Preparative fractionation of DNA restriction fragments by reversed phase column chromatography

(restriction endonucleases/purification of DNA sequences/base composition/λp*lac* 5 DNA)

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ABSTRACT   Reversed phase column chromatography on RPC-5 resin was used to fractionate milligram quantities of DNA fragments generated by restriction endonucleases. Fractionation was on the basis of size, the presence or absence of sticky ends, and at least one as yet undetermined property.

We are interested in the role of DNA structure and properties in gene regulation (1–5). We wish to extend our previous studies (refs. 2 and 3 and Chan, Dodgson, and Wells, unpublished data) on the properties of the *lac* operator by isolating large quantities of DNA restriction fragments that contain *lac* genetic control elements (6, 7), such as the operator, promotor, and the catabolite gene activator protein binding site. Chemical, physical, and biological investigations on these defined genetic regions may provide new insights into the molecular mechanism of gene expression. This approach requires large quantities of homogeneous DNA restriction fragments. This paper describes the use of reversed phase column chromatography for the purification of milligram quantities of DNA restriction fragments.

Reversed phase column chromatography was originally developed for high-resolution fractionation of tRNAs (8, 9) and has been used for other RNA fractionations (10). Recent studies with a homologous series of single-stranded DNA oligomers showed that molecules (up to approximately 30 nucleotides in length) differing by one nucleotide could be separated (5). The fractionation of three representative mixtures of large duplex DNAs is described below.

MATERIALS AND METHODS

DNA. λp*lac*5cI$_{857}$S$_7$ DNA was prepared from the lysogen *Escherichia coli* M1733 (gift of I. Nes) by the previously described procedure (2) with the following modifications: 1-liter cultures contained 5 g of NaCl, 10 g of Bacto-Tryptone, and 5 g of yeast extract. After cells were harvested by centrifugation, they were resuspended in 0.1 the volume of the original culture in a solution of 50 mM NaCl, 10 mM MgSO$_4$, and 20 mM Tris·HCl (pH 7.4) and were lysed by shaking for 20 min at 37° with 2% (vol/vol) CHCl$_3$ and 0.25 μg/ml of DNase I (Worthington Biochemical). After phenol and ether extraction, the DNA was dialyzed exhaustively against and stored in 10 mM Tris·HCl (pH 7.4), 0.1 mM EDTA.

Cleavage of DNA with Bacterial Restriction Endonucleases. A sample of λp*lac* DNA was treated at 37° in 50 mM Tris·HCl (pH 7.9), 5 mM MgCl$_2$, and 0.5 mM dithiothreitol with sufficient *Haemophilus aegyptius* (*Hae*) III (gift of R. W. Blakesley) to give complete digestion in 20 hr, as determined by gel electrophoresis. Alternatively, a sample of the DNA in

Abbreviations: bp, base pairs; *Hae* III, *Hin*d II + III, and *Eco*RI, restriction endonucleases from *Haemophilus aegyptius, H. influenzae,* and *Escherichia coli,* respectively.

6.6 mM Tris·HCl (pH 7.5), 8.7 mM MgCl$_2$, and 35 mM NaCl was digested with *H. influenzae* R$_d$ (*Hin*d) II + III, prepared essentially as described (11), at 37° for 20 hr.

For digestion with *Eco*RI, the mixture contained 100 mM Tris·HCl (pH 7.4), 5 mM MgCl$_2$, 130 mM NaCl (12), λp*lac* 5 DNA, and sufficient *E. coli* (*Eco*) RI (gift of R. Jorgensen and W. S. Reznikoff) to give complete digestion in 20 hr at 37°. Each mixture was dialyzed into equilibration buffer (10 mM Tris-acetate, pH 7.4, 1.4 M sodium acetate) in preparation for column chromatography.

RPC-5 Column Chromatography. The DNA samples were loaded through a Milton Roy Minipump onto a 135 × 0.9 cm glass column containing RPC-5 resin (lot no. 7; Miles Laboratories). The column was equipped with high-pressure fittings and Teflon lines. The resin had been degassed before packing at 400–500 lb./inch^2 (2.8–3.5 MPa). After the sample was loaded, a 2-liter degassed linear gradient of 1.4–1.8 M sodium acetate containing 10 mM Tris-acetate (pH 7.4) was applied at 0.4 ml/min with 200–400 lb./inch^2 (1.4–2.8 MPa) at room temperature. Fractions of 4 ml were taken.

Gel Electrophoresis. Gel electrophoresis was performed directly on 0.005–0.05 ml of each column fraction mixed with an equal volume of 50% glycerol, 0.2 mg/ml of bromophenol blue, 0.1 M EDTA, 0.2% sodium dodecyl sulfate, and 0.6 × Peacock's buffer (13). Five percent polyacrylamide gels (containing 0.25% bis-acrylamide) were prepared in Peacock's buffer (13) containing 25% glycerol, 0.03% *N,N,N',N''*-tetramethylethylenediamine, and 0.1% ammonium persulfate. Gels were electrophoresed in Peacock's buffer at 80 or 200 V until the dye reached the tip of the gel. Gels were stained with ethidium bromide solution and photographed under UV light. The 0.7% agarose gel electrophoresis was described previously (14).

RESULTS

Fractionation of *Hae* III fragments

λp*lac* 5 DNA, when fully digested with *Hae* III restriction endonuclease, produced more than 50 fragments varying in size from less than 6 × 10^4 to 1.9 × 10^6 daltons. Fragment sizes were estimated by polyacrylamide gel electrophoresis in comparison to φX174 replicative form DNA digested with *Hae* III (15) and λp*lac* 5 DNA (16) digested with *Hin*d II + III. *Hae* III produces "blunt end" cleavages at G-G↓-C-C sites (17).

Fig. 1 shows that the *Hae* III fragments eluted from RPC-5 over a range of 190 fractions. The smallest fragments [fewer than 100 base pairs (bp) in fractions 20–40] eluted first and the largest (3200 bp in fractions 188–200) eluted last. All fragments were purified substantially, but only a few approached homogeneity: 3200 bp in fractions 188–194, 1500 bp in fractions 178–184, 2130 bp in fractions 170–174, 1890 bp in fractions 160–162, and 1190 bp in fractions 156–158. Since electropho-
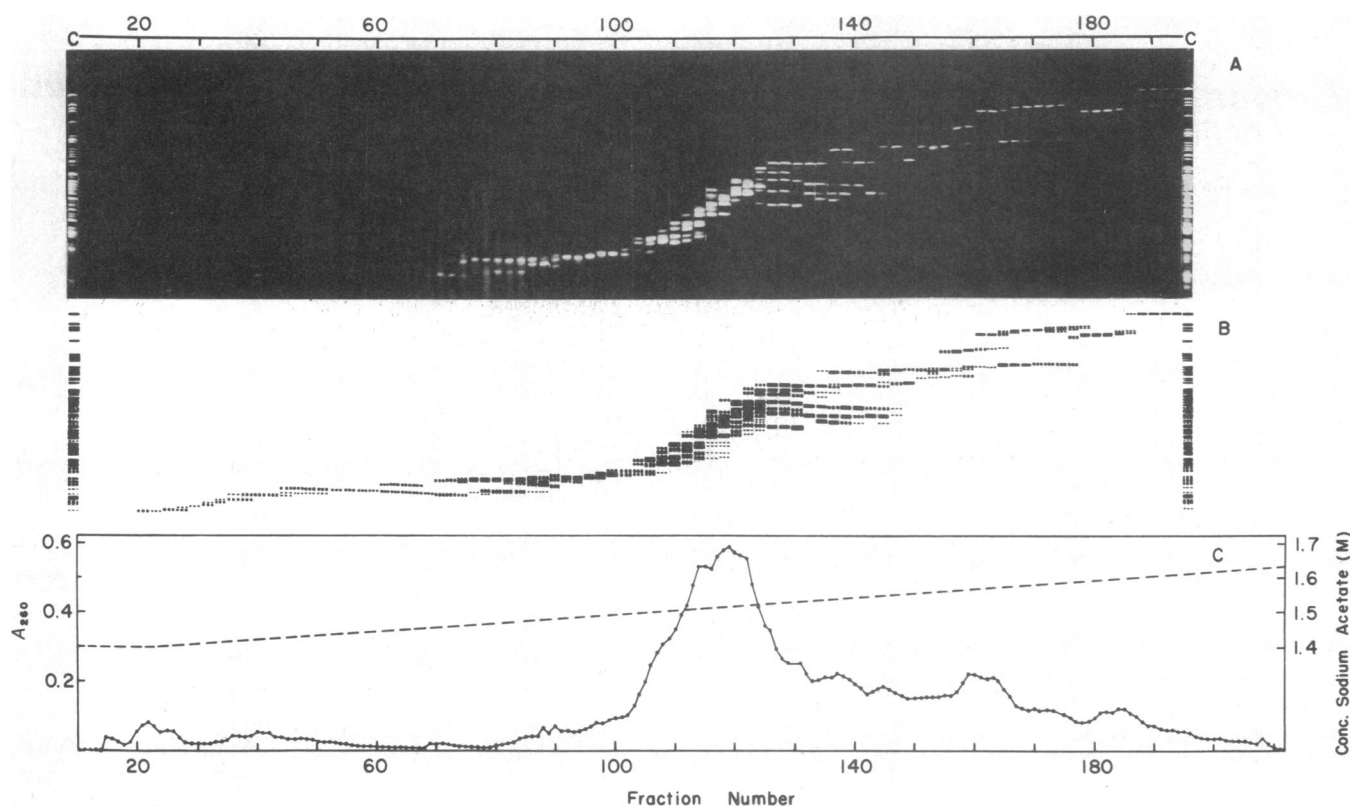
FIG. 1.   Fractionation of *Hae* III digest of λp*lac* 5 DNA by column chromatography on RPC-5. 122 A_{260} units of the digest in 15 ml were loaded onto the column and fractionated as described in *Materials and Methods*. (1 A_{260} unit is that amount of material that gives an absorbance of 1 when dissolved in 1 ml of solvent with a light path of 1 cm.) (A) Five percent polyacrylamide cylindrical gels on even-numbered fractions of the eluant of reverse phase column chromatography. Control gels (marked C) at the extreme right and left sides are unfractionated digests. Gels 60–80 have a faint background from incomplete destaining. (B) Artist's conception of panel A. Dotted lines indicate faint bands. (C) A_{260} elution profile of the eluant of reverse phase column chromatography. No absorbing material eluted elsewhere in the gradient. Total recovery of A_{260} was 80%. The dashed line indicates sodium acetate concentration.

resis of the complete digest on 5% polyacrylamide gels did not resolve most of the fragments, identification of individual bands was difficult. Nonetheless, 30–40% difference in molecular

weight was estimated to be sufficient for essentially complete resolution.

Factors other than molecular weight affected the elution

Table 1.   Reversed phase chromatographic properties of DNA fragments containing "sticky ends"

Gel position of fragments containing "sticky ends" (cm)*	No. of base pairs	Fraction no.
1.7	3580	130–142
2.2	2500	146–154
3.1	1450	140–150
3.3	1350	120–130
3.6	1200	116–126
4.5	910	102–112
6.6	520	110–112
7.8	375	90–96

* The gel positions (from Fig. 2) of the fragments containing "sticky ends" from *Hin*d III cleavages were determined by comparing the electrophoresis patterns (not shown) of λp*lac* 5 DNA digested by only *Hin*d II (gift of W. S. Reznikoff) with that digested with *Hin*d II + III. Complete agreement was observed with previous studies (16, 19). Molecular weights of the fragments were described previously (16). The fraction numbers were taken from Fig. 2. There were also "blunt ended" fragments of approximately 375 and 520 bp. The latest eluting fragments in these cases were presumed to carry the "sticky ends".

Table 2.   Nucleotide composition of fractionated *Eco*RI fragments

Fragment molecular weight (×10⁻⁶)*	Buoyant density (g/ml)†	Calcu-lated G+C content (%)	Order of elution from RPC-5 (Fig. 3)
14.11 (12.0 + 2.11)	1.705	52	1
3.71	1.700	47	2
4.35	1.698	45	5
3.57	1.697	44	4
4.67	1.696	43	3
30.4 (intact λp*lac* 5 DNA)	1.701	48	Not applicable

* *Eco*RI fragments were fractionated by column chromatography on RPC-5 (Fig. 3). The fraction containing each fragment in the most homogeneous state was dialyzed against 1 mM potassium phosphate (pH 7.6) and concentrated 10-fold by lyophilization. Fragment molecular weights were reported previously (14).
† Analytical buoyant density analyses (courtesy of J. E. Larson) were performed in a Spinco model E ultracentrifuge as described (21) with either (dA)$_n$ or (dA-dT)$_n$·(dA-dT)$_n$ as density markers. The values listed are ± 1 mg/ml. The density of the 3.71 × 10⁶ fragment was measured as a shoulder on the profile of the 1.705 g/ml peak.
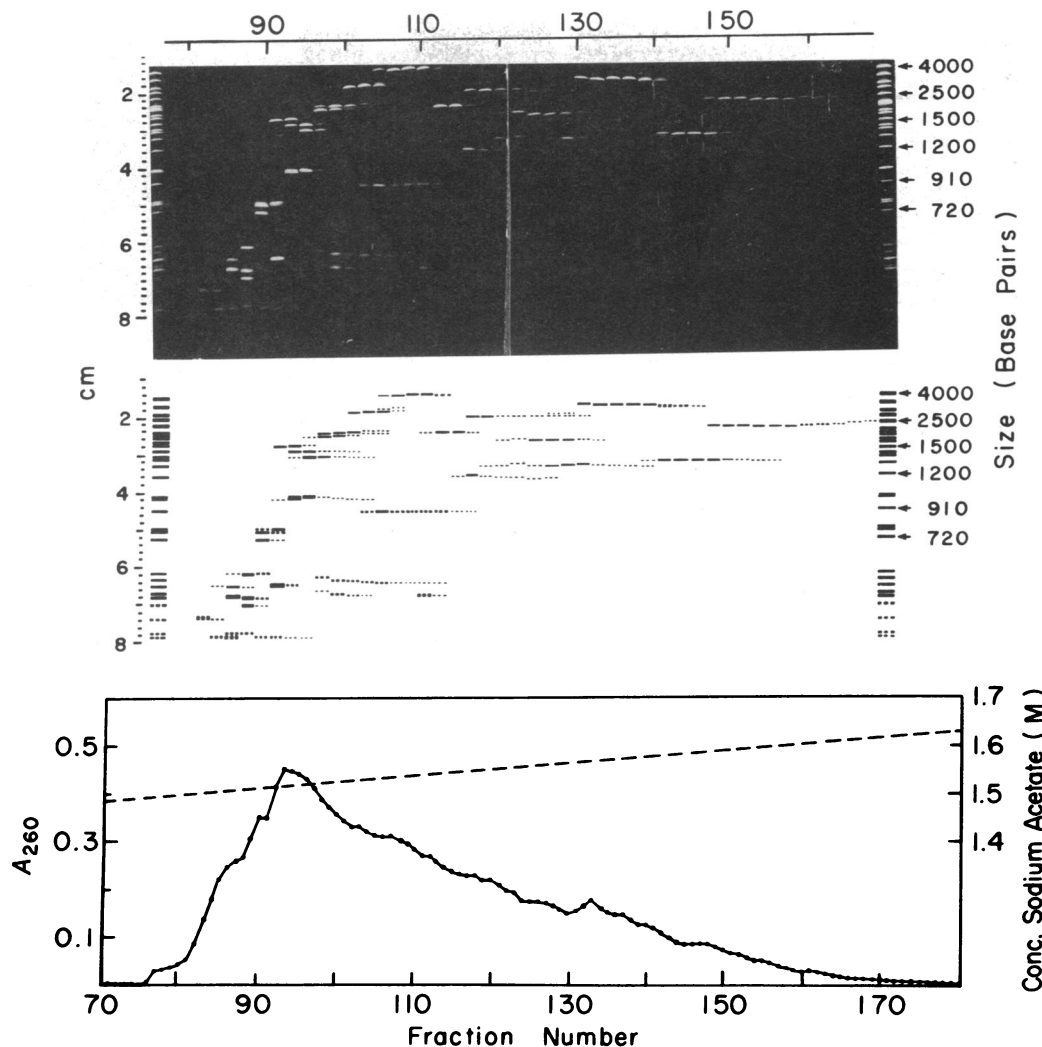
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FIG. 2.   Fractionation of *Hin*d II + III digest of λp*lac* 5 DNA by RPC-5 chromatography. 96 A_{260} units of the digest in 12 ml were loaded onto the column and chromatographed on an RPC-5 column as described in *Materials and Methods*. (Upper panel) Four percent polyacrylamide gel electrophoretic analysis of odd-numbered fractions from the column. The gels at the extreme right and left are on unfractionated digests. The scale at the left is an arbitrary measure for ascertaining the position of fragments. (Middle panel) Artist's conception of the upper panel. Dotted lines indicate faint bands. (Lower panel) A_{260} elution profile. Eighty-five percent of the loaded DNA appeared in this part of the profile, and no absorbance was detected elsewhere.

pattern. Fig. 1 shows numerous instances where fragments of essentially the same electrophoretic mobility were widely separated by reversed phase column chromatography. For example, the four fragments of similar size (approximately 750–850 bp) that are the sixth to ninth largest fragments [see control (C) gels] were readily separated into four components (fractions 136–138, 144–146, 152–160, and 164–174). Also, the second, third, and fourth largest fragments (1500–2130 bp) did not elute in order of increasing size from RPC-5. Rather, the third largest eluted first, followed by the second, then the fourth largest. In addition, between fractions 120 and 150 (250–700 bp) there are numerous other examples where fractionation is influenced by a property other than relative size.

Fractionation of *Hin*d II + III fragments

Thirty-eight fragments were produced by complete digestion of λp*lac* 5 DNA with a mixture of *Hin*d II + III. The average size of fragments from this digest was larger than from the *Hae* III digest. *Hin*d II gives a "blunt cut" at G-T-Py�*-Pu-A-C sites, whereas *Hin*d III cleaves at A⁺-A-G-C-T-T sites to give "sticky ends" of four nonpaired nucleotides (18).

Fig. 2 shows the fractionation of *Hin*d II + III fragments by reversed phase column chromatography. In general, the smaller fragments eluted first, as was found for the *Hae* III digest (Fig. 1). However, Fig. 2 shows a pronounced separation for some fragments having similar sizes, as judged by gel electrophoresis. Table 1 lists the fragments with ends produced by *Hin*d III. In every case, these "sticky ended" fragments eluted at higher salt concentrations than "blunt ended" fragments of approximately the same size.

Also, there are "blunt ended" fragments of similar sizes which separate widely during reversed phase column chromatography. This behavior is the same as observed for *Hae* III fragments.

Fractionation of *Eco*RI fragments

*Eco*RI endonuclease produces only six fragments on digestion of λp*lac* 5 DNA, all containing "sticky ends". The recognition site is G⁺-A-A-T-T-C (20). Fragment sizes were reported (14). Under our conditions the two fragments containing the 12 bp cohesive ends of λ DNA (2.1 × 10⁶ and 12 × 10⁶ daltons) remain annealed and behave as a 14.1 × 10⁶ dalton fragment.
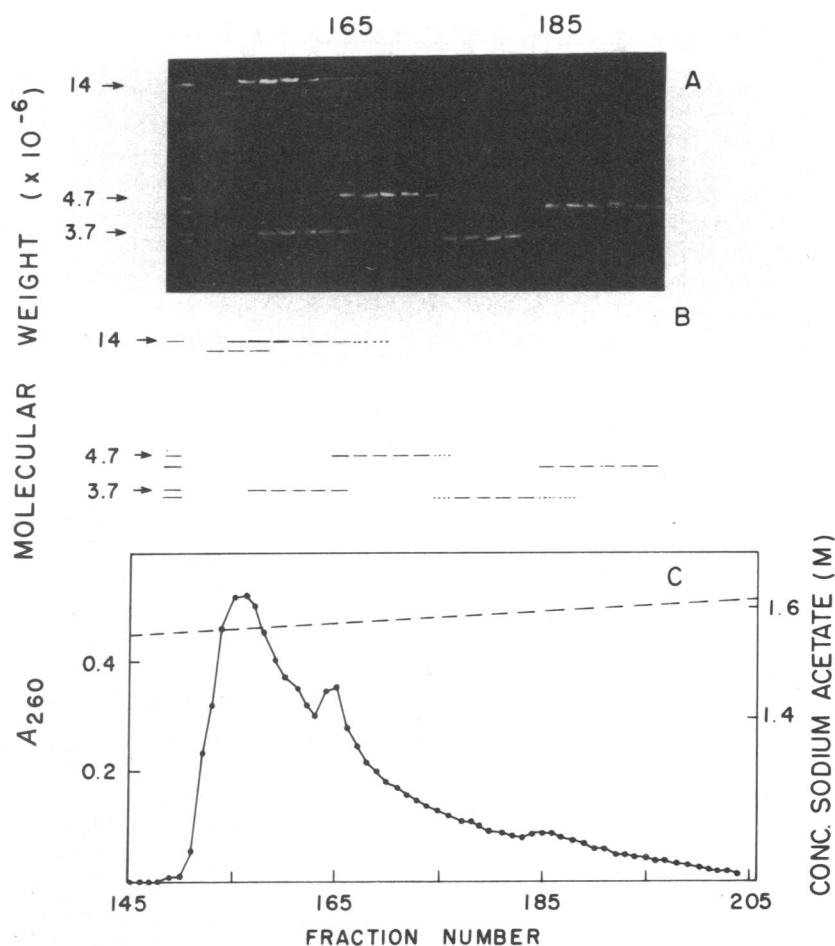
FIG. 3. Fractionation of an *Eco*RI digest of λp*lac* 5 DNA by RPC-5 chromatography. 50 A_{260} units of digested DNA in 7.0 ml was chromatographed on an RPC-5 column as described in *Materials and Methods*. (A) Agarose gel electrophoresis (0.7%) on odd-numbered fractions. (B) Artist's conception of panel A. (C) A_{260} elution profile of the eluant. A recovery of 77% was found; no UV absorbing material was found in fractions before 145.

Only a small amount of dissociated 12×10^6 dalton fragment was observed. Fig. 3 shows that, unexpectedly, the *Eco*RI fragments do not elute according to increasing molecular weight. Instead, the largest (14.1×10^6) and the 3.7×10^6 fragments elute almost simultaneously. The other fragments elute later in an unpredicted order.

To determine if the nucleotide composition was influencing elution properties, we performed analytical CsCl buoyant density analyses on the fractionated fragments. The results in Table 2 show no apparent correlation between base composition and elution order. This does not rule out a base composition effect, but does indicate that another as yet undefined factor is involved.

DISCUSSION

Duplex DNAs elute from RPC-5 predominantly in order of increasing molecular weight. We have chromatographed DNAs of between 6×10^4 and 14×10^6 daltons without observing upper or lower size limits. Factors other than molecular weight that affect the elution pattern appear with increasing frequency at higher molecular weight. Neither length nor base composition dictates the elution properties of *Eco*RI fragments; the reason for this behavior is uncertain at present. Elucidation of these factors may provide new information on the structural intricacies (1) of DNA.

"Sticky ended" fragments elute at substantially higher salt

concentrations than "blunt ended" fragments of the same size. This behavior may be due to self-association of the "sticky ends" to give oligomers or due to an association of the nonpaired nucleotides to the RPC-5. The latter hypothesis is consistent with the observation (5) that single-stranded DNA elutes at much higher salt concentration than double-stranded DNA of the same length. In either case, chromatography on RPC-5 affords a sensitive assay for "sticky ends".

Workers in numerous laboratories, including our own, have experienced difficulty redigesting DNA recovered from gels after electrophoresis. To determine if this was also a problem with reversed phase column chromatography, fractionated *Eco*RI fragments, prepared as in the legend to Table 2, were recombined in the original molar ratios and digested under standard conditions with each of the following restriction enzymes: *H. haemolyticus* I, *H. influenzae* R_f, *H. parainfluenzae* II (gifts of W. S. Reznikoff), *Hae* III, and *Hin*d II + III. The polyacrylamide gel electrophoresis patterns of these digests were identical with control double digests (data not shown).

As found with tRNA (22), the nature of the eluting salt influences the fractionation. KCl was tested in place of sodium acetate as the eluting salt (data not shown). Digests of λp*lac* 5 DNA with *Hae* III or with *Hin*d II + III eluted between 0.6 and 0.8 M KCl with the same order of elution of fragments as found in Figs. 1 and 2, respectively. However, the resolution was inferior. When *Eco*RI fragments were eluted with KCl solution, a single sharp unresolved peak was observed (data not shown).

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Experiments are in progress to determine if the base pair specific binding agents, netropsin (23) and actinomycin (24), can be used to alter the elution pattern (G. T. Horn, S. C. Hardies, and R. D. Wells, unpublished).

Reversed phase column chromatography may be used to prepare DNA fragments for sequencing; physical, spectroscopic, and chemical studies on defined genetic loci; detailed binding studies of proteins involved in gene expression (such as repressors and RNA polymerase); DNA heteroduplex studies; and genetic engineering. Ideally, a fragment from a simple digest can be purified by one passage through RPC-5. In Fig. 3, for example, many fractions contain a single EcoRI fragment. Of course, more complicated separations can be made by a series of restriction digestions with different nucleases, each followed by reversed phase column chromatography. From a single run, a 10- to 20-fold purification of any given fragment can be expected. Such a step might be advantageous prior to the use of another fractionation method. Since fragments of the same electrophoretic mobility often separate by reversed phase column chromatography, this technique followed by preparative gel electrophoresis should be an improvement over either method alone. Also, reversed phase column chromatography could be used analytically to separate separate fragments that coelectrophorese.

Some advantages of reversed phase column chromatography are high capacity, ease of recovery of the DNA in a state of high purity, and the capacity to separate some fragments that are unresolvable by electrophoresis. The method does not require ligand binding activity; therefore fragments that do not contain strong and specific binding sites can be purified by the same procedure. However, reversed phase column chromatography does not produce the resolution of gel electrophoresis, and it is not as rapid for analytical purposes. Because of its advantages, reversed phase column chromatography is well suited for use in large-scale preparations of DNA restriction fragments.

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