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Preparative separation of the complementary strands of DNA restriction fragments by alkaline RPC-5 chromatography

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ABSTRACT

High pressure reversed phase chromatography (RPC-5) at pH 12 was used for preparative separation of the complementary strands of the smaller DNA fragments which are generated by the Hae III restriction endonuclease digestion of Col El DNA. A single high pressure RPC-5 chromatographic step at neutral pH served to purify duplex fragments 70, 172, 250 and 440 base pairs long; each of these yielded two elution peaks upon chromatography under alkaline denaturing conditions.

INTRODUCTION

There are a number of reasons for seeking to separate the complementary strands of DNA restriction fragments, including sequence determination[1], formation of specific hybrid double helices, chemical modification of individual strands, and physical studies of the properties of single stranded DNA of defined sequence. Equilibrium density gradient sedimentation, for example in alkaline cesium salts, usually is the method of choice for strand separation of high molecular weight samples, but cannot be used for small restriction fragments because the sedimentation band width is too large. Recently, small restriction fragments have been separated into their component strands by polyacrylamide gel electrophoresis of samples denatured by treatment with alkali[1,2]. This technique has excellent resolving power, but its yield is restricted by the low concentration of DNA allowed in the alkaline sample which is loaded on the gel; high concentrations renature rapidly as the separated strands enter the neutral pH environment of the gel.

Our use for separated complementary DNA strands requires larger amounts than can be conveniently prepared by gel electrophoresis, forcing us to seek a chromatographic technique to accomplish the purpose. Reversed phase chromatography, especially the RPC-5 system[3-6], has successfully been used to purify a variety of nucleic acids, including DNA restriction fragments[7-9]. Because the RPC-5 organic phase is a quaternary amine, and the support is a

chemically inert polychlorotrifluoroethylene, we reasoned that the column should be stable in solutions which are sufficiently alkaline to cause DNA denaturation. In addition, we were encouraged to use RPC-5 chromatography for initial purification of duplex fragments because of the excellent separations, clearly better resolved than published profiles[7,8], obtained by P. Cole (private communication) using the same RPC-5 resin as that available in our laboratory and already used extensively by us for fractionation of oligoribonucleotides.

MATERIALS AND METHODS

Materials. The restriction endonuclease from Haemophilus aegyptius, Hae III (GG ↓ CC), was purchased from New England Biolabs. RNAse A (type 1-A) and protease (type IV) were obtained from Sigma.

RPC-5 resin was prepared by coating[4] polychlorotrifluoroethylene powder (Plaskon CTFE 2300, Allied Chemical Company) with methyltrialkyl (C_8-C_{10}) ammonium chloride (Adogen 464, Ashland Chemical Company). Unfortunately, Plaskon 2300 is no longer commercially available, and a possible substitute grade, Plaskon 2100, has been found unsuitable for RPC-5 chromatography (unpublished observations). Several laboratories are currently searching for an effective replacement for Plaskon 2300; the limited quantities now in use should not be discarded, but retained for recoating.

Large scale preparation of Col E1 DNA. Colicin producer (JC 411 thy^-) cells (gift of Dr. W.D. Rupp) were grown in M9-glucose medium to a density of 2-3 x 10^8 cells/ml ($A_{550} \approx 0.6$), a freshly prepared solution of chloramphenicol in ethanol was added to a final concentration of 170 μg/ml, and the culture was shaken for 20 hours at 37°C. The isolation procedure was a modification by Bastia[11] of that described by Clewell[12]. As an additional modification, the SDS supernatant was treated with pancreatic RNAse (20 μg/ml) and protease (200 μg/ml) for 1 hour at 37°C prior to equilibrium density gradient sedimentation.

Cleavage of DNA with Hae III. The Hae III digestion was carried out at 37°C, pH 7.5, in a buffer containing 6.6 mM Tris-HCl, 10 mM $MgCl_2$, 6.6 mM β-mercaptoethanol, 20 mM NaCl, and 100 μg/ml gelatin. Sufficient enzyme was used to give complete digestion in 15 hours, as determined in advance by gel electrophoresis of small-scale trial reaction mixtures. The digestion reaction was quenched by addition of EDTA to a final concentration of 10 mM, which was followed by sequential digestions first with pre-boiled pancreatic RNAse (20 μg/ml) and then with protease (~ 35 μg/ml), in each case for 1 hour

at 37°C. The mixture was dialyzed exhaustively against TE buffer (10 mM Tris, 1 mM EDTA, pH 7.8) containing 250 mM NaCl in preparation for loading on RPC-5 columns.

Preparation of columns and solutions. The separation of duplex fragments was carried out on a 0.9 x 105 cm Cheminert glass column (LC-9MA-4B) purchased from Laboratory Data Control. A 0.3 x 50 cm Altex microbore column (Anspec Company) was used for alkaline strand separation. A Milton Roy minipump, maximum pressure rating 1000 psi, was used at roughly 300-400 psi to obtain the indicated flow rates. An Altex UV monitor, equipped with their Biochemical detector, was used to follow the elution profile of all columns.

All column solutions were thoroughly degassed prior to use. TE buffer containing the indicated gradients of NaCl was used for separation of duplex fragments. Alkaline chromatography utilized 12 mM NaOH and the indicated gradients of KCl; it is our experience that NaCl and KCl can be used interchangeably on RPC-5 columns. Alkaline solutions were prepared and degassed immediately prior to use, and the stirred gradient flask was kept under an N_2 atmosphere, to avoid uptake of CO_2 and consequent pH drop. All columns were run at room temperature, approximately 20-25°C.

DNA samples for alkaline chromatography were first dialyzed against 0.25 M KCl, 0.5 x TE buffer, and made alkaline by direct addition of 1 M NaOH to a final pH of approximately 12.2. The denatured DNA was immediately loaded onto the microbore RPC-5 column, which previously had been equilibrated with 0.25 M KCl, 12 mM NaOH. The column was then washed with a few column volumes of equilibration buffer, during which some UV absorbing material eluted. Judging by UV spectra, this material is not nucleic acid. Blank runs on the dialysate buffer yielded the same peak, which we believe arises from an EDTA-metal complex.

A very shallow linear gradient of KCl (< 2 mM/ml) in 12 mM NaOH was needed to separate the complementary strands. A quick test was generally performed with a few μg of each new fragment investigated, using a steep salt gradient. This preliminary elution profile was then used to pick the correct boundaries for the gradient used in the preparative strand separation. After elution the peaks were pooled and neutralized immediately. Our yields were about 75-80% of the loaded DNA, with about 10-15% of the material eluting at the start of the gradient. We do not know the reason for this loss.

Gel electrophoresis. Five percent polyacrylamide slab gels (29:1,

acrylamide:bisacrylamide) were run according to Maniatis et al.[13] in their TBE buffer. Samples were prepared by overnight dialysis of 1-5 μg DNA against 0.5 x TE buffer, concentration to 100-300 μl by rotary evaporation, and ethanol precipitation in 2% sodium acetate and a two-fold added volume of 95% ethanol. The precipitate was dissolved in 10-25 μl of 0.1 x TBE, 10% glycerol, 0.05% bromophenol blue. Better resolution of the larger fragments was achieved on discontinuous acrylamide gels of 3.6% and 15% (20:1, acrylamide:bisacrylamide) in 40 mM Tris-acetate, 10 mM EDTA, pH 7.8. All gels were electrophoresed at 120-150 volts. Following staining with ethidium bromide solution, the visible ethidium fluorescence was photographed under UV excitation.

RESULTS

Fractionation of Col E1 - Hae III fragments. Colicin E1 plasmid (Col E1) is a closed circular DNA with a molecular weight[14] of 4.2×10^6. The restriction endonuclease Hae III cuts Col E1 DNA into 14 fragments, whose nomenclature[15] and size (determined by co-electrophoresis with Hae III fragments from the sequenced[16] DNA of ϕX174 bacteriophage) are listed in Table I.

Figure 1 shows the elution profile of a neutral pH RPC-5 column loaded

Table I. Colicin E1 DNA-Hae III Limit Digest.

Fragment[15]	Size/base pairs
A	1100[a]
B	1050[a]
C,D	910[a]
E,F	440[b]
G,H	400[b]
I	250[b]
J	172[b]
K	85[b]
L,M	70[b]
N	43[b,c]

[a]Size estimated from the fraction of Col E1 DNA in these bands[15] and the size of the smaller fragments.

[b]Size determined by co-electrophoresis with sequenced[16] Hae III fragments from ϕX174 DNA.

[c]Size determined by direct sequencing.[18]

with 7 mg of total Col E1 - Hae III digest and eluted with a 1600 ml linear gradient from 0.48 to 0.70 M NaCl. All of the smaller fragments (I-N) are well resolved, including the two 70 base pair fragments M and L (we have arbitrarily called the one eluted first M and the other L) which have identical electrophoretic mobilities. Also separated is fragment E (440 base pairs). Since our interest is mainly in the smaller fragments, we have not tried to resolve fragments A-H any further, but we believe that with shallower gradients some further fractionation may be achieved. Aliquots of pooled peaks were concentrated as described in Materials and Methods, and applied to a 3.6-15% discontinuous polyacrylamide gel. The high purity of each chromatographic peak is apparent from the gel patterns in Figure 2.

RPC-5 chromatography in alkaline medium. When we initially considered using RPC-5 for the separation of complementary strands we had to find a denaturant in which RPC-5 was stable. The first DNA denaturing agent tried was 1.1 mM methylmercuric acetate in the standard borate buffer[17]. Batchwise binding tests suggested the formation of a very tight DNA-$MeHg^+$ and RPC-5 complex: we were not able to elute the sample with even very high sodium sulfate concentrations.

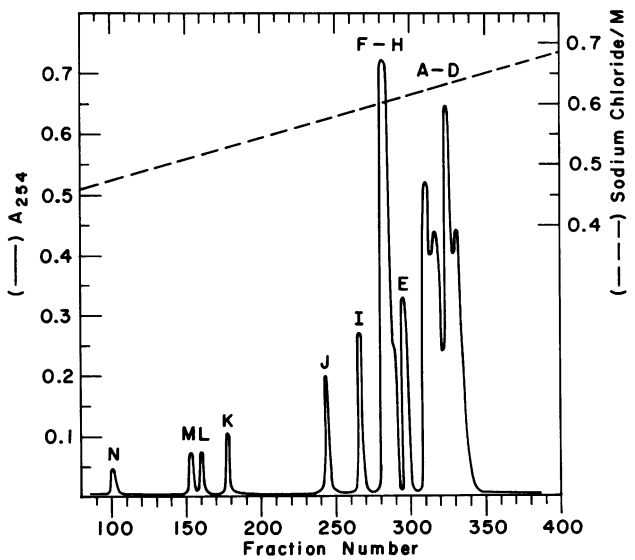

Figure 1. Fractionation of Hae III digest of Col E1 DNA by high pressure RPC-5 chromatography. Seven mg of the digest in 47 ml were loaded. The linear NaCl gradient (1600 ml) was from 0.48 M to 0.70 M in TE buffer, and the flow rate was 96 ml/hr at a pressure of about 350 psi. Four ml fractions were collected.

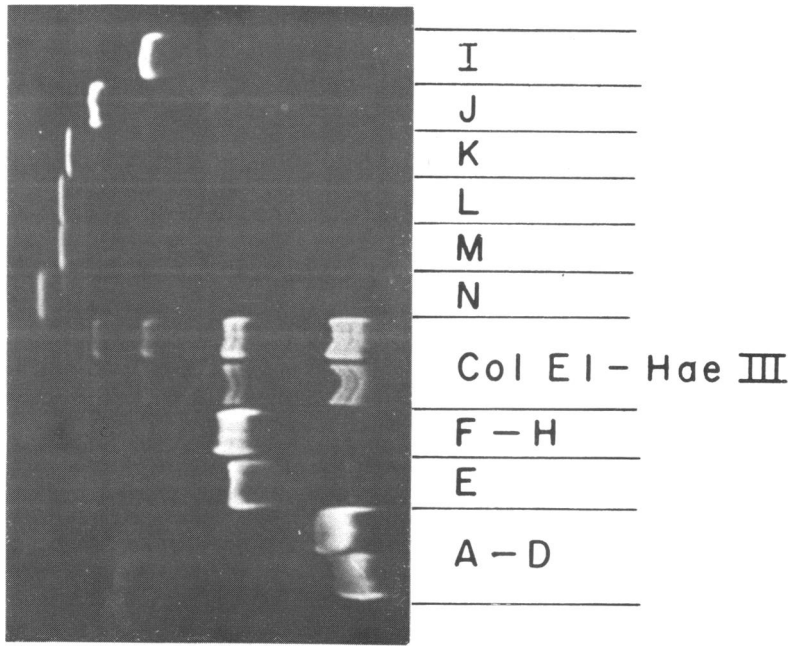

Figure 2. Discontinuous polyacrylamide slab gel (3.6-15%). Aliquots from peaks in Figure 1 were prepared for gel electrophoresis as described in Materials and Methods. Electrophoresis is from right to left.

Next we found that small DNA fragments bind to RPC-5 in 0.1 M NaOH, eluting in surprisingly low salt concentrations (vicinity of 1 M KCl). Reduction of the alkali concentration to 12 mM (pH = 12-12.1) retained the denatured state, but lowered the risk of alkali-catalyzed modification of the DNA sample.

Figure 3 shows the profiles for separation of the complementary strands of fragments a) I (250 base pairs), b) J (172 base pairs) and c) L (70_{late} base pairs). A photograph of a 5% polyacrylamide gel showing each strand and a renatured double stranded sample (annealed at 60° for 15 minutes) of I and J is shown in Figure 4. It is evident that the renatured fragments exhibit electrophoretic mobilities identical to those of the original fragments. We have also been able to separate the complementary strands of fragment E (440 base pairs).

The shaded sections of the elution profiles in Figure 3 show how the individual peaks were pooled. The choice was made to optimize yield, while keeping the estimated contamination of the complementary strand below 10%,

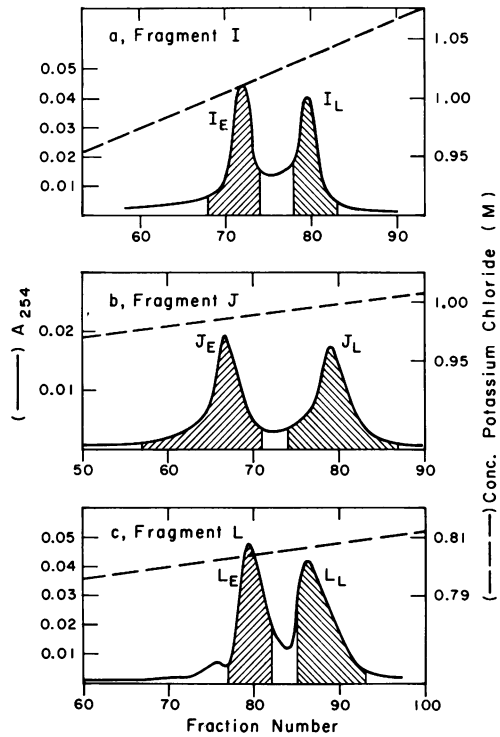

Figure 3. Strand separation by high pressure RPC-5 chromatography in alkali. Samples were denatured by the direct addition of 1N NaOH to a final pH of ~ 12.2. Linear gradients (150 ml) were run at the indicated salt concentrations in 12 mM NaOH. The flow rate was 20-25 ml/hr at an approximate pressure of 380 psi. Fractions of 1-1.5 ml were collected. The shaded areas were pooled immediately and neutralized by the addition of 1/100 (v/v) of 100 x TE and 1/125 (v/v) of 1N HCl. The subscripts E and L (for early and late) are used to represent the order of elution of the complementary strands of any fragment.

as judged by the separation from the adjacent elution peak. The gel photograph in Figure 4 shows that detectable amounts of duplex result from contamination by the other strand, but the apparent amount of this is greatly exaggerated by the much higher sensitivity of duplex than single strand for the ethidium stain. Because of the large difference in electrophoretic mobility between duplex and single strands demonstrated in Figure 4, it is a simple matter to remove the contaminating strand by preparative gel electrophoresis of a single strand sample which has been returned to neutral pH and annealed to convert all of the contaminating complementary strand to duplex.

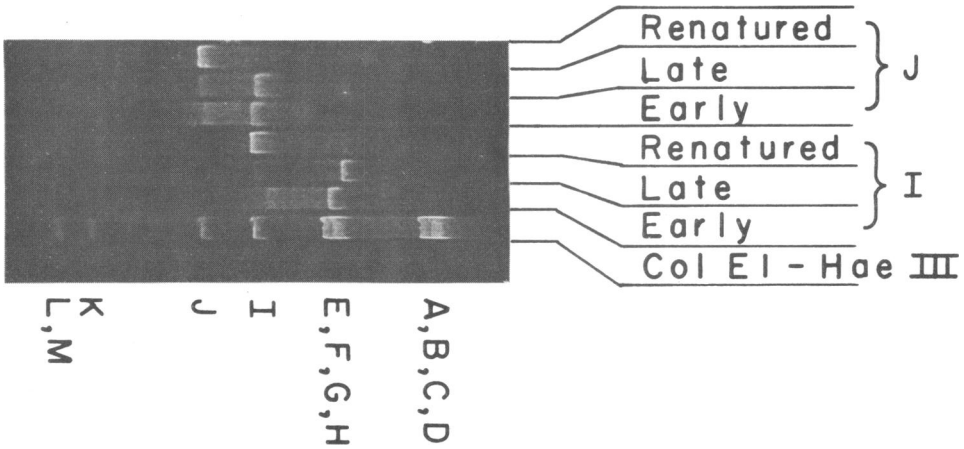

Figure 4. Five percent polyacrylamide slab gel in TBE. Equal amounts of any 2 complementary strands were mixed and annealed at 60° for 15 minutes. Aliquots from the renatured sample as well as the separated strands were prepared for gel electrophoresis as described in Materials and Methods. Electrophoresis is from right to left.

DISCUSSION

Our results demonstrate a new chromatographic system for separation of the complementary strands of DNA fragments on a preparative scale. We found no loss of resolution for fragments as large as 440 base pairs, but we did not test larger fragments because we had no purified duplex samples longer than 440 base pairs. We have loaded as much as 150 μg of fragment J on a 0.3 x 50 cm column without any loss of resolution, from which we expect that use of larger columns will permit strand separation to be carried out on mg quantities, assuming that such amounts of purified duplex are available.

A major limitation of RPC-5 chromatography in both neutral and alkaline solutions lies in the fact that the basis of separation is not well understood. This lack of knowledge makes it difficult to predict the resolution one may expect in the separation of closely sized double stranded fragments, and also in the strand separation for any new fragment. As can be seen by comparison of the strand separation profiles of fragments J and L (Figures 3b and 3c), the resolution varies from one fragment to another, possibly depending on base composition, or the presence of specific sequences, or both. Aside from these limitations, RPC-5 chromatography in alkaline as

well as neutral conditions presents a new and promising method for the rapid isolation of single and double stranded DNA fragments for use in biochemical and biophysical studies.

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