Journal of Chromatography, 336 (1984) 3—14
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2290

REVIEW

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF DNA

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CONTENTS

1. INTRODUCTION

The science community has witnessed a veritable explosion in our knowledge of many facets of DNA in the past eight years. Enormous advances have been made in our understanding of DNA structure and DNA—protein interactions, organization and expression as well as developmental regulation and gene transfer. These developments may be attributed to the establishment of innovative techniques in four areas: gene cloning, restriction enzymology, DNA sequencing, and high-performance liquid chromatography (HPLC) of restriction fragments. Each of the first three techniques will be reviewed briefly followed by a discussion of HPLC of DNA. The first three techniques are of such importance that Nobel Prizes have been awarded to the discoverers in recent years.

0378-4347/84/$03.00

1.1. Gene cloning

The development of gene cloning has been of paramount importance for our capacity to focus scientific attention of certain DNA segments for biochemical and genetic studies. The impact of gene cloning on present and future studies in the molecular biology of gene expression may be likened to the impact of chromatography on chemistry, biology, and physics at approximately the turn of the century. Simply stated, gene cloning is the process of inserting any given DNA segment (for example, from plant, viral, bacterial, or mammalian source) into a suitable vector which can be readily replicated and manipulated by genetic and biochemical techniques [1—5]. In general, investigators have used procaryotic cells as hosts although animal cells are used in some cases. Owing to the extreme power of cloning, it is possible to identify in pure form one given DNA fragment out of a population of many thousands of contaminating fragments. One requirement for successful gene cloning is a method of identification of the desired fragment. In general, this is accomplished by hybridization analyses, immunological identification of gene products, biochemical or genetic functions, etc.

It has been stated that virtually any DNA segment can be cloned today as long as the investigator has a procedure for identifying it. Virtually every leading biological journal today is comprised of a number of papers which embody studies on cloned molecules. Excellent reviews exist on the procedures involved [1—5] as well as on specific systems which have been cloned. A very brief listing of some of the thousands of gene systems which have been cloned are the following: DNA segments which undergo conformation transitions, bacteriophage promoters, animal tumor virus (SV40 and polyoma) promoters, ribosomal RNA genes, heat shock genes from monkey cells, promoters and hormone regulatory sequences in mouse mammary tumor viruses, immunoglobin genes, genes involved in recombination, nitrogen fixation genes from *Klebsiella*, cytochrome genes, growth hormone genes, human interferon genes, regulatory genes from *Dictyostelium*, genes for human lymphokines, histone genes, animal cell metallothionein genes, and human and murine antigen genes, just to name a few. Excellent reviews on these subjects exist [1, 2].

1.2. Restriction endonucleases

The second major development which has facilitated great strides in our recent understanding of DNA is the isolation and characterization of type II restriction endonuclease [6—9]. More than 210 restriction endonucleases have been purified and their recognition sites identified. These enzymes provide precise scalpels for dissecting DNA at specific base pair sequences. These tools are important for mapping sequences relative to each other and thereby aligning biochemical and genetic functions. Furthermore, these enzymes have been invaluable for DNA sequencing and gene cloning studies. Restriction enzymes have made possible experiments that were not even dreamed about just a few years ago.

Restriction endonucleases generally cleave base pair sequences that contain two-fold axes of symmetry. The types of ends which are produced are blunt ends (no nucleotide overhangs), two, three, or four nucleotide sticky ends. DNA fragments can be readily separated on the basis of length by gel electro-

phoresis. Even small differences (1—3%) in length between related DNA molecules can be readily detected by this procedure. The electrophoretic mobility of a DNA fragment is inversely proportional to the logarithm of the number of base pairs up to a certain limit. Whereas these gels have extremely high resolving power and sensitivity (a few micrograms), it is difficult to purify quantities of DNA fragments by this procedure (see HPLC description below).

1.3. Sequence determinations

DNA sequencing studies are routinely performed in a large number of laboratories by basically two procedures: the Maxam—Gilbert chemical cleavage method or the Sanger 2′,3′-dideoxy DNA polymerase method [6]. By either of these procedures, it is possible to routinely sequence approx. 200 base pairs per day per investigator. The largest intact genomes which have been sequenced to date include human mitochondrial DNA [10], bacteriophage lambda DNA [11], and adenovirus DNA [12]. The total number of base pairs known at the present time from all systems is over $2 \cdot 10^6$ and the number continues to rise at a rate of about $1 \cdot 10^6$ base pairs per year. The capacity of the DNA biochemists to readily establish the base pair sequence of

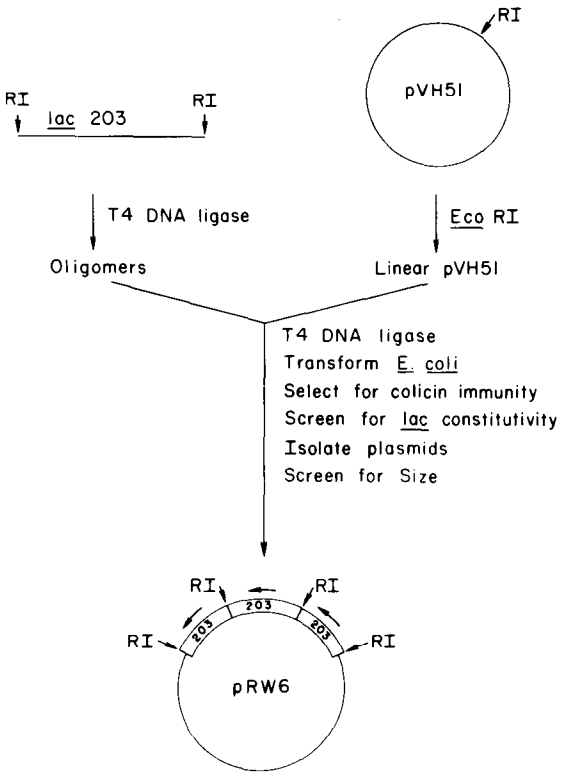

Fig. 1. Cloning of multiple inserts into vector. Multiple copies of the 203-bp fragment containing the *Escherichia coli* lactose operator and promoter were ligated to form oligomers. These oligomers were then ligated into the *Eco* RI site of pVH51. After suitable transformation and screening, it was possible to characterize a recombinant plasmid containing three copies of the 203-bp insert. All fragments are oriented in the same direction. For further details, see ref. 18.

a genome under study has transformed molecular genetics from the less precise field of genetics to the area of well characterized biochemical molecules. Today, a large number of DNAs are as precisely defined chemically as small peptides or steroids.

1.4. HPLC: interrelationship with other techniques

The fourth important technique is HPLC of DNA. This technique was originally developed in order to purify quantities of restriction fragments in order to perform spectroscopic and biochemical studies to evaluate the role of DNA structure in gene regulation [13]. Purifications were originally performed on RPC-5 [14—16], but this support has now been effectively replaced with NACS (Nucleic Acid Chromatography System, Bethesda Research Labs. trade name). These procedures have been invaluable for obtaining milligram quantities of restriction fragments for studying conformational features such as right-handed to left-handed structural transitions [17]. The most effective overall schemes for fragment purification and characterization involve the combined use of all four techniques (gene cloning, restriction enzymology, DNA sequencing, HPLC). Fig. 1 shows a general cloning scheme for a 203-bp (base pair) fragment containing a promoter and an operator for the *Escherichia coli* lactose gene. It is possible to clone multiple copies of this fragment, thus providing a higher yield of insert fragment for a given amount of plasmid. Fermentation technology is used [18, 19] to prepare pound quantities of cells containing the recombinant DNA. Procedures have been devised [18, 19] for isolating gram quantities of pure plasmid DNA from these cells. Thus, it is

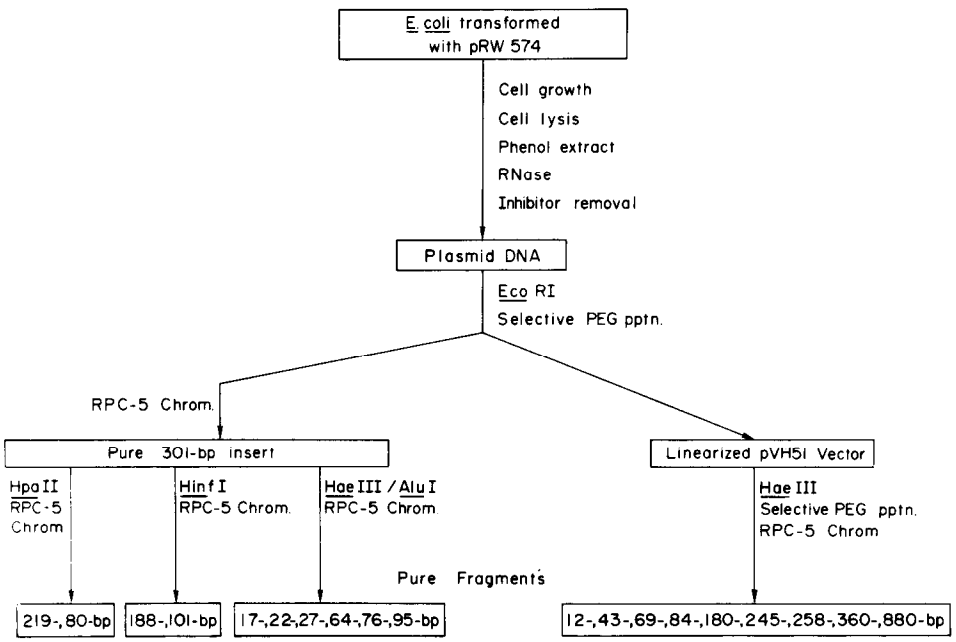

Fig. 2. Overview of purification schemes of restriction fragments. In addition to isolation of the pure 301-bp insert from pRW574 as shown, the 95-bp insert was purified from pRW554. Reprinted with permission from ref. 18.

possible to isolate milligram quantities of the fragment from the vector by HPLC [14—43].

An alternative general procedure is shown in Fig. 2; in this case, the insert, which is 301 bp in length was separated from the pVH51 vector by selective precipitation. Individual restriction fragments were resolved after suitable digestions of the 301-bp insert. When the linearized pVH51 vector was cleaved with a restriction enzyme, partial fractionation of the fragments into sub-classes could be accomplished by selective precipitation. Further fractionation then was accomplished on HPLC columns to obtain the pure individual fragments [18].

HPLC on RPC-5 or NACS is most useful in conjunction with gene cloning methods since recombinants can be devised which optimize the resolution of the column. However, cloning alone will not provide a single pure restriction fragment; another technique (HPLC) must be used. Thus, neither procedure alone is adequate.

2. HPLC OF DNA

HPLC of DNA has been reviewed previously [14, 16, 21, 44]. Thus, only an overview of the types of separations will be discussed. Previous reviews [14, 16, 21, 44] covered the literature on fractionations of oligonucleotides, tRNAs, and polynucleotides. This review will focus on duplex restriction fragments.

HPLC is the only efficient method available for isolating milligram quantities of DNA restriction fragments in homogeneous form. The technique is rapid, reliable, employs modest equipment, and can be used for a variety of applications (e.g. fractionation of fragments, separation of digested genomic DNA, isolation of plasmids from cell lysates, fractionation of short single-stranded oligonucleotides, fractionation of supercoiled DNA, transfer RNA, ribosomal RNA, separation of complementary strands of DNA restriction fragments, and other applications).

A number of biological studies have been performed on microgram amounts of fragments. For this purpose, it is acceptable to elute the fragments from a polyacrylamide gel. However, it is advantageous or necessary for other studies to have milligram quantities of a pure fragment. Some of these studies are: protein binding, ultraviolet (UV), Raman, and NMR spectroscopy, hydrodynamic studies, etc. The technique is optimum for fragments shorter than approx. 1000 bp but satisfactory resolution is achieved for longer fragments [14—44] if the size differential is sufficiently great.

The impressive feature of HPLC on RPC-5 or NACS is the scale of feasible purifications. Single-column fractionations have been routinely performed to give 10,000 times as much pure fragments as obtained by gel electrophoresis. Also, the order of elution of fragments is not always the same found by gel electrophoresis. Thus, RPC-5 column chromatography can completely resolve duplex restriction fragments of the same size in some cases [14, 16, 20, 21].

2.1. Fractionation of insert from vector

HPLC has been successfully used for the separation of up to 120 mg total of

UV-absorbing material when the goal is to separate a relatively small insert from a vector which is 10—20 times its size. Obviously, the resolving power of RPC-5 or NACS for large-scale preparations is significantly enhanced if it is possible to remove the major part of contaminating DNA by a fractional precipitation prior to the fine separation on HPLC.

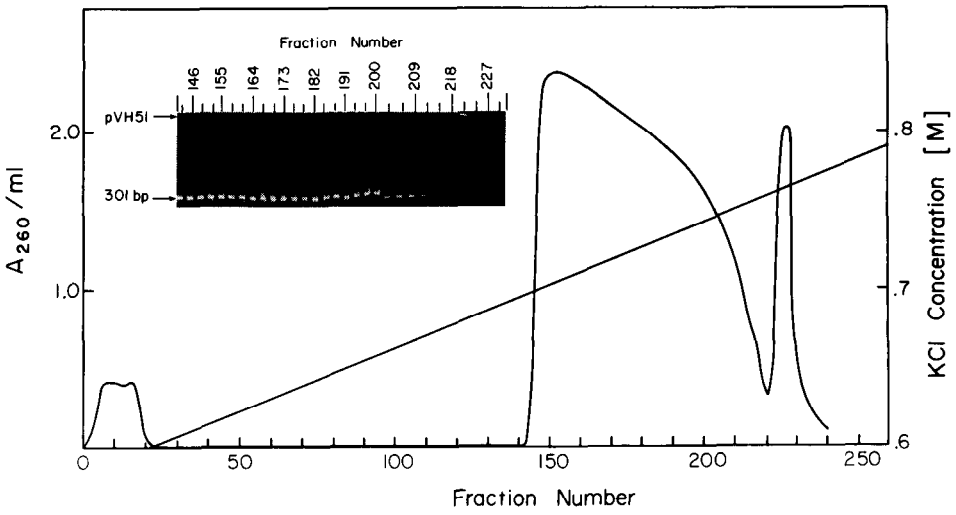

Fig. 3. Elution profile of the RPC-5 column fractionation of the 301-bp insert from the residual vector DNA. A 85 × 2.5 cm RPC-5 column thermostated at 45°C was loaded with 120 mg of UV-absorbing material and developed with a linear 3-l gradient from 0.5 to 0.8 *M* potassium chloride containing 10 m*M* Tris—HCl (pH 8.0) and 0.1 m*M* EDTA. Fractions (12 ml) were collected at a flow-rate of 1.7 ml/min. The insert shows the 5% polyacrylamide gel analysis of some of the UV-absorbing fractions. No DNA bands were found in the flow-through peak. The 301-bp fragment containing fractions were collected, evaporated until potassium chloride started to precipitate, then dialyzed against 10 m*M* Tris—HCl (pH 8.0) and 0.1 m*M* EDTA, further evaporated to a DNA concentration of 2 mg/ml, and redialyzed against the same buffer. The fragment was stored in this state at —20°C. The total amount of the 301-bp fragment purified in this step was 87.5 mg. Reprinted with permission from ref. 18.

Fig. 3 shows the separation of an *Eco* RI digest of pRW574 containing a tetrameric insertion of a 301-bp fragment originating from the *E. coli* lactose genetic control region [18]. The reaction products are thus a 301-bp fragment and the vector DNA which is approx. 3850 bp long [18]. Prior to chromatography on RPC-5 or NACS, the main amount of the vector DNA was removed by a fractionated precipitation using polyethylene glycol (PEG). The separation of the 301-bp fragment from the remaining vector DNA was complete and the yield of pure 301-bp DNA was 87.5 mg in this particular run. Similar separations with smaller amounts of DNA were also performed omitting the fractionated PEG precipitation.

2.2. Fragment purification

Fig. 4 shows a typical fractionation on an analytical system [20]. The sample was a *Hae* III digest of a miniCol E1 derivative, pRZ2, which gave 17 fragments ranging in size from 43 to 850 bp, including three 425-bp fragments.

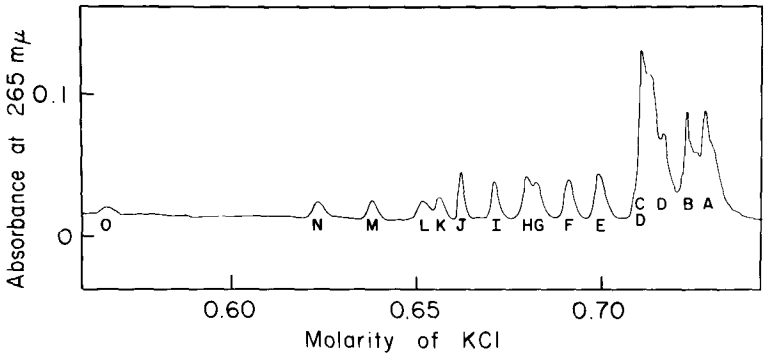

Fig. 4. Separation of DNA fragments generated by a *Hae* III digest of pRZ2 DNA on RPC-5. Fractionation of the digest was performed at 43° C and pH 6.8 using a 40 × 0.15 cm column. The column was eluted with a 40-ml gradient from 0.55 to 0.8 *M* potassium chloride containing 10 m*M* Tris—HCl, pH 6.8, and 0.1 m*M* EDTA. The flow-rate was around 0.22 ml/min. UV-absorbing fractions were analyzed on 5% polyacrylamide gels. The 789-bp fragment was loaded on every tube for calibration. The fragments are designated by letters, their sizes are the following: A 850; B 575; C 465; D 425 (three fragments); E 255; F 203; G 180; H 169; I 135; J 117; K 102; L 98; M 85; N 69; O 43; This figure shows the elution profile. Reprinted with permission from ref. 20.

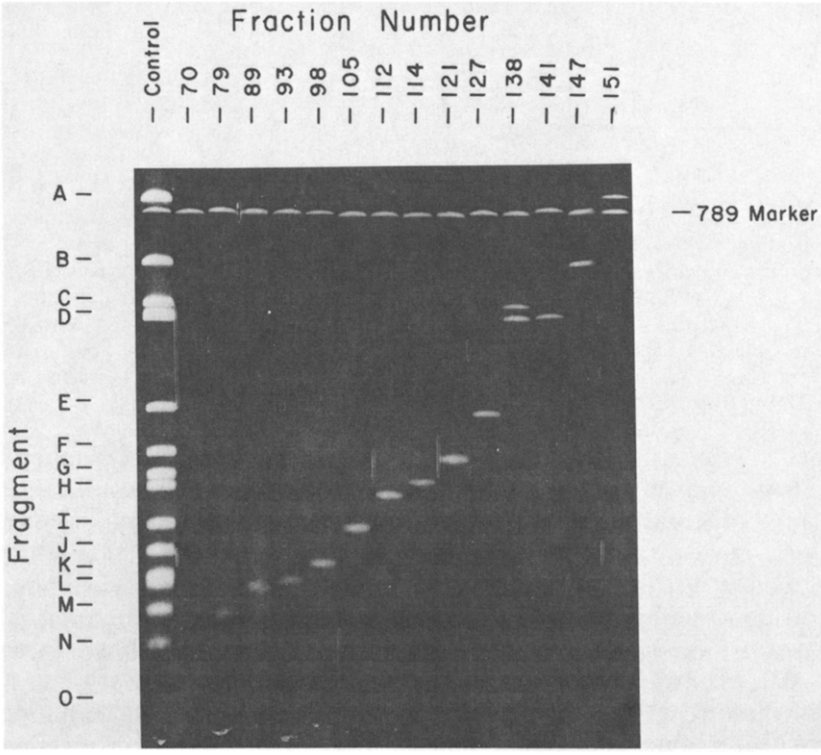

Fig. 5. Polyacrylamide gel analyses of elution pattern shown in Fig. 4. The contents of the fractions were determined by gel electrophoresis. Fragment sizes are listed in the legend to Fig. 4. Reprinted with permission from ref. 20.

Fig. 4 shows that virtually all 17 fragments can be separated from each other when using a potassium chloride gradient at pH 6.8 at 43°C. Even the 98- and 102-bp fragments, which differ in size by as little as 4%, were separated. Fig. 5 shows the polyacrylamide gel electrophoretic analyses of fractions from the HPLC column. Excellent resolution was observed to provide quite pure restriction fragments.

Fig. 6 demonstrates the excellent resolving power of HPLC for small double-stranded DNA fragments. The 301-bp fragment was digested with *Alu* I and *Hae* III to yield a stoichiometric mixture of 17-, 22-, 27-, 64-, 76-, and 95-bp fragments which were separated completely in one step on RPC-5 (or NACS). Although the load (approx. 0.6 mg DNA per ml RPC-5) for this separation is in the range of the upper capacity limit, the separation obtained under these conditions was nearly complete. All six fragments were separated with only very little overlap between the 64- and 76-bp fragments (peaks D and E).

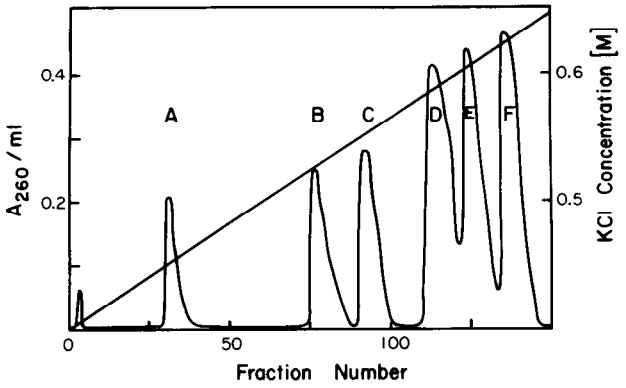

Fig. 6. RPC-5 elution profile of the *Hae* III/*Alu* I double digest of the 301-bp fragment. DNA (12 mg consisting of 17-, 22-, 64-, 76-, and 95-bp fragments was loaded onto a 25 cm × 1 cm RPC-5 column which was thermostated at 45°C. The column was developed with a 2-l gradient from 0.4 to 0.75 *M* potassium chloride containing 10 m*M* Tris—HCl (pH 8.0) and 0.1 m*M* EDTA. Fractions of 9 ml were collected at a flow-rate of 0.65 ml/min; 6% polyacrylamide gel analysis of the UV-absorbing fractions revealed that peaks A—F contained the pure fragments in the order of increasing size. The first small peak (fraction 3) does not contain DNA. Reprinted with permission from ref. 18.

There are many impressive features of HPLC of DNA as a general technique. The equipment is relatively inexpensive, the resins are stable and reusable, purifications are efficient and do not require extended time periods, reproducibility is excellent, recovery is extremely high (generally greater than 95%), and fragments purified by this method appear to contain no deleterious contaminants which inhibit further enzymatic, spectroscopic or biological studies. At least fifteen determinations have been applied to evaluate these properties [14, 16, 21, 44].

A number of factors have been evaluated which affect separations including the following: elution conditions, chain length, length and time of protruding ends, nucleotide composition, and other factors [14, 16, 21].

2.3. Separation of complementary strands

Under some conditions it is possible to separate the complementary strands

of modest size (less than approx. 500 bp) DNA fragments on alkaline HPLC columns. Fig. 7 shows the separation of the complementary strands of a 210-bp fragment by *Hind* II digestion of ϕX174 replicative form DNA. Purification of the duplex 210-bp fragment was described [14]. The strand-separation technique [14] is readily reproducible under the conditions tested (i.e. using 7.5 mg of duplex DNA fragment). In some cases, investigators have experienced difficulty in the fractionation of complementary strands of trace quantities of radioactively labeled DNA restriction fragments; however, others have made substantial progress in resolving these problems [44].

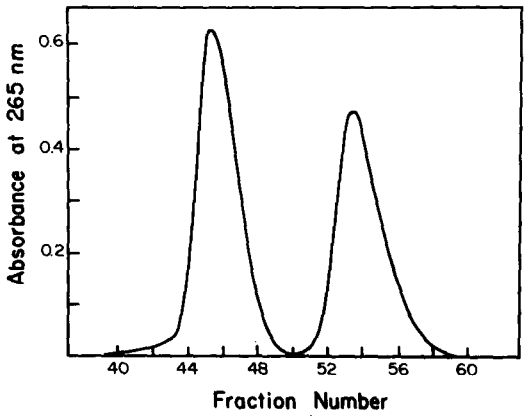

Fig. 7. Separation of the complementary strands of a 210-bp restriction fragment. The 210-bp *Hin* dII fragment from ϕX174 replicative form DNA was isolated by RPC-5 chromatography and dialyzed versus 0.25 *M* potassium chloride, 5 m*M* Tris (pH 7.4), 0.5 m*M* EDTA. The pH of the solution was raised to 12.5 by the addition of 1 *M* sodium hydroxide, and 7.5 μg of the fragment (in 0.2 ml) was loaded onto a 200 mm × 1.5 mm RPC-5 column equilibrated with 0.25 *M* potassium chloride, 12 m*M* sodium hydroxide (pH 12.5). All solutions were degassed immediately before use and kept under a nitrogen atmosphere to maintain the high pH. The column was washed with 2 ml of the equilibration buffer and was eluted with a 20 ml linear gradient of 0.90—0.95 *M* potassium chloride in 12 m*M* sodium hydroxide (pH 12.5) at 0.23 ml/min and 150 p.s.i. Fractions of 0.2 ml were collected. The elution profile was continuously monitored at 265 nm. The separated strands eluted between 0.92 and 0.93 *M* potassium chloride. Appropriate fractions were pooled and dialyzed, and aliquots were analyzed on 5% polyacrylamide gels. Reprinted with permission from ref. 14.

2.4. Recent developments

In the past several years, a number of important developments have been made. First, an alternate source of resin (NACS) was produced which is at least as good as the original RPC-5. Secondly, a fractionation of the resin material according to particle size into four classes (NACS-12, -20, -37, -52) was effected. This fractionation according to size has permitted development of resins which do not require high pressure. NACS-12 provides the highest resolution purification of nucleic acids but requires a high-pressure pump (requiring 1.3—33 bars). NACS-20 and -37 may be employed with a peristaltic pump and NACS-52 may be used in the gravity flow mode. Each of these fractionated resins has slightly different optimum uses. It is possible to screen clones, purify samples (such as after end labeling), concentrate samples, extract nucleic acids

from agarose or polyacrylamide gels, using minicolumns with gravity flow. Other separation may require a peristaltic pump or a high-pressure pump including separation of high-molecular-weight single-stranded DNAs, fractionation of complementary strands, fractionation of genomic DNAs, RNAs, or oligonucleotides.

Thirdly, prepacked columns have been developed which may be used in either a gravity flow mode or may be attached to a Pipetman for a wide variety of routine manipulations in the molecular biology laboratory, such as removal of small unreacted molecules from polynucleotide kinase or reverse transcriptase reactions, extraction of nucleic acids from gel pieces, or rapid screening of supercoiled DNA.

3. PROSPECTS FOR THE FUTURE

It is likely that this technique will be used much more widely in the future for a number of the following applications.

(1) In cases where it is necessary to isolate milligram quantities of defined segments of chromosomes, RPC-5 or NACS column chromatography is the only available high-resolution technique at the present time. Specific applications are the following. (a) The isolation of defined regions of complex chromosomes (such as eucaryotic chromosomal DNAs) in order to partially fractionate the genome. The fractionation may be followed by hybridization with specific probes such as RNA transcripts or viral nucleic acids. (b) The isolation of defined segments of genomes, such as operators, promoters and origins of DNA replication, etc., for biochemical and physical studies. (c) Isolation of regions of certain infectious agent genomes which cannot be cloned under the present NIH guidelines.

(2) The separation of some fragments from other fragments of similar chain length but with different base compositions. It is not possible to accomplish these separations, even on small scale, by polyacrylamide gel electrophoresis.

(3) The separation of some fragments which have approximately the same size (and therefore cannot be separated on gels) but one of which contains an *Eco* RI or *Hind* III end and the other has ends which are or behave like blunt ends.

(4) Separation of the complementary strands of DNA restriction fragments.

(5) The high-resolution fractionation of single-stranded oligonucleotides. To the best of our knowledge, this is the only high-resolution technique for separating quantities of oligonucleotides. Separations of up to approx. the 60 mer [44] have been observed in some cases (diethylaminoethyl cellulose chromatography loses resolution at approx. the 10—15 mer). These fractionations are very important for synthetic studies on oligonucleotides as well as for the isolation of single-stranded oligomers such as intermediates in DNA replication (i.e. RNA primers or short Okazaki fragments). For example, note the spectacular separations of Van Roode and Orgel [45] of oligomers which contain 2′—5′ internucleotide bonds from molecules with 3′—5′ bonds.

(6) As a probe for AT-rich fragments or for AT-rich regions within GC-rich fragments. In addition, present studies indicate that fragments which bind more tightly to the column contain known regulatory regions [14, 23, 25]. If further

work bears out this correlation, these properties could be used as an indication of biological function.

Potential future uses of RPC-5 or NACS are the following.

(1) The separation of DNAs which have small differences in their content of non-paired nucleotides, such as (a) DNA containing a short nick or gap. (b) Intermediates in DNA replication, such as growing fork regions which may contain several non-paired nucleotides. (c) Transcription complexes consisting of DNA template, which is predominantly in the duplex form, but with a nascent RNA still attached. (d) Intermediates in DNA recombination. (e) The separation of covalently closed circular DNAs containing different numbers of supercoiled turns. (f) Replicative form I from form II DNA. (g) DNAs with frayed ends (i.e. possibly produced purposely by Exo III or the lambda exonuclease) versus identical fragments which are fully double stranded.

(2) The separation of certain protein—nucleic acid complexes (such as nucleosomes or transcription complexes) from naked DNA.

A major commercial development of HPLC of DNA for the future will be in the area of automated analyses for developing routine clinical instruments. It is rather simple to automate the analyses of fragments, DNAs etc. from a column whereas it is rather difficult to automate the analyses from gels. Thus, it is likely that a variety of types of instruments will be developed for analyzing complex viral genomes, etc. using this methodology.

4. ACKNOWLEDGEMENTS

This work was supported by grants from the National Institutes of Health (GM30822) and the National Science Foundation (83-08644). Part of this work was conducted at the Department of Biochemistry, University of Wisconsin, Madison, WI 53706, U.S.A. A number of very talented and dedicated scientists in my laboratories have contributed to the developments in HPLC described herein; their names are listed in references.

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