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Preparative fractionation of DNA restriction fragments by high pressure column chromatography on RPC-5

Arthur Landy,[1] Carl Foeller,[1] Rosalie Reszelbach,[2,3] and Bernard Dudock[2]

[1]Division of Biology and Medicine, Brown University, Providence, RI 02912, USA

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ABSTRACT

High pressure liquid chromatography on the RPC-5 reversed-phase ion exchange system has been shown to have several potential applications as an initial high capacity step in the isolation of specific DNA restriction fragments. The fractionation of a Hinc II digest of λ DNA, which contains 35 fragments with "flush ends" ranging in size from 3×10^6 to 7×10^4 daltons, has been used as a model system. Under certain conditions there are some restriction fragments whose elution relative to other fragments is different on RPC-5 chromatography than it is on gel electrophoresis. In some special circumstances it is possible to obtain satisfactory yields (60-70%) of a pure restriction fragment after a single passage through an RPC-5 column.

INTRODUCTION

The combination of type II restriction enzymes and the fractionation of DNA digestion products by gel electrophoresis has rendered the mapping and analysis of specific DNA regions a routine procedure (for a recent review, see ref. 1). There are however certain situations in which a supplementary high capacity procedure for fractionating DNA restriction fragments would be of great use. The two most likely applications for such a procedure would be in the isolation of very large quantities of a fragment for biophysical studies, or in certain studies with complex eukaryote DNAs that require starting with very large amounts of DNA. An even wider range of applications would exist if the supplementary procedure also had fractionation properties which were different from, or at least not identical to, those of gel electrophoresis.

Although there are a number of high capacity methods that have been applied to the fractionation of DNA (e.g., see ref. 2 for a review, and also 3 and 4), we felt that it

would be useful to further explore this problem with special reference to the problem of isolating restriction fragments. We report here experiments which show that the RPC-5 reversed-phase ion exchange column chromatography system developed by Pearson et al.[5] has considerable potential in the large scale fractionation of restriction fragments. These columns, which were originally developed for the separation of individual tRNA species, consist of an inert resin support (polychlorotrifluoroethylene) coated with a C_8-C_{10} trialkylmethylammonium chloride adsorbent called Adogen 464. A general discussion of both the practical and theoretical aspects of this system in relation to the fractionation of nucleic acids can be found in refs 6 and 7.

For tRNA molecules there are many features, such as modified bases and unusual conformations, which may enhance the chromatographic resolution of different species. For DNA it is not clear what features other than total charge are relevant. However as will be shown in this report, under certain conditions there are some restriction fragments whose elution relative to other fragments is different on RPC-5 chromatography than it is on gel electrophoresis. This feature of the RPC-5 system should extend the number of situations in which it can be profitably employed as an initial high capacity step in the isolation of specific DNA restriction fragments.

MATERIALS

Bacteriophage λ was prepared from the heat-inducible lysogen C190/λcI857 S7 and the DNA extracted as described previously.[8]

The restriction endonuclease Hinc II was prepared as described previously.[8] This enzyme produces fragments with flush ends and has the same substrate recognition site as Hind II $\overset{GTPy\downarrow PuAC}{CAPu\downarrow PyTG}$.[8,9] The strain H. influenzae Rc (obtained from Grace Leidy) is the most convenient source for this restriction activity since in the H. influenzae strain Rd it must be separated from the Hind III enzyme which produces staggered cuts at the sequence $\overset{A\downarrow AGCTT}{TTCGA\uparrow A}$.[10] The restriction endonuclease Eco RI which produces staggered ends at the

sequence $\overset{\downarrow}{G}AATTC$ 11 $CTTAA\overset{\uparrow}{G}$ was prepared from E. coli strain RY13 (obtained from Herb Boyer) according to the procedure of Greene et al.[12]

Plaskon (polychlorotrifluoroethylene) was obtained from Allied Chemical Corp., and Adogen 464 (C_8-C_{10} trialkylmethylammonium chloride) was obtained from Ashland Chemical Co. Stainless steel chromatography columns were made as described previously[13] and Milton Roy pumps with a maximum pressure rating of 1000 lb/inch^2 were used to obtain the indicated flow rates.

METHODS

The Hinc II digests were carried out in 6.6 mM Tris (7.9), 6.6 mM β-Mercaptoethanol, 6.6 mM $MgCl_2$, 60 mM NaCl at 37°C.

The DNA concentration was approximately 300 µg/ml and the amount of enzyme used was determined for each DNA preparation by titrating for a complete digestion. Similar titrations were done for the Eco RI digests which were carried out in 100 mM Tris (7.5), 50 mM NaCl, 5 mM $MgCl_2$, 0.0005% gelatin. The digestions were terminated by the addition of EDTA to a concentration of 0.02 M. The solutions were brought to a final concentration of 0.1% Sarkosyl NL97 (Geigy), 50 mM Tris (7.9), 200 mM NaCl and were then extracted with one-half volume of water-saturated phenol. The DNA was precipitated with two volumes of cold 95% ethanol and after centrifugation at 23,300 g for 15 min at -10°C, the pellets were washed once with cold 75% ethanol plus 0.2 M NaCl. The precipitates were thoroughly dried, dissolved in 10 mM Tris (7.9) and the OD_{260} taken to determine the final DNA concentration.

The Plaskon was coated with Adogen according to method C of Pearson et al.[5] The columns (0.2-0.4 mm x 100 cm) were packed as described previously and were reused several times without any loss of resolution.[13] (All column buffers contained 1 mM $Na_2S_2O_3$--a carryover from the tRNA column buffers and not really necessary for DNA restriction fragments.) The indicated amount of DNA sample for each column was dissolved in 0.5 ml of the starting buffer and layered on top of the column bed. Concave salt gradients were generated by using two buffer chambers of different diameters.[13] The concentration of standard

components in the column buffers, i.e. Tris (7.6), Na-acetate (4.5), $MgCl_2$ or EDTA, as indicated in the figure legends, was always 10 mM. The columns were run with flow rates of 0.4-1.0 ml/min at pressures of 200-400 psi. One ml fractions were collected and the OD_{260} for each fraction measured. Recoveries from the columns were 70-90% of the OD_{260} input.

Polyacrylamide gel electrophoresis in EC model 470 slab gels with circulating coolant, and staining of the gels with "Stains-all" was carried out as described previously.[8] Several different gel conditions were used to obtain optimal resolution of different fragment sizes. The 2% gels contained 1/20 and the 5% gels contained 1/30 N,N'-methylenebisacrylamide. In addition, the 2% gels contained 0.5% agarose. All gels were run with the circulating coolant at 2°C (unless indicated otherwise) for the voltages and times specified.

Photographs of the stained gels were analyzed as described below to facilitate a graphic comparison of the different column fractions and the different columns. Within a single lane the relative amount of material in each fragment was estimated by eye and this figure was converted to relative numbers of moles by dividing by the molecular weight of the fragment. The "relative mole percent" was calculated by: (moles of a particular fragment/total moles of all fragments in that lane) x 100. In those cases where two or three fragments were not resolved in the test gels, they were treated as a single fragment of intermediate molecular weight. In the schematic conceptions (Figs. 2, 4, and 6), these unresolved fragments have been indicated by a tent under the bar.

RESULTS AND DISCUSSION

To investigate the potential of the RPC-5 reversed-phase ion exchange high pressure liquid chromatography system as a preparative fractionation procedure for DNA restriction fragments a Hinc II digest of λ DNA was used as the test system. This particular digest was chosen for several reasons. First of all, it yields a wide range of fragment sizes, from approximately 3×10^6 to 7×10^4 daltons (see Table 1). Second, the digest is quite complex (e.g., see Figure 1) and therefore

Table I

Molecular Weight Ranges for Groups of Fragments in the λ Hinc II Digestion Profile

Fragment Group	Size Range (daltons x 10^{-5})
A	29.1 - 17.8
B	13.8 - 9.76
C	8.65 - 7.15
D	5.54 - 4.37
E	3.33 - 2.62
F	2.34 - 1.88
G	1.32 - 1.20

provides a severe and informative test of the capabilities of the chromatography system. A final attractive feature of this digest, although it is not critical to the experiments reported here, was the fact that we had been mapping these DNA fragments with respect to each other and with respect to known markers on the λ genome (Robinson and Landy, in preparation). The mapping experiments were actually carried out on Hind II+III digests of λ DNA. However, in order to avoid the complication of some fragments having the protruding 5' termini which are generated by the Hind III cleavages,[10] the experiments reported here were carried out using Hinc II which has the same cleavage specificity as Hind II.[8]

To facilitate cross reference between the results reported here and the mapping information for these fragments, we have retained the nomenclature of the Hind II+III digestion profiles. There are 10 restriction fragments in the Hind II+III digestion profile which have either one or both ends cut by Hind III and hence are missing from the Hinc II digestion profile. These fragments account for the discontinuities in the Hinc II nomenclature at A2, B4, B8, C1, C3, C4, D1, E4, F3, G3, and G4. These "missing" Hind II+III fragments appear in the Hinc II digest as a part of some larger fragments which are of course not found in the Hind II+III profile. These Hinc II-unique fragments have been named from the next largest Hind II+III fragments by affixing a small letter suffix: A1a, A1b, B7a, B9a, C3a, and E2a.

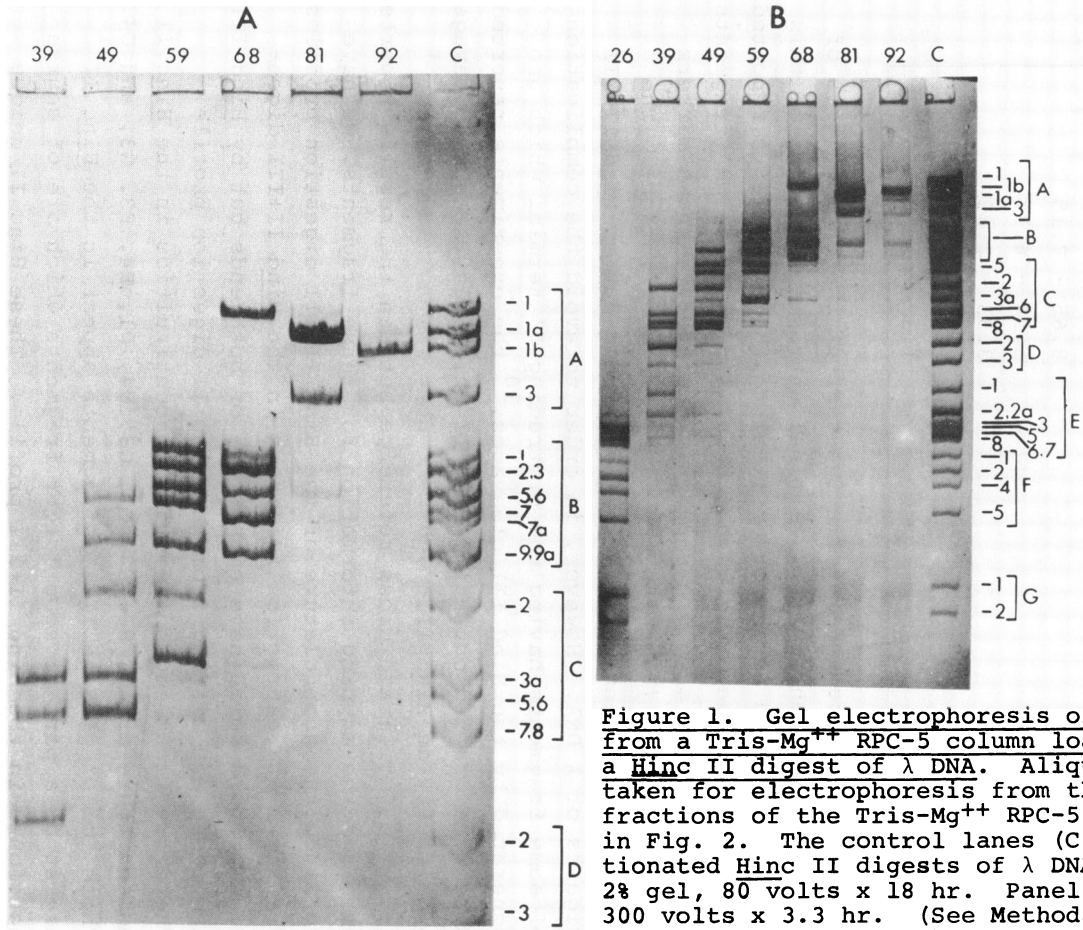

Figure 1. Gel electrophoresis of fractions from a Tris-Mg^{++} RPC-5 column loaded with a Hinc II digest of λ DNA. Aliquots were taken for electrophoresis from the indicated fractions of the Tris-Mg^{++} RPC-5 column shown in Fig. 2. The control lanes (C) are unfractionated Hinc II digests of λ DNA. Panel A: 2% gel, 80 volts x 18 hr. Panel B: 5% gel, 300 volts x 3.3 hr. (See Methods for details of gel electrophoresis).

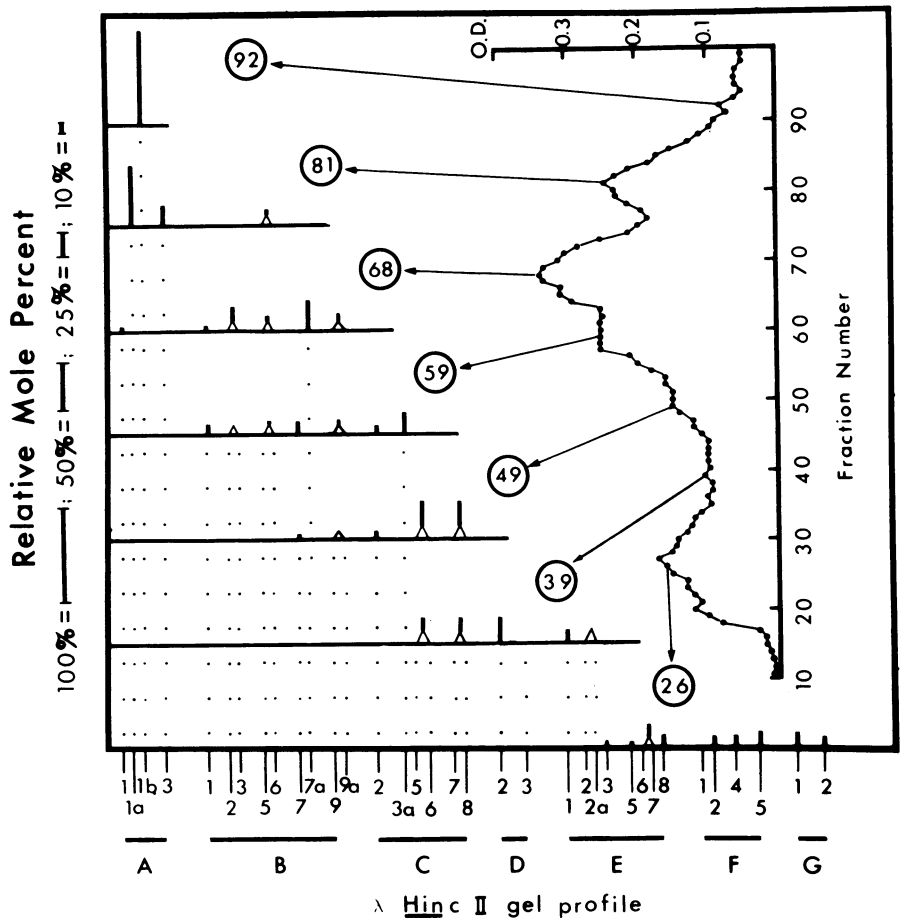

Figure 2. Elution profile of a Tris-Mg^{++} RPC-5 column and a schematic comparison of the distribution of λ Hinc II restriction fragments in each of the indicated column fractions. The RPC-5 column was prepared and loaded with 600 μg of a Hinc II digest of λ DNA as described in Methods. The concave NaCl gradient was from 0.57 M (90 ml) to 0.65 M (60 ml), and the flow rate was 0.4 ml/min. Tris (7.6) and $MgCl_2$ were present in all buffers at 10 mM. Each horizontal panel corresponds to the indicated column fraction. The calculated relative mole percent for the restriction fragments in each fraction is based upon visual estimates of the band intensities from a single lane of the gels shown in Fig. 1. (See Methods.) The scale for the relative mole percent values is given along the ordinate.

The size ranges for each of the seven "groups" of fragments in the Hinc II λ DNA digestion profile are shown in Table 1. When the electrophoresis conditions are altered, in order to optimize the resolution of different size fragments, the shape of the digestion profile is also altered. Under certain conditions the relative shifts in electrophoretic mobility of some fragments are sufficiently extreme as to result in an inversion of their positions in the profile--nevertheless, the fragments still retain their "canonical" designations. Experiments describing these electrophoretic shifts will be reported elsewhere (Marini, Robinson, and Landy, in preparation).

The gel profiles generated by running aliquots of individual fractions from a Tris-Mg^{++} RPC-5 column (see Methods) are shown in Fig. 1 alongside unfractionated control Hinc II digests of λ DNA. The most striking feature of the column fractionation is that in general the overall order of elution of restriction fragments is the same as their order of migration in gel electrophoresis. This of course is not unexpected since the ion-exchange properties of the column should retard the fragments in proportion to their total negative charge. Closer examination of the gel profiles, however, reveals that this correspondence between gel position and elution position does not hold true for all of the restriction fragments. As will be discussed further below, some fragments are dramatically shifted from their "expected" elution position.

In comparing the gel profiles of the different column fractions, it must be kept in mind that the different gel lanes do not necessarily contain the same weight of DNA or the same number of mole equivalents of intact λ DNA. Optimum resolution and detection of the largest fragments in the profile requires "underloading" of the gels, whereas analysis of the smallest fragments in the profile calls for "overloading." Furthermore, in analyzing a column fraction which contains only 1 or 2 fragments (e.g., fraction 92 in panel A of Fig. 1), it is necessary to load the gel with less weight of DNA than in the case of a column fraction which

contains a larger number of fragments (e.g., fraction 59 in Panel A of Fig. 1).

To facilitate the comparison of different fragments in a single column or between different columns, a schematic representation of the composition of each column fraction has been used. The parameter called "relative mole percent" is the number of moles of a restriction fragment in a single column fraction relative to the total number of moles of all restriction fragments in that column fraction (see Methods). This schematic representation of the data from Fig. 1 is shown in Fig. 2 along with RPC-5 column elution profile. Each horizontal panel in Fig. 2 shows the relative distribution of the total moles of restriction fragments present in the indicated column fraction.

This type of data summary does not depend upon an analysis of every column fraction and is not influenced by differences in the recoveries from one column fraction to the next. Most important however is the fact that it facilitates the comparison of column fractions which have been analyzed under different gel conditions and with different amounts of material. In the experiments reported here, we have used this type of data summary only as an approximation of the composition of each column fraction. If a column fraction contained only a single restriction fragment, the relative mole percent for that fragment would be 100%. If a column fraction contained 35 fragments in equal molar amounts, the relative mole percent for each fragment would be 3%--that is the relative mole percent for each fragment in an unfractionated Hinc II digest of λ DNA.

In any given column fraction most of the Hinc II λ restriction fragments are absent, i.e., have a relative mole percent which is very much smaller than 3%. Because of the limitations on the scale of the schematic diagrams in Figs. 2, 4, and 6, no fragments present at less than a relative mole percent of 4% could be plotted. However, careful examination of the gels shows that this is not a serious limitation, since none of the RPC-5 columns yielded a profile

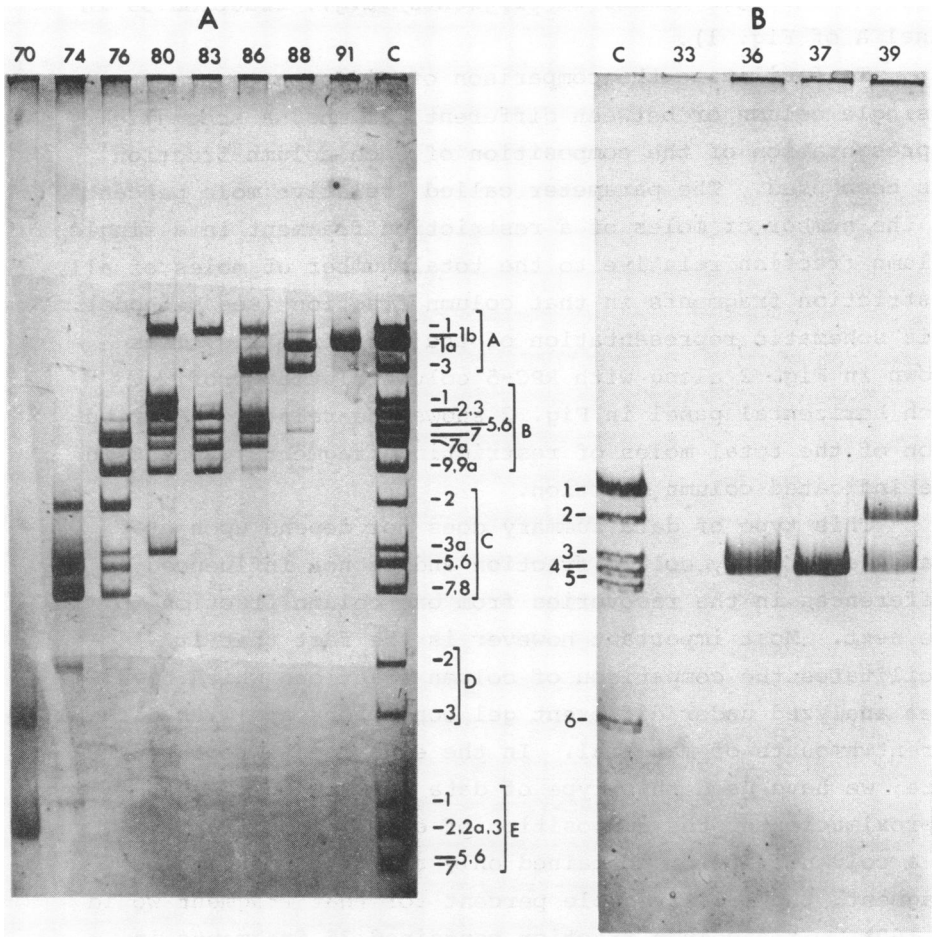

Figure 3. Gel electrophoresis of fractions from two different RPC-5 columns. Panel A: Aliquots were taken for electrophoresis from the indicated fractions of the Acetate-Mg^{++} RPC-5 column shown in Fig. 4. This 2% gel was electrophoresed at 200 volts for 5 hr. as described in Methods. Panel B: Samples were taken from the indicated fractions of a Tris-Mg^{++} RPC-5 column which had been loaded with 200 µg of an *Eco* RI digest of λ DNA. The NaCl gradient was from 0.57 M (90 ml) to 0.65 M (60 ml) and the flow rate was 1 ml/min. This 2% gel was electrophoresed at 60 volts for 43 hours with the circulating coolant at 20°C. The control lane (C) is an unfractionated *Eco* RI digest.

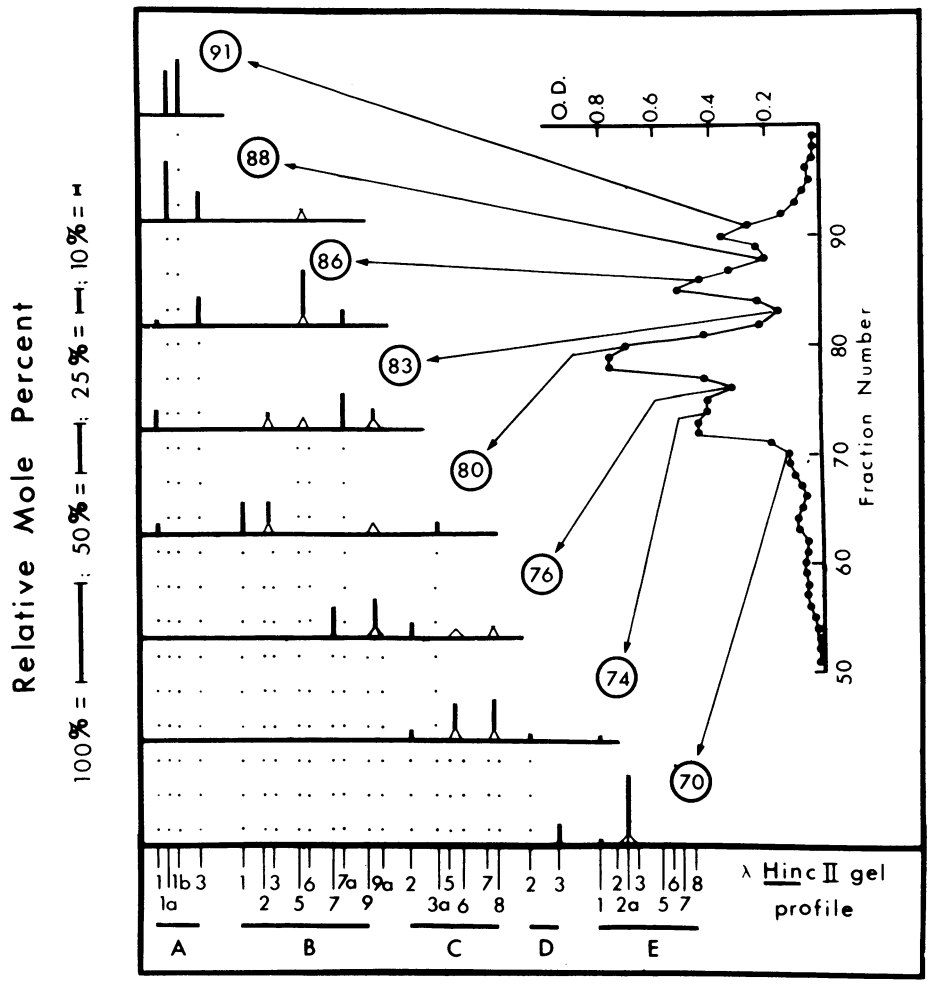

Figure 4. Elution profile of an Acetate-Mg^{++} RPC-5 column and a schematic comparison of the distribution of λ Hinc II restriction fragments in each of the indicated column fractions. The RPC-5 column was prepared and loaded with 550 µg of a Hinc II digest of λ DNA as described in Methods. The concave NaCl gradient was from 0.56 M (90 ml) to 0.67 M (60 ml) and the flow rate was 0.9 ml/min. Sodium acetate (4.5) and $MgCl_2$ were present in all buffers at 10 mM. The calculated relative mole percent for the restriction fragments in each fraction is based upon estimates of the band intensities from a single lane of the gel shown in Panel A of Fig. 3. The scale for the relative mole percent values is given along the ordinate.

in which a large number of bands were present in low molar amounts--as would be the case if all or a large portion of the digest eluted from the column in a continuous broad smear.

When the RPC-5 column is run at pH 4.5 with Na-acetate buffer instead of Tris at pH 7.6, there is no significant change in the relative order of elution of the restriction fragments although there is an alteration in the general shape of the OD_{260} profile (see Figs. 3 and 4). The order of elution of the restriction fragments is also unaltered if the Mg^{++} is replaced by EDTA in the column buffer. However, there does appear to be an improvement in the recovery of the smallest fragments under these conditions (see Figs. 5 and 6).

Although there is a general correlation between gel position and elution position for most of the restriction fragments, this pattern is not adhered to equally by all of them. One of the most striking discrepancies between the relative positions in the column eluate and the gel profile is for the largest fragments, the A group. Fragment A1, which is the slowest moving fragment in a 2% high or low voltage gel (see Methods for gel conditions) elutes ahead of all the other A fragments on the RPC-5 column (see Figs. 4 and 6). Fragment A1a, which on a 2% low voltage gel runs slower than A1b, elutes in front of A1b on the RPC-5 column. This is seen equally well in any of the RPC-5 columns. It should be pointed out that the three fragments in the A group are among a class of fragments which show specific shifts in their relative electrophoretic mobilities under different gel conditions (to be published elsewhere). We do not know precisely which properties of these restriction fragments are responsible for their unusual electrophoretic behaviour or whether they are even the same properties which are responsible for the relative migration differences on RPC-5 and gel electrophoresis. Thus far there is not a simple relationship between the two sets of unusual behaviour for these fragments, and not all fragments which are unusual in one situation are necessarily unusual in the other.

The "deviant" elution pattern is not a property confined

to the large fragments. It is also observed for smaller fragments such as C3a, which elutes from the RPC-5 column later than "expected" on the basis of its relative position in the gel electrophoresis profile (this is best seen in the two low pH columns shown in Figs. 4 and 6).

Another example of a "late" eluting fragment is B7a. In addition to showing the property of "late" elution, this fragment provides a good example of how the RPC-5 column fractionation can be used to complement the fractionation which is obtained by gel electrophoresis. The two fragments, B7 and B7a, migrate in gels so close together that it is very difficult to separate them on preparative scale gels. On the RPC-5 columns, however, B7 and B7a are surprisingly well resolved (this is most pronounced in the two low pH columns shown in Figs. 4 and 6).

A possibly similar and even more dramatic situation is seen with fragments B5 and B6. These two fragments run together as a double band on all types of gels tested thus far. The only way they have been "resolved" is in the course of mapping experiments with λ deletion mutants which are missing one of the two fragments. Because B5 and B6 are not resolved in the test gels they have been plotted as a single fragment in the schematic diagrams (see Methods). It can be seen that in the low pH EDTA column the B5,6 band is resolved into two components (see Fig. 6). Fractions 66, 68, and 71 contain one of the components; nothing is found in fractions 74 and 76, and in fractions 81 and 84 the second component appears. The simplest interpretation of this elution pattern is that B5 and B6 have been separated on the RPC-5 column. To determine which component is B5 and which is B6 would require comparisons of the restriction digestion profiles of those λ deletion mutants which were used to identify and map B5 and B6 (Robinson and Landy, in preparation). This interpretation is consistent with the fact that we have never seen what we know to be a single restriction fragment elute in two different places on the RPC-5 columns. A very similar example from the same RPC-5 column, of two restriction fragments which are better resolved on the column than on the test gels

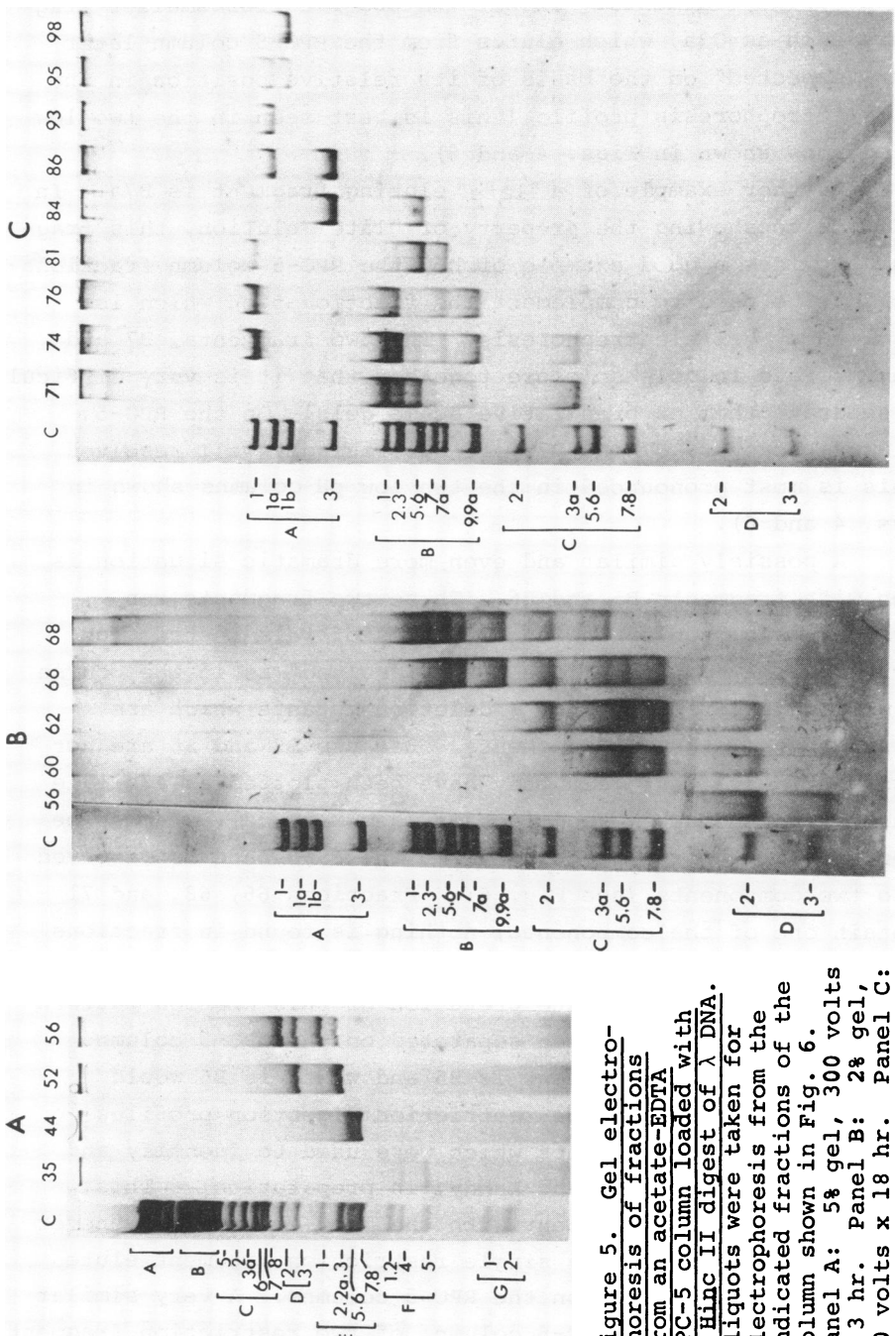

Figure 5. Gel electrophoresis of fractions from an acetate-EDTA RPC-5 column loaded with a Hinc II digest of λ DNA. Aliquots were taken for electrophoresis from the indicated fractions of the column shown in Fig. 6. Panel A: 5% gel, 300 volts x 3 hr. Panel B: 2% gel, 80 volts x 18 hr. Panel C: 2% gel, 80 volts x 18 hr.

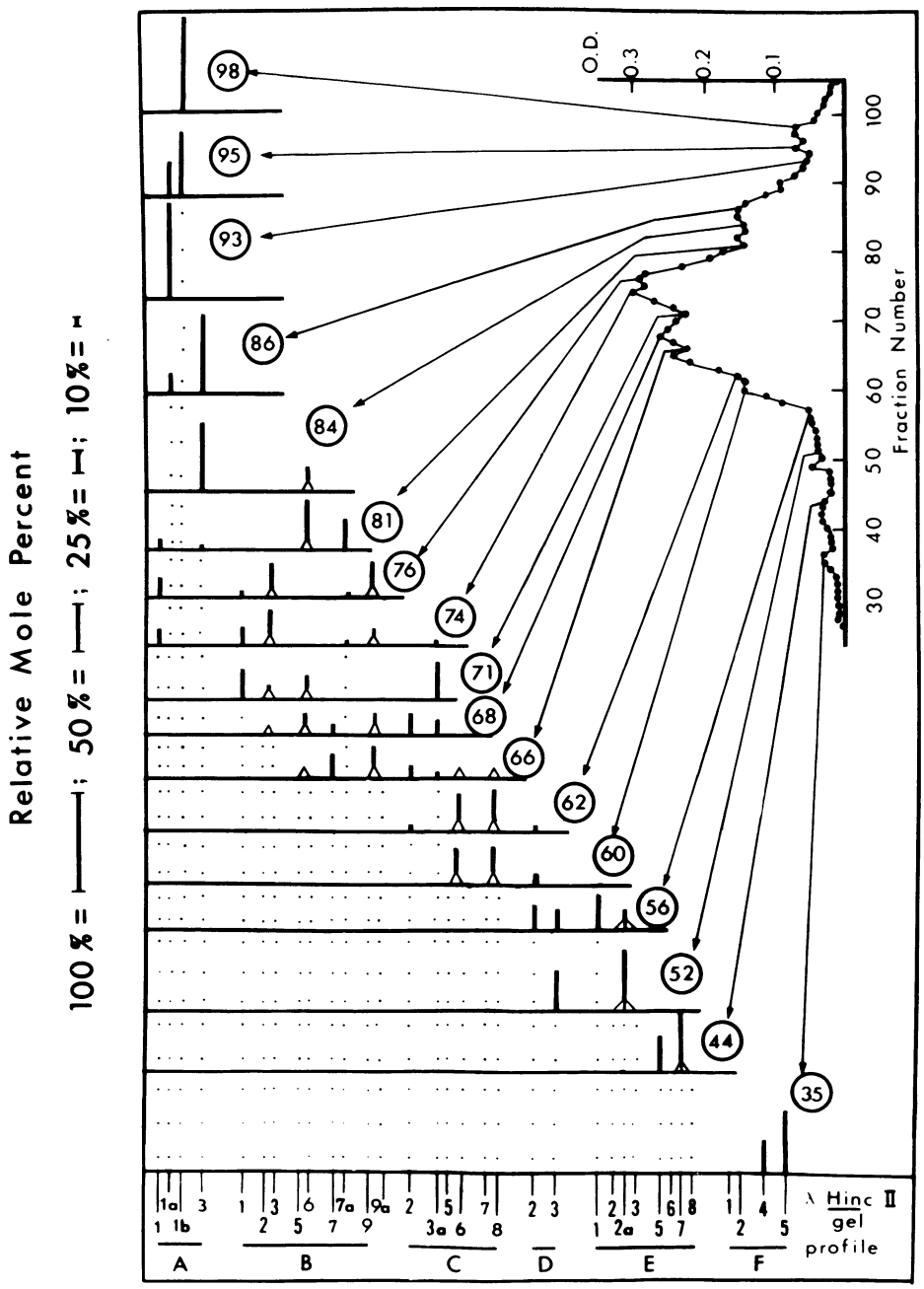

Figure 6. Acetate-EDTA RPC-5 column. The column was loaded with 650 µg of a λ Hinc II digest and eluted at a flow rate of 0.7 ml/min. with a NaCl gradient from 0.62 M (90 ml) to 0.72 M (60 ml) (containing Na-acetate (4.5) and EDTA at 10 mM). (See Fig.4.)

are fragments B9 and B9a. One of the two elutes in fractions 66 and 68; nothing is found in fraction 71, and the other fragment elutes in fractions 74 and 76.

In the practical application of these columns to fractionate large quantities of restriction fragment digests, the extent to which recovery will be sacrificed to increase purity is of course dependent on the ultimate requirements of the system and the nature of any second step purification which will be used. Although we have found the RPC-5 column chromatography system to be superior to any of the several other column systems we have tested for fractionating DNA restriction fragments, it cannot be considered a high resolution technique. Rather, its potential usefulness is as a preparative procedure whose capacity is limited only by the size of the column. Although we have not specifically tested the capacity of our columns for DNA fragments, the capacity of this size column (see Materials) for fractionating tRNAs is on the order of 5-10 mgs.

At its present stage of development the most common application of the RPC-5 system will probably be as the first high capacity step in a two (or more) step purification scheme. The most effective way in which it can be applied is in a situation where a large amount of DNA from one restriction digest is fractionated on the column. The appropriate fractions are pooled and then digested with a second enzyme. Having greatly reduced the total amount of DNA to be handled, the second digest can be fractionated by gel electrophoresis. With such a scheme the quantity of starting DNA is no longer a practical limitation. Another present application of the RPC-5 column is to those special instances where a fragment which must be isolated in large quantities has a sufficiently unique behaviour, under certain column conditions, to be isolated free of other relevant contaminants in a digest. One example of such a fragment is shown in Panel B of Fig. 3. When an Eco RI digest of λ DNA is fractionated on a Tris-Mg^{++} RPC-5 column, the Eco RI fragment 4 elutes ahead of all the others and can be obtained virtually pure with approximately 70-80% recovery.

Further work with the RPC-5 system will hopefully increase its usefulness and extend the areas in which it can be profitably employed. Two areas which should receive attention are the application of these columns to the isolation of satellite DNA; and their use in conjunction with a number of highly base pair-specific DNA ligands such as some of the phenazinium dyes. [4]

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[2]Department of Biochemistry, SUNY at Stony Brook, Stony Brook, NY 11794, USA

[3]Present address: Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20014, USA

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Note added in proof:

We have just learned of similar experiments by Hardies and Wells designed to purify preparative quantities of a restriction fragment from λplac DNA, by RPC-5 chromatography.