2–5 are convex gradients (2 is the most convex gradient available in the Waters software); curve 6 is a linear gradient.

Gen-Pak FAX column

Prototype Gen-Pak FAX columns were initially supplied by Waters to the Iowa State University Nucleic Acid Facility for testing. The column is now available commercially. The Gen-Pak FAX column is a DEAE anion-exchange column with a matrix consisting of methacrylate polymer with a 2.5 μm particle size[19]. The data presented here were generated with a 10 cm × 4.6 mm column (Waters 15490).

The Gen-Pak FAX column must have sufficient time to re-equilibrate to starting conditions in between runs (at least 30 min at 0.5 ml/min flow-rate). Also, this re-equilibration time must be kept constant. If the interval between runs is more or less that 5% of the total re-equilibration time, memory peaks will appear in the chromatograph. In addition, the column must be cleaned daily according to the manufacturer's instructions.

Gel analysis

Aliquots of DNA from the HPLC fractions were electrophoresed on 0.7 or 1% agarose gels using TPE buffer (0.08 *M* Tris–phosphate, pH 8.0, 0.002 *M* EDTA)[25]. The loading buffer contained 100 m*M* EDTA, 50% (v/v) glycerol, and 0.25% Bromophenol Blue[25]. Electrophoresis was performed at 75–95 mA for the times specified. The gels were stained with ethidium bromide in TPE buffer and photographed with Polaroid Type 55 P/N film using a Polaroid MP4 camera. The UV light source, equipped with a Wratten gelatin filter, No. 23A, was a Fotodyne Fotosystem 1000.

Synthetic oligonucleotides were radioactively end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase[26], then electrophoresed on a 10% denaturing polyacrylamide gel[25] for 4 h at 1800 V, 35 W, and 20 mA. Kodak XAR film was exposed for 15 h on the completed gel, then developed by standard procedures.

Rotavirus RNA was analyzed by polyacrylamide gel electrophoresis (PAGE) using Laemmli gels[27] with 3.5% stacking and 7.5% resolving gels. The gels were run at 14 mA for 14 h and stained with silver stain as described by Herring *et al.*[28].

Recovery of nucleic acids from the column

Based on A_{260} recovered from pooled fractions compared with A_{260} injected on the column, recoveries were greater than 80–90% and approached 100% when the column was new.[19] The loss of A_{260} absorbing material appeared to be the result of binding to inlet column frits rather than binding to the column matrix[19].

RESULTS

Isolation and purification of DNA restriction fragments

Potentially one of the most important applications of HPLC technology to nucleic acid research is in the rapid isolation and purification of cloned DNA inserts[29–36]. Several HPLC procedures for separation of double-stranded restriction fragments have been described previously[4,11,14] but resolution of fragments in the

size range from 0.5 to 20 kb (the most common sizes of cloned DNA inserts) was relatively limited. To test the Gen-Pak FAX column for its ability to resolve restriction fragments in this size range, various plasmid DNA molecules were digested with appropriate restriction enzymes, and the resulting digests subjected to HPLC. An A_{260} profile demonstrating base-line separation of a 3.7-kb from a 14-kb restriction fragment (10.3 kb difference) is shown in Fig. 1a. Gel electrophoretic analysis of aliquots of fractions collected from the separation depicted in Fig. 1a was carried

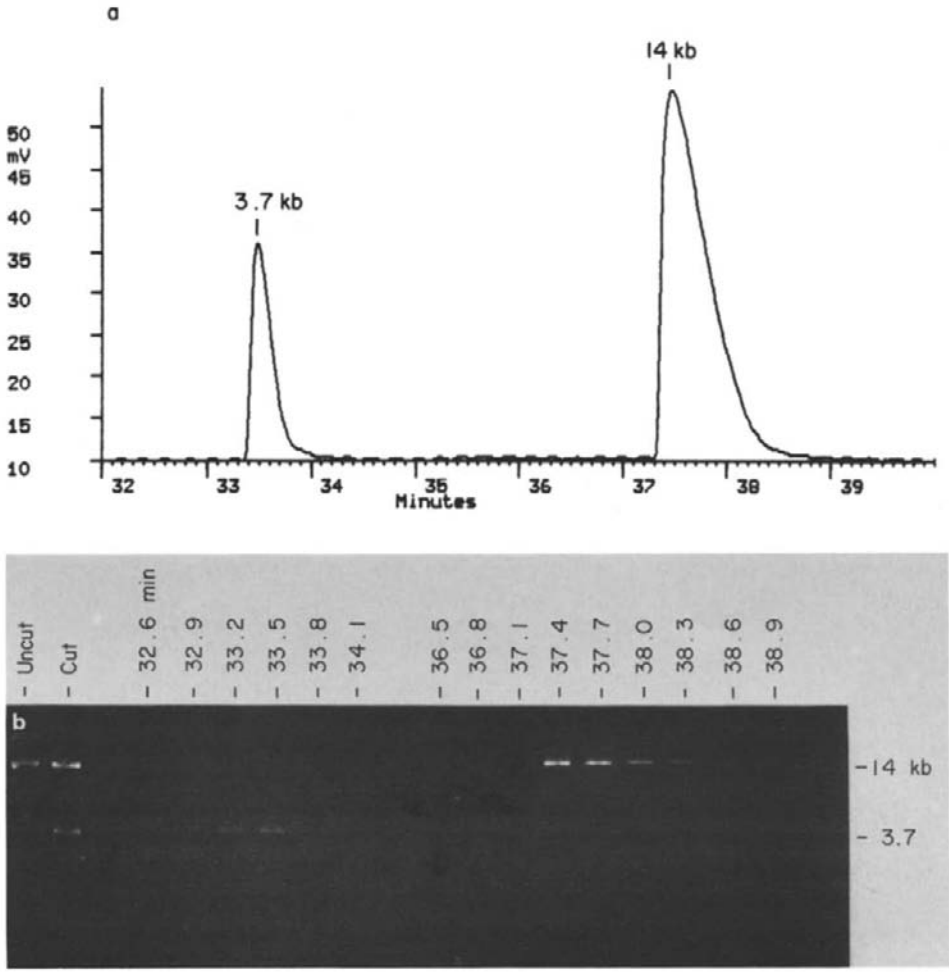


Fig. 1. HPLC separation of two DNA restriction fragments: 3.7 and 14 kb. (a) A 1.5-μg DNA sample consisting of a mixture of 3.7-kb and 14-kb DNA restriction fragments, generated by digestion of pXG$\alpha\beta$1 DNA with Eco R1 endonuclease was injected onto the Gen-Pak FAX column. Flow-rate, 0.5 ml/min; pressure, 1608 p.s.i.; temperature, 22°C. Eluent A was buffer C and eluent B was buffer D. A 20-min linear gradient (curve 6) from 100% A to A–B (20:80) was run. Fractions were collected every 0.3 min. The retention times of the 3.7-kb and 14-kb fragments were 33.5 and 37.5 min, respectively. (b) Aliquots (30 μl) of each fraction in the peak area were subjected to electrophoresis on a 1% agarose gel. The gel was stained with ethidium bromide and photographed. The first two lanes contain uncut pXG$\alpha\beta$1 DNA and pXG$\alpha\beta$1 DNA digested with Eco R1, respectively, the other lanes are labeled with their elution times.

out. As shown in Fig. 1b, the two restriction fragments were cleanly separated from each other and no cross contamination was detected. Similar baseline resolution of 2.9- and 6.1-kb fragments (3.2 kb difference) is shown in Fig. 2a. No cross contam-

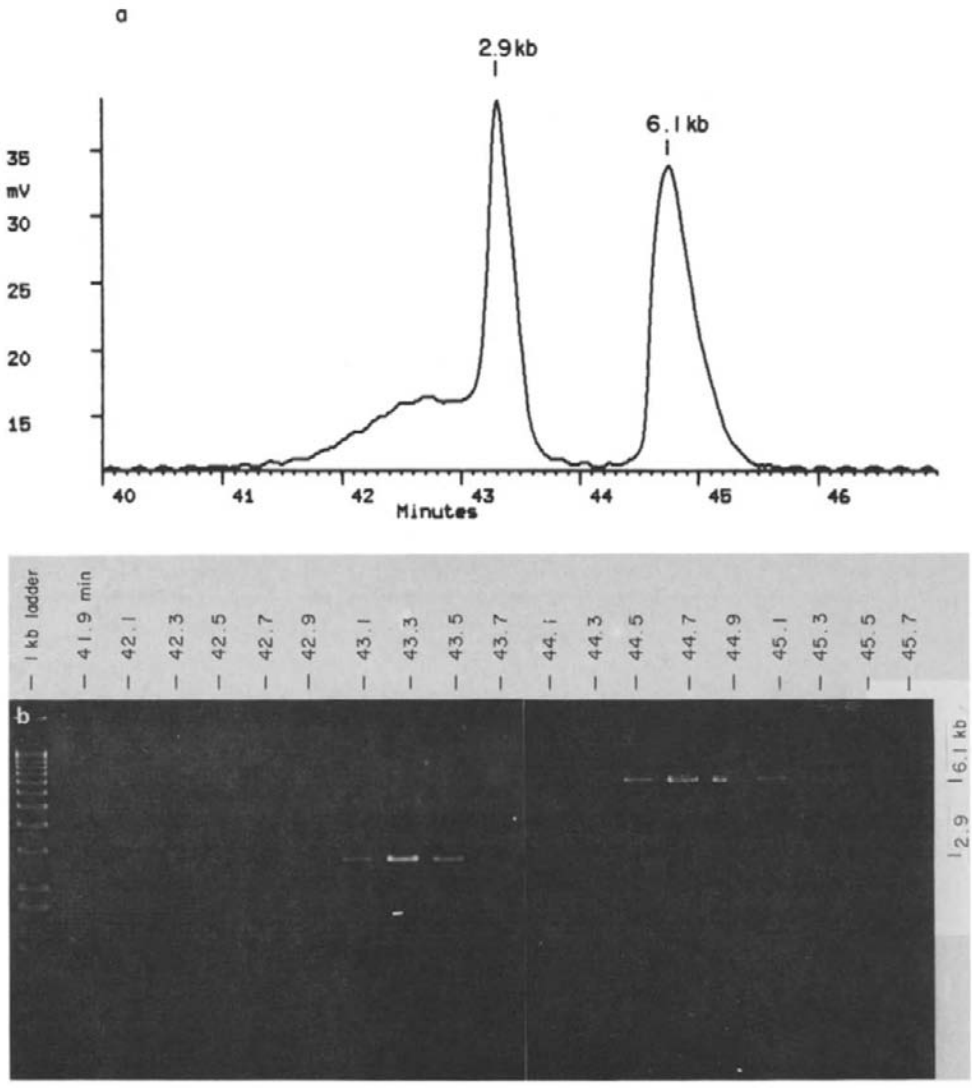


Fig. 2. Separation of 2.9- and 6.1.-kb DNA restriction fragments. (a) Approximately 0.5 μg of a mixture of 2.9-kb and 6.1-kb DNA restriction fragments, generated by digestion of p1934 DNA with Sal I restriction endonuclease, were subjected to HPLC. The sample was injected onto a 10 cm × 4.5 mm Gen-Pak FAX column. Eluent A was buffer C and eluent B was buffer D. A 20-min linear gradient from A–B (55:45) to A–B (30:70) was run. Flow-rate, 0.5 ml/min; pressure, 2280 p.s.i.; temperature, 22°C. Fractions were collected every 0.2 min. The retention times of the 2.9- and 6.1-kb fragments were 43.5 and 45.0 min, respectively. The A_{260} absorbing material at 41–43 min is of uncertain origin. (b) A 30-μl aliquot of each 0.2-min fraction in the peak area was analyzed by electrophoresis on a 1% agarose gel. The gel was stained with ethidium bromide and photographed. The first lane contains a double-stranded 1-kb ladder as a size marker; the other lanes are labeled with their elution times.

ination of these fragments was detected by gel analysis as shown in Fig. 2b. Baseline resolution of relatively small restriction fragments of 0.66 and 2.2 kb (1.54 kb difference) is shown in Fig. 3a. These data suggest that HPLC on Gen-Pak FAX satisfactorily resolves restriction fragments in the size ranges in which most cloned inserts are found.

To evaluate the effective capacity of the column, we examined the effect of varying the amount of DNA injected on the resolution of a 1.6-kb from a 2.6-kb restriction fragment. As shown by comparing Fig. 3b and c, significant broadening of the peaks and loss of resolution were observed when 16 μg of DNA was injected (Fig. 3c). Additional analyses suggest that baseline resolution of restriction fragments throughout the size range from 0.1 kb to at least 50 kb can be obtained with slight modifications in the gradient conditions used[19]. Moreover using the 10 cm × 4.6 mm column, amounts up to 20 μg of DNA can be fractionated in a single run.

Purification of supercoiled plasmid DNA molecules

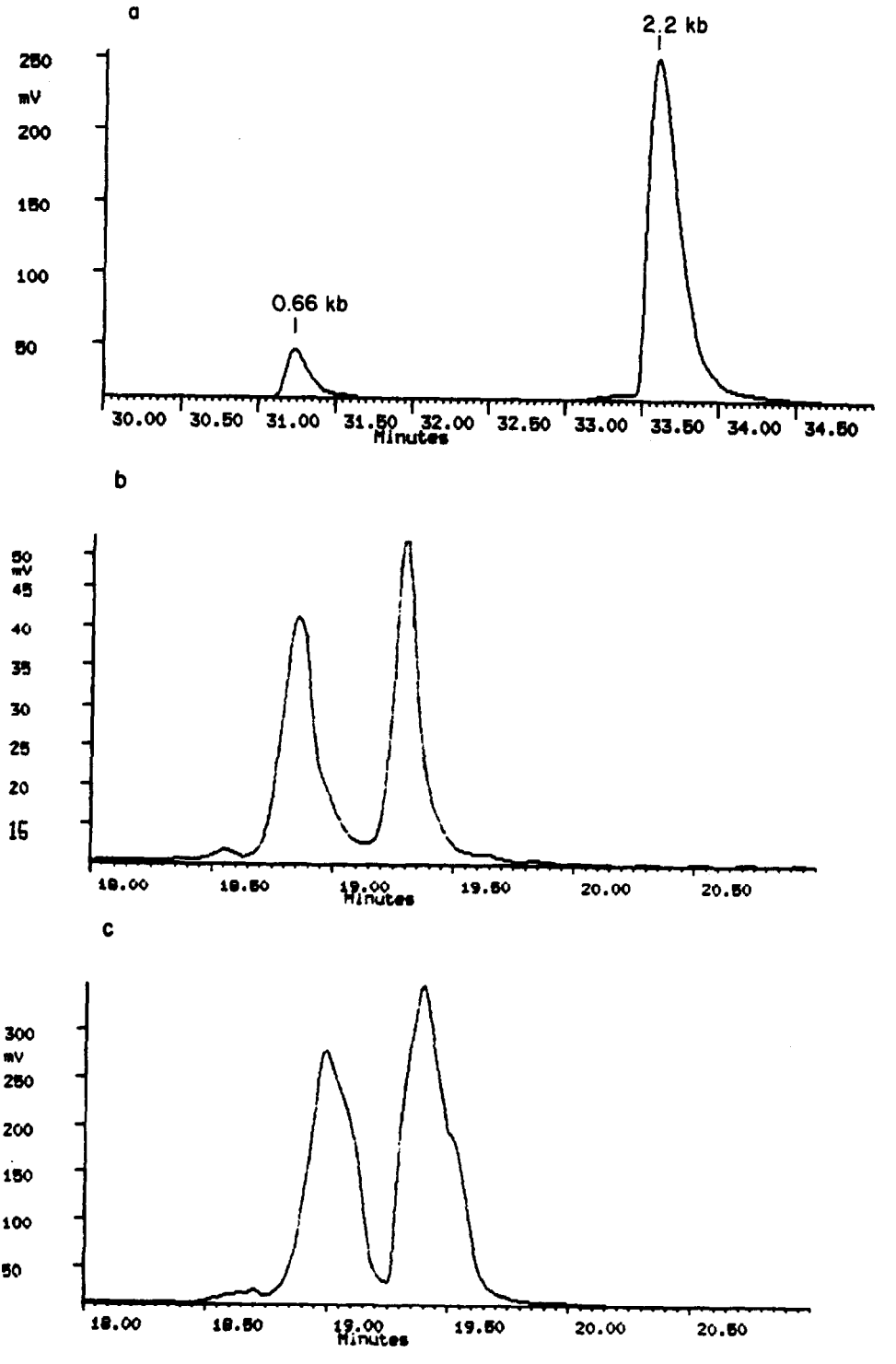
The purification of supercoiled plasmid DNA molecules is another important, frequently performed task to which HPLC technology has been applied[3,6–9,13,15]. As shown in Fig. 4a, supercoiled (form I) and nicked (form II) plasmid replicative form (RF) DNA molecules were readily separated from single-stranded DNA of the same length. Gel analysis of fractions from this profile are shown in Fig. 4b. There was no cross-contamination of the single- and double-stranded forms, but the various conformational isomers of double-stranded DNA were not completely resolved. Improved resolution of form II (nicked), form I (supercoiled) and form III (linear) conformational isomers was obtained as shown in Fig. 4c, but gel analysis still showed some cross-contamination (data not shown). The ability to obtain relatively clean populations of conformational isomers should prove useful for analyzing the effect of conformation on biological function.

Separation of topological isomers of covalently-closed circular DNA molecules

Reaction of supercoiled (form I) DNA with DNA topoisomerase I generates a population of topological DNA isomers containing varying numbers of superhelical turns. In two separate runs, supercoiled (form I) DNA and fully relaxed (form IV) DNA were subjected to HPLC on Gen-Pak FAX. As shown in Fig. 5a (an overlay of the two runs), the retention times for the two forms differed by 45 s. Gel analysis of fractions from the two profiles (Fig. 5b) showed that the supercoiled and fully relaxed forms potentially were resolved. A mixture of supercoiled and relaxed forms was then subjected to HPLC and the two forms were separated on a single run as shown in Fig. 5c. Topological isomers containing an intermediate number of superhelical turns were also partially resolved. These results suggest that it is feasible to develop quantitative HPLC assays for DNA topoisomerase activities.

Purification of synthetic oligonucleotides

One of the most frequent applications of HPLC in nucleic acid research is the purification of synthetic oligonucleotides[2]. A major difficulty with many current procedures is that molecules containing more than 30 or 40 nucleotides are often not fully separated from failure sequences and contaminants. Also, current procedures often require detritylation, extensive desalting and/or dialysis before the oligonu-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cleotide can be used. As shown in Fig. 6, HPLC on Gen-Pak FAX results in separation of the primary synthesis product (74-base oligonucleotide) from failure sequences on the basis of oligonucleotide size. Using appropriate gradient conditions, a 128-base oligonucleotide was similarly separated from the $n-1$ (127-base) and the $n+1$ (129-base) failure sequences[19]. The resulting purified oligonucleotides, in phosphate or Tris buffers, can be used directly after dilution or dialysis to the appropriate sodium chloride concentration.

Purification of circular single-stranded DNA for use in automated DNA sequencing systems

Single-stranded circular M13mp18 DNA, prepared as described in Experimental, was subjected to HPLC on a Gen-Pak FAX column. As shown in Fig. 7, single-stranded circular M13mp18 molecules were purified away from low levels of contaminating double-stranded M13mp18 replicative form DNA (Fig. 4a) and from most high-molecular-weight DNA (Fig. 10). The resulting single-stranded DNA was used as in a sequencing reaction with DNA polymerase I (Klenow fragment) and dye-labeled primers, and analyzed on a Applied Biosystems 370A DNA sequencing system[37]. The resulting sequence data were at least as accurate as control sequencing data. Thus, the Gen-Pak FAX column can provide an alternative to current purification methods of single-stranded circular M13 templates for DNA sequencing.

Isolation of double-stranded genomic RNA fragments

The genomes of many double-stranded RNA viruses consist of discrete, short genomic RNA fragments. Purification of an individual genomic fragment for use in genetic and molecular biological experiments is often a tedious procedure with low yields. As shown in Fig. 8, the eleven porcine rotavirus genomic RNA fragments were resolved into overlapping size classes. Based on similar results with DNA restriction fragments, it should be possible to resolve individual fragments within each size class using shallower gradients.

Purification of isoaccepting species of tRNA

Methods for the purification of milligram amounts of single-stranded isoaccepting tRNA molecules are needed for biophysical and biochemical analysis of

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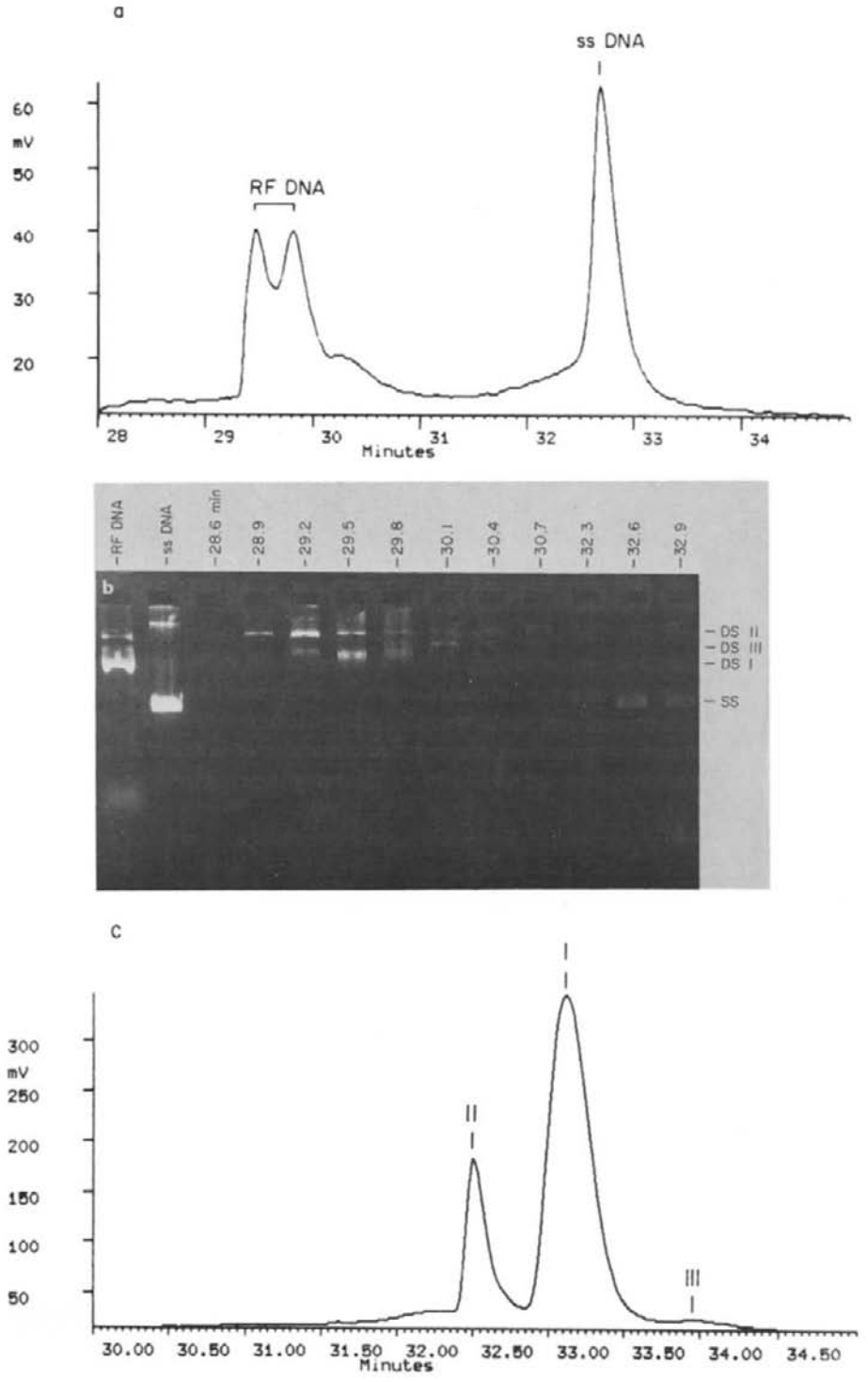
Fig. 3. Separation of 2.2-kb vector from its 0.6-kb insert. (a) Approximately 6 μg of a mixture of 2.2-kb size vector and 0.6-kb insert were injected onto the Gen-Pak FAX column. Flow-rate, 0.5 ml/min; pressure, 978 p.s.i.; temperature, 60°C. Eluent A was buffer E, and eluent B was buffer F. A 30-min. slightly convex gradient (curve 5) from A–B (55:45) to A–B (25:75) was run. The 0.6-kb fragment eluted at 31.2 min, the 2.2-kb vector eluted at 33.6 min. (b) 1 μg of SBPSB105 DNA, consisting of 1.6- and 2.6-kb restriction fragments, was injected onto a Gen-Pak FAX column. Flow-rate, 0.5 ml/min; pressure, 1152 p.s.i.; temperature, 35°C. Eluent A was buffer E and eluent B was buffer F. A 5-min gradient from A–B (55:45) to A–B (15:85), then at 20 min a convex gradient (curve 2) from A–B (15:85) to A–B (10:90) was run. The retention times of the 1.6-kb and 2.6-kb were 18.9 and 19.3 min, respectively. (c) 16 μg of SBPSB105 DNA, consisting of 1.6- and 2.6-kb restriction fragments, were injected into a Gen-Pak FAX column. Flow-rate, 0.5 ml/min; pressure, 858 p.s.i.; temperature, 35°C. Eluent A was buffer E and eluent B was buffer F. A 5-min linear gradient from A–B (55:45) to A–B (15:85), then at 20 min a convex gradient (curve 2) from A–B (10:90), was run. The retention times of the 1.6-kb and 2.6-kb fragment were 19.0 and 19.4 min, respectively.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tRNA dynamics. As shown in Fig. 9, our preliminary results of HPLC of *E. coli* tRNA indicate partial separation of at least 17 species.

Fractionation of complex mixtures of nucleic acids

A mixture of single-stranded circular DNA, double-stranded replicative form DNA, and *E. coli* DNA was subjected to HPLC. As shown in Fig. 10, this mixture was fractionated into partially overlapping but well defined classes of molecules. This suggests that effective purification strategies for relatively minor DNA components in complex mixtures of nucleic acids can be devised.

DISCUSSION

Merion[19] has shown that HPLC on a Gen-Pak FAX column achieved complete resolution of all of the double-stranded DNA fragments in the BRL 1-kb DNA ladder and of a Hind III digest of bacteriophage lambda DNA. The theoretical resolution of the Gen-Pak FAX column, which extends from 0.1 kb to 200 kb was exploited in the current experiments to obtain complete resolution of 3.7- from 14-kb restriction fragments (Fig. 1), of 2.9- from 6.1-kb fragments, and of 0.66- from 2.2-kb fragments (Fig. 3). Similar results have been obtained with restriction fragments ranging from 0.125 kb to >100 kb. The resolution of the column is such that the 2.027-kb restriction fragment from a Hind III digest of bacteriophage lambda can be separated from the 2.322-kb fragment. Moreover, only slight modifications in the sodium chloride gradient are necessary for baseline separations in different size ranges. The effect of base composition of the fragments on resolution was less noticeable than with other HPLC columns[2]. The 10 cm × 4.6 mm column currently available commercially has an effective capacity of 10–20 μg of DNA before resolution begins to decrease (Fig. 3c).

Supercoiled double-stranded plasmid DNA was readily separated from single-stranded circular DNA (Fig. 4a) and from conformational isomers (Fig. 4c). Somewhat better results were obtained when plasmids were first chromatographed on a Protein-Pak DEAE 5PW column (Waters), then chromatographed on the Gen-Pak FAX column[19].

Supercoiled DNA that had been incubated with DNA topoisomerase I eluted

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Fig. 4. Preparative HPLC of single-stranded circular DNA: separation from double-stranded replicative form; separation of double-stranded conformational isomers. (a) 10 μg of a one-to-one mixture of M13mp18 replicative form (double-stranded) and M13mp18 single-stranded were injected onto a 10 cm × 4 mm Gen-Pak FAX column. Flow-rate, 0.5 ml/min; pressure, 1002 p.s.i.; temperature, 60°C. Eluent A was buffer E and eluent B was buffer F. A 20-min linear gradient (curve 6) from A–B (55:45) to 100% B was run. 0.3-min fractions (0.15 ml) were collected. The retention times for the RF DNA and the single-stranded DNA were approximately 29.6 and 32.7 min, respectively. (b) 25-μl aliquots of each fraction were subjected to gel electrophoresis in 0.7% agarose in Tris borate, EDTA buffer for 4 h. Lanes 1 and 2 contain the unmixed replicative and single-stranded forms of M13mp18, respectively. (c) 20 μg M13mp18 double-stranded DNA were injected onto a 10 cm × 4 mm Gen-Pak FAX column. Flow-rate, 0.5 ml/min; pressure, 2500 p.s.i.; temperature, 22°C. Eluent A was buffer A and eluent B was buffer B. A 30-min linear gradient (curve 6) from A–B (60:40) to A–B (20:80) was run. 0.1-ml fractions were collected. The retention times of relaxed or nicked circular M13mp18 (form II) DNA was 32.5 min, of supercoiled M13mp18 (form I) was 33.1 min, and of linear M13mp18 (form III) was 34.0 min.

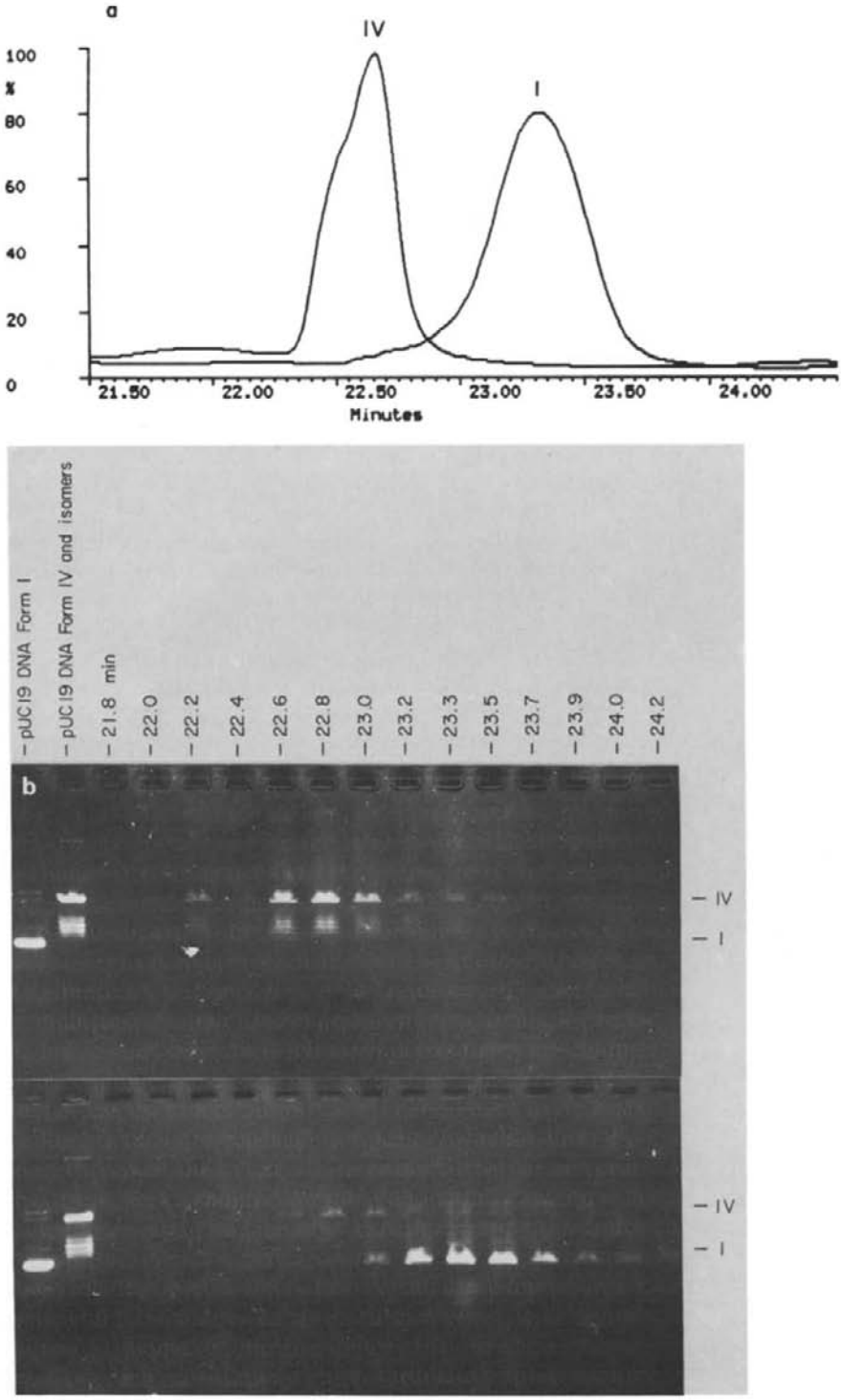
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Fig. 5.

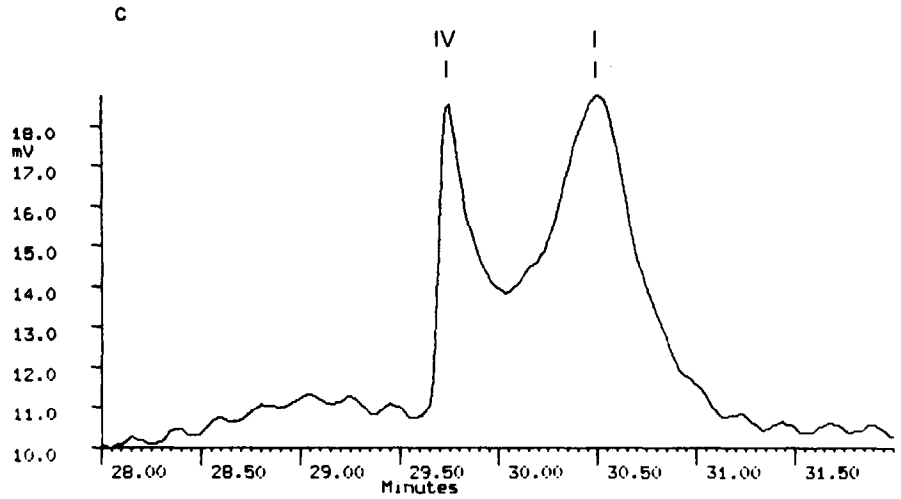
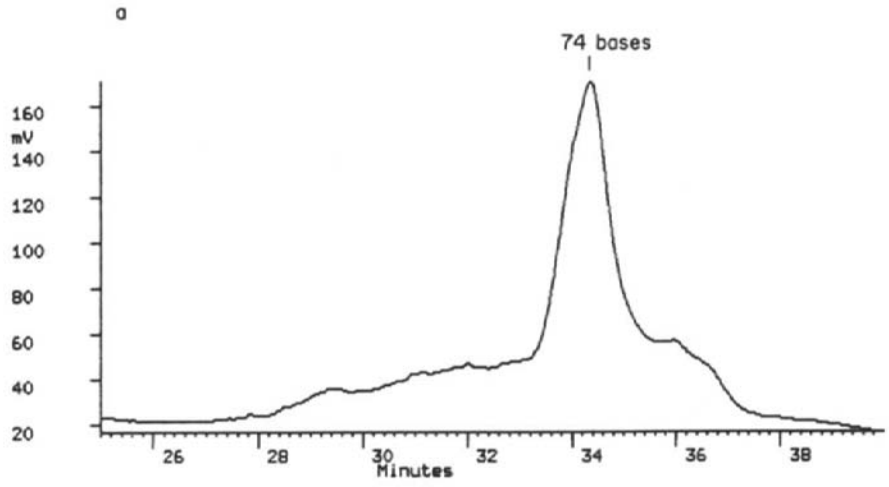


Fig. 5. Topological isomers of circular plasmid DNA molecules. (a) 10 μg of pUC19 DNA in 10 m*M* Tris–HCl, pH 8.0, 1 m*M* EDTA were injected onto a 10 cm × 4 mm Gen-Pak FAX column. Flow-rate, 0.5 ml/min; pressure, 1608 p.s.i.; temperature, 22°C. Eluent A was buffer A and eluent B was buffer B. A 30-min linear gradient (curve 6) from A–B (60:40) to A–B (10:90) was run. The retention time of supercoiled pUC19 DNA was 23.3 min based on the A_{260} profile. 10 μg of pUC19 DNA were incubated for 30 min at 37°C with 10 units of *Xenopus laevis* DNA topoisomerase I, processed as in Experimental, then injected and analyzed as above. The retention time of the relaxed pUC19 DNA was 22.7. The profiles of the two HPLC runs were overlaid in this figure for ease in comparison. 70 μl (0.2 min) fractions of each gradient were collected starting at 22 min. (b) (Top) 30-μl aliquots of each fraction of the HPLC analysis of the DNA topoisomerase I–pUC19 reaction were subjected to electrophoresis on a 1% agarose gel for 4.5 h. The gel was stained with ethidium bromide and photographed. The first two lanes contain supercoiled (form I) pUC19 DNA and fully relaxed (form IV) pUC19 DNA standards, respectively. The ethidium bromide stained material migrating between forms I and IV are topological isomers of pUC19 DNA containing intermediate numbers of superhelical turns. (Bottom) 30-μl aliquots of each fraction of the HPLC analysis of supercoiled pUC19 DNA were also subjected to electrophoresis on the same gel. (c) 2 μg of an equal mixture of supercoiled pUC19 (form I) and topoisomerase reacted (form IV) DNA was injected onto a Gen-Pak FAX column. Flow-rate, 0.5 ml/min; pressure, 1552 p.s.i.; temperature, 35°C. A 30-min slightly convex gradient (curve 5) from A–B (55:45) to A–B (5:95) was run. The retention times of the form IV and form I pUC19 DNAs were 29.8 and 30.5 min, respectively.

at a different time than did the supercoiled (form I) DNA (Fig. 5a). The resolution was sufficient to permit quantitative kinetic analysis of the rate of disappearance of the supercoiled (form I) species and subsequent appearance of the fully relaxed (form IV) species (Fig. 5c). Topological isomers with intermediate numbers of superhelical turns were only partially resolved, although further optimization of gradient conditions may permit isolation of individual topological isomers.

Although a number of HPLC columns exist for the purification of synthetic oligonucleotides, the results in Fig. 6 suggest that the Gen-Pak FAX column has some advantages. Oligonucleotides as long as 128 nucleotides have been successfully purified[19]. The base composition had a significantly smaller effect on retention time than with other columns[2]. Moreover, the purified product eluted in a buffered salt solution that permitted most further manipulations without extensive dialysis or other procedures.



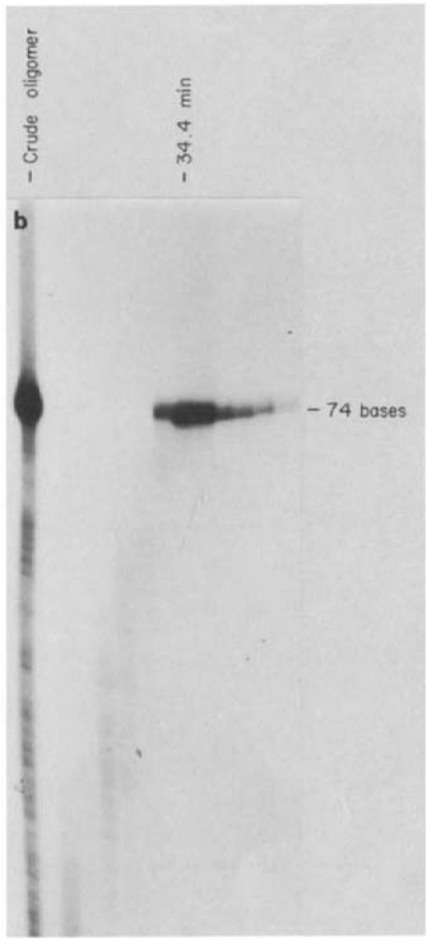


Fig. 6. HPLC of synthetic oligonucleotides. (a) 10 μg of a 74-base long synthetic oligonucleotide were injected onto a Gen-Pak FAX column. Flow-rate, 0.5 ml/min; pressure, 1500 p.s.i.; temperature, 22°C. Eluent A was buffer C and eluent B was buffer D. A 30-min convex gradient (curve 5) from 100% A to A–B (40:60) was run. Fractions were collected every 0.2 min. The retention time of the 74-base synthetic oligonucleotide was 34.4 min. The sequence of the oligonucleotide was: 5'-GTC-AAA-AAG-GGG-ATC-CGA-TGG-CAG-ACG-ACG-ATG-ACG-ACG-ATG-ACG-ACG-ATG-ACT-ACT-AGA-AGC-TTA-TCG-ATG-AT-3'. (b) 2 μl of the T4 polynucleotide kinase reaction mixture (see Experimental) were loaded on a strand separating 10% polyacrylamide gel. Lane 1 is the crude oligo input. Lanes thereafter correspond to elution time 30.2 min and so on, 0.6 min apart. The peak of the oligonucleotide corresponds best to lane 8 (34.4 min).

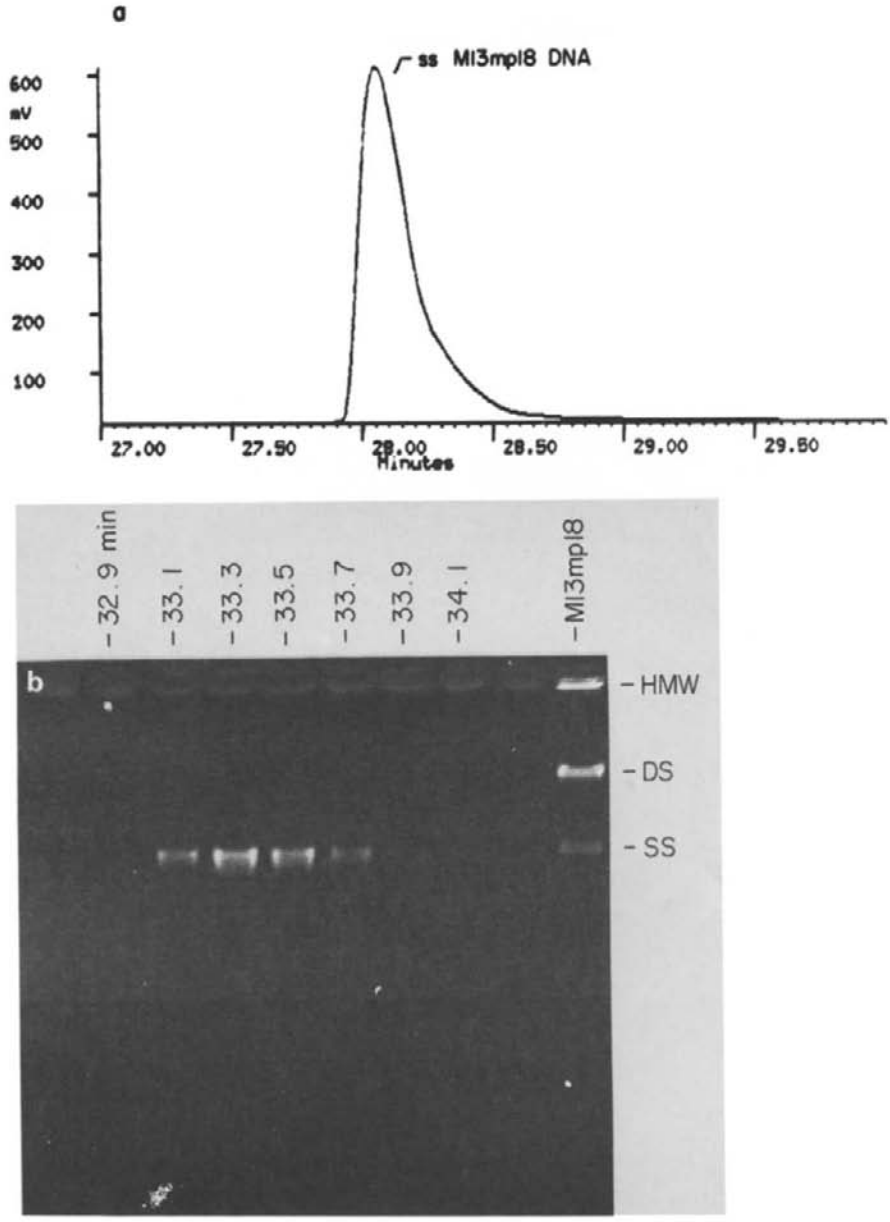
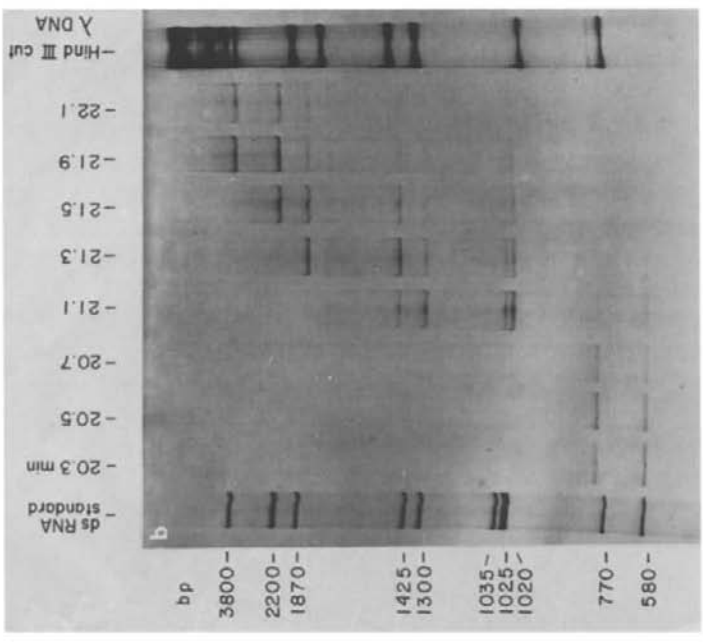


Fig. 7. HPLC purification of single-stranded DNA. (a) Approximately 18 μg of single-stranded M13mp18 DNA were injected onto a 10 cm × 4 mm Gen-Pak FAX column. Flow-rate, 0.5 ml/min; pressure, 882 p.s.i.; temperature, 60°C. A 30-min linear gradient (curve 6) from A–B (55:45) to A–B (20:80) was run. Eluent A was buffer E and eluent B was buffer F. The retention time of the single-stranded DNA was 28.1 min. The peak fractions were dialyzed and used as a sequencing template. The reactions were analyzed on an Applied Biosystems 370A sequencer (data not shown). (b) Approximately 25 μg of single-stranded M13mp18 were injected onto a Gen-Pak FAX column. Flow-rate, 0.5 ml/min; pressure, 1620 p.s.i.; temperature, 22°C. A 30-min linear gradient (curve 6) from A–B (60:40) to A–B (20:80) was run. Eluent A was buffer A and eluent B was buffer B. The retention time of the single-stranded DNA was 33.3 min (profile not shown). A 30-μl aliquot of each fraction in the peak area was subjected to electrophoresis. The gel was stained with ethidium bromide and photographed. The right lane contains a sample of the input DNA and the other lanes are labeled with their elution times.



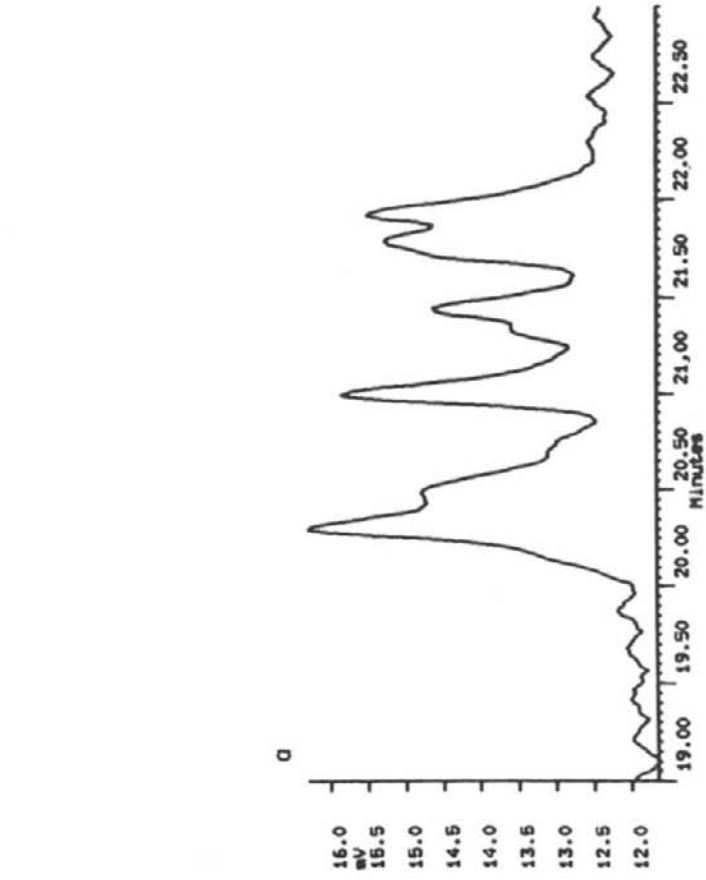


Fig. 8. Preparative HPLC of porcine rotavirus double-stranded genomic RNA. (a) Approximately 0.4 μg of a double-stranded porcine rotavirus RNA sample was injected onto the Gen-Pak FAX column. Flow-rate, 0.5 ml/min; pressure, 2256 p.s.i.; temperature, 22°C. Eluent A was buffer A and eluent B was buffer B. First a 5-min linear gradient (curve 6) from A–B (55:45) to A–B (45:55) was run, followed by a 60-min linear gradient (curve 6) from A–B (45:55) to A–B (30:70). 0.1-ml fractions were collected. The retention time of the smallest double-stranded RNA fragment was 20 min. The largest double-stranded RNA fragment eluted from the column at 22 min. (b) 30 μl of each fraction of the HPLC analysis were subjected to electrophoresis on a 7.5% polyacrylamide gel at 14 mA for 14 h. The gel was stained with silver stain. Lane 1 contains unfractionated RNA as a size marker (it is not the input to the column) and lane 10 contains lambda DNA restricted with Eco RI and Hind III as a molecular weight standard. The sample lanes are labeled with their HPLC elution times.

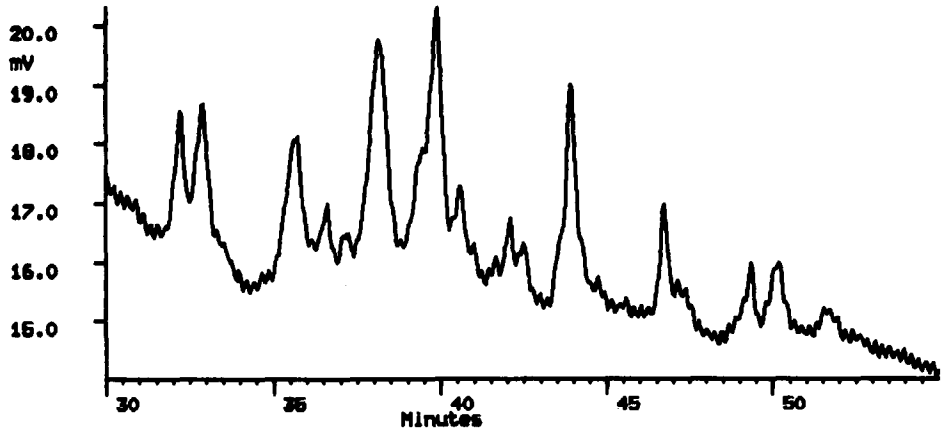


Fig. 9. Analytical HPLC of *E. coli* tRNA. 3 μg of a sample of *E. coli* tRNA were injected onto a 10-cm Gen-Pak FAX column. Flow-rate, 0.5 ml/min; pressure, 2208 p.s.i.; temperature, 22°C. Eluent A was buffer E and eluent B was buffer F. First a 10-min linear gradient (curve 6) from 100% A to A–B (60:40) was run, then at 20 min a 30-min linear gradient (curve 6) from A–B (60:40) to A–B (40:60) was run. Preliminary experiments fix the retention times of the tRNA fragments to between 30 and 53 min.

Circular M13 DNA is frequently used for both cloning (replicative form) and sequencing (single-stranded form), and is currently the preferred vector for automated DNA sequencing systems[37,38]. The Sanger method of sequencing, particularly as carried out for the automated systems, requires highly purified single-stranded DNA, free from even traces of contaminating host cell DNA. Purification is currently a labor- and time-intensive process, often involving extended ultracentrifugation and gel electrophoresis. With the increasing use of automated sequencing systems in centralized service facilities, confirmation of the purity of the input DNA from multiple laboratories becomes a prime consideration. Single-stranded circular DNA was readily prepared on Gen-Pak FAX (Fig. 7a). The DNA was then used successfully in automated DNA sequencing systems with only one dialysis step.

HPLC on Gen-Pak FAX columns is not limited to DNA. Promising but preliminary separations of double-stranded linear rotarivus genomic RNA fragments in the size range of 0.3–3 kb have been obtained (Fig. 8). A number of (so far undefined) tRNA species have also been resolved by preparative HPLC on the Gen-Pak FAX column (Fig. 9). Even complex mixtures can be fractionated (Fig. 10).

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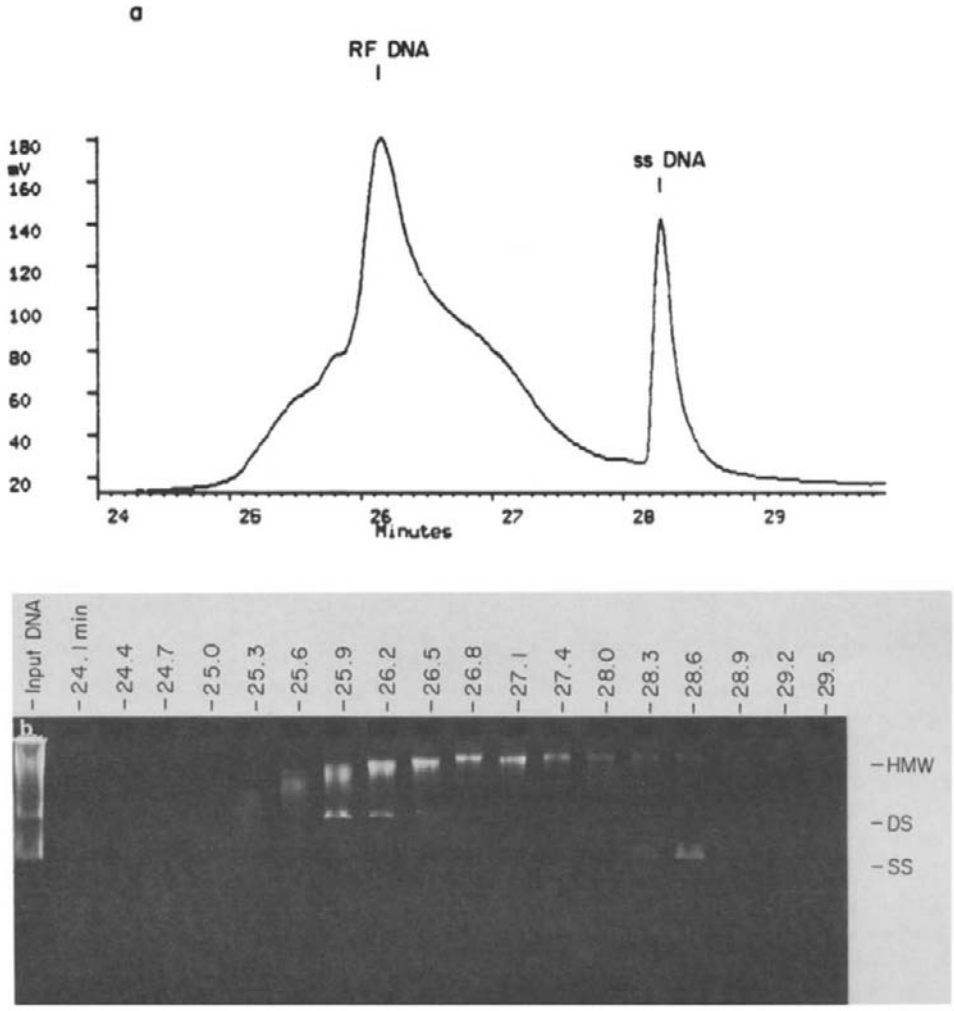

Fig. 10. Separation of double- and single-stranded circular DNA contaminated with high molecular weight DNA. (a) 15 μg of an equal mixture of M13mp18 RF, M13mp18 single-stranded, and *E. coli* genomic DNA were injected onto a Gen-Pak FAX column. Flow-rate, 0.5 ml/min; pressure, 1056 p.s.i.; temperature, 60°C. Eluent A was buffer E and eluent B was buffer F. A 20-min linear gradient (curve 6) from A–B (55:45) to 100% B was run. The retention times of the replicative form and single-stranded DNAs were 26.2 and 28.3 min, respectively. Fractions were collected every 0.3 min (0.15 ml). (b) 30-μl aliquots of each fraction in the peak areas were run on a 1% agarose gel for 4 h, then stained with ethidium bromide. Lane 1 is the mixture of input DNA; the other lanes are labeled with their respective elution times.

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