THE CLEAVAGE OF ϕX174 DNA WITH RESTRICTION ENDONUCLEASES

Thesis by

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To

my Father

on his 60th birthday

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Abstract

Restriction endonucleases from Hemophilus influenzae (Hin), H. parainfluenzae (Hpa) and H. aegyptius (Hae) have been prepared and purified from contaminating exonuclease activities. Using ϕX174 RFI as substrate, Hin produces 13 specific fragments, Hpa produces 8 and Hae produces 11 fragments. These have been analyzed by polyacrylamide gel electrophoresis and their molecular size determined to be within the range of 1600 to 70 base pairs. The 5' end group of the fragments produced by the action of Hin on ϕX RF have been identified to be adenine (50-57%), and guanine (30-40%), whereas in the case of Hpa, preliminary results show that a majority of the fragments terminate at the 5' end with cytosine.

By analyzing partial digestion products, as well as overlapping sets of fragments produced by two different restriction enzymes, the physical order of the three sets of DNA fragments produced by hydrolysis of ϕX RF by the Hin, Hpa and Hae restriction endonucleases have been determined. This analysis has been facilitated by the adaptation of a continuous electro-elution device to separate and recover individual DNA restriction enzyme fragments.

The physical cleavage map has been in turn related to the ϕX genetic map by the fact that many ϕX174 mutations are available and that methods have been developed by which restriction enzyme fragments may be assayed to determine which genetic loci they contain.

With the availability of the ϕX174 cleavage map, it is possible to locate the unique, methylated cytosine of the ϕX genome. Bacteriophage ϕX DNA is labeled in vivo with [^{3}H-methyl]methionine and used as

template for *in vitro* synthesis of the complementary strand. The resultant RF DNA is then cleaved in separate experiments with the *Hin* and *Hae* enzymes. The DNA fragments are analyzed by polyacrylamide gel electrophoresis. It is concluded that the single methyl group in the viral DNA is located at a specific region of the ϕX174 genome, very likely in gene H.

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Chapter I

General Introduction

Bacteria, despite their small size, have an effective mechanism for defense against invading DNAs. Restriction enzymes--enzymes that can recognize and cleave foreign DNA--are part of this defense mechanism. These enzymes are extremely specific; each one acts only within certain well-defined base sequences in double-stranded DNA molecules.

Since the original report of the isolation of a restriction endonuclease from E. coli K by Meselson and Yuan (1968), a number of such enzymes have been purified from various strains of bacteria, including several specified by intracellular virus (Meselson and Yuan, 1968; Haberman et al., 1972) or by plasmids (Hedgpeth et al., 1972; Yoshimori, 1971).

Often, a restriction enzyme is a component of a modification-restriction (M-R) system. (For reviews, see Arber and Linn, 1969; Boyer, 1971; Meselson et al., 1972). The M-R system consists of two enzymatic components, a modification enzyme and a restriction endonuclease, sharing a similar (or identical) recognition specificity.

The presence of a host M-R system may be detected in an organism by biological or biochemical means. According to the original criteria of Luria and Human (1952), an M-R system exists in a certain strain of bacteria A if a virus grown in some other strain B plates with reduced efficiency on A relative to B; at the same time, virus recovered from strain A plates with full efficiency on A, but after regrown on B, it again displays reduced efficiency on A.

The biochemical bases for these biological phenomena are now relatively better understood. Phage grown on strain B is restricted in A because the viral DNA does not have the modification pattern of

strain A. DNAs lacking appropriate modification are recognized by a site-specific restriction endonuclease and double-stranded cleavage of such DNA results. Unmodified viral DNA occasionally escapes cleavage and gives rise to progeny virus which receive the host specific modification and thus are unrestricted in A. However, subsequent cloning of the A modified virus back in the B bacterial strain will again yield progeny virus which does not carry the A specific modification and will be restricted in A.

Biochemically, restriction endonucleases can be identified by the fact that they recognize and cleave foreign DNA into specific short fragments while they remain inactive on the (presumably) modified host DNA. Recently, several new restriction enzymes have actually been isolated by this criterion (Smith and Wilcox, 1970; Gromkova and Goodgal, 1972; Middleton *et al.*, 1972).

Restriction enzymes are widely distributed throughout the bacteria world. However, the enzymes of *Escherichia coli* and *Hemophilus influenzae* are the best characterized. These enzymes can be subdivided into two classes, depending on whether they require the cofactors adenosine triphosphate, S-adenosyl methionine and magnesium ions for their activity (type I), or whether they require only magnesium ions (type II).

The restriction enzymes first isolated by Meselson and Yuan (1968) and Linn and Arber (1968) from *E. coli* K and B are examples of the type I enzymes. These enzymes, *Eco*B and *Eco*K, are the enzymes responsible for the restriction-modification specificity associated with *E. coli* B and K. These enzymes have been intensely purified

and they introduce a limited number of double-strand cleavages in unmodified DNA, in the presence of the cofactors (Arber and Linn, 1969; Boyer, 1971; Roulland-Dussoix and Boyer, 1969).

Type I enzymes are large and complex in structure; their molecular weights have been estimated to be around 2-4.5 x 10^5 daltons. They are composed of three different kinds of peptide subunits, one for restriction, another for modification and a third for both modification and restriction which determine the specificity of the DNA site recognition (Meselson et al., 1972).

Recently, it has been found that although endonuclease B attaches to DNA only at a specific site, the same site recognized by the methylase, it does not appear to cut the molecule there; i.e., the enzyme may move along the DNA before cleaving it (Horiuchi and Zinder, 1972). It is not known whether specific base sequences are required at the actual cleavage sites.

The type II enzymes are smaller and simpler in subunit composition than the type I enzymes. Moreover, they appear to be more specific in their cleavage sites. Examples of these are the restriction endonucleases from several Hemophilus strains.

Kelly and Smith (1970) determined the sequence of bases at the cleavage site for the restriction enzyme isolated from H. influenzae (Hin). The sequence is as follows:

5'.....GpTp Py ↓ pPupApC.....3'

3'.....CpAp Pup ↑ PypTpG.....5'

It consists of six bases, with ambiguity in the basis in the center of the sequence. Either purine (adenine or guanine) or

pyrimidine (cytosine or thymine) may occupy the positions indicated. The outstanding characteristic of this base sequence is that the two strands are identical in this region. This has led to the speculation that this symmetry may be essential for the function of the enzyme-cleavage, at equivalent points, of two DNA strands of opposite polarity.

The sequences susceptible to two additional type II restriction enzymes, EcoRI and EcoRII, both genetically controlled not by the E. coli chromosome but by drug resistance transfer factors, RI and RII, have been determined (Dugaiczyk et al., 1974; Bigger et al., 1973). (Drug resistance transfer factors are plasmids, DNA molecules that replicate independently of the bacterial chromosome and they may be transferred between bacterial cells.) The substrate sequence for endonuclease EcoRI is as follows:

5'.....(A/T)pG ↓ pApApTpTp Cp(T/A).....3'

3'.....(T/A)pC pTpTpApAp ↑ Gp(A/T).....5'

This nucleotide sequence is also symmetrical but, unlike that of the endonuclease Hin substrate, the base ambiguity is at the ends of the sequence where either adenine or thymine are permissible. One significant feature of the cleavage point is that the cuts made by the EcoRI enzyme are staggered in such a way that "cohesive" ends are formed. This means that any DNA fragments produced by the action of endonuclease EcoRI can be joined together. Because of this, restriction endonucleases appear to have great potential value for the construction of new plasmid species by joining DNA molecules from different sources. Cohen et al. (1973) have reported the first successful construction of biologically functional bacterial plasmids in vitro, using

the EcoRI enzyme.

A similar situation has been found at the cleavage site of EcoRII (Bigger et al., 1973). In fact, nucleotide sequences at the cleavage sites of over 5 type II restriction enzymes have recently been determined and they all possess symmetry about the center of the sequence recognized (K. Murray, R. J. Roberts, unpublished results).

Therefore, it is conceivable to suggest that the mode of action of these enzymes is based on the symmetric character of the DNA substrate. Restriction enzymes may act by loosening the double helix in the vulnerable sequence causing the formation of two rotationally symmetrical hairpin loops on opposite strands. These loops may be envisaged to fit nicely into the active site(s) of the enzyme molecule. The enzymes would then proceed to carry out the hydrolysis process at specific points. In order for the enzymes to act in this fashion, it would be expected that restriction enzymes may consist of (at least) two identical subunits. Some of the restriction enzymes which have been purified and studied do indeed confirm to this expectation, at least structurally. For example, the Hin enzyme has been found to consist of 4 identical subunits, each of a molecular weight of 25,000 daltons (P. H. Johnson, personal communication).

It has become evident that the specificity, especially of the type II restriction enzymes, makes them invaluable tools for studying DNA structure and function and for mapping the genetic and physical features of the chromosomes. The importance of these theoretical and practical applications has in turn stimulated the search for new restriction enzymes with different specificities. Over 16 of them have

now been found and being characterized (Sugisaki and Takanami, 1973; Takanami, 1973; R. J. Roberts, personal communication).

Because of the variety of organisms from which they are isolated, some kind of species-strain designation for each new enzyme is a definite necessity. Therefore, a unified system of nomenclature for restriction endonucleases has been devised (Smith and Nathans, 1973). This nomenclature has been followed in this thesis; Hin refers to the restriction enzyme isolated from H. influenzae, Hpa from H. parainfluenzae, and Hae from H. aegyptius. In some special case, sub-classification of these enzymes are essential. For example, Hin_dII refers to the restriction enzyme isolated from H. influenzae, strain d and specifically relates to the modification and restriction system II (of which there are three in H. influenzae [Roy and Smith, 1973]).

During the last two years, many new discoveries have resulted from the application of restriction endonucleases to the study of DNA and chromosome structures. Progress has been made particularly in the area of understanding the animal viral DNAs as well as their mode of replication (Danna and Nathans, 1971, 1972; Nathans and Danna, 1972; Adler and Nathans, 1973; Mertz and Davis, 1972; Morrow and Berg, 1972; Mulder and Delius, 1972; Pettersson et al., 1973; Huang et al., 1973) and transcription (DeFilippes, 1972; Khoury and Martin, 1973).

Restriction enzymes with different specificities generate overlapping fragments of DNA. Such overlapping fragments are prerequisites for mapping the genome. Using this technique, Danna et al. (1973) have constructed a physical map of the arrangements of the

fragments in the SV40 genome. A similar physical map for polyoma DNA has also been recently determined (Griffin et al., 1974). Such physical maps are preliminary information to develop a functional map showing the location of genes and intracistronic regions.

To achieve exactly that goal, the genome of the bacteriophage ϕX174 offers several distinct advantages.

First, the small size of the genome (about 5500 nucleotides) simplifies the number of fragments produced and therefore makes their complete characterization more feasible.

Secondly, it is relatively easy to prepare quantitative amounts of homogeneous ϕX DNA for enzyme digestion. [Since restriction enzymes do not cut single-stranded DNA, the replicative form of ϕX (RF) is used].

Thirdly, and most importantly, there already exists a detailed genetic map of ϕX174 (Benbow et al., 1974) and with mutants in almost all of its cistrons. Also, techniques have already been worked out for the determination of the genetic loci contained by any DNA fragment (Weisbeek and Van de Pol, 1970; Hutchison and Edgell, 1971).

Edgell et al. (1972) and Middleton et al. (1972) have reported results using the restriction endonucleases from H. influenzae and H. aegyptius to cleave ϕX174 RF DNA into specific fragments and have begun mapping some of the fragments on the ϕX174 genetic map by genetic complementation assays (C. A. Hutchison, personal communication). They have also presented preliminary results identifying three promotor regions of the ϕX174 genome (Chen et al., 1973).

Concurrently, our laboratory has also been preparing restriction enzymes from various Hemophilus strains. Some of the work involving the use of the Hpa enzyme to cleave ϕX DNA into specific fragments have been published (Johnson et al., 1973). The details of the enzyme isolation procedure and gel electrophoresis technique are discussed in this thesis.

In this laboratory, we have wanted to obtain a cleavage map of the ϕX174 genome for use in the study of DNA replication, of transcription in vivo and in vitro, and eventually for sequencing some of the most interesting regions on the genome. Particular points of interest such as the unique methylation group may also be localized through the use of restriction enzymes. A recent communication from our laboratory has reported the localization of the specific discontinuity of the late RF molecules during single strand synthesis of ϕX DNA (Johnson and Sinsheimer, 1974). The earlier stage of DNA replication has also been investigated with the Hin enzyme (A. Zuccarelli, Ph.D. Thesis, Caltech).

In the work reported in this thesis, we have adapted a continuous electro-elution system for the convenient separation and recovery of DNA fragments from gels (Lee and Sinsheimer, 1974a), we have completely ordered three sets of Hemophilus fragments on the ϕX174 genetic map, and we have localized the unique methylation group of ϕX in the gene H regions (Lee and Sinsheimer, 1974b, 1974c). This work is described in greater detail in the following chapters.

Restriction endonucleases have unquestionally proven to be a breakthrough in DNA technology. They have already been put into

application to study eukaryotic chromosomes, to give information about DNA sequence arrangements (Mowbray and Landy, 1974). It is also theoretically possible to isolate particular genes by cleaving chromosomes with the right combination of restriction enzymes. The provision of a variety of specific hydrolases to degrade DNA in a specific manner may finally open up the difficult field of DNA sequencing.

References

Adler, S. P., and D. Nathans (1973) Biochim. Biophys. Acta 299:177-188.

Arber, W., and S. Linn (1969) Ann. Rev. Biochem. 38:467-500.

Benbow, R. M., A. J. Zuccarelli, G. Davis, and R. L. Sinsheimer (1974) J. Virol. 13:898-907.

Bigger, C. H., K. Murray, and N. E. Murray (1973) Nature New Biol. 244:7.

Boyer, H. W. (1971) Ann. Rev. Microbiol. 25:153.

Chen, C. Y., C. A. Hutchison, III., and M. H. Edgell (1973) Nature New Biol. 243:233-236.

Cohen, S. N., A. C. Y. Chang, H. W. Boyer, and R. B. Helling (1973) Proc. Nat. Acad. Sci. 70:3240-3244.

Danna, K. J., and D. Nathans (1971) Proc. Nat. Acad. Sci. 68:2913-2917.

Danna, K. J., and D. Nathans (1972) Proc. Nat. Acad. Sci. 69:3097.

Danna, K. J., G. H. Sack, and D. Nathans (1973) J. Mol. Biol. 78: 363-376.

DeFilippes, F. M. (1972) Biochim. Biophys. Acta 272:125-129.

Dugaiczyk, A., J. Hedgpeth, H. W. Boyer, and H. M. Goodman (1974) Biochemistry 13:503-512.

Edgell, M. H., C. A. Hutchison, III., and M. Sclair (1972) J. Virol. 9:574-582.

Griffin, B. E., M. Fried, and Q. Cowie (1974) Proc. Nat. Acad. Sci. 71:2077-2081.

Gromkova, R., and S. H. Goodgal (1972) J. Bacteriol. 109:393-409.

Haberman, A., J. Heywood, and M. Meselson (1972) Proc. Nat. Acad. Sci. 69:3138.

Hedgpeth, J., H. M. Goodman, and H. W. Boyer (1972) Proc. Nat. Acad. Sci. 69:3448.

Horiuchi, K., and N. D. Zinder (1972) Proc. Nat. Acad. Sci. 69: 3220-3224.

Huang, E. S., J. E. Newbold, and J. S. Pagano (1973) J. Virol. 11: 508-514.

Hutchison, C. A., III., and M. H. Edgell (1971) J. Virol. 8:181-189.

Johnson, P., A. S. Lee, and R. L. Sinsheimer (1973) J. Virol. 11: 596-599.

Johnson, P. H., and R. L. Sinsheimer (1974) J. Mol. Biol. 83:47-61.

Kelly, T. J., and H. O. Smith (1970) J. Mol. Biol. 51:393-409.

Khoury, G., and M. A. Martin (1973) J. Mol. Biol. 78:377-389.

Lee, A. S., and R. L. Sinsheimer (1974a) Analyt. Biochem. 60: 640-644.

Lee, A. S., and R. L. Sinsheimer (1974b) Proc. Nat. Acad. Sci., 71: 2882-2886.

Lee, A. S., and R. L. Sinsheimer (1974c) J. Virol., in press.

Linn, S., and W. Arber (1968) Proc. Nat. Acad. Sci. 59:1300-1306.

Luria, S. E., and M. L. Human (1952) J. Bacteriol. 64:557-569.

Meselson, M., and R. Yuan (1968) Nature 217:1110-1114.

Meselson, M., R. Yuan, and J. Heywood (1972) Ann. Rev. Biochem. 41: 447.

Mertz, J. E., and R. W. Davis (1972) Proc. Nat. Acad. Sci. 69:3370.

Middleton, J. H., M. H. Edgell, and C. A. Hutchison, III (1972) J. Virol. 10:42-50.

Morrow, J. F., and P. Berg (1972) Proc. Nat. Acad. Sci. 69:3365.

Mowbray, S. L., and A. Landy (1974) Proc. Nat. Acad. Sci. 71: 1920-1924.

Mulder, C., and H. Delius (1972) Proc. Nat. Acad. Sci. 69:3215.

Nathans, D., and K. J. Danna (1972) J. Mol. Biol. 64:515.

Pettersson, U., C. Mulder, H. Delius, and P. A. Sharp (1973) Proc. Nat. Acad. Sci. 70:200-204.

Roulland-Dussoix, D., and H. Boyer (1969) Biochim. Biophys. Acta 195:219-229.

Roy, P. H., and H. O. Smith (1973) J. Mol. Biol. 81:427-444.

Smith, H. O., and K. W. Wilcox (1970) J. Mol. Biol. 51:379-391.

Smith, H. O., and D. Nathans (1973) J. Mol. Biol. 81:419-423.

Sugisaki, H., and M. Takanami (1973) Nature New Biol. 246:138.

Takanami, M. (1973) FEBS Letters 34:318.

Weisbeek, P. J., and J. H. Van de Pol (1970) Biochim. Biophys. Acta 224:328-338.

Yoshimori, R. (1971) Ph.D. Thesis, University of California, San Francisco, 73 pp.

Chapter II

The Isolation and Purification of Restriction Enzymes from the *Hemophilus influenzae*, *H. parainfluenzae* and *H. aegyptius* Cells

Introduction

A number of bacteria are capable of recognizing and degrading foreign DNA. Meselson and Yuan (1968) first reported the purification of a restriction endonuclease from Escherichia coli K12. Another restriction endonuclease was isolated from Hemophilus influenzae, strain Rd, by Smith and Wilcox (1970). The essentials of the purification procedure of this enzyme have been described (Smith and Wilcox, 1970). However, many crucial observations during the entire procedure remained in unpublished form. Here, we describe in greater detail each of the isolation steps and an additional DEAE step for removal of contaminating exonuclease activity.

Materials and Methods

I. Strains of Cells

a) H. influenzae strain Rd was obtained from H. O. Smith, and is a non-encapsulated, nonpathogenic strain. The cells are non-motile, gram-negative and occurred as small rods of size 0.2-0.3 μ by 0.5-2.0 μ, about 1/2 to 1/3 the size of E. coli C.

b) H. parainfluenzae was obtained from Sol Goodgal and originally from G. Leidy. General characteristics are the same as H. influenzae except that Hpa cells tend to form short chains in saturated cultures.

c) H. aegyptius, ATCC 11116, was obtained from C. Hutchison, III., originally from American Type Culture Collection, Rockville Rd.

Stocks of a, b and c are stored in ampules at -20°C in the complete BHI medium, 50% glycerol.

II. Medium

a) Bacto brain heart infusion broth (dehydrated): (BHI is purchased from Difco Laboratories, Detroit, Michigan.) 37 gm BHI in 1 liter of distilled water. The water is autoclaved, cooled, and BHI added. The mixture is briefly autoclaved for 10-15 min at 121°C. Prolonged heating diminishes the ability to support Hemophilus growth.

b) DPN stock: (From Yeast, Grade III, purchased from Sigma Chemical Co., St. Louis, Mo.) 10 mg/ml in distilled water. The stock is filter sterilized and stored at 4°C.

c) Hemin stock: (Obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio) 0.1 gm of hemin is dissolved in 100 ml of 4% 2,2',2"-nitriloethanol, and is heated to 65°C for 15 min to sterilize. Stock is stored at 4°C.

d) Complete BHI broth for Hin and Hae: For a liter of BHI broth, 10 ml of hemin stock and 0.2 ml DPN stock are added.

e) Complete BHI for Hpa: For a liter of BHI broth, 0.2 ml of DPN stock is added. No hemin supplement is necessary.

III. Buffers

a) 0.05 M Tris, pH 7.4, 1 mM glutathione.

b) 1 M NaCl, 0.02 M Tris, pH 7.4, 0.01 M β-mercaptoethanol, 10% glycerol.

c) 0.01 M potassium phosphate buffer, pH 7.4, 10% glycerol.

d) 6.6 mM Tris, pH 7.4, 6.6 mM $MgCl_2$, 6.6 mM β-mercaptoethanol.

IV. General Procedure for the Purification of the Hemophilus Endonucleases

The procedure adopted is a modification of the purification method previously described by Smith and Wilcox (1970), and the scheme is outlined in Figure 1.

a) Preparation of inoculum: For a 4 liter Hemophilus cell culture, an inoculum of 400 ml is prepared. The cells are grown to a log phase of about 3×10^8 cells/ml.

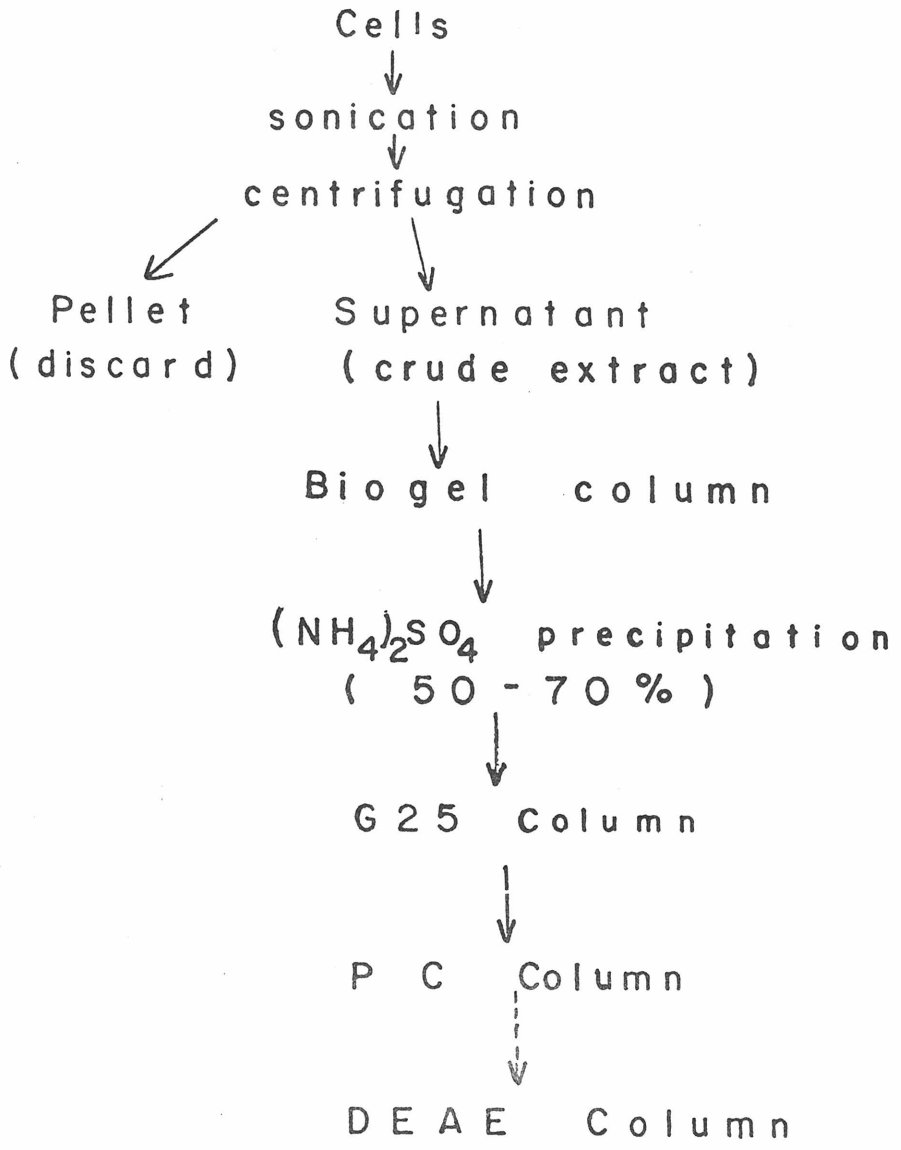
The stock culture used for inoculum is strictly screened for contamination by other bacteria. For this purpose, the culture is plated out on BHI plates with or without the Hemophilus required co-factors. Only those cultures completely free of contamination are used.

b) Preparation of Hemophilus cells: 4 to 12 liters of Hemophilus cells are grown in complete BHI medium with vigorous aeration at 37°C to about 10^9 cells/ml. Antifoam is added whenever necessary. The generation time for the three strains of Hemophilus cells used is around 40 min. The cells are then collected by centrifugation at 4°C at 6K (rpm) for 20 min, washed once with 500 ml of buffer a and recentrifuged at 6K for 20 min. The cells may be stored at -20°C until ready for extraction of enzyme.

c) Sonication: Before sonication, the cells are thawed, and resuspended homogeneously in about 10-30 ml of buffer a (4°C) by use of an Omni-mixer. The cell suspension is transferred to a small, stainless steel beaker, and a 10 µl aliquot of the mixture is removed for an initial OD_{650} reading. While being cooled by a salt ice mixture

FIGURE 1

ISOLATION OF RESTRICTION ENDONUCLEASES FROM HEMOPHILUS CELLS



surrounding the beaker, the cells are sonicated for a total of 10 min with a Branson sonifier at maximum ampere output. For solution volumes exceeding 10 ml, a standard tip is used for sonication. In case of smaller volumes, a microtip is used. Sonication is usually performed at half to one minute durations to prevent heating of the cell suspension to higher than 7°C. The extent of sonication is monitored by reading the OD_{650} of the cell culture during sonication. When a plateau is reached, cell rupture is complete. The lysis process is also checked by looking at the cells under the light microscope.

All subsequent steps are performed at 4°C.

d) Centrifugation: The sonicated cell mixture is then dispensed into type 30 rotor centrifuge tubes, and subjected to centrifugation at 27K for 1 hr. The cell pellets are discarded and the enzyme is contained in the supernatant (crude extract).

e) Biogel A 0.5 column: A Biogel A 0.5 column (2.5 cm x 48 cm) is prewashed with 10 column volumes of buffer b. Crude extract from culture volumes up to 12 liters can be applied to this column size.

The supernatant obtained from the sonicated mixture is adjusted to 1 M NaCl by the addition of solid sodium chloride before applying to the top of the Biogel column. Right after the supernatant has completely entered the column, an equal volume of buffer b is added to the column top to rinse off the sides. Elution is carried out at 1 ml/min using buffer b and 6 ml fractions are collected until the amber colored crude extract is completely eluted off from the column. Column fractions are assayed for endonuclease activity by the "clot assay" (described in section IVb); the OD_{260}, OD_{230} readings are taken with

buffer b as blank.

The enzymes normally elute from the Biogel column between volumes of 180-230 ml, at the OD_{230}/OD_{260} peak. Fractions containing endonuclease activity with A_{230}/A_{260} ratio higher than 3 are pooled.

f) Ammonium sulfate precipitation: The pooled fractions are diluted with two volumes of 0.02 M Tris, pH 7.4, and stirred in an ice bath. Solid ammonium sulfate (Special Enzyme Grade, purchased from Schwarz/Mann, Orangeburg, New York), is added slowly to obtain a 0-50% precipitate. The 0-50% supernatant is further precipitated with ammonium sulfate to obtain a 50-60% precipitate. The supernatant is reprecipitated with ammonium sulfate to obtain a 60-70% precipitate. The 50-60% and the 60-70% precipitates which contain the major enzyme activity are combined and redissolved in a minimum volume (about 5 to 10 ml) of buffer c. It is not necessary to adjust the pH during ammonium sulfate precipitation.

g) G25 Sephadex desalting column: A column bed volume five times greater than the sample volume is used. The column is equilibrated with buffer c. The enzyme (amber colored) elutes in the excluded volume and is diluted about three-fold in this step.

h) Phosphocellulose column: Phosphocellulose (P11) is charged in the following way: P11 powder is suspended in twice its volume of distilled, deionized water and allowed to settle. The supernatant is decanted off. This process is repeated several times until the resin is washed to a white color. Then, the phosphocellulose is treated with 0.5 N NaOH for 30 min, washed thoroughly with distilled water in a Buchner funnel until the pH becomes neutral. The phosphocellulose is then treated

with 0.5 N HCl for 30 min. After thorough rinsing with distilled water it is resuspended in 0.01 M potassium phosphate buffer, pH 7.4, and ready for use. A small phosphocellulose column (1 x 5 cm) is poured and equilibrated with buffer c. The maximum capacity of the phosphocellulose column is about 100 mg protein/ml column volume. The G25 elute which contains the desalted sample is directly applied onto the PC column at a rate of 4 ml/hr and the material which does not bind to the column is collected in a flask. The column is then eluted stepwise with three column volume portions of buffer c, containing increasing molarities of KCl from 0, 0.1 M, 0.2 M to 1.0 M. The pump speed is 36 ml/hr and one column volume fractions are collected. The peak of activity for Hin and Hpa is eluted at 0.2-0.4 M KCl, whereas Hae elutes at 0.7 M KCl. These fractions can be directly used for digestion and were stored at 4°C.

i) DEAE column: Contaminating exonuclease activity in some of the phosphocellulose fractions may be removed by chromatography on DEAE-cellulose (Whatman DE52). The column is equilibrated with 0.05 M NaCl, 0.01 M potassium phosphate, pH 7.4, 10% glycerol. The PC column enzyme fraction is applied at 10 mg of protein per ml of column bed volume. Elution is stepwise with three column volumes each of 0.05 M, 0.1 M, and 0.2 M NaCl in buffer c at 28 ml/hr. One column volume fractions are collected and assayed for both endonuclease and exonuclease activity. For Hpa, the endonuclease elutes at 0.05 M NaCl, whereas the exonuclease activity elutes after 0.1 M NaCl. The DEAE fractions containing the endonuclease activity can be used without further concentration.

V. Assays of Endonuclealytic Activity

a) Viscometry: (Smith and Wilcox, 1970) DNA viscosity measurements are performed at 30°C in an Ostwald viscometer having a flow time for buffer of approximately 68 seconds. The viscometer is filled with 3.5 ml of assay buffer, containing 40 µg/ml of calf thymus DNA (Worthington, stock solution = 1.4 mg/ml) in buffer d. After addition of 10-50 µl of enzyme fractions, flow time measurements (t) are made at several time intervals. At the end of the experiment (i.e., when t becomes constant), 50 µg of pancreatic DNase are added. After 5 min, the final flow time measurement (t_o) is taken. The DNA viscosity is expressed as specific viscosity, $\eta sp = (t/t_o) - 1$. Specific viscosity measurements are plotted against time on semi-logarithm paper as fractional values of zero-time value. One unit of enzyme activity is defined as that amount which produces a decrease in the DNA specific viscosity of 25% in 1 min under the conditions described above.

b) Clot assay: An easy qualitative assay for large numbers of samples (Kelly and Smith, personal communication).

The reaction mixture consists of 0.1 ml of buffer d, 20 µl of phenolized salmon sperm DNA (Sigma, 1.5 mg/ml in 0.01 M NaCl, 0.04 M NH_4OH, pH 9, solubilized without stirring), 50 mM NaCl (for Hin), 20 mM NaCl (for Hpa and Hae), 10-20 µl of enzyme fractions. Incubation is at 37°C for 30 min. 50 µl of 0.3% BSA, 0.2 ml of cold 10% TCA are added. Control without enzyme should contain flocculent precipitates of DNA. Presence of endonuclease in reaction mixture causes the formation of a finely-divided precipitate.

c) Polyacrylamide slab gel electrophoresis: Fractions collected from stepwise elution from the phosphocellulose and DEAE columns can be tested for both endonuclease and exonuclease activity by this method since the enzyme fractions are relatively free from contaminating host DNA and other protein. 0.1 µg of P^{32} φX RF (∿10,000 cpm) is digested in separate tubes in the buffer d by 10 µl aliquots of different column fractions. Incubation is at 37°C. The reaction is stopped by the addition of EDTA. The sample is then adjusted to 1% SDS, 15% sucrose before layering on a 4 or 5% polyacrylamide gel slab. After electrophoresis, the gel is dried and autoradiographed (See Chapter V). The gel profiles of the ^{32}P φX RF substrates after hydrolysis by various enzyme fractions are compared. Endonuclease activity is detected by the appearance of discrete bands of φX RF fragments, and exonuclease activity is present when the radioactivity applied has disappeared from the gel after electrophoresis.

VI. Exonuclease Activity Assay

The reaction mixture consists of 20 µl of P^{32} φX RF or sonicated P^{32} *E. coli* DNA (∿10^5 cpm/µg, 10 µg/ml), 5 µl of 10 times concentrated buffer d, and 20 µl of enzyme fractions. Pancreatic DNase is used in one tube as a control. The mixture is incubated at 37°C for 1-2 hr. EDTA is added to 10 mM to stop the reaction. 100 µl of calf thymus DNA (2.5 mg/ml), 50 µl of 0.3% BSA, and 200 µl of 10% TCA are then added. The mixture is cooled to 0°C. The supernatant is separated from the DNA precipitate by centrifugation at 10K for 10 min. 200 µl of the supernatant are spotted on a Whatman 3MM filter disc, dried in oven and counted in toluene-liquid counting fluid.

Results

I. Preparation of Crude Extract

From 4 liters of culture grown to 10^9 cells/ml, about 10 gm wet weight of cells are obtained. These cells are resuspended in Tris buffer and subjected to sonication. The extent of sonication is monitored by reading OD_{650} of aliquots taken out from the sonicating mixture and diluted them 1:100 into "isotone," a saline buffer. As shown in Table 1, after 8 min of sonication, the OD_{650} reading reaches a plateau, indicating that most of the cells are lysed. The drop of OD_{650} correlates with the observation of lysed cells under the phase microscope. The cell debris is removed by centrifugation at 27K for 1 hr. The supernatant, which is yellowish and clear, already contains detectable endonuclease activity.

II. Viscometric Assay for Restriction Endonuclease Activity in Crude Extract

Smith and Wilcox (1970) have demonstrated that the assay result is valid even on crude extracts. Since no activity is found in crude extracts against homologous DNA, the extract apparently contains a nuclease which is specific towards the foreign DNA. Figure 2 shows the results of a series of viscometric measurements on addition of a crude extract to calf thymus DNA. Two distinct rates of reaction are evident by the shape of the curve, where a sharp fractional decrease in η_{sp} proceeds logarithmically with time until the value is below 0.7, after which the decrease becomes less rapid. The above observation

Table 1

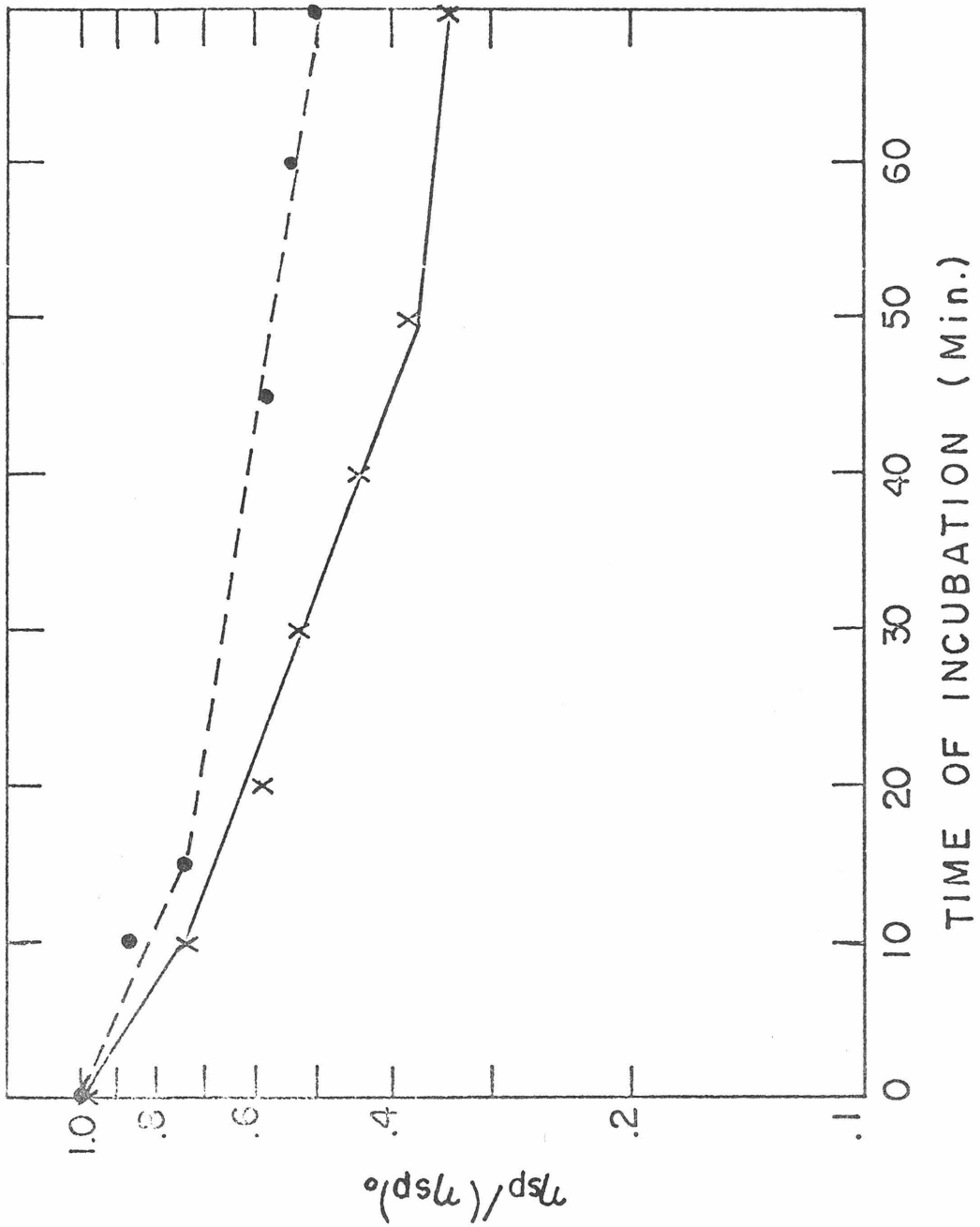
Sonication of *Hemophilus* Cell* Suspension

Time (min)	OD_{650} of 1/100 dilution of cells into isotone	Temperature
0	0.37	3-5°C
5	0.12	3-5°C
7.5	0.06	3-5°C
10.0	0.08	5-7°C

*Value taken from an Hae enzyme preparation. 8 gm of wet cells were resuspended in 12 ml of 0.02 M Tris, pH 7.4, 1 mM glutathione. 10 µl aliquots were taken at various time intervals.

FIGURE 2. Effect of a sonicated cell extract from H. influenzae on the specific viscosity of calf thymus DNA.

A viscometer containing 3.6 ml of calf thymus DNA at a concentration of 40 µg/ml in buffer d (-●—●-) were equilibrated at 30°C. The initial specific activity (ηsp) was taken before the addition of the enzyme. At zero time, 50 µl of the crude extract was added. Measurements of the specific viscosity (ηsp) were then taken at intervals and were plotted as fractional values of the zero time specific viscosity $(\eta sp)_o$. (*-*-*), 50 mM NaCl was included in buffer d.


$\eta_{sp}/(\eta_{sp})_o$
1.0
.8
.6
.4
.2
.1
0
10
20
30
40
50
60
TIME OF INCUBATION (Min.)

suggests that there may be more than one endonuclease acting on the DNA substrate, one of which has a faster reaction rate than the other. The addition of 50 mM NaCl to the reaction mixture enhances the Hin endonuclealytic activity(s) by approximately 2-fold, as shown in Figure 2.

The proportionality between initial rate of decrease in nsp and the amount of extract added provides a quantitative assay for the enzyme.

III. Biogel A 0.5 Column

As the crude extract is layered onto the Biogel column at 1 M NaCl, two zones of intense yellow color very rapidly separate out on the column length with the front one running much faster, leaving in between a relatively clear zone. The front zone consists mainly of large molecular weight host DNA which is excluded from the Biogel 0.5 A column, and contributes to the first peak of OD_{260} reading. The Hemophilus enzymes are normally eluted after the first huge OD_{260} peak but before the appearance of a second OD_{260} peak, which consists of low molecular weight host proteins and nucleic acids. The Biogel 0.5 M (200-400 mesh) has an operating range from 10,000-500,000 M.W. The Hin enzyme has been found to consist of four subunits, each of molecular weight 23,000 (P. H. Johnson, personal communication). A very convenient way to localize the enzyme peak is to obtain the OD_{230}/OD_{260} ratio of the Biogel column fractions. The enzyme activity has been found to coincide with the peak of the OD_{230}/OD_{260} ratio, which is as high as 4-5 and is 80-fold above the background (Fig. 3).

FIGURE 3. Biogel chromatography of the crude extract.

45 ml of a Hemophilus parainfluenzae crude extract containing 5,800 A_{260} units and adjusted to 1 M NaCl were applied to a 2.5 x 48 cm column equilibrated in buffer b. The elution and analysis procedures were described in Materials and Methods.

(-o—o-) A_{260} units/ml; (-●—●-) A_{230}/A_{260}.

Endonuclease activity as detected by the "clot assay" was represented by the hatched area.

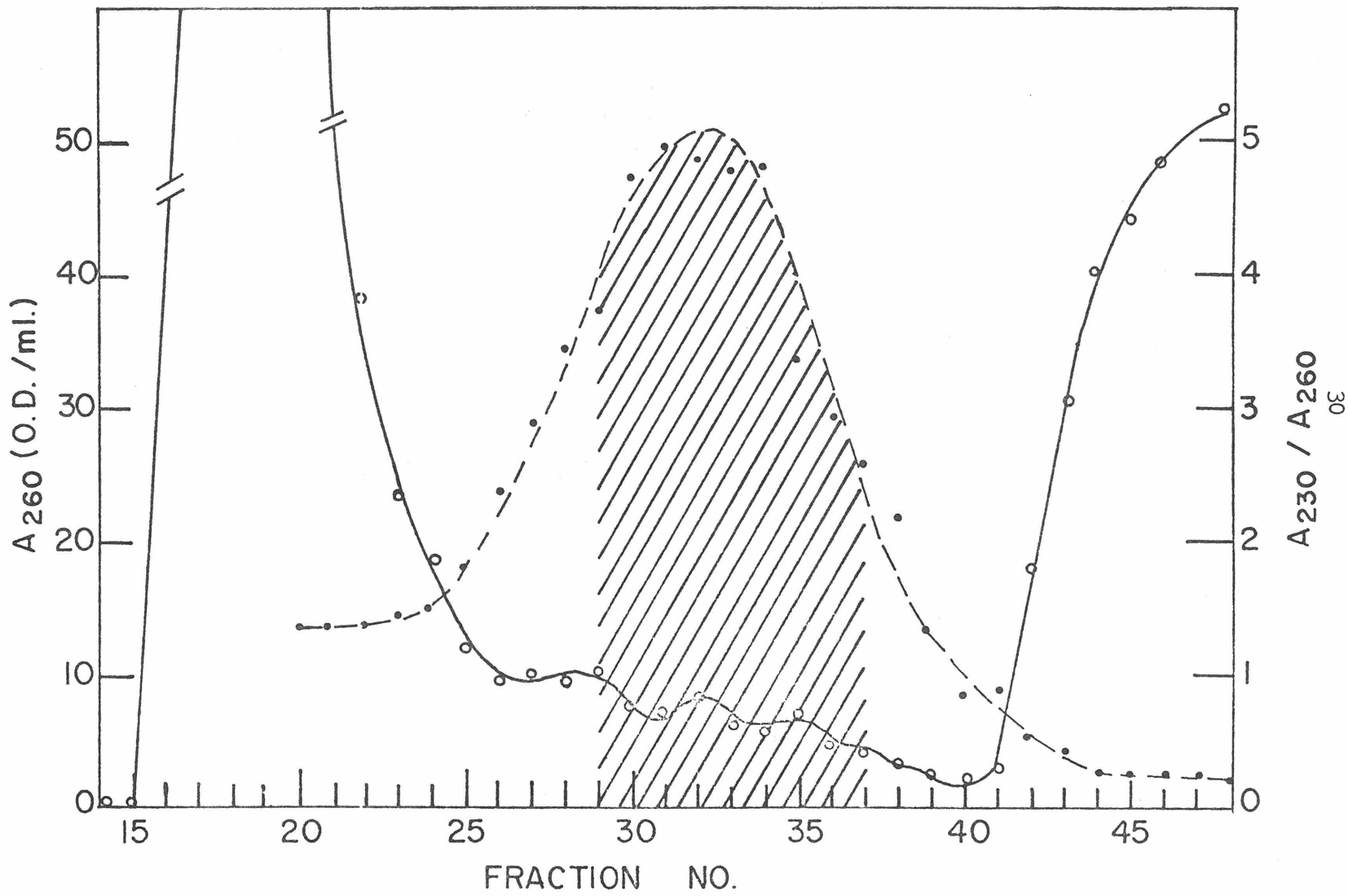
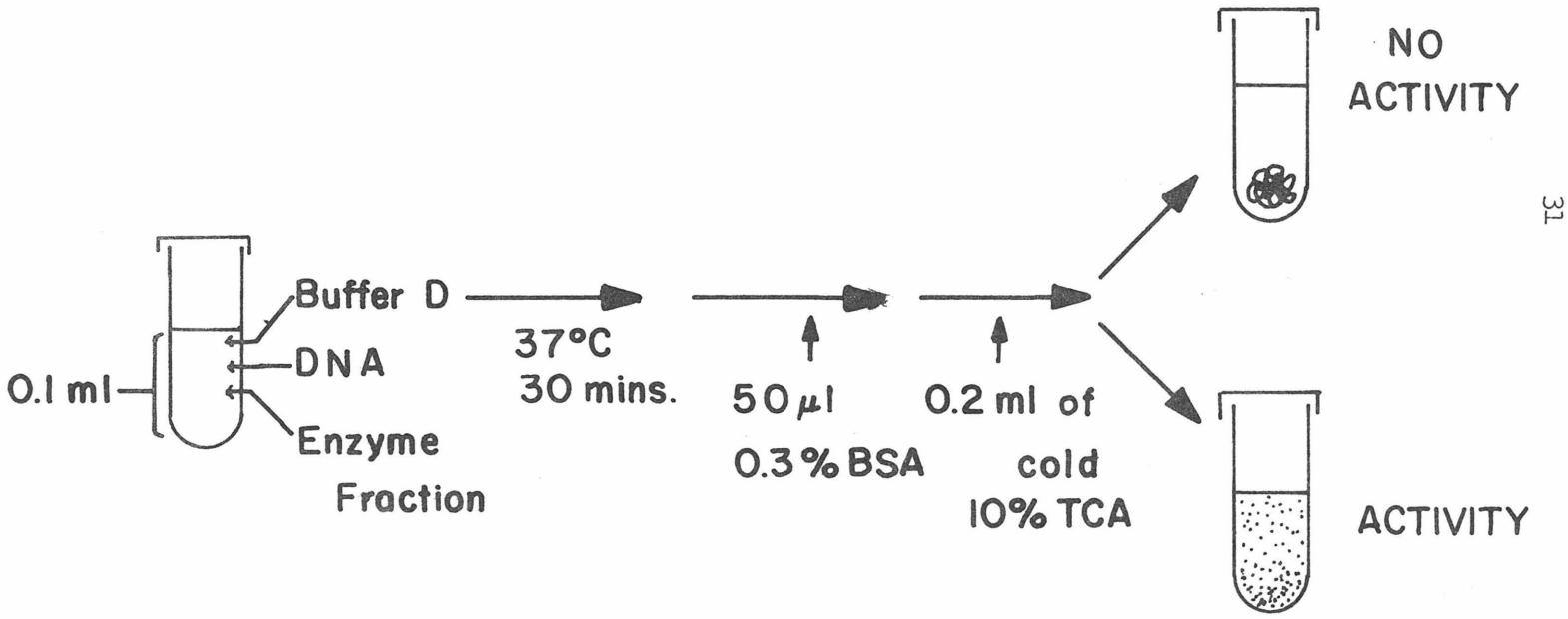

50
40
30
20
10
0
A260 (O.D./ml.)
5
4
3
2
1
0
A230 / A260
15
20
25
30
35
40
45
FRACTION NO.

FIGURE 4

Schematic Diagram for the Clot Assay



An easy and simple qualitative assay for a large number of column fractions is the "clot assay" described in Materials and Methods and is shown in Figure 4. Only 20 µl from each of the 6 ml column fractions are required to produce a fine precipitate. It should be noted that the aliquot should be diluted to below 50 mM Na^+ in the assays since high Na^+ concentration is known to inhibit some restriction enzymes, e.g., Hpa II was inhibited by >50 mM NaCl (Sharp et al., 1973).

IV. Ammonium Sulfate Precipitation

The scheme of ammonium sulfate precipitation steps for the Biogel column fractions from Figure 3 is presented in Figure 5. The amount of ammonium sulfate added each time to attain the required concentration is calculated from Table 2. Most of the enzyme activity is found in the 50-70% precipitate for Hin and Hae preparations, however, for Hpa preparations, some activity is found in the 0-50% precipitate. This observation is in agreement with the findings of Gromkova and Goodgal (1972). However, the majority of enzyme activity is found in the 50-70% precipitate.

V. G-25 Desalting Column

The enzyme, which is amber colored, is excluded while the ammonium sulfate trails behind. The enzyme is diluted about threefold.

a) PC column: Figure 6 shows a typical stepwise elution profile of a phosphocellulose column. The majority of the amber colored component which co-purified with the enzyme does not bind to the column. When

FIGURE 5

AMMONIUM SULFATE PRECIPITATION

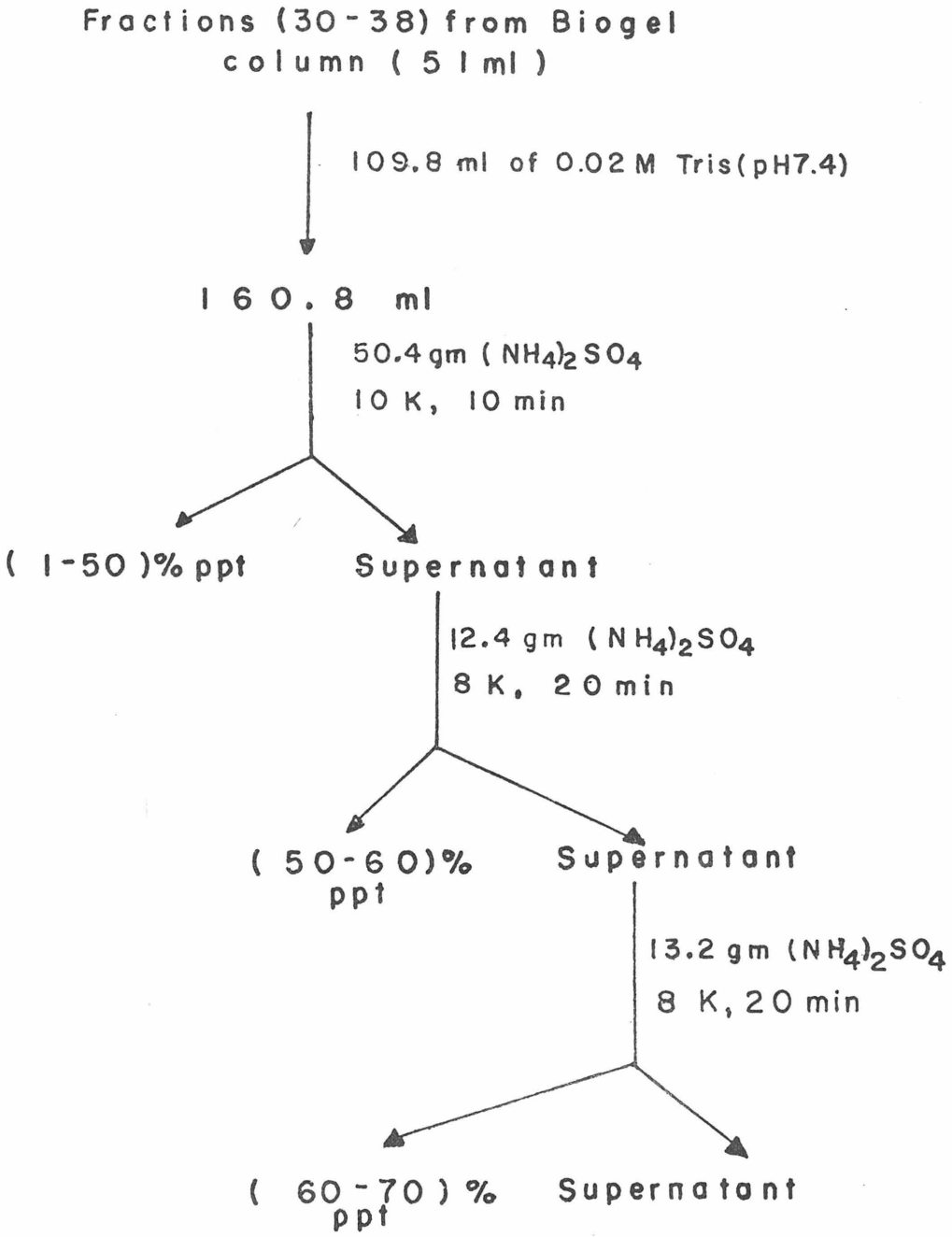


Table 2

Final Concentration of Ammonium Sulfate, % Saturation, at 0°C

Grams Solid Ammonium Sulfate to be Added to 1 liter of Solution

Initial concentration of ammonium sulfate, % saturation, at 0°C	10	20	25	30	33	35	40	45	50	55	60	65	70	75	80	90	100
0	53	109	138	168	187	199	231	264	298	333	369	407	445	485	527	614	707
10		54	83	112	130	142	173	205	238	272	308	344	381	421	461	546	637
20			28	56	74	85	116	147	179	212	246	282	318	356	395	477	566
25				28	45	57	87	117	149	182	215	250	286	323	362	443	531
30					17	28	58	88	119	151	185	219	254	291	329	409	495
33						11	40	70	102	133	166	200	235	272	310	389	474
35							29	59	89	121	154	188	223	259	296	375	460
40								29	60	91	123	156	191	226	263	341	424
45									30	61	92	125	159	194	230	307	389
50										30	62	94	127	162	198	273	354
55											31	63	95	129	165	239	318
60												31	64	97	132	205	283
65													32	65	99	171	248
70														32	66	136	212
75															33	102	177
80																68	141
90																	71

Values were taken from Data for Biochemical Research, edited by Dawson et al. (1969), pg. 616. Oxford University Press.

FIGURE 6. Phosphocellulose chromatography of the 50-70% ammonium sulfate precipitate.

15 ml of the G-25 desalted ammonium sulfate precipitate from an H. aegyptius culture containing 400 A_{280} units were applied to a 7 ml phosphocellulose column equilibrated in buffer c. The elution procedure was described in Materials and Methods. (●-●-●-●) OD_{230}/ml.

Page 35 is missing.

each PC column fraction is assayed for enzyme activity, it is commonly found that the enzyme eluted at more than one salt concentration. The variable results observed on the PC columns may be due to differences in different batches of P11, and, perhaps, the way they have been charged. However, the Hpa and Hin activities are mostly eluted around 0.2-0.4 M KCl. The majority of the Hae activity is eluted at 0.7 M KCl.

VII. DEAE column: Some enzyme fractions are heavily contaminated with exonuclease activities, e.g., in a 0.3 M KCl elute of Hpa preparation, 10-14% of the P^{32} ϕX RF are rendered acid soluble after incubation with the enzyme aliquots (2.6 µg protein) at 37°C for 1 hr. To remove the exonuclease activity, an aliquot of the 0.3 M KCl PC column eluate is diluted with 0.01 M potassium phosphate buffer, pH 7.4, so that the final K^+ concentration is at or below 0.05 M K^+ before applying to the DEAE column. The DEAE fractions which contain endonucleolytic activity but not exonuclease activity can be stored in the phosphate buffer, with 10% glycerol at 4°C.

Discussion

The main problem with the isolation procedure is to obtain pure cultures of Hemophilus cells. Since Hemophilus cells are cultured in Brain Heart Infusion medium, which is a very rich medium, contamination by other bacteria such as E. coli occurs very easily unless strict aseptic procedures are followed. However, due to the cofactor requirement of Hemophilus cells, the purity of inoculum or stock cultures can be conveniently checked by plating out the cells on plates with or

without the cofactor supplement.

Once the cells are obtained, the isolation procedure is relatively easy and straightforward. The Biogel column is highly reproducible. The PC column yields variable results with different batches of PC. The DEAE step is only necessary to remove the exonuclease activity.

Since the publication of the Smith and Wilcox procedure (1970), there have been reports of alternative procedures to purify the Hpa restriction enzyme (Sharp et al., 1973; DeFilippes, 1974). The Sharp procedure to separate Hpa I (M.W. 65,000) and Hpa II (M.W. 25,000) on one Biogel column has been attempted in this laboratory once with no success. The reason for this is unknown but may be due to different origins of Hemophilus parainfluenzae cells used for the isolation of the enzyme. The association properties of the Hpa enzymes may differ slightly in different cell strains. The other procedure has not been tested.

References

DeFilippes, F. M. (1974) Biochem. Biophys. Res. Commun. 58:586-596.

Gromkova, R., and S. H. Goodgal (1972) J. Bacteriol. 109:393-409.

Meselson, M., and R. Yuan (1968) Nature 27:1110-1114.

Sharp, P., B. Sugden, and J. Sambrook (1973) Biochemistry 12:3055-3061.

Smith, H. O., and K. W. Wilcox (1970) J. Mol. Biol. 51:379-391.

Chapter III

Production of Specific Fragments of ϕX174 DNA by the *Hin*, *Hpa* and *Hae* Restriction Enzymes

Introduction

One powerful approach to study the structure and function of the ϕX174 genome is to use restriction enzymes, which are sequence specific endonucleases, to produce unique DNA fragments containing particular parts of the ϕX genome. Recent reports of Edgell et al. (1972) and Middleton et al. (1972) have presented preliminary results of the use of restriction endonucleases from Hemophilus influenzae (Hin) and Hemophilus aegyptius (Hae) to cleave ϕX174 RF molecules into specific fragments. Here we describe the analysis on polyacrylamide gels of specific ϕX fragments produced by the Hin, Hae enzymes and, in addition, by the H. parainfluenzae enzyme (Hpa). The 5' terminal nucleotide sequence of the Hin and Hpa fragments have been determined by the action of polynucleotide kinase in the presence of γ AT^{32}P.

Materials and Methods

I. Phage and Bacterial Strains

a) Escherichia coli H502 (thy^-, uvr A, endo I^-, su^-) was used as the host strain for ϕX174 am3.

b) ϕX174 am3 is an amber mutant of cistron E with a lysis defective phenotype (Hutchison and Sinsheimer, 1966). The phage stock is the kind gift of Dr. P. Johnson and has been prepared from a single plaque. The titer is determined by assay on the amber suppressor strain E. coli HF4714. Wild type revertants are detected by parallel plating on E. coli C; their frequency is less than 10^{-5}.

II. Media

a) Minimal medium is that described by Knippers et al. (1969) and is prepared in a modified method. For one liter of minimal medium, 6 gm KCl, 3 gm NaCl, 1.1 gm NH_4Cl and 12 gm of Tris base are dissolved in 400 ml of water (Solution I). 3 gm of amino acid mixture (Nutritional Biochemical) are dissolved in 500 ml of H_2O by boiling (Solution II). After it is cooled to room temperature, solution I is added. Then 1 ml of 1 M $MgSO_4$, 1 ml of 10 mg/ml thiamine-HCl, 2 ml of 1 M $CaCl_2$ are added and mixed thoroughly (Solution III). The solution is then adjusted to pH 7.5 with concentrated HCl and brought to a total volume of one liter with distilled water before autoclaving. Before inoculation, solution III is supplemented with 10 ml of 20% glucose, 10 ml of 0.2 M sodium phosphate, pH 8, and 1 ml of 2 mg/ml thymine per liter of medium.

III. Preparation of H^3-ϕX RF

Freshly cloned H502 has been obtained from Dr. R. Rohwer and an overnight culture in 10 ml of minimal medium supplemented with 2 µg/ml thymine is used as inoculum for a 100 ml culture. When the cell density reaches 3×10^8/ml, after vigorous aeration at 37°C, chloramphenicol is added to a final concentration of 30 µg/ml. Five minutes later, ϕX174 am3 is added to multiplicity of 10 phage/cell. After 2 min, 5 mCi of H^3-thymidine (Schwartz Bioresearch) is added. The culture is incubated at 37°C for another 2 hr.

The following procedure has been adapted from Johnson and Sinsheimer (1974). The cells are collected by centrifugation, washed

once with 0.05 M Tris-HCl, 0.005 M EDTA, pH 8.0, and suspended in 10 ml of the same buffer. Lysozyme is added to give a final concentration of 0.2 mg/ml and the mixture incubated at 0°C for 40 min. Subsequently, pronase (nuclease free, Calbiochem) and sodium dodecyl sulfate are added to a final concentration of 200 µg/ml and 0.8%, respectively. Incubation is continued for an additional 6 hr at 37°C. The solution is adjusted to 1 M NaCl and incubated overnight at 0°C to precipitate selectively the high molecular weight DNA (Hirt, 1967). The sodium dodecyl sulfate DNA precipitate is removed by centrifugation for 30 min in a SS34 rotor of the Sorvall centrifuge. The supernatant is diluted with an equal volume of Tris-EDTA.

Freshly distilled phenol is equilibrated with an equal volume of Tris-EDTA and the excess aqueous phase is removed. The pH of the phenol is adjusted to around 8 by addition of a few drops of 1 N NaOH. The supernatant is extracted once with an equal volume of phenol for 15 min at room temperature by vigorous rotation. The DNA is precipitated from the aqueous phase by adding 2 volumes of isopropanol, 0.1 volume of 3 M sodium acetate (pH 5.5) and cooling to -20°C for overnight.

The DNA precipitate is collected by centrifugation at 25K for 1 hr in an SW27 rotor, dissolved in 5 ml of Tris-EDTA and dialyzed against 3 changes of 1.5 liters of 0.1 M Tris, pH 8, 1 mM EDTA, for 15 hr. Two µg of heat treated (80°C for 15 min) pancreatic ribonuclease A (Sigma, 2 mg/ml of 0.01 M Tris, pH 7.5) are added per A_{260} unit and the solution incubated at 37°C for 1 hr. The final volume of the mixture is 6.5 ml.

An additional 3.5 ml of the dialysis buffer is added to adjust the final volume to 10 ml. To this, 9.2 gm of cesium chloride, and 0.2 ml of 10 mg/ml freshly prepared ethidium chloride solution are added and mixed well. The solution is equally dispensed into 2 Type 50 cellulose nitrate tubes, which are completely filled with baytol oil and capped. Centrifugation is carried out at 40K for 60 hr, at 5°C. Peaks corresponding to RFI and RFII are pooled separately. The ethidium bromide is removed by passing the pooled fractions over a Dowex 50 X-2 column (0.5 cm x 10 cm), and the DNA eluted with 10 x SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate). The DNA is then precipitated with alcohol and redissolved in 1 ml of Tris-EDTA. To remove residual cesium chloride which may coprecipitate with the DNA, the resuspended DNA is dialyzed against 6 liters of Tris-EDTA at 4°C. The specific activity of the RFI prepared by the above method is approximately 5×10^4 cpm/μg; it is free from host DNA contamination.

IV. Polyacrylamide Gel Electrophoresis

Electrophoretic analysis of ϕX174 digests are carried out in 4 to 5% polyacrylamide gels. The buffers and methods used are modifications of those described by Loening (1967). The stock solutions are a) 5A electrophoresis buffer, composed of 0.2 M Tris, 0.1 M sodium acetate, 5 mM EDTA, 0.5% SDS (pH 7.8), b) acrylamide stock--15 gm acrylamide and 0.75 gm bisacrylamide are dissolved in 100 ml H_2O and stored in dark bottle with aluminum wrap outside at 4°C. Stock solutions should not be kept more than one month. c) Stock solution of N,N,N',N',tetramethylethylenediamine (TEMED), d) solid ammonium

persulfate. b), c) and d) are purchased as an electrophoresis kit from Bio-Rad Laboratories, California, and are all stored at 4°C.

Plexiglas tubes of internal diameter 1 cm and of 20 cm in length are routinely used to prepare cylindrical gels to resolve ϕX174 restriction enzyme fragments.

To prepare a 1 x 20 cm 5% polyacrylamide gel, 6 ml of acrylamide stock are well mixed with 8.4 ml of distilled water. 3.6 ml of 5A buffer are added and the solution gently swirled. Then 3 drops of TEMED and 6 drops of freshly prepared ammonium persulfate solution (0.1 gm/ml water) are added and well mixed. The gel solution is immediately poured into a vertically clamped plexiglas tube, the bottom of which is sealed by a piece of parafilm or a rubber disc.

When plexiglas tubes are used, considerable streaking of the sample along the sides of the tube may occur since the acrylamide may not adhere well to the surface of the tube. To avoid loss of sample through such streaking, a sample well is constructed in the upper end of the gel by insertion of a tapered plexiglas rod into the agar while it solidifies.

Within 10 min to 30 min after the addition of TEMED and ammonium persulfate to the acrylamide solution, the gel should solidify and turn opaque.

The gel is then ready to be used; the parafilm wrap at the bottom is replaced by tying a piece of dialysis membrane around the end of the gel to prevent it from falling out of the tube.

Usually, gels are prerun in 1A (5A diluted 4 times its volume with water) electrophoresis buffer for half an hour in a

cylindrical gel apparatus to remove ions remaining from polymerization.

The DNA sample is adjusted to 1% SDS and 15% sucrose (Bromophenol blue is added to 1% in some cases) before applying it into the well. Electrophoresis is normally performed at room temperature at a constant potential of 50 V; running time is 1 hr per cm of gel length.

V. Scintillation Counting

To quantitate the radioactivity, gels are frozen and cut into 1 mm segments by using the Mickle gel slicer (Brinkman Instruments). Gel slices are each solubilized with 10 ml of toluene-Liquifluor containing 10% NCS (Amersham-Searle), incubated in the dark at 60°C for 5 hr, then at room temperature for 2 hr. The radioactivity is measured in a Beckman liquid scintillation counter.

VI. End Group Determination

ϕX RF DNA (>98% form I) is degraded by endonuclease Hpa in a reaction mixture consisting of 7 mM Tris (pH 7.4), 7 mM mercaptoethanol, 50 mM $MgCl_2$, 50 mM NaCl, 12 µg of DNA, 125 µliters enzyme (0.1-0.5 units); total volume, 150 µliters. Incubation is at 37°C for 14 hr. The reaction with endonuclease Hin is the same except the $MgCl_2$ concentration was 7 mM. The reaction is stopped by incubation at 0°C with two-fold excess EDTA. The reaction mixture is adjusted to 0.1 M Tris (pH 8.0), 1 unit of alkaline phosphatase (Worthington; purified free from endonuclease activity) added per ml in a volume of 200 µliters, and incubated at 37°C for 45 min. The phosphatase is inactivated by addition of ethylene-glycotetraacetic acid to 6.5 mM followed

by incubation for 7 min at 65°C (Theodore Live, personal communication).

The reaction volume is adjusted to 300 µliters and 10 mM $MgCl_2$, 20 mM mercaptoethanol, 1.7 mM potassium phosphate, 70 mM Tris (pH 8.0), 15 nmol of $\gamma AT^{32}P$ per ml (20 mCi/µmol, ICN), and 80 units of polynucleotide kinase are added per ml. (Polynucleotide kinase is obtained from P. H. Johnson and is purified from T4 phage-infected cells by Richardson's procedure (1965), modified by employing DNA-cellulose and Biogel exclusion.) The reaction mixture is incubated at 37°C for 1 hr; the reaction is terminated by a final concentration of 50 mM EDTA and pyrophosphate.

The labeled DNA is separated from the unreacted ATP by exclusion chromatography on a porous, glass bead column (1 by 38 cm; Sigma G-240-250, approximately exclusion limit 100,000) and then precipitated at -20°C for 12 hr by addition of two volumes of isopropanol and sodium acetate, pH 5.5, to 0.3 M. The precipitate is collected by centrifugation and dissolved in 100 µliters of 25 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 200 µg of pancreatic DNase I (Worthington WDPC grade) per ml. Incubation is at 37°C for 30 min. 10 µliters of 1 M glycine (pH 9.5) and 5 µliters of venom phosphodiesterase (Worthington, purified by the procedure of Sulkowski and Laskowski, 1971, and dissolved in water at 5 mg/ml) are then added, and incubation is continued for 30 min. The reaction is stopped by addition of EDTA to 50 mM, and the volume is reduced to 10 µliters by evaporation.

The sample is subjected to electrophoresis on a strip of Whatman 3MM paper for 60-80 min at 4 kV with a buffer composed of 5% acetic acid, 0.5% pyridine, 10 mM EDTA, pH 3.5. The paper is dried and cut

into 1 cm stripes and the radioactivity is measured in toluene-Liquifluor in a Beckman scintillation counter. Lambda DNA is used as a control in these experiments and is treated identically, except the reaction with endonucleases is omitted. The total incorporations of ^{32}P in the endonuclease Hpa and endonuclease Hin reactions are approximately the same, ± 10%. Control experiments are performed without endonuclease, and there is no significant incorporation.

Results

Figure 1 shows the fractionation of a Hin limit digest of ^{3}H-labeled ϕX174 am3 RFI DNA by 5% polyacrylamide gel electrophoresis. R1, R2, R3, R4, R8 and R9 are separated very well to baseline. The peaks are sharp and well-defined. The total ^{3}H counts under each band are summed and plotted against their relative mobilities (Fig. 1, insert). Peak R6 contains three distinct tracts very similar in size; therefore contains three times the amount of counts as the other band. Peak R7 contains two distinct tracts, which can be partially separated into two peaks by a longer electrophoresis time. The biphasic nature of the curve may be due to variations in proportions of thymine in small fragments.

The resultant fragments derived from a Hpa digestion on ϕX RF as analyzed by 5% polyacrylamide gel electrophoresis are shown in Figure 2. Improved separation of P1 and P2 can be obtained by using gels of lower acrylamide concentration. The positions and proportion of the peaks do not change, indicating that a limit digest of the DNA has been produced. The insert in Figure 2 shows that there is a

FIGURE 1. Polyacrylamide gel electrophoresis of ϕX174 RF DNA fragments produced by H. influenzae restriction endonuclease (Hin).

The reaction mixture contained 0.9 μg ^{3}H labeled RFI DNA, 7 mM Tris (pH 7.4), 7 mM-mercaptoethanol, 7 mM $MgCl_2$, 50 mM NaCl, 10 μl of the Hin enzyme, in a total volume of 100 μl. Incubation was at 37°C for 12 hr. The reaction was stopped by adding EDTA to 10 mM. Sucrose and sodium dodecyl sulfate were added to final concentrations of 5% and 0.5%, respectively. The mixture was incubated at 37°C for 15 min and layered on a 1 cm x 20 cm 5% polyacrylamide gel. Electrophoresis was at 60V for 20 hr. Mobility is the distance of migration in millimeters at the end of the run.

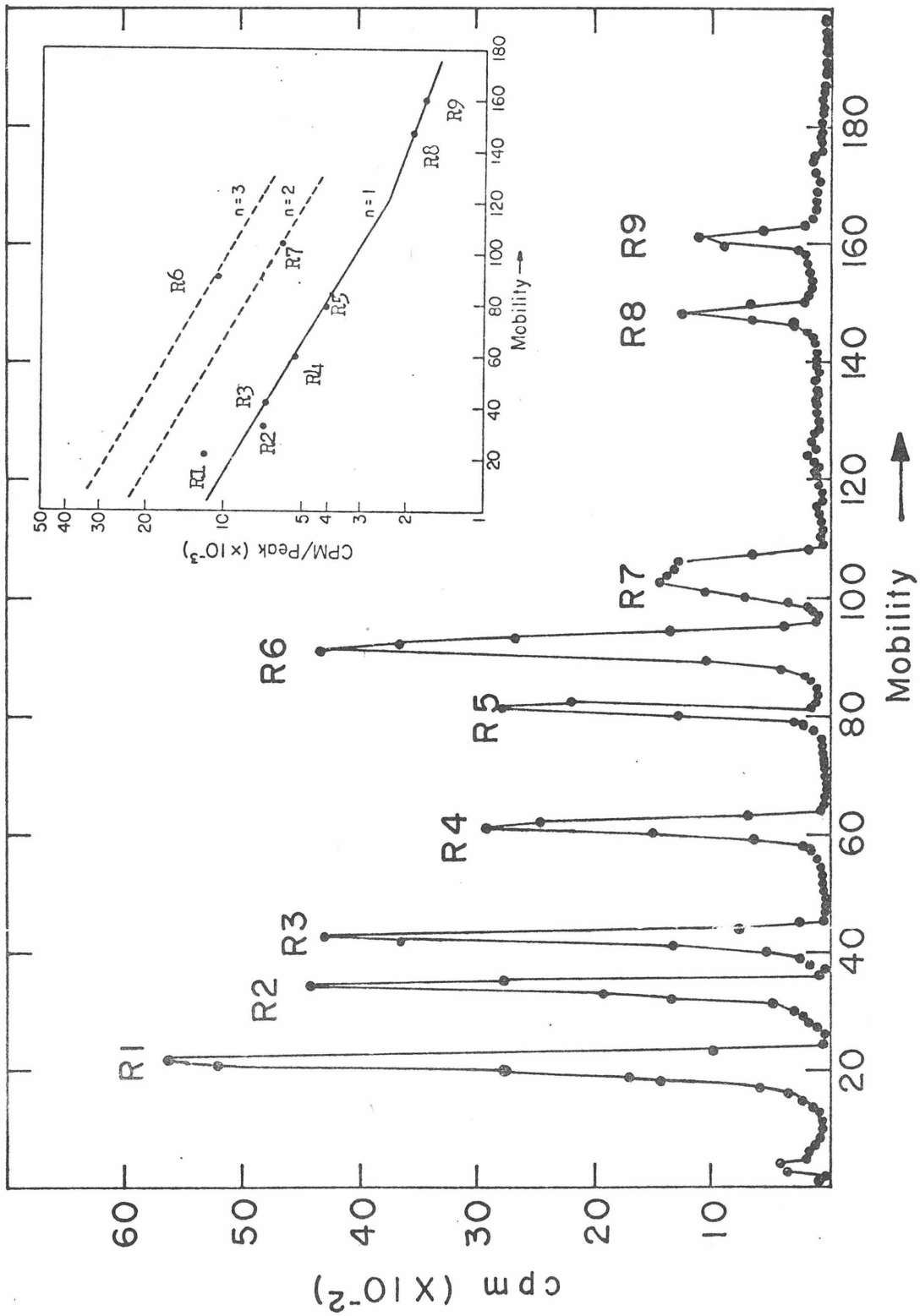
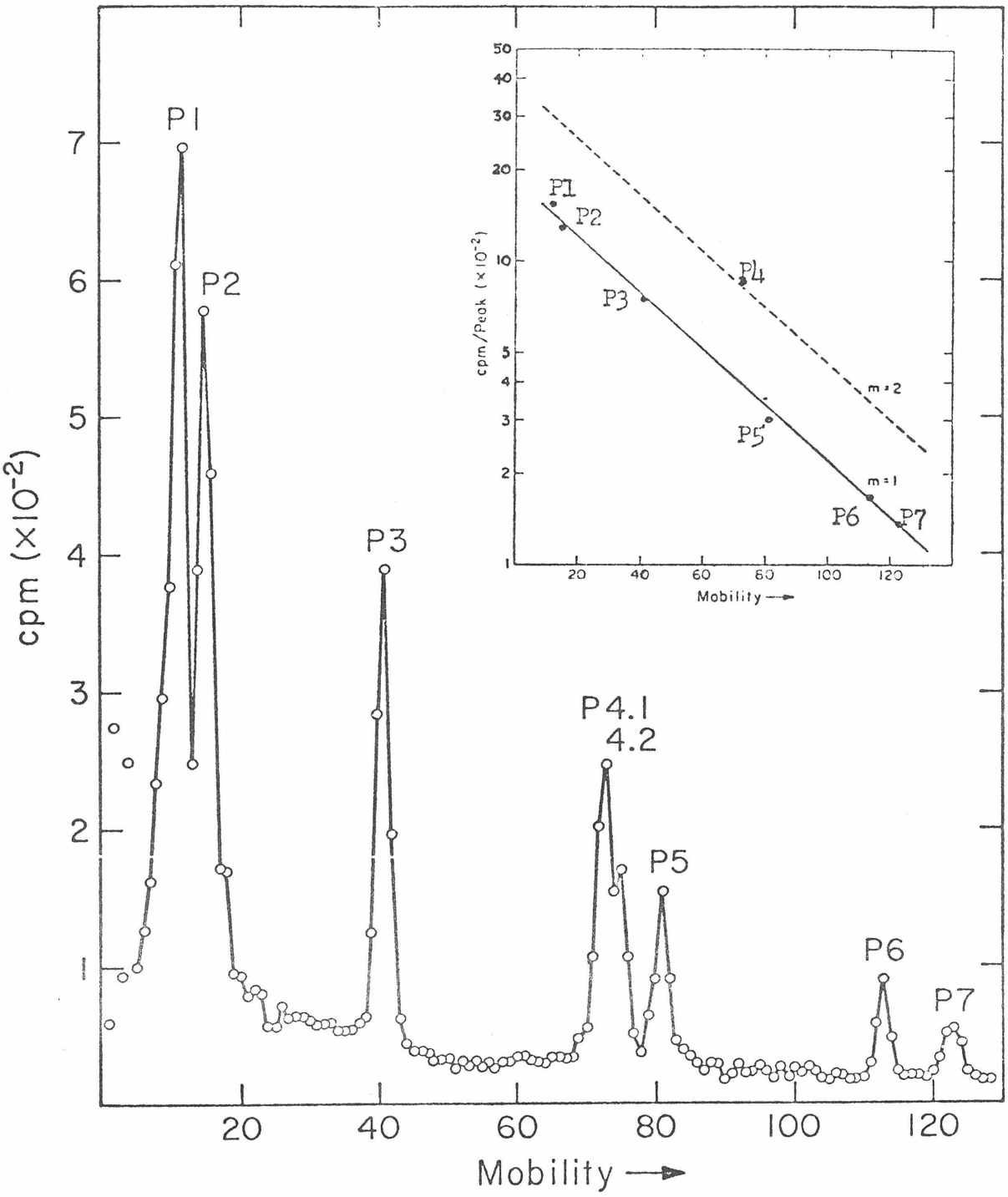

cpm (X 10⁻²)
Mobility
R1
R2
R3
R4
R5
R6
R7
R8 R9
CPM/Peak (x 10⁻³)
Mobility
n=1
n=2
n=3

FIGURE 2. Polyacrylamide gel electrophoresis of ϕX174 RF DNA fragments produced by H. parainfluenzae restriction endonuclease (Hpa).

The digestion mixture consisted of 0.1 µg of ^{3}H-RFI, 7 mM Tris (pH 7.4), 7 mM mercaptoethanol, 50 mM $MgCl_2$, total volume, 100 µl. A 20 µl amount of Hpa was added at zero time and again at 2 hr. Digestion was at 37°C for 10 hr. To stop reaction, EDTA was added to 0.05 M. Sucrose and sodium dodecyl sulfate were added to 5% and 0.5% final concentrations, respectively. The mixture was incubated at 37°C for 10 min and layered on a 5% polyacrylamide gel column (1 x 20 cm). Electrophoresis was at a constant voltage of 45 V for 14 hr. Mobility is the distance of migration in millimeters at the end of the run.


P1
P2
P3
P4.1
4.2
P5
P6
P7
cpm (X10⁻²)
Mobility
cpm/Peak (x10⁻²)
m=2
m=1
P4

linear relationship between the logarithm of the integrated counts in each peak and the electrophoretic mobility. Fragment P4 contains twice the counts expected, suggesting the presence of two distinct tracts of similar size. A partial separation of these two fragments can be obtained with longer electrophoresis time.

The restriction enzyme from Hemophilus aegyptius cleaves ϕX174 into another set of distinct fragments which were fractionated on a 4% polyacrylamide gel (Fig. 3). Z6 consists of 2 tracts of near identical size and contain twice the amount of counts (Fig. 3, insert).

Table 1 shows the size of the endonuclease fragments calculated from the fraction of total counts present in each peak, and expressed as a percentage of ϕX174 RF DNA. These are in close agreement with the molecular size determined by mobility during polyacrylamide gel electrophoresis, using as standards the known molecular size of the Hin limit products R9 and R10 as reported by Maniatis et al. (1973) (see Chapter V).

In order to differentiate the Hpa enzyme cleavage site from that of the Hin enzyme, the restriction enzyme fragments produced by the above two enzymes in separate experiments were end labeled with polynucleotide kinase, in the presence of $\gamma AT^{32}P$.

Chemical evidence for a difference in the recognition site for endonuclease Hpa and endonuclease Hin is presented in Table 2. The nucleotide sequence of the recognition site for endonuclease Hin was determined to be G T Py ↓ Pu A C (Kelly and Smith, 1970), where the center dinucleotide is ambiguous and the arrow indicates the point of cleavage. We find consistent results with ϕX174 RF DNA as substrate

FIGURE 3. Polyacrylamide gel electrophoresis of ϕX174 RF DNA fragments produced by H. aegyptius restriction endonuclease (Hae).

The reaction mixture contained 0.3 µg of ^{3}H labeled RFI DNA, 7 mM Tris (pH 7.4), 7 mM $MgCl_2$, 7 mM mercaptoethanol, 3 µl of the Hae enzyme, in a total volume of 14 µl. Incubation was at 37°C for 4 hr. The reaction was stopped by adding EDTA to 10 mM. Sucrose and sodium dodecyl sulfate were added to final concentration of 15% and 1% respectively. The mixture was layered on a 1 x 19 cm 4% polyacrylamide gel. Electrophoresis was at 50 V for 15 hr. Mobility is the distance of migration in millimeters at the end of the run.

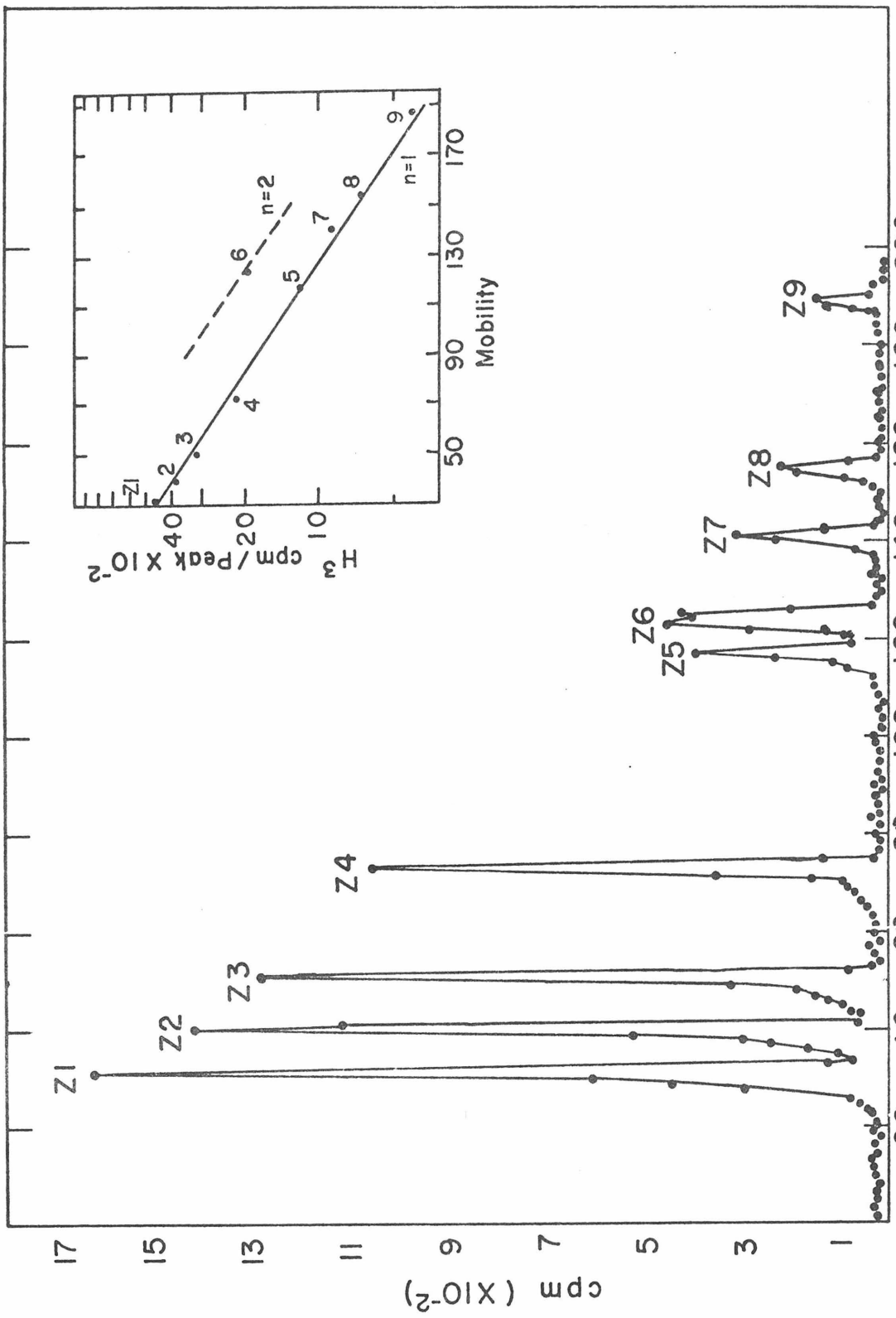

cpm (X10-2)
Mobility
Z1
Z2
Z3
Z4
Z5
Z6
Z7
Z8
Z9
H3 cpm/Peak X10-2
n=1
n=2
Mobility

Table 1

Digestion of ϕX174 RF with Hemophilus Restriction Endonuclease

Hin fragment*	Estimated molecular size†	Hae fragment*	Estimated molecular size†	Hpa fragment*	Estimated molecular size†
R1	20.8	Z1	25.3	P1	30.3
R2	13.0	Z2	20.6	P2	25.4
R3	12.2	Z3	16.6	P3	15.0
R4	9.8	Z4	11.3	P4**a	17.0
R5	7.9	Z5	6.1	b	
R6**a	18.9	Z6**a	10.1	P5	6.0
b		b		P6	3.3
c		Z7	4.6	P7	2.7
R7**a	9.8	Z8	3.4		
b		Z9	1.8		
R8	4.2				
R9	3.4				
Total	100%		100%		100%

*Fragment numbers correspond to those indicated in Figure 1, 2 and 3.

**These fragments contain multiple tracts very similar in size, each tract is designated by a, b, c, etc.

†These values were calculated from the fraction of total ^{3}H-counts in each peak and were expressed as percentage of the total ϕX RF DNA. Note that R10 and Z10 were electrophoresed out of the gel and were not included in the above calculation.

Table 2

A Comparison of the 5'-Terminal Nucleotides of ϕX174 RF DNA Fragments Produced by Endonuclease Hpa and Endonuclease Hin

Substrate	Restriction enzyme	Mol % nucleotide at 5' ends			
		A	T	G	C
ϕX174 RFI	Hpa	<1	<1	<1	97
ϕX174 RFI	Hin	55	6	35	4
T7*	Hin	60	0	40	0
Lambda	none	44	7	45	4

*These values were taken from Kelly and Smith (1970).

for endonuclease Hin, that 55% of the 5' terminal nucleoside is adenosine, 35% being guanosine (Table 2). The enzyme preparation has a very low, but detectable, level of exonuclease contamination which might account for the production of 10% pyrimidines. The data indicate that the recognition site for endonuclease Hpa is different since only deoxycytidylic acid is found at the 5' ends of fragments produced by this enzyme. The presence of a single, unique nucleotide at the 5' ends of these fragments suggests that the recognition site for endonuclease Hpa may not contain an ambiguous dinucleotide as does the site for Hin.

Discussion

With the use of highly labeled [^{32}P] ϕX RF, it has been found that smaller fragments, R10 and Z10, exist in addition to the known, major fragments discussed before. Their sizes are determined by their electrophoretic mobilities to be 80 and 73 base pairs respectively (Chapter V).

In comparing the results presented here to those of the previously published data of Edgell et al. (1972) on Hin ϕX fragment, and Middleton et al. (1972) on Hae ϕX fragments, the number of fragments of limited digests obtained in either case are in agreement with each other, as are the fragment size assignments of the medium sized fragments, e.g., R4, Z4 (around 500 base pairs). However, in case of large fragments, e.g., R1, Z1, and particularly for the smallest fragments, e.g., R9, Z9, there are considerable differences. In the previously published polyacrylamide gel profiles, R8, R9 and Z8 and Z9 are hardly

recognizable over the background; it would therefore be very difficult to assign accurate mass measurements to them. The fact that previous fragment sizes for R8, R9, Z8 and Z9 are only 50% of our values may also be explained by the possible action of contaminating exonuclease in earlier enzyme preparations.

Our finding that the Hpa enzyme produces fragments terminated specifically by deoxycytidylic acid suggested that the Hpa and Hin enzymes are not identical, since the cleavage site for the Hin enzyme is known to be G T Py ↓ Pu A C. However, to avoid exonuclease activity, only a very brief Hpa enzyme digestion has been performed. Conceivably we have only end-labeled a partial digest. A more rigorous experiment would be required to determine the terminal nucleotides of each limit Hpa fragment, instead of a mixture of fragments.

It has recently been reported by Sharp et al. (1973) that the Hpa enzyme is actually a mixture of two enzymes, Hpa I and II. Hpa I cleaves SV40 at three sites which appear to coincide with 3 of the 11 cleavage points attacked by the Hin enzyme, whereas Hpa II cleaves SV40 at a single, unique site. Our Hpa preparation cleaves SV40 into 4 bands, and therefore contains both Hpa I and II.

In addition, the Hin enzyme was further resolved into two components (Roy and Smith, 1973), Hin_dII and Hin_dIII. The recognition site determined by Kelly and Smith (1970), using T7 DNA, is that for Hin_dII. Hin_dII cleaves ϕX RF into 13 fragments, whereas Hin_dIII does not appear to be able to hydrolyze ϕX RF (P. Johnson, personal communication).

Since the cleavage site for $\underline{Hin}_dII$ is known to be G T Py Pu A C, one would expect three to four such sites according to statistical estimations if this site is randomly distributed over ϕX. The high number of occurrences of this site over the small genome of ϕX remains unclear.

References

Edgell, M. H., C. A. Hutchison, III., and M. Sclair (1972) J. Virol. 9:574-582.

Hirt, B. (1967) J. Mol. Biol. 25:365-369.

Hutchison, C. A., III., and R. L. Sinsheimer (1966) J. Mol. Biol. 18: 429-477.

Johnson, P. H., and R. L. Sinsheimer (1974) J. Mol. Biol. 83:47-61.

Kelly, T. J., and H. O. Smith (1970) J. Mol. Biol. 51:393-409.

Knippers, R., A. Razin, R. David and R. L. Sinsheimer (1969) J. Mol. Biol. 45:237-263.

Loening, V. E. (1967) Biochem. J. 102:251-257.

Maniatis, T., and M. Ptashne (1973) Proc. Nat. Acad. Sci. 70:1531-1533.

Middleton, J. H., M. H. Edgell, and C. A. Hutchison, III. (1972) J. Virol. 10:42-50.

Richardson, C. C. (1965) Proc. Nat. Acad. Sci. 54:158-165.

Roy, P. H., and H. O. Smith (1973) J. Mol. Biol. 81:427-444.

Sharp, P. A., B. Sugden, and J. Sambrook (1973) Biochemistry 12: 3055-3061

Sulkowski, E., and M. Laskowski, Sr. (1971) Biochim. Biophys. Acta 240:443-447.

Chapter IV

The Recovery of DNA Restriction Enzyme Fragments

Introduction

Bacterial restriction enzymes are deoxyribonucleases which cleave double-stranded DNA at specific sites and produce unique, high molecular weight fragments (Smith and Wilcox, 1970; Johnson et al., 1973; Middleton et al., 1972). These enzymes provide a most powerful tool for studying the structure and function of DNA. The principal method for separation of the DNA fragments is electrophoresis through acrylamide or agarose gels (Aaij and Borst, 1972).

When fragments are to be isolated for further analysis, several methods can be used. Continuous elution from long cylindrical gels is by far the most convenient way. Here, we discuss the various methods, and in particular describe a continuous electro-elution system modified from that designed by Popescu et al. (1971) and demonstrate how the system is used to analyze a mixture of specific DNA fragments produced from bacteriophage ϕX174 DNA by a restriction enzyme from Hemophilus aegyptius.

Methods and Results

I. Diffusion from Gel Slices

The DNA fragments are first separated by polyacrylamide gel electrophoresis, and their locations are detected either by autoradiography of the wet gel (in case of a vertical slab gel) or by Cerenkov counting of cylindrical gel slices. The segments corresponding to the bands are excised from the frozen slab gel or the cylindrical gel slices from a DNA band are pooled.

The DNA is then eluted by immersion of gel slices in two successive volumes of 1 ml of 0.1 x SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate) at room temperature for 48 hr. The DNA is then precipitated with ethanol.

There are several disadvantages to this method. First, even after incubation for 48 hr at room temperature, only 30-50% of the cpm from the larger fragments (size over 600 base pairs) have been recovered. Secondly, acrylamide from the gel slices also diffuses out with the DNA and would coprecipitate with the DNA fragments after alcohol precipitation. Since acrylamide is highly soluble in aqueous buffer, it can be tolerated when it goes into solution with the DNA on resuspension in buffer after alcohol precipitation. However, the presence of acrylamide in DNA fragment samples is highly undesirable for hybridization or genetic assay experiments later on.

II. Electrophoresis from Gel Slices

The gel slices containing the DNA band are packed into a small cylindrical tube and the DNA fragments are electrophoresed out into small dialysis bags attached to the end of the tubes.

It is crucial that the gel slices be compactly packed, otherwise the current will go through the buffer channels among the gel slices, and the DNA will not be electrophoresed out. Also, large DNA fragments have very slow electrophoretic mobilities in polyacrylamide gels (e.g., R1 migrated at a rate of 0.1 cm/hr at 60 V in 5% gel). It would be a tedious and time consuming process if a large number of DNA fragments were to be processed this way.

The major advantage offered by cutting up the gel containing DNA bands before elution is that bands very similar in size (e.g., R6 and R7, Z5 and Z6), which otherwise would be hard to separate by other methods, can be isolated individually in one step.

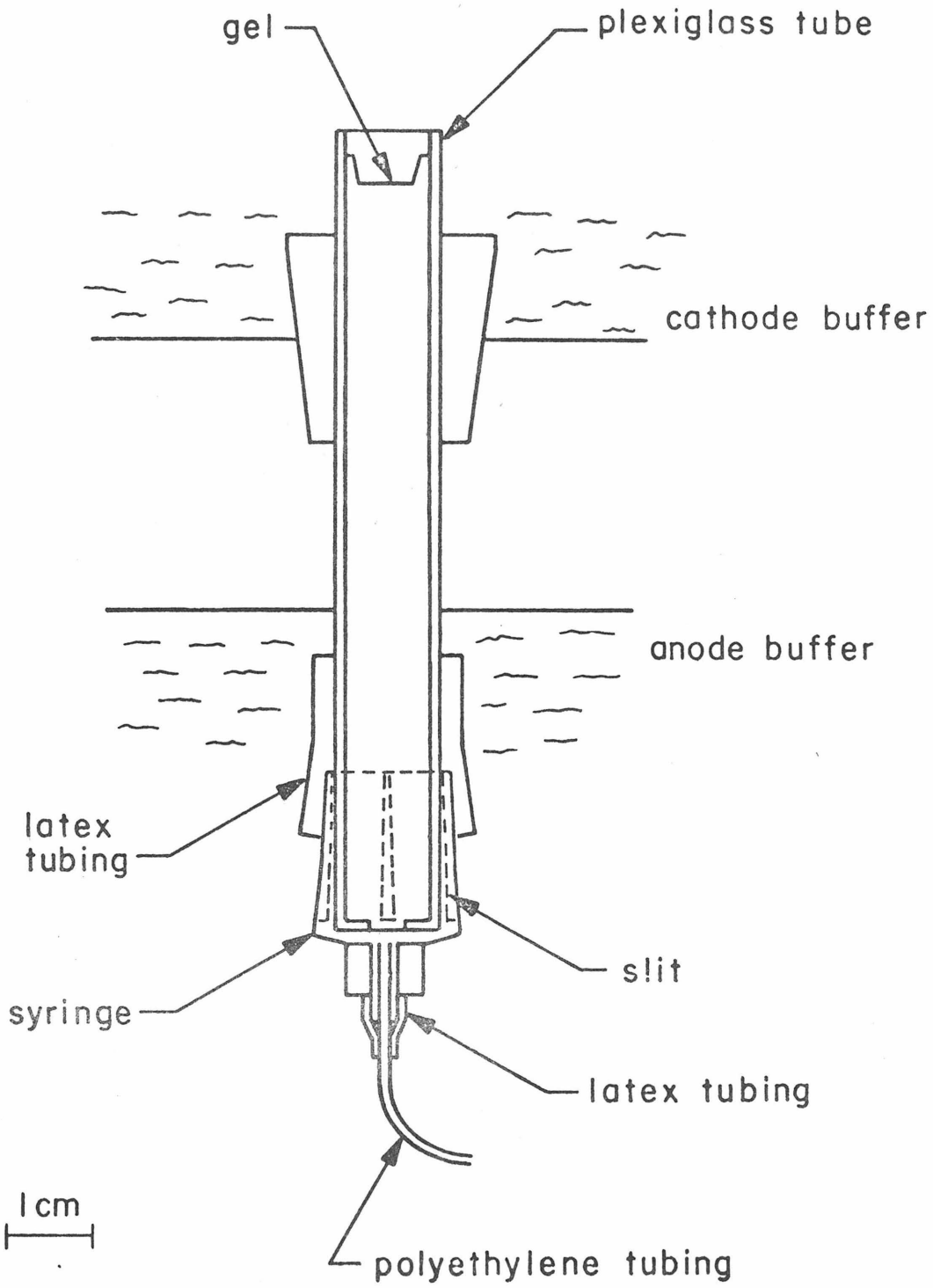
III. Continuous Electro-Elution

2.5% agarose gels are prepared by dilution of a 4% (4 gm/100 ml buffer) agarose stock (Sigma Co.) with electrophoresis buffer (0.04 M Tris, pH 7.8, 0.02 M sodium acetate, 1 mM EDTA). When plexiglas tubes are used, considerable streaking of the sample along the sides of the tube may occur since the agarose does not adhere well to the surface of the tube. To avoid loss of sample through such streaking, a well is constructed in the upper end of the gel by insertion of a tapered plexiglas rod into the agar while it solidifies.

To hold the gel inside the plexiglas tube during electrophoresis, we replace the cheesecloth used previously with a small annular ring (0.3 cm wide) glued onto the end of the plexiglas tube.

For plexiglas tubes of 1 cm (i.d.) by 12 cm, an adaptor is constructed from the bottom 1.8 cm of a 10 ml disposable plastic syringe. As shown in Figure 1, four vertical slits, each 1 mm wide and 1.5 cm long, are cut along its sides. A PE 200 intramedic polyethylene tubing (Clay Adams Co.) is fitted onto the syringe nozzle. The plexiglas tube is inserted into the adaptor and held there by a piece of latex tubing. To minimize dilution of the fragments as they elute from the gel, the end of the gel is positioned only 0.3 cm above the nozzle. The tube is then placed in a standard cylindrical gel

FIGURE 1. Schematic diagram of the continuous electro-elution apparatus.



apparatus, with the adaptor completely submerged in the anode buffer chamber.

Gels are prerun for 30 min. Samples (in 15% sucrose) containing DNA fragments are layered directly into the well of the agarose gel, and electrophoresed at constant voltage using the conditions described in the legend to Figure 2. A peristaltic pump on the outlet tubing provides a constant flow rate.

Figure 2 shows the elution profile of ϕX174 DNA fragments produced by the restriction enzyme from Hemophilus aegyptius (Hae). All the DNA fragments were eluted from the gel in about 15 hr with near 100% recovery. Fragments Z1, Z2, Z3, and Z4 (respective sizes are 1200, 1050, 870 and 600 base pairs) were separated to baseline. Although some loss of resolution of similarly sized fragments, e.g., Z5 and Z6 (320 and 290 base pairs) occurs, polyacrylamide gels of the appropriate concentration or a longer agarose gel can be used to separate them further in a second run, e.g., a 1 x 10 cm 5% polyacrylamide gel will separate Z5, Z6, Z7 and Z8 into distinct peaks.

The peak fractions were pooled into an SW41 polyallomer tube. 0.1 volume of 3 M sodium acetate, pH 5.5, and 2 volumes of isopropanol were added and the tube stored at -20°C for at least 8 hr.

The DNA was collected by centrifugation at 38K for one hour and re-electrophoresed in a 4% polyacrylamide slab gel. Slab gel electrophoresis is performed using the technique described by Studier (1973). Figure 3a shows the results of an analytical gel electrophoresis of ϕX174 fragments recovered by continuous elution. All fragments re-electrophoresed at their normal position, showed no evidence

FIGURE 2. Elution profile of ϕX174 DNA fragments produced by the restriction enzyme from Hemophilus aegyptius (Hae). 60 µl of ^{32}P ϕX RF digestion mixture was adjusted to 1% SDS, 15% sucrose, and 1% bromphenol blue, and layered in the sample well of a 2.5% agarose gel (1 x 11 cm). After 1 hr at 15 V, 2.5 mA, the sample was run at 55 V, 8 mA. 22-drop fractions (0.5 ml) were collected, beginning when the dye was 1 cm above the end of the gel. Pump speed was 5.5 ml/hr. ^{32}P radioactivity was monitored as Cerenkov radiation in a liquid scintillation counter. The anode is at the left.

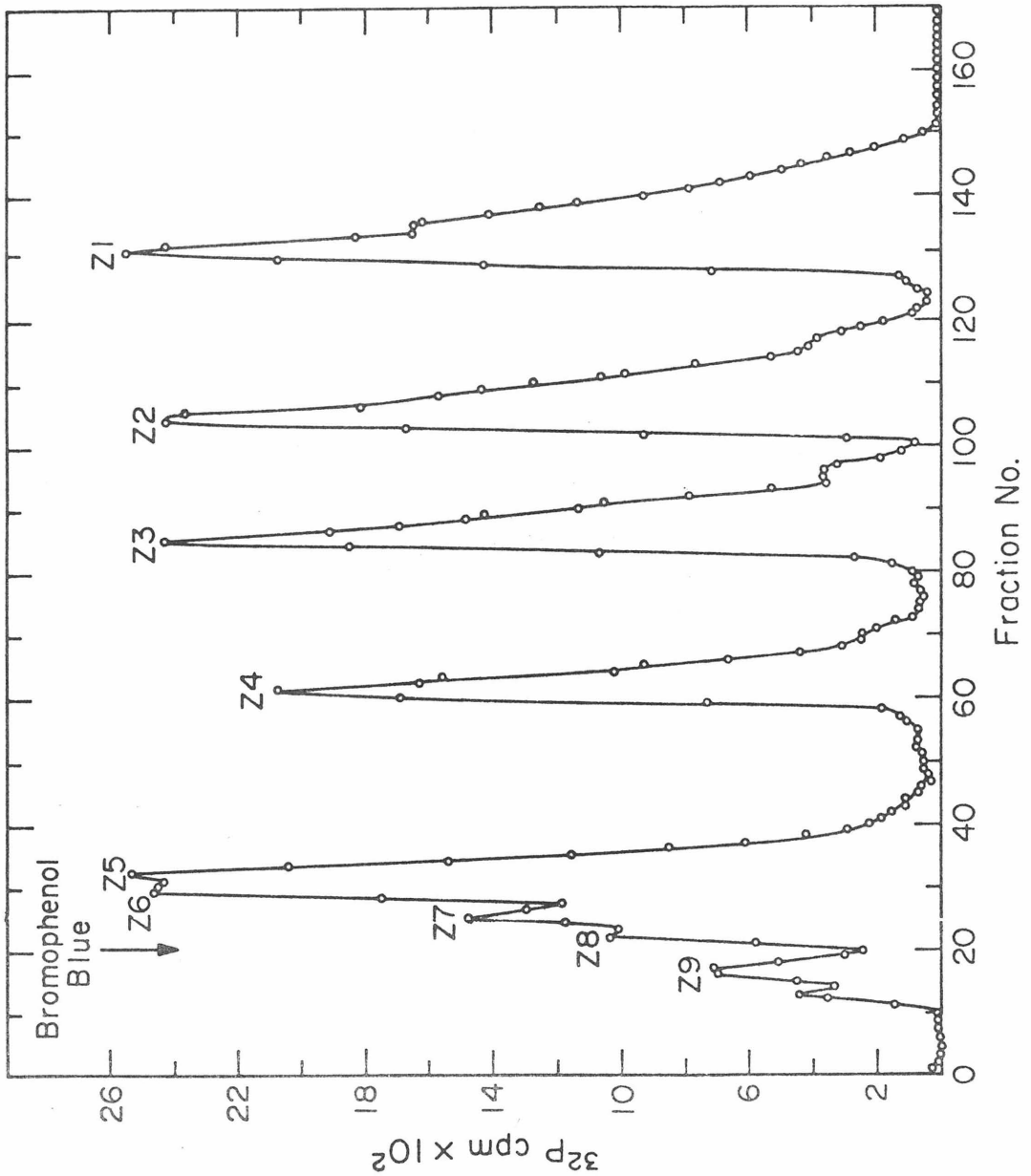
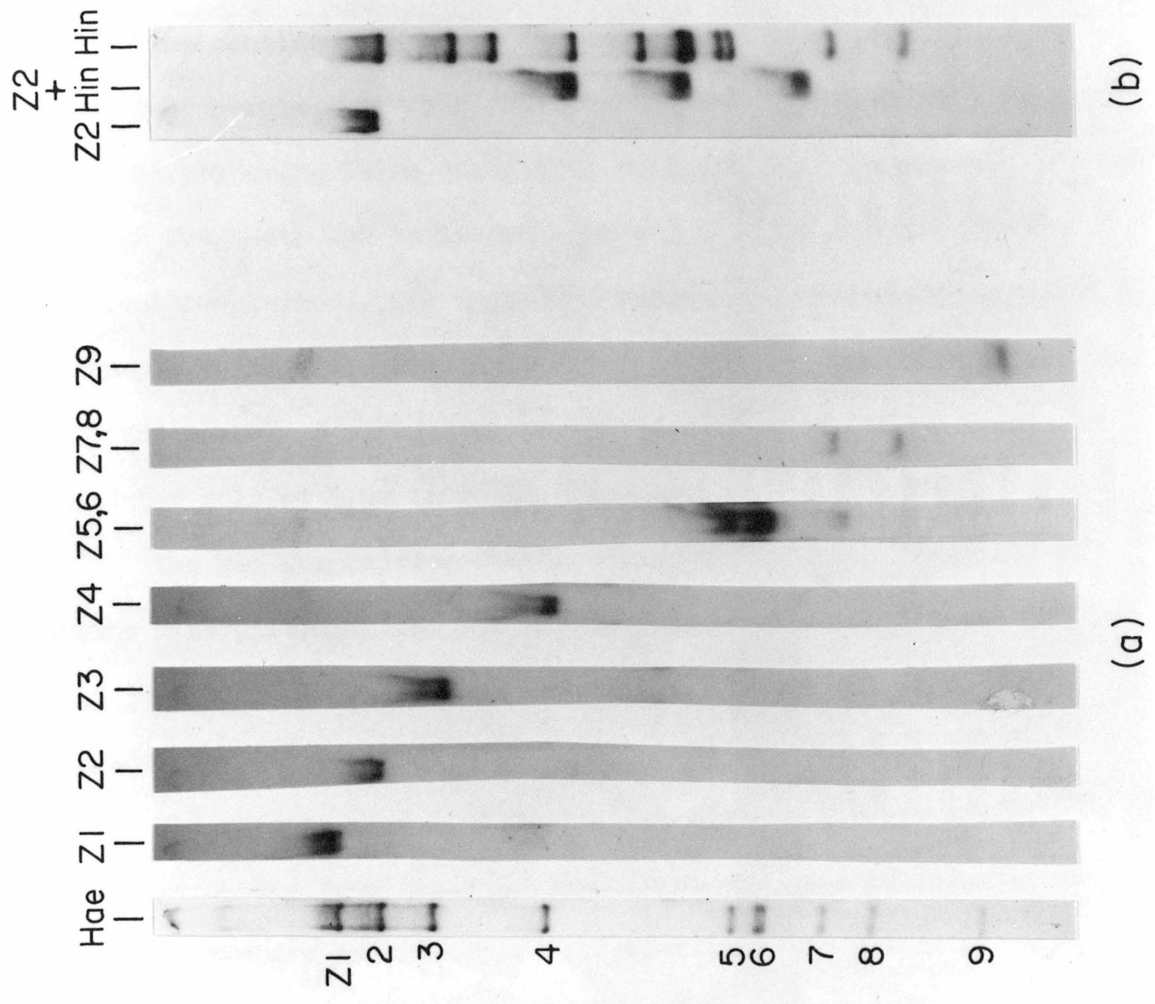

Z1
Z2
Z3
Z4
Z5
Z6
Z7
Z8
Z9
Bromophenol Blue
^{32}P cpm x 10^2
Fraction No.
0
20
40
60
80
100
120
140
160
2
6
10
14
18
22
26

FIGURE 3. Analytical gel electrophoresis of ϕX174 fragments recovered by continuous elution. Respective fractions containing the fragments were pooled and alcohol-precipitated at -20°C. The DNA was collected by centrifugation and redissolved in 40 µl of 0.005 M Tris, pH 7.4, which was then split into two aliquots, one for direct re-electrophoresis, the other for further digestion with the restriction enzyme from Hemophilus influenzae (Hin). Samples were made to 1% SDS and 15% sucrose. Electrophoresis was for 7 hr at 50 V on a 4% polyacrylamide slab gel (14 x 12 x 0.15 cm). After electrophoresis the gel was dried and the autoradiogram was exposed for 12 hr (see Chapter V).

(a) Individual fragments were electrophoresed with complete Hae digest marker.

(b) Digestion of isolated Z2 fragment with Hin.


Hae
Z1
Z2
Z3
Z4
Z5,6
Z7,8
Z9
Z1 2 3 4 5 6 7 8 9
(a)
Z2 + Z2
Hin Hin
(b)

of degradation as the result of electro-elution or precipitation, and were perfectly redigestible with a second restriction enzyme (Fig. 3b). Fragments Z1, Z2, Z3, Z4 and Z9 were completely separated and free from contamination from other fragments.

Discussion

The continuous electro-elution method is extremely simple to set up and operate. It will prove to be very useful as well as convenient for preparing large quantities of individual fragments. 100 µg of ϕX RF fragments can be loaded onto a 1 x 15 cm agarose gel with excellent resolution. The degree of resolution is largely affected by the sample volume; the smaller the sample volume, the sharper are the resultant peaks. A sample volume not greater than 100 µl is recommended for gels of 1 cm internal diameter.

The gel composition can be varied to separate fragments of various size distribution. To separate large partial digests of size range 30-50% of ϕX RF, a lower percentage agarose gel (e.g., 1.5%) can be used.

The agarose used must be of pure electrophoresis grade. Agarose purchased from Sigma Co. and Sekem Co. gave satisfactory results. To prepare an agarose stock solution, cold electrophoresis buffer is added to the agarose and the mixture is heated in a capped tube in a boiling water bath. After the agarose is dissolved, the contents of the tube should be well mixed to obtain a homogeneous stock solution.

In our system the use of a sample well enhances recovery of DNA fragments primarily by eliminating the problem of streaking along the sides of the gel tube. The high conductivity of this continuous electrophoresis system, due to the large area of contact between the bottom of the gel and the electrophoresis buffer, provides good separation of fragments at relatively low voltage, therefore minimizing heating effects. In addition, almost the whole gel is immersed in the buffer chambers, which automatically equilibrate with the room temperature. Therefore, no cooling device is necessary for this setup. The pumping speed used for elution can be as low as 4-5 ml/hr without loss of sample into the anode buffer chamber. A slow pumping speed and collection of small volume fractions minimizes the dilution of the eluting fragments.

References

Aaij, C., and P. Borst (1972) Biochim. Biophys. Acta 269:192-200.

Johnson, P., A. S. Lee, and R. L. Sinsheimer (1973) J. Virol. 11: 596-599.

Middleton, J. H., M. H. Edgell, and C. A. Hutchison, III (1972) J. Virol. 10:42-50.

Popescu, M., L. H. Lazarus, and N. Goldblum (1971) Analyt. Biochem. 40:247-253.

Smith, H. O., and K. W. Wilcox (1970) J. Mol. Biol. 51:379-391.

Studier, W. (1973) J. Mol. Biol. 79:237-248.

Chapter V

Mapping the *Hpa*, *Hin* and *Hae* Fragments on the ϕX174 Genome

Introduction

The genome of bacteriophage ϕX174 is a covalently closed circular molecule consisting of a single strand of about 5500 nucleotides (Sinsheimer, 1959). Several bacterial restriction deoxyribonucleases have been described which cleave the double-stranded, circular ϕX174 replicative form DNA into specific fragments (Middleton et al., 1972; Johnson et al., 1973; Edgell et al., 1972). These enzymes are particularly useful since they hydrolyze DNA at specific sites, therefore producing fragments containing particular parts of the genome. In order to use these specific fragments to study the replication and transcription of ϕX, as well as other functions of the genome, it is necessary to order these fragments with respect to the ϕX genetic map (Benbow et al., 1974). By analysis of partial digestion products and of overlapping sets of fragments, we have ordered physically the three sets of DNA fragments produced by hydrolysis of ϕX RF by the restriction endonucleases from Hemophilus influenzae, H. aegyptius and H. parainfluenzae.

The physical cleavage map has been in turn related to the ϕX genetic map by the fact that many ϕX174 mutations are available, and that methods have been developed by which restriction enzyme fragments may be assayed to determine which genetic loci they contain (Weisbeek and Van de Pol, 1970; Hutchison and Edgell, 1971). On the basis of these results, a detailed cleavage map of the ϕX174 genome has been constructed with respect to the cleavage sites of all three enzymes.

Nomenclature. The restriction enzyme(s) isolated from Hemophilus influenzae is referred to as Hin; the ϕX RF fragments generated from Hin digestion are R1, R2, etc. (where R1 is the largest fragment); Hpa represents the restriction endonuclease activity from H. parainfluenzae, the fragments generated are designated as P1, P2, etc. The restriction endonuclease from H. aegyptius is denoted as Hae, producing a set of Z fragments from ϕX RF. When fragments very similar in size are generated from a single digest, the designations a, b, c are used, e.g., the R7 band consists of two fragments very similar in size and R7a refers to the larger, R7b to the smaller. Unique fragments generated by double digestions are designated as U_X, where X is its size in base pairs.

Materials and Methods

I. Preparation of Uniformly Labeled ϕX [^{32}P]-DNA

[^{32}P]-ϕX RF (10^6 cpm/μg) was a kind gift from Dr. P. H. Johnson.

II. Hemophilus Restriction Endonucleases

H. influenzae strain Rd was obtained from H. O. Smith, H. parainfluenzae from G. H. Sack and H. aegyptius from C. A. Hutchison, III. The purification procedures for the restriction endonucleases from these Hemophilus strains were described in Chapter II. The phosphocellulose column fractions containing the bulk of the enzyme activity from each of the three Hemophilus strains were used without further purification; they were stored at 4°C in 10% glycerol.

III. Digestion of ϕX RF DNA with Restriction Endonucleases

For the preparation of a complete digest, [^{32}P]-ϕX RF DNA (0.2 µg) was incubated at 37°C with 5 µl of Hemophilus endonuclease in a volume of 30 µl reaction mixture containing 7 mM Tris (pH 7.4), 7 mM $MgCl_2$ and 7 mM β-mercaptoethanol. 50 mM NaCl was included in the H. influenzae restriction enzyme digestion mixtures. For both H. parainfluenzae and H. aegyptius restriction enzymes the final reaction mixture contained 20 mM NaCl. Under these conditions, digestion was usually complete in less than 2 hr. With each preparation of enzyme, a series of enzyme concentrations and incubation times was used to determine the optimum conditions for complete digestion of ϕX RF. EDTA was added to 0.01 M to terminate the reactions.

To obtain incomplete digests of [^{32}P]-labeled ϕX RF, both the ratio of enzyme to DNA and the time of incubation were varied, but incubation was carried out in the standard buffer described above.

IV. Slab Gel Electrophoresis

A standard vertical gel electrophoresis apparatus with power cables, set of glass plates, combs and spacers was purchased from Aquebogue Machine and Repair Shop, New York. Before use, the glass plates were siliconized, cleaned with alcohol, distilled water and allowed to air dry after a final rinse of methanol. The spacers were lightly greased and were held in position between the glass plates with clamps on either side of the plates. Modelling clay was used as a stand to support and seal the bottom of the set of glass plates. Polyacrylamide gel solution was then poured into the space between the two

glass plates through a small funnel. Any air bubbles trapped in gel solution could be rapidly removed by stirring the solution gently with a piece of PE 50 intramedic polyethylene tubing. Before the gel polymerized, a comb containing the appropriate number of wells was lowered into the gel solution and was gently removed after the gel solidified. The wells were flushed with electrophoresis buffer. The slab gel was then mounted onto the gel apparatus and held in place with clamps on either side. To prevent leaking of buffer from cathode chamber to the anode buffer, the side of the glass plate which was in contact with the gel apparatus was lightly greased and the upper edge sealed with molten agar. The gel was prerun for 30 min at 40 V before use. Before electrophoresis, the reaction mixtures containing DNA samples were adjusted to 1% SDS and 15% sucrose. The digests were then subjected to electrophoresis for 6 to 10 hr at a constant voltage of 40 V in 4 or 5% polyacrylamide vertical slab gels (14 x 12 x 0.15 cm) in a buffer consisting of 0.04 M Tris, 0.02 M sodium acetate, 1 mM EDTA, 0.1% SDS (pH 7.8).

For qualitative analysis of digestion products, the gel was dried under vacuum at 100°C by Maizel's modification (1971) of the method described by Fairbanks et al. (1965). The dried gel was then placed in direct contact with Kodak RP54 X-ray medical film. Usually the DNA bands in the autoradiogram could be detected after an overnight exposure.

V. Recovery of DNA Fragments

Two methods were used to elute DNA fragments from gels. In

the first method ^{32}P DNA bands on 3% polyacrylamide vertical slab gels were excised and immersed in 0.1 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate). Alternately, DNA fragments were recovered from continuous electro-elution from 2.5% cylindrical agarose gels. Details of the method were described in Chapter IV, and have been published (Lee and Sinsheimer, 1974a).

After alcohol-precipitation, the DNA fragments were redissolved in 40 µl of 0.005 M Tris (pH 7.4).

VI. Genetic Assays for Restriction Enzyme Fragments

Nonradioactive DNA fragments recovered from continuous electro-elution was a gift of Dr. L. Smith. About 0.1 µg of each DNA fragment in 20 µl of 0.005 M Tris (pH 7.4) was transferred to a siliconized glass tube (6 x 50 mm). The tubes were placed in a boiling water bath for three minutes to denature the DNA, and then quenched in ice. Immediately, 20 µl of amber mutant DNA (0.1 µg) was added to each tube. The reannealing mixture (volume 40 µl) was adjusted to a final 2 x SSC salt concentration. The tubes were sealed with parafilm, placed in a 57°C water bath or oven for 2-3 hr and then incubated at room temperature for 30 hr.

20 µl of the reannealed fragments from each tube was diluted with 0.05 M Tris, pH 8.1, to 0.2 ml in a fresh tube (1 x 10 cm). An equal volume of spheroplast of E. coli KT prepared by the method of Guthrie and Sinsheimer (1963) was added and gently mixed. E. coli KT is an amber permissive strain obtained from Dr. M. Hayashi. Infected spheroplasts were incubated at 37°C for 4-5 hr, lysed by freezing and thawing three times and selectively assayed for wt phage by plating on

E. coli C. The total phage yield was assayed on E. coli 4714.

Results

I. Molecular Size Estimates of DNA Fragments

We have prepared the above three endonucleases and used them to hydrolyze [^{32}P]-labeled ϕX RF. The resultant fragments were separated on 4% polyacrylamide gels.

The molecular sizes of the fragments were calculated from their relative mobilities using a plot relating log molecular size to R_f in a 4% polyacrylamide gel (P. H. Johnson, personal communication), and using as standards the known molecular sizes of the Hin limit products R9 and R10 as reported by Maniatis and Ptashnel (1973). The sizes of the larger fragments (e.g., P1 and P2) were also estimated by summation of the molecular sizes of the products derived from them by degradation with a second enzyme. Table 1 summarizes the molecular size estimates thus obtained of ϕX RF fragments produced by cleavage with the three Hemophilus restriction endonucleases. These values are in good agreement with the relative yield of each fragment from uniformly labeled [^{32}P]-DNA.

II. Order of ϕX Hpa Fragments by Analysis of Partial Digest Products

The general approach for ordering of the Hpa fragments was the separation by either polyacrylamide or agarose gel electrophoresis of [^{32}P]-labeled fragments obtained by incomplete digestion of ϕX RF with the Hpa restriction enzyme. Each partial product was subsequently eluted and redigested with excess Hpa enzyme to produce the final digestion products, and thereby establish a linkage group.

Table 1

Molecular Size Estimates of ϕX174 RF Fragments Produced by Cleavage with Hemophilus Restriction Endonucleases

Hin Fragments	Size in Base Pairs	Hae Fragments	Size in Base Pairs	Hpa Fragments	Size in Base Pairs
R1	1000	Z1	1200	P1	1600
R2	760	Z2	1050	P2	1400
R3	670	Z3	870	P3	720
R4	510	Z4	600	P4*a	400
R5	400	Z5	320	b	390
R6*a	350	Z6*a	290	P5	350
b	345	b	285	P6	215
c	340	Z7	230	P7	185
R7*a	300	Z8	190		
b	290	Z9	115		
R8	205	Z10	73		
R9	155				
R10	80				
Total	5405		5223		5260

*These fragments contain multiple tracts very similar in size, each tract is designated by a, b, c, etc.

FIGURE 1. Examples of partial and complete digests of [^{32}P]-labeled ϕX RF with restriction endonuclease from H. parainfluenzae.

(a) Effect of incubation time on the generation of partials. 2.0 µg of [^{32}P]-labeled ϕX RF (5 x 10^5 cpm/µg) were incubated in a volume of 20 µl with 1 µl of Hpa restriction enzyme under standard conditions. 3 µl aliquots of the reaction mixture were removed after 5 min incubation (1), 10 min (2), 20 min (3), 30 min (4), 1 hr (5), 7 hr (6). A complete Hpa digest marker (Hpa) was included. Samples were applied to a 5% slab gel and were electrophoresed at 47 V for 10 hr.

(b) Separation of Hpa partials on 3% polyacrylamide gel (bottom 1 cm is 10% gel). 10 µg of [^{32}P]-labeled ϕX RF (10^5 cpm/µg) were incubated for 30 min with 5 µl of Hpa restriction endonuclease in a volume of 0.15 ml. Partials A-G were separated on a 3% gel at 45 V for 16 hr.

(c) Redigestion of partial digests products A-G eluted from 3% gel. For each sample, the partial product (A, B, etc.) and the redigested partial product (a, b, etc.) were electrophoresed on a 4% gel with a complete Hpa digest (Hpa). Electrophoresis was carried out at 50 V for 6 hr.

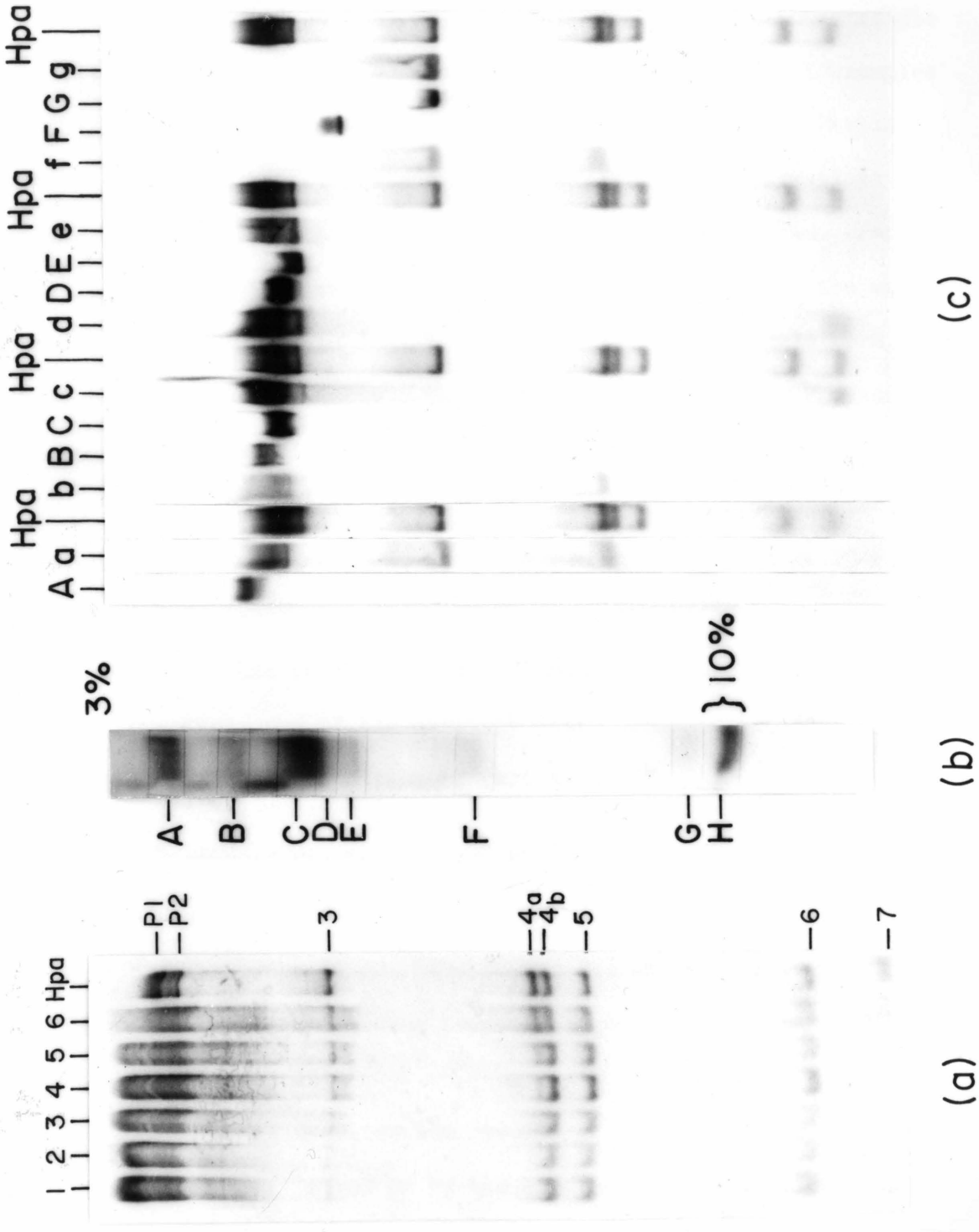

Hpa
1 2 3 4 5 6 Hpa
P1
P2
3
4a
4b
5
6
7
(a)
3%
10%
A
B
C
D
E
F
G
H
(b)
Hpa
A a b B C c d D E e f F G g
Hpa
Hpa
Hpa
(c)

Figure 1a shows the effect of incubation time on the generation of partials. Several partial digests were prepared, the intermediate products purified (Figure 1b), and then redigested. Several examples of the products of redigestion are presented in Figure 1c. Partial product A, for instance, yielded P1, P3 and P4a upon redigestion; partial B yielded only P1 and P4a; partial F yielded P3 and P4a. Partial C yielded P2 and P7 on redigestion. Partial D was actually the same as partial C. Some products isolated from the preparative gels were, in fact, already limit products. Thus, sample E was actually P2, and sample G, P3; these yielded no other fragments upon redigestion.

Table 2 summarizes the data from all partial products analyzed. The order of fragments P1, P3, P4a, and P5 can be deduced as P5-P1-P4a-P3 from overlapping positions. However, the exact order of P7-P6 cannot be determined using this set of conditions for partial digestion; P4b was invariably one of the earliest limit products produced and was not obtained by the redigestion of partials.

III. Redigestion of Hin Fragments with Hpa Restriction Enzyme

To resolve the remaining ambiguity in the order of Hpa fragments (i.e., the order of P2, P4b, P6 and P7), as well as to order the Hin fragments, we have digested terminal digest Hin fragments with the Hpa restriction enzyme.

Figure 2a illustrates the results of a simultaneous digestion (to completion) of [^{32}P]-ϕX RF by the Hin and Hpa restriction enzymes as analyzed on 4% polyacrylamide gels. The bands corresponding to R2 and R7a disappeared in the double digest. When the yield of cpm of the

Table 2

Redigestion of Partial Digestion Products with Hemophilus parainfluenzae restriction endonuclease (Hpa)

Partial #	Hpa Products	ϕX Fragment
A	1, 3, 4a, 5	5 1 4a 3
B	1, 3, 4a	1 4a 3
C	1, 4a	1 4a
D	3, 4a	4a 3
E	2, 7	(7)2
F	2, 7, 6	(7)2(6)
G	1, 4a, 5	5 1 4a

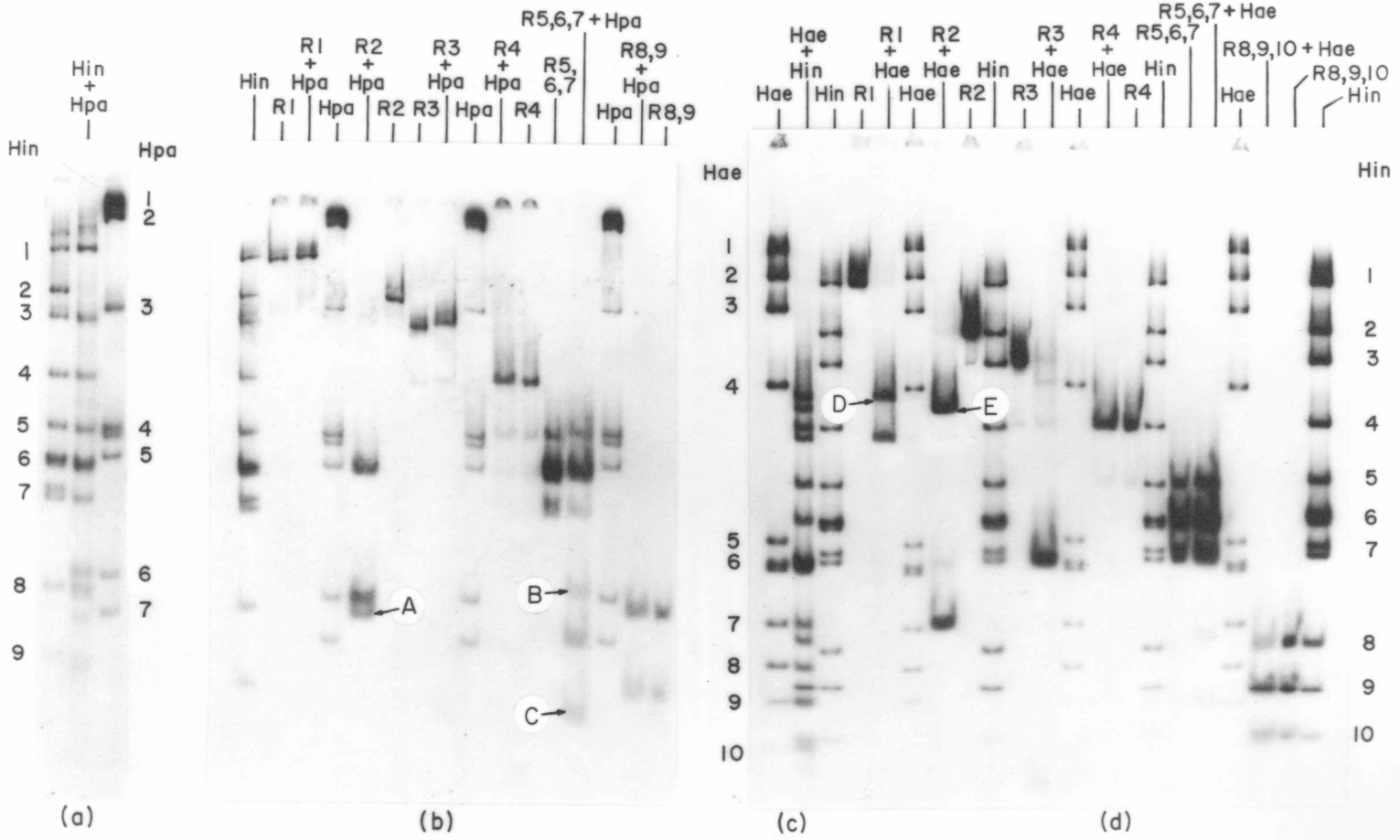
FIGURE 2. Analysis of [^{32}P]-labeled ϕX RF digests produced by restriction endonucleases from H. influenzae, H. parainfluenzae, and H. aegyptius.

(a) Digestion of ϕX RF with H. influenzae and H. parainfluenzae enzymes. A Hin digest is at the left and a Hpa digest is at the right. The middle column is a digest produced by simultaneous digestion with Hpa and Hin enzymes. Electrophoresis was carried out on a 5% gel at 40 V for 9 hr.

(b) Digestion of isolated Hin fragments with H. parainfluenzae enzyme. The undigested fragments (R1, etc.) and the redigested fragments (R1 + Hpa) were electrophoresed with complete digest markers (Hin) and (Hpa) as in (a). The arrows designate the new unique fragments, A = U195, B = U220, C = U145.

(c) Digestion of ϕX RF with H. influenzae and H. aegyptius enzymes. On the left is a complete Hae digest on ϕX RF, and the right is a Hin digest. The middle column is a digest with both enzymes at the same time. Electrophoresis was carried out in a 4% gel (bottom 1 cm is 10% gel) at 50 V for 6.5 hr.

(d) Redigestion of individual Hin fragments with the Hae enzyme. Electrophoresis conditions were the same as in (c). D = U590, E = U540.


Hin + Hpa
Hin
Hpa
R1 + Hpa
R2 + Hpa
R3 + Hpa
R4 + Hpa
R5,6,7 + Hpa
R8,9 + Hpa
R5,6,7 + Hae
R8,9,10 + Hae
Hae + Hin
R1 + Hae
R2 + Hae
R3 + Hae
R4 + Hae
R8,9,10
A
B
C
D
E
(a)
(b)
(c)
(d)

double digestion products was quantitated, one of the fragments in the R6 band (presumably R6a as judged from the slab gel) was also missing. In addition, unique fragments (U220, U195, and U145 indicated by arrows in Fig. 2b) were obtained.

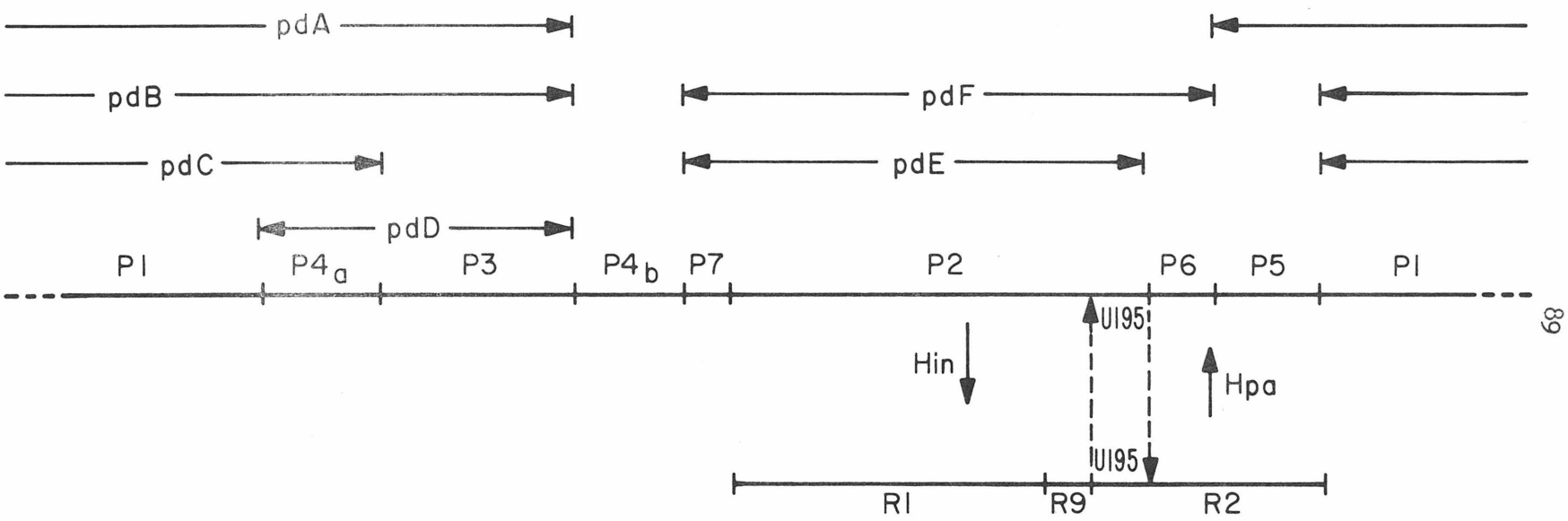
On isolation of individual Hin fragments and digestion of these with Hpa, as shown in Figure 2b, R1, R3, R4, R5, R8 and R9 were uncleaved. Fragment R2 yielded P5 and P6 and a unique piece (U195). This latter piece also derived uniquely from P2, when the reciprocal digestions were made (Table 3). This result led us to conclude that P5, P6 and P2 are linked. Combining these data with those in Table 2, the (circular) order of the Hpa fragments was resolved (Fig. 3).

When a mixture of R5, R6 and R7 was digested with the Hpa restriction enzymes, R5, two of the R6 components and R7b were not cleaved. However, at least three small fragments of size 220, 185 and 145 base pairs were produced from R6 and R7a. By separate digestion of R6 and R7 with the Hpa enzyme, it can be shown that the R6 component yields P7 (185 base pairs) and the unique 145 base pair piece; R7a yields the unique piece of 220 base pairs and a small fragment of 80 base pairs. The results of the Hin fragments redigested with the Hpa enzyme are summarized in Table 3.

IV. Redigestion of Hpa Fragments by Hin

To further order the Hin fragments, we have determined which Hin fragments could be derived from the individual Hpa fragments. The results of such digestions are in Table 4.

FIGURE 3. Derivation of the Hpa cleavage map.



pd, partial digest fragment (Hpa) from Table 2;

P, complete digest fragment (Hpa);

R, complete digest fragment (Hin);

U195, unique fragment of 195 base paris from double digest.

Table 3

Digestion of Hin Fragments with Hpa and Hae

Hin Fragment	Size*	Products with Hpa†	Product Size*	Products with Hae†	Product Size*
R1	1000	ΔP2	1000‡	ΔZ1 ΔZ4	590 480 1070
R2	760	P5 P6 ΔP2	350 215 195 760	ΔZ1 ΔZ2	540 230 770
R3	670	ΔP1	670‡	Z6a Z6b small piece	290 285 57 632
R4	510	ΔP1	510‡	ΔZ2	510‡
R5	390	ΔP4a	390‡	ΔZ3	390‡
R6a	350	ΔP4b P7	145 185 330	Z8 ΔZ4	180 120 300
R6b	345	ΔP3	345	Z7 ΔZ3	220 115 335
R6c	340	ΔP1	340‡	ΔZ2	340‡
R7a	300	ΔP4b Small piece	220 80 330	ΔZ5 (?)	290
R7b	290	ΔP3	290‡	ΔZ3	290‡
R8	205	ΔP1	205‡	Z10 small piece	73 75 63 211
R9	155	ΔP2	155‡	ΔZ1	155‡
R10	80	N.D.		N.D.	

Footnotes to Table 3

*Molecular size in base pairs was estimated from relative mobility in polyacrylamide gels.

†A part of a fragment is denoted by Δ, e.g., ΔP2 means part of fragment P2.

‡These fragments were uncleaved.

N.D. = not determined.

Table 4

Redigestion of Hpa Fragments by Hin and Hae

Hpa Fragment	Size*	Products with Hin†	Product Size*	Products with Hae†	Product Size*
P1	∿1600	R3	670	Z6a	290
		R4	510	Z6b	285
		R6	340	ΔZ2	850
		R8	205	Z9	115
			1725		1540
P2	∿1400	R1	1000	ΔZ1	1000
		R9	155	ΔZ4	500
		ΔR2	195		1500
			1350		
P3	720	R6	345	ΔZ3	450
		R7b	290	Z7	230
			635		680
P4a	400	R5	400	ΔZ3	400‡
P4b	390	ΔR7a	220	ΔZ5 (?)	255
		ΔR6	145	ΔZ8	120
			365		375
P5	350	ΔR2	350‡	ΔZ2	245
				ΔZ1	110
					355
P6	215	ΔR2	215‡	ΔZ1	215‡
P7	185	ΔR6	185‡	Cleaved to <100	

*Molecular size in base pairs was estimated from relative mobility in polyacrylamide gels.

†A part of a fragment is denoted by Δ, e.g., ΔR2 means part of fragment R2.

‡These fragments were uncleaved.

P1, when redigested with the Hin restriction enzyme, yields R3, R4, one component of R6 and R8. P2 yields R1, R9, and the same unique fragment (U195) which is derived from R2 when digested with Hpa (Fig. 2b). P3 yields one component of R6 and R7b; P4b yields two new unique fragments identical with those derived upon digestion of R6 plus R7a by Hpa.

For some fragments (e.g., P3), the sum of product molecular size is slightly less than its molecular size estimated from relative mobility. It is possible that an additional small fragment (less than 100 base pairs) was derived upon redigestion, but was electrophoresed out of the gel and thus unaccounted for.

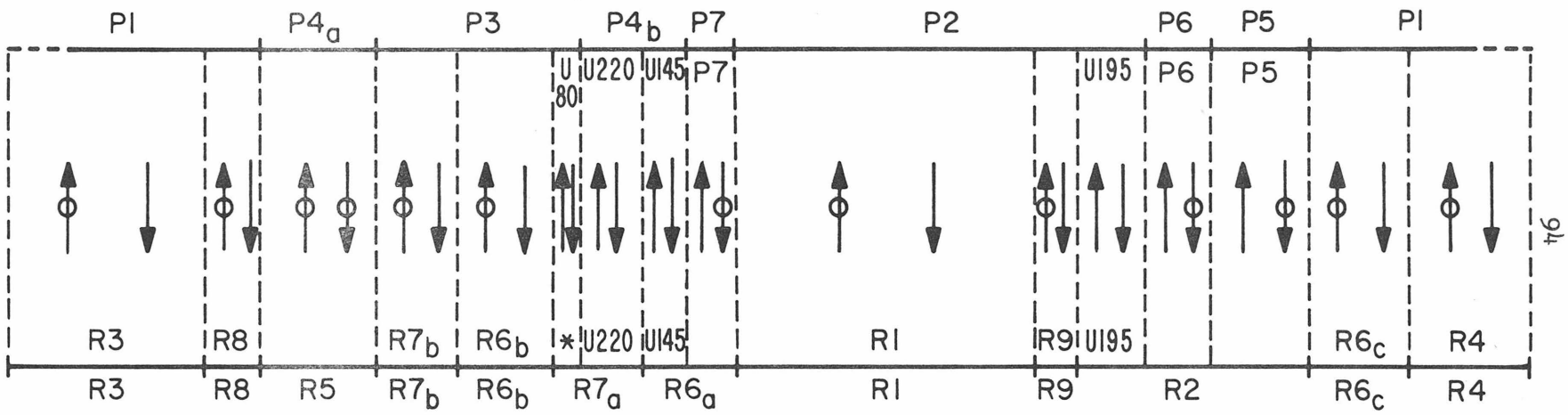
A unique situation arose in the relationship of P4a and R5, which have identical electrophoretic mobilities on polyacrylamide gels. Neither P4a or R5 are cleaved by the other enzymes; however, only one fragment of size of R5 (or P4a) was obtained from a simultaneous Hin and Hpa digestion. This led us to conclude that P4a and R5 are identical.

Combining the above results, a partial order of the R fragments can be derived as shown in Figure 4.

V. Redigestion of Hae Fragments with Hpa and Hin

The restriction enzyme from H. aegyptius cleaves ϕX RF into 11 specific fragments; Z6 consists of two fragments of similar size. One approach to resolve the linear order of the four Hin fragments derived from P1, the order of R7b and R6, and the order of R1 and R9 is to isolate individual Hae fragments and redigest them with Hpa and Hin restriction enzymes, as shown in Figure 5.

FIGURE 4. Relationship between Hpa and Hin fragments.



↑, Hin fragments redigested with the Hpa enzyme;

↓, Hpa fragments redigested with the Hin enzyme;

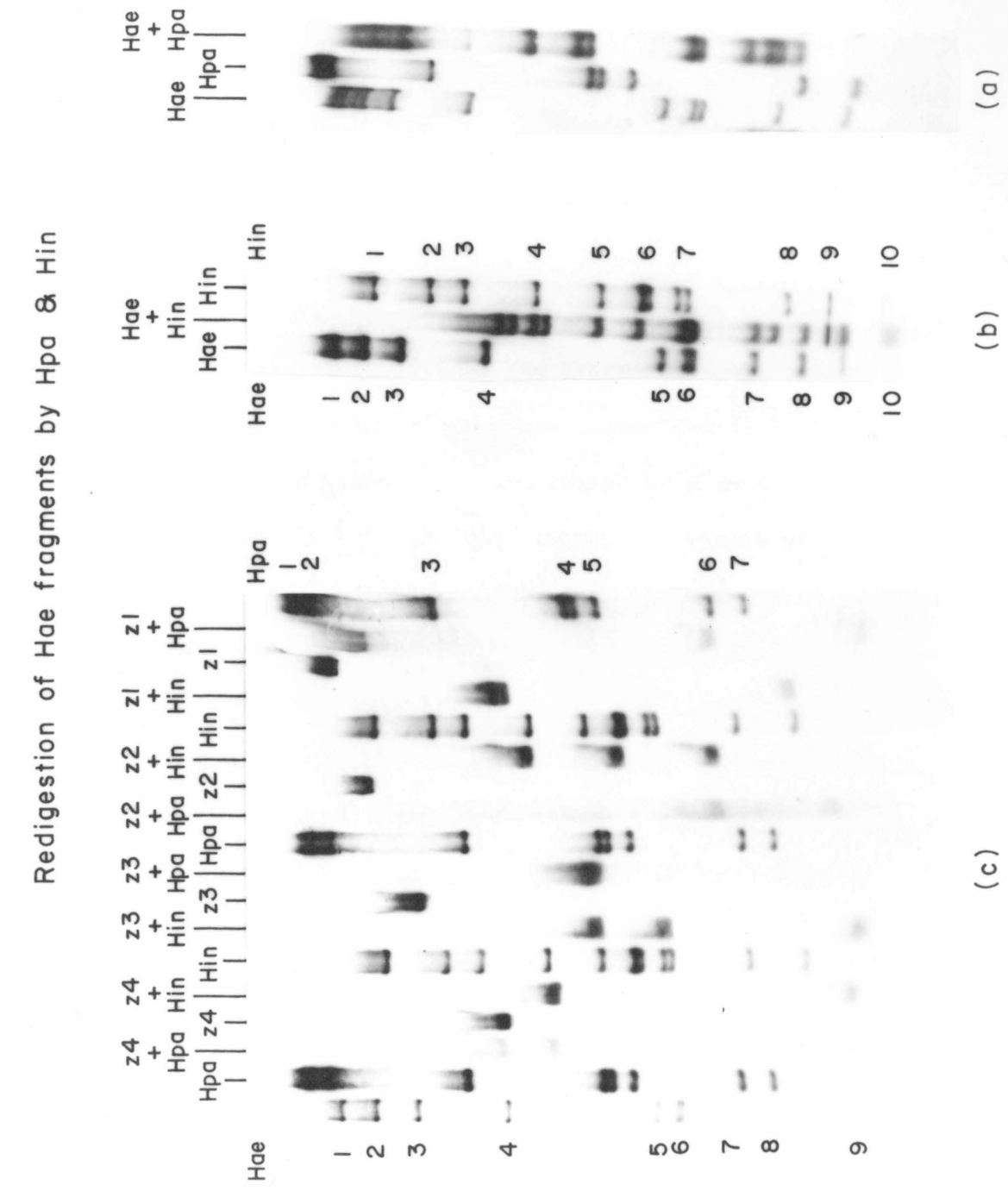
↕, uncleaved.

FIGURE 5. Analysis of [^{32}P]-labeled ϕX RF digests produced by restriction endonucleases from H. influenzae (Hin), H. parainfluenzae (Hpa), and H. aegyptius (Hae).

(a) Digestion of ϕX RF with Hpa and Hae. A Hae digest is at the left and a Hpa digest in the middle. The column at the right is a digest produced by simultaneous digestion with the Hae and Hpa enzyme. Electrophoresis was carried out on a 5% gel at 35 V for 13 hr.

(b) Conditions were the same as described in Figure 2c.

(c) Digestion of isolated Hae fragments with Hpa and Hin. The undigested fragments (Z1, etc.), and the redigested fragments (Z1 and Hpa), (Z1 and Hin), were electrophoresed with complete digest marker (Hin), (Hpa) and (Hae). Electrophoresis was carried out on a 4% gel (bottom 1 cm is 10% gel) at 50 V for 7 hr.


Redigestion of Hae fragments by Hpa & Hin
Hae + Hpa
Hae | Hpa
(a)
Hae + Hin
Hae | Hin
Hae
Hin
(b)
z4 + Hpa
z4 + Hpa Hin
z4 | z4 | Hin
z3 + Hin
z3 + Hpa Hin
z3 | z3 | Hpa
z2 + Hpa
z2 + Hpa Hin
z2 | z2 | Hin
z1 + Hin
z1 + Hpa Hin
z1 | z1
z1 + Hpa
Hpa | Hpa
Hae
Hpa
(c)

When [^{32}P]-labeled ϕX RF is simultaneously digested with the Hae and Hpa restriction enzymes and the resultant digestion products analyzed, P4a, P6, Z6a, Z6b, Z7, Z9 and eight additional major unique fragments (U1000, U850, U500, U450, U255, U245, U120 and U110) can be identified.

Figure 5b illustrates the result of a double digestion of ϕX RF with the Hin and Hae restriction enzymes. It is evident that R1, R2, R3, as well as Z1, Z2, Z3, Z4 and Z5 were cleaved.

The results of individual Hae fragments redigested with either the Hpa or Hin restriction enzymes are summarized in Table 5. The identity of unique fragments was determined by digestion of individual Hpa and Hin fragments with the Hae restriction enzyme and analysis of overlapping sets of fragments. Additional linkage groups of Hin fragments are derived from redigestion of large Hae fragments, e.g., from Z1 it can be concluded that R1, R9 and R2 are linked, in that order.

It is also possible to deduce the order of Hae fragments from redigestion data of individual Hpa fragments with the Hae restriction enzyme (Table 4) (except for Z10 which was electrophoresed out of the gel). The relationship of the Hpa and Hae fragments is illustrated in Figure 6.

To confirm the redigestion data, individual Hin fragments were digested with the Hae restriction enzyme (Figure 2d). The reciprocal digestion results are in agreement with each other. For instance, Z1 was cleaved by the Hin restriction enzyme into two large fragments (U590 and U540) and R9 (Fig. 5c). Figure 2d illustrates that the U590 fragment is derived from R1, the U540 piece from R2, and R9 is

Table 5

Redigestion of Hae Fragments by Hpa and Hin

Hae Fragment	Size*	Products with Hin†	Product Size*	Products with Hpa†	Product Size*
Z1	1200	ΔR1 ΔR2 R9	590 540 155 1285	ΔP2 P6 ΔP5	1000 215 110 1325
Z2	1050	R4 R6 ΔR2	510 340 230 1080	ΔP5 ΔP1	245 850 1095
Z3	870	R5 R7b ΔR6 Small piece	400 290 115 75 880	P4a ΔP3	400 450 850
Z4	600	ΔR1 ΔR6	480 120 600	ΔP2 ΔP7	500 100 600
Z5	320	ΔR7a (?) Small piece	290 ∿30 320	ΔP4b Small piece	255 70 325
Z6a	290	ΔR3	290‡	ΔP1	290‡
Z6b	285	ΔR3	285‡	ΔP1	285‡
Z7	230	ΔR6	220	ΔP3	230‡
Z8	190	ΔR6	190‡	ΔP4b Small piece	120 70 190
Z9	115	ΔR3 ΔR8	57 63 120	ΔP1	115‡
Z10	73	ΔR8	73‡	N.D.	N.D.

Footnotes to Table 5

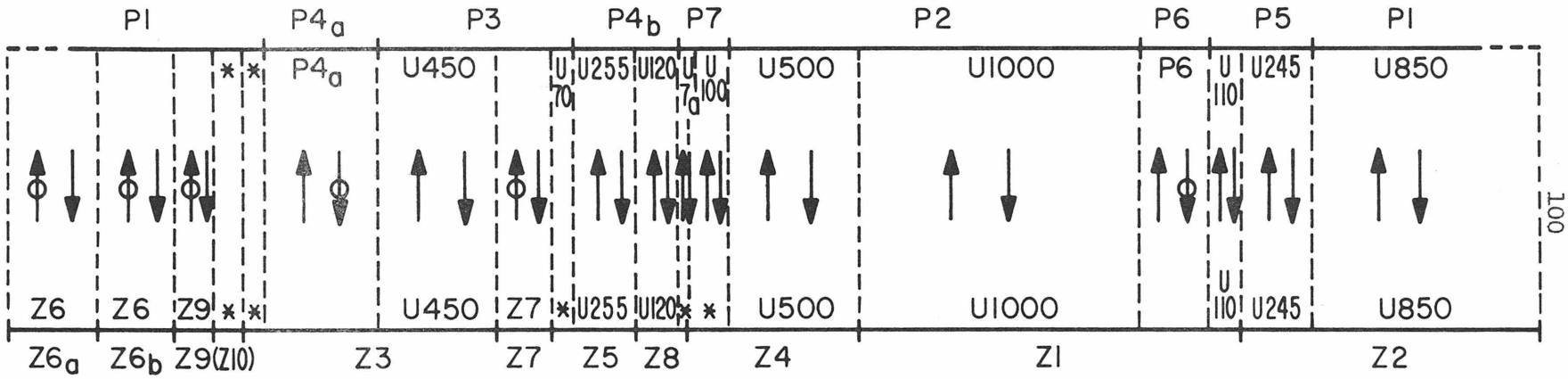
*Molecular size in base pairs was estimated from relative mobility in polyacrylamide gels.

†A part of a fragment is denoted by Δ, e.g., ΔR2 means part of fragment R2.

‡These fragments were uncleaved.

N.D. = not determined.

FIGURE 6. Relationship between Hpa and Hae fragments.



↑, Hae fragments redigested with the Hpa enzyme;

↓, Hpa fragments redigested with the Hae enzyme;

↕, uncleaved.

uncleaved. The relationship between the Hin and Hae fragments is illustrated in Figure 7.

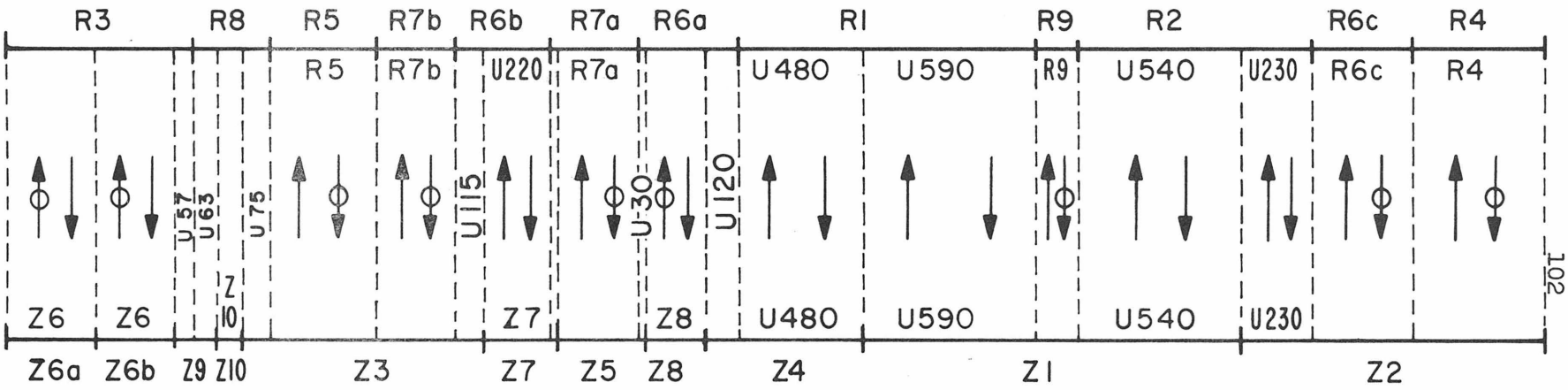
The results of the genetic assays carried out for some of Hin and Hae fragments are shown in Table 6. The efficiency with which these fragments can complement amber mutants is similar to that attained by others (M. Hayashi, personal communication). It was found that R6 and Z7 contain the am10 locus in gene D, and R5 to contain am16 in gene B. These results were in agreement with the findings of Hutchison et al. (personal communication). It was surprising to find that there were some, though minimal amounts, of wild type resulting from annealing other fragments to these amber mutants (e.g., R5, R8 to am10). Possibly these fragments contained trace amounts of degradation products of R6 as a contaminant during the isolation procedure.

We can summarize all data in a single cleavage map of the ϕX genome, incorporating the various cleavage sites and molecular size estimates of the fragments (Fig. 8).

The assignment of several fragments, e.g., R6c, R4, R8, Z5, Z9 and Z10, deserves comment. From our redigestion data alone, the position of R6c and R4 can be interchanged and R8 can be on either side of R3. However, a partial order of Hin fragments has been determined by Johnson and Sinsheimer (1974) using E. coli DNA polymerase I in an extended repair reaction with uniquely gapped late RF II molecules. Their results indicate that R8 is between R3 and R5.

The position of Z5 is considered tentative since it was cleaved by both Hpa and Hin. The assignment was based on the production of an identical unique fragment from P4b and Z5 on redigestion with the

FIGURE 7. Relationship between Hin and Hae fragments.



↑, Hae fragments redigested with the Hin enzyme;

↓, Hin fragments redigested with the Hae enzyme;

↕, uncleaved.

Table 6

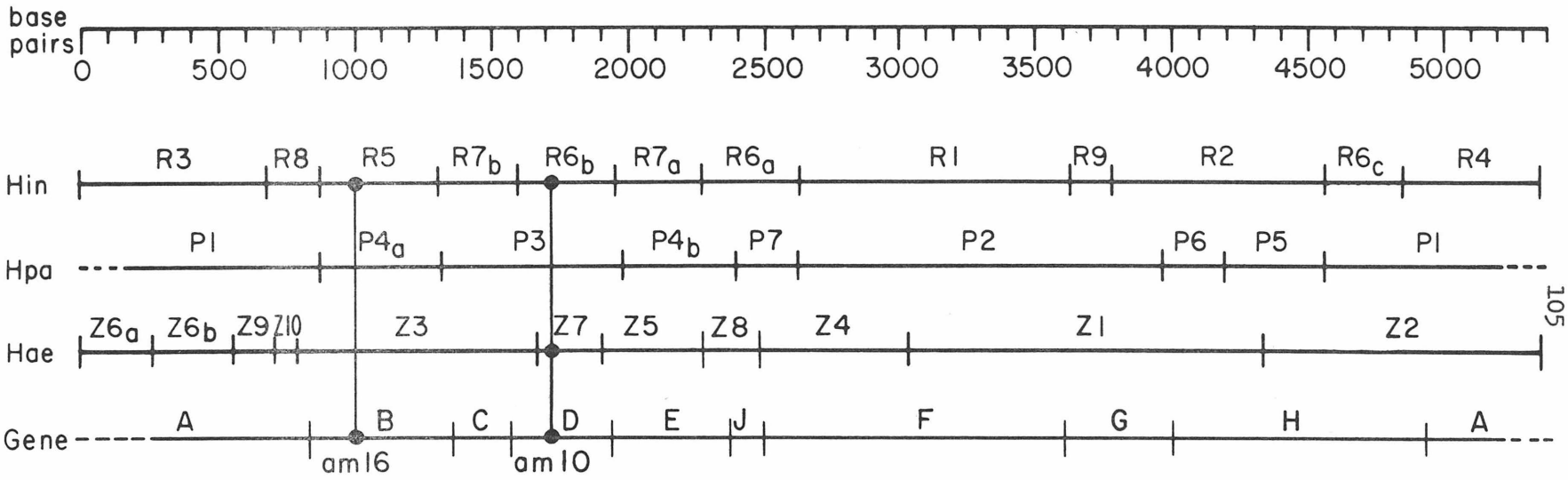
Hybridization of Restriction Enzyme Fragments

to Viral Mutant DNA

Fragment	Viral DNA*	Titer		(w.t./total) %
		w.t.	total	
R1 (0.10 µg)	gene D, am10	0	8×10^4	0
R2 (0.10 µg)	gene D, am10	0	1.2×10^4	0
R5 (0.10 µg)	gene D, am10	7×10^2	1.3×10^5	0.5
R6 (0.15 µg)	gene D, am10	1.3×10^4	1.3×10^5	10.0
R6 (0.30 µg)	gene D, am10	1.9×10^4	1.5×10^5	13.0
R8 (0.10 µg)	gene D, am10	1×10^3	1.6×10^5	0.6
R9 (0.10 µg)	gene D, am10	8×10^2	1.9×10^5	0.4
Z7 (0.10 µg)	gene D, am10	1.9×10^4	3.6×10^5	6.3
Z7 (0.20 µg)	gene D, am10	1.2×10^4	2.8×10^5	4.2
Z7 (0.10 µg)	gene B, am16	5×10^2	1.3×10^5	0.5
R5 (0.10 µg)	gene B, am16	1.9×10^4	5×10^4	41.0
R5 (0.15 µg)	gene B, am16	3.6×10^4	7×10^4	51.0
R8 (0.05 µg)	gene B, am16	1.8×10^3	1.3×10^5	1.4
R9 (0.05 µg)	gene B, am16	6×10^2	4×10^4	1.7
buffer alone		0	0	0

*Viral mutant DNA was a kind gift of Dr. R. Dennin.

FIGURE 8. Restriction enzyme fragment map of ϕX174. The molecular size and physical order of the ϕX DNA fragments produced by cleavage with the Hin, Hpa and Hae has been published (Lee and Sinsheimer, 1974b). The order and size of the ϕX genes shown in the map was assigned according to results of Benbow et al. (1974). Using genetic assays for ϕX fragments, R5 was found to complement am16 in gene B, R6b and Z7 to am10 in gene D. The assignment of other fragments to the ϕX genetic map can be inferred but can only be considered as tentative.


base pairs
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
Hin
R3
R8
R5
R7b
R6b
R7a
R6a
R1
R9
R2
R6c
R4
Hpa
P1
P4a
P3
P4b
P7
P2
P6
P5
P1
Hae
Z6a
Z6b
Z9
Z10
Z3
Z7
Z5
Z8
Z4
Z1
Z2
Gene
A
B
C
D
E
J
F
G
H
A
am16
am10
105

other enzyme.

Both P1 and P5, on redigestion with the Hae enzyme, yield a fragment very similar in size to Z9. However, R2, from which P5 was derived (Fig. 2b), did not yield Z9 when treated with the Hae enzyme. On the other hand, R3 was cleaved into at least three fragments by the Hae enzyme, namely the two Z6 fragments and a small piece of 57 base pairs. R8, which is adjacent to R3, yielded 2 bands when digested with the Hae enzyme. The sizes of the fragments in these bands are 75 (equivalent to Z10) and 63 base pairs. The larger band (U75) probably consists of two tracts very similar in size. In addition, Z9 was cleaved by the Hin restriction enzyme into two fragments, U57 and U63. This could only occur if Z9 and Z10 are located between R3 and R5 as shown in Figure 8. The sum of the two small fragments derived from R3 (U57) and R8 (U63) on cleavage with the Hae enzyme adds up to the size of Z9. Our assignment of Z9 and Z10 is in agreement with the partial order of Z fragments determined by L. K. Miller (personal communication) using in vitro repair synthesis of gapped late RF II molecules.

Discussion

The physical map of ϕX as shown in Figure 8 is based on cleavage of ϕX RF by three bacterial restriction endonucleases: the enzymes from H. influenzae, H. parainfluenzae, and H. aegyptius. The Hpa enzyme we used in our experiments hydrolyzed SV40 into four specific fragments as reported by Sack and Nathans (1973). It was reported by Sharp et al. (1973) that the Hpa restriction enzyme contained two

enzymes, Hpa I and II.

The methods we have used to order these DNA fragments are by analysis of contiguities in partial digestion products or by analysis of overlaps among the fragments produced by restriction enzymes with different specificities. Overlaps are studied by redigestion of fragments produced by one enzyme with another enzyme of different specificity. Alternatively, DNA fragments can be ordered by electron microscopy or hybridization techniques. However, the exact relationship of cleavage sites of enzymes of different specificity can be most accurately determined by the physical ordering method that we used.

We have confirmed the physical order of the Hpa fragments by using two individual Hpa fragments (P1 and P2) as specific primers for in vitro DNA synthesis after annealing the complementary strand of the fragments to ϕX viral DNA (unpublished results). Briefly, the partial RF thus formed was incubated with DNA polymerase and nucleoside triphosphates. The nucleotide incorporated was pulse labeled with [^{32}P]dXTP and the distribution of label within the completed double-stranded molecules was determined by analysis of specific fragments produced by the Hpa enzyme. A gradient of labeling was found such that the Hpa fragment adjacent to the priming fragment (e.g., P5 in the case of P1 as primer) contained the highest [^{32}P]-label. Using R fragments as specific primers for in vitro DNA synthesis, R6c was found to be located between R2 and R4, R8 between R3 and R5, and that R10 is between R9 and R2.

In a partial restriction enzyme fragment map for ϕX reported by Chen et al. (1973), R2 was positioned within the Z1 fragment.

However, our redigestion data indicated that R2 was cleaved by the Hae restriction enzyme into two pieces. This result is consistent with the double digestion data described earlier by Middleton et al. (1972), and the electron microscopy data presented by Chen et al. (1973). Our physical assignment of Z1 and R2 would indicate that the left end of R2 contains a sequence protected by RNA polymerase.

With the availability of a detailed genetic map of ϕX174 (Benbow et al., 1974) and the genetic loci of R5, R6 and Z7 known, it is possible to correlate other fragments with the ϕX genetic map. So far, the genetic assignments shown in Figure 8 have been consistent with results of genetic assays performed in other laboratories (C. Hutchison and P. Baas, personal communication).

The availability of ordered sets of specific fragments of ϕX is proving useful in the analysis of replicating intermediates of ϕX. For example, the location of gapped molecules during ϕX replication was studied with the use of Hin restriction enzyme fragments (Johnson and Sinsheimer, 1974). Smaller fragments derived from double and triple enzyme digestions would be useful for isolating small fragments of the genome for DNA sequencing.

References

Benbow, R. M., A. J. Zuccarelli, G. Davis, and R. L. Sinsheimer (1974) J. Virol. 13:898-907.

Chen, C. Y., C. A. Hutchison, III., M. H. Edgell (1973) Nature New Biol. 243:233-236.

Edgell, M. H., C. A. Hutchison, III, and M. Sclair (1972) J. Virol. 9:574-582.

Fairbanks, G., Jr., C. Levinthal, and R. H. Reeder (1965) Biochem. Biophys. Res. Commun. 20:293.

Guthrie, G. D., and R. L. Sinsheimer (1963) Biochim. Biophys. Acta 72:290-297.

Hutchison, C. A., III., and M. H. Edgell (1971) J. Virol. 8:181-189.

Johnson, P., A. S. Lee, and R. L. Sinsheimer (1973) J. Virol. 11: 596-599.

Johnson, P. H., and R. L. Sinsheimer (1974) J. Mol. Biol. 83:47-61.

Lee, A. S., and R. L. Sinsheimer (1974a) Analytical Biochemistry 60: 640-644.

Lee, A. S., and R. L. Sinsheimer (1974b) Proc. Nat. Acad. Sci. 71:2882-2886.

Maizel, J. V., Jr. (1971) Meth. Virol. 5:180-247.

Maniatis, T., and M. Ptashne (1973) Proc. Nat. Acad. Sci. 70:1531-1533.

Middleton, J. H., M. H. Edgell, and C. A. Hutchison, III (1972) J. Virol. 10:42-50.

Sack, G. H., Jr., and D. Nathans (1973) Virology 51:5-7.

Sharp, P. A., B. Sugden, and J. Sambrook (1973) Biochemistry 12: 3055-3061.

Sinsheimer, R. L. (1959) J. Mol. Biol. 1:43-53.

Weisbeek, P. J., and J. H. Van de Pol (1970) Biochim. Biophys. Acta 224:328-338.

Chapter VI

The Location of the 5-Methylcytosine Group on the Bacteriophage ϕX174 Genome

Introduction

Methylation of DNA occurs in viruses, bacteria and higher organisms, and has been the topic of intensive study. A possible role of DNA methylation in cell differentiation has been discussed (Scarano et al., 1967). Other evidence suggests that methylation is involved in the host cell phenomena of DNA modification and restriction (Arber and Linn, 1969). Experiments carried out in bacteria have demonstrated that methylation of DNA is related to DNA replication, and that this process normally occurs in the nascent DNA strand close to or at the replication point (Billen, 1968; Lark, 1968a,b).

The small circular DNA of bacteriophage ϕX174 provides a convenient system for the study of DNA methylation. Experiments previously reported from this laboratory showed that the only methylated base present in ϕX174 DNA is 5 methylcytosine, occurring at a density of one per DNA molecule. In addition, this minor base was found exclusively in the dinucleotide fraction of the pyrimidine isopliths, suggesting that it is present in a non-random distribution in the ϕX genome (Razin et al., 1970). Further studies on the process of in vivo DNA methylation during the infection of E. coli C with ϕX174 revealed that methylation of ϕX occurs on the nascent DNA strand of the replicating intermediates involved in the synthesis of progeny single-stranded DNA (Razin et al., 1973).

The use of restriction enzymes to cleave ϕX174 DNA into specific fragments had been previously described (Edgell et al., 1972; Middleton et al., 1972; Johnson et al., 1973) and a cleavage map of

the ϕX174 genome by these enzymes has been prepared (Lee and Sinsheimer, 1974b). In this paper, we report the results of restriction enzyme digestions of the methyl-labeled viral DNA (after its conversion to the replicative form by *in vitro* synthesis in presence of nucleoside triphosphates and *E. coli* polymerase I) and the location of the methyl group in the genome.

Materials and Methods

I. Phage and Bacterial Strains

E. coli strain. *Escherichia coli* C416 (pu^-, met^-, arg^-, try^-) was used as the host strain.

Phage strain. A lysis-defective mutant of phage ϕX174, *am3* was used.

II. Medium

The growth medium used was the TPG medium of Sinsheimer *et al.* (1962) supplemented with (/ml) 50 μg of 18 amino acids, 4 μg each of L-methionine and L-cysteine; 4 μg thymine and 10 μg adenosine.

III. Reagents

[^{3}H]-methyl methionine (specific activity 2.6 Ci/mmole) was purchased from Schwartz Bioresearch Inc. (Orangeburg, New York).

[α-^{32}P]dXTP (specific activity ~100 Ci/mmole) was a product of New England Nuclear (Boston, Massachusetts).

Oligonucleotide $(pApG)_4$ was purchased from Collaborative Research Inc. (Waltham, Massachusetts).

IV. Enzymes

E. coli DNA polymerase I was the generous gift of Dr. Arthur Kornberg. Hemophilus influenzae and H. aegyptius restriction enzymes were prepared by modifications (Johnson et al., 1973) of the method previously described by Smith and Wilcox (1970). The bulk of enzyme activity from H. aegyptius was eluted at 0.7 M KCl from the phosphocellulose column. The phosphocellulose column fractions were used without further purification and were stored at 4°C in 10% glycerol.

V. Preparation of [^{3}H-methyl]-viral DNA

[^{3}H-methyl]-viral DNA was prepared by two different labeling conditions with the same results.

In the first method, E. coli C416 cells were grown to 5 x 10^8 cells/ml in a 500 ml culture in the growth medium described above. The cells were then washed once with growth medium (without methionine) and resuspended in the original volume of growth medium, including all supplements except the methionine concentration was adjusted to 2.8 µg/ml and the thymine concentration increased to 8 µg/ml. After 20 min of aeration at 37°C, the cells were infected with am3 (multiplicity of infection = 10). 5 min after infection, 10 mCi of [^{3}H-methyl]methionine (2.6 Ci/mmole) was added to the culture. The final concentration of L-methionine was 4 µg/ml. 2 hr later, the cells were collected by centrifugation.

Alternately, a 100 ml culture of E. coli C416 was grown up to 5 x 10^8 cells/ml. The cells were washed free of methionine and resuspended in same volume of growth medium, including all supplements except methionine.

After 15 min of aeration at 37°C, 10 mCi of [^{3}H-methyl] methionine (2.6 Ci/mmole) was added. The final concentration of methionine was 5 µg/ml, carrier-free. 10 min later, the cells were infected with am3 (multiplicity of infection = 10). Incubation was at 37°C for 2 hr with aeration.

The DNA of the progeny virus was prepared as described by Razin et al. (1970). After alcohol precipitation, the viral DNA was resuspended in 70 µl of 0.01 M Tris (pH 8), 0.001 M EDTA, and stored at -10°C.

VI. In Vitro Synthesis of the Complementary Strand of Methyl-Labeled DNA

The reactions were carried out in 130 mM Tris pH 7.4, 13 mM KCl and 5 mM $MgCl_2$. The reaction mixture (300 µl) contained 0.25 µmole each of dCTP, dGTP, dTTP and dATP, and 0.16 nmole [α-^{32}P]dXTP (108 Ci/mmole), 0.04 µmole (in nucleotides) of methyl-labeled DNA, 0.003 µmole of $(pApG)_4$ as primer (K. Grohmann, personal communication), and 15 units of DNA polymerase I. The progress of synthesis was followed by measuring the accumulation of acid insoluble radioactivity with time. The mixture was incubated at 15°C for 9 hr, at which time repair synthesis was complete (Dumas et al., 1971). Reaction was stopped by addition of EDTA to 10 mM.

VII. Polyacrylamide Gel Electrophoresis

Before electrophoresis, the digestion mixture was adjusted to 1% SDS and 15% sucrose. The mixture was placed on the sample well (Lee and Sinsheimer, 1974a) of a cylindrical polyacrylamide gel. The

electrophoresis buffer consisted of 0.04 M Tris, 0.02 M sodium acetate, 1 mM EDTA, 0.1% SDS, pH 7.8. Conditions of electrophoresis are described in the figure legends. To quantitate the radioactivity, gels were frozen and cut into 1 mm segments by using the Mickle gel slicer (Brinkman Instruments). Gel slices were solubilized with toluene-Liquifluor containing 9% NCS (Amersham-Searle), 1% H_2O and the radioactivity was measured in a Beckman liquid scintillation counter.

Results

The discovery of an unique methylcytosine group in the progeny phage ϕX DNA by Razin et al. (1970) prompted us to localize it with respect to restriction enzyme fragments and thereby on the ϕX genetic map.

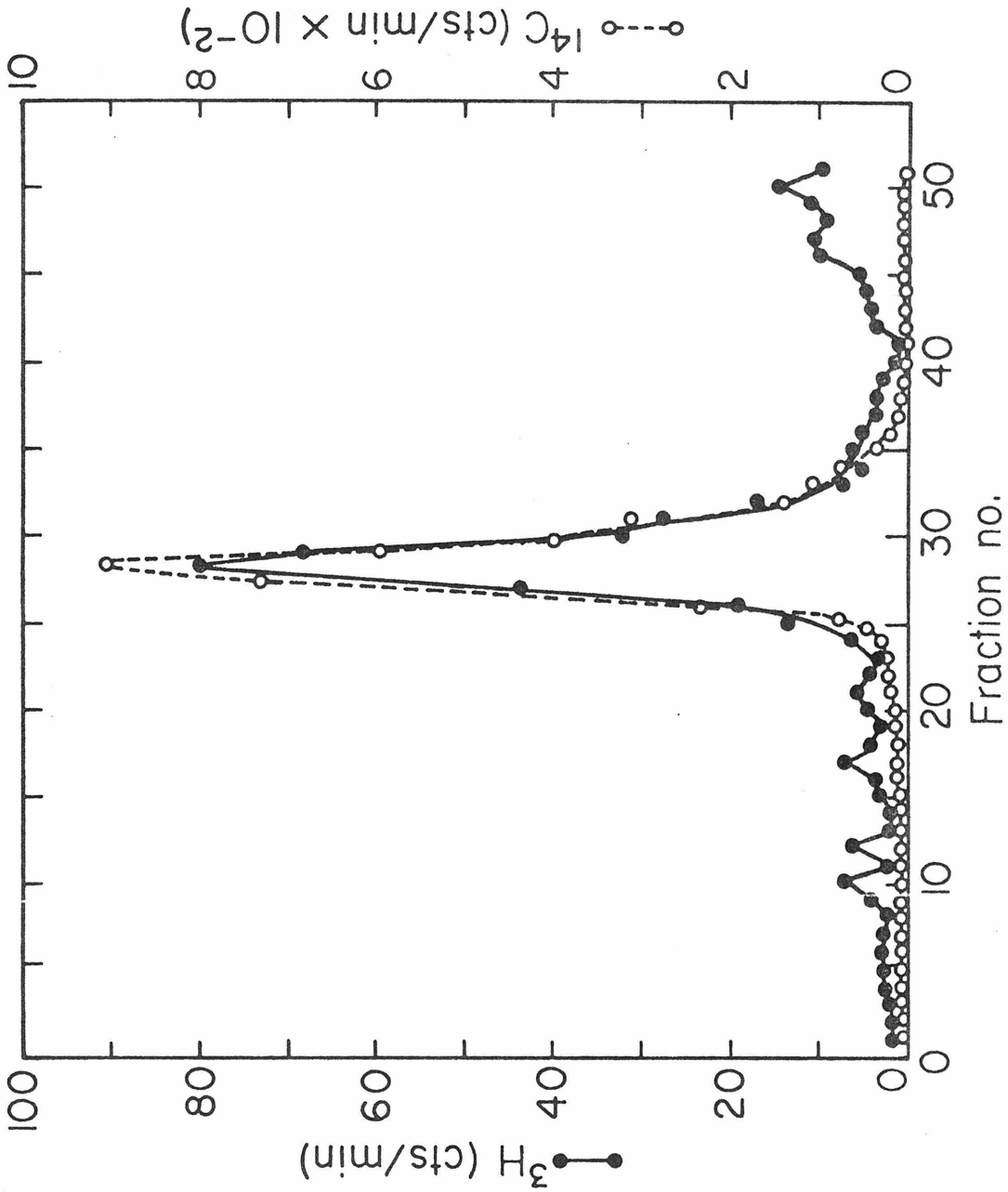
I. Sedimentation Profile of the Methyl-Labeled DNA

To verify the purity of the methyl-labeled DNA prepared by the above methods, viral DNA samples were sedimentated through an alkaline cesium chloride gradient as shown in Figure 1. The sedimentation profiles of viral DNA prepared by either labeling condition were identical and co-sedimented in a single sharp band with the ^{14}C single-stranded phage DNA marker. About 5% of the ^{3}H-radioactivity was found at the top of the gradient, probably indicative of the presence of a small amount of low molecular weight contaminant.

II. In Vitro [^{3}H-Methyl]RF

In order to use the Hemophilus restriction endonucleases to cleave ϕX DNA into specific fragments, the DNA must be in the double

FIGURE 1. Zone sedimentation of methyl-labeled DNA through an alkaline CsCl gradient. ϕX viral DNA was labeled in vivo with [^{3}H-methyl] methionine and isolated as described in Materials and Methods. 16 µl of the labeled DNA dissolved in Tris-EDTA was adjusted to 0.1 M KOH, 0.01 M EDTA, and then layered onto a preformed, linear gradient of CsCl (ρ = 1.20-1.35 gm/ml) in 4.8 ml of 0.1 M KOH, 0.01 M EDTA. Centrifugation was carried out at 4°C, 55K for 120 min in an SW65 rotor. 0.1 ml fractions were collected from the bottom of the tubes onto 2.3 cm GF/C filters. The filters were dried and counted in toluene-Liquifluor scintillation fluid. Sedimentation is from right to left. The ^{14}C single-strand phage DNA marker was run in a separate tube and is included in the same sedimentation profile for comparison. ●——●, ^{3}H; o——o, ^{14}C.


^{3}H (cts/min)
^{14}C (cts/min x 10^{-2})
Fraction no.
0
20
40
60
80
100
0
2
4
6
8
10
0
10
20
30
40
50

stranded form. Therefore, methyl-labeled viral DNA was used as template for *in vitro* synthesis of the complementary strand using *E. coli* DNA polymerase I, in the presence of nucleoside triphosphates. The nucleotide incorporated was labeled with [α-^{32}P]dXTP. Under the conditions described in Materials and Methods, one-fold synthesis was usually complete in about 7 hr at 15°C, when the incorporation of the ^{32}P-radioactivity reached a plateau level.

This *in vitro* RF DNA was separated from unreacted [α-^{32}P]dXTP by exclusion chromatography on a porous, glass bead column. As shown in Figure 2, the RF DNA was eluted as a single narrow peak well separated from the unreacted [α-^{32}P]dXTP which trailed behind. The elution procedure was usually complete within an hour. (The glass bead column can be regenerated for further use by 2 hr of washing with the acetate buffer.)

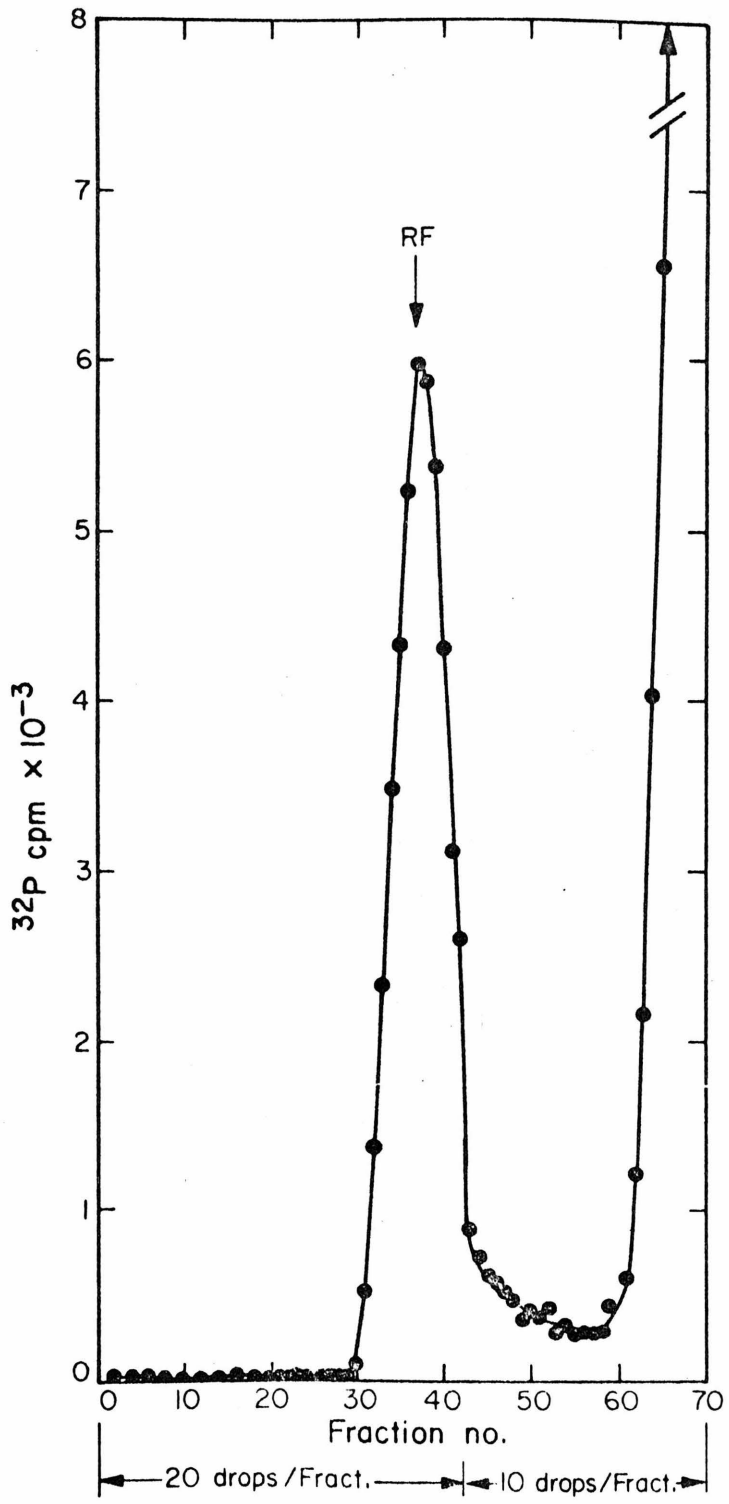
The peak fractions containing the RF DNA were pooled into two SW41 polyallomer tubes. Two volumes of absolute alcohol were added, the solution was well mixed, and stored at -20°C for at least 8 hr.

The DNA was collected by centrifugation at 35K for 1 hr and resuspended in 50 µl 0.005 M Tris 7.4 for the *Hemophilus* restriction enzyme digestions.

III. Digestion of In Vitro Methyl-Labeled RF with Hemophilus influenzae Restriction Endonuclease

The *H. influenzae* restriction enzyme (*Hin*) produces specific cleavage in double-stranded DNA at a sequence of six nucleotides, G T Py ↓ Pu A C (Kelly and Smith, 1970). The fragments produced by

FIGURE 2. Exclusion chromatography of *in vitro* RF DNA on porous, glass bead column. A 1 cm x 54 cm column of porous glass bead (Sigma G2000-50, pore size 2000 Å) was washed with 50 ml of 0.2 N NH_4OH, then 250 ml of 0.1 M sodium acetate, 1 mM EDTA, pH 6.5. The *in vitro* synthesis reaction mixture (300 µl) was adjusted to 0.2% SDS, 15% sucrose and applied to the column. Elution was carried out at 4°C, at a rate of 36 ml/hr with the acetate buffer described above. Fractions of 20 drops (∿0.5 ml) were collected and ^{32}P-radioactivity was monitored as Cerenkov radiation in a liquid scintillation counter.


RF
^{32}P cpm x 10^{-3}
Fraction no.
20 drops/Fract.
10 drops/Fract.

the action of this enzyme on the replicative form of ϕX174 have been characterized (Edgell et al., 1972; Lee and Sinsheimer, 1974b). The R fragments range in size from 1,000 to 80 base pairs. The R6 band contains three distinct tracts (R6a, b, c), very similar in size, and R7 contains two distinct tracts, R7a and R7b which can be partially separated into two peaks on 5% polyacrylamide gels.

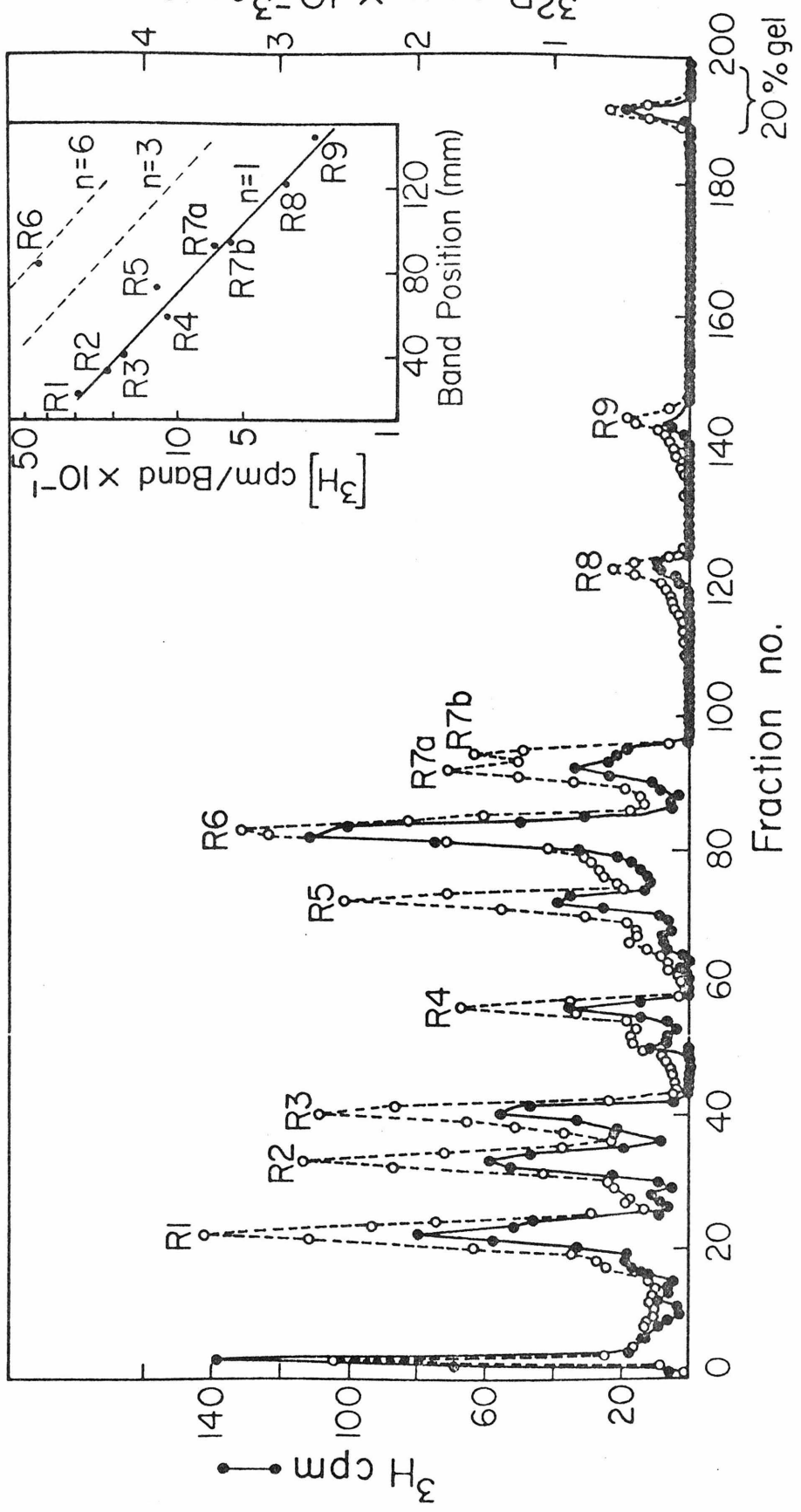
The in vitro RF containing a [^{3}H-methyl]-viral strand and a [α-^{32}P]dTTP labeled complementary strand was digested with the restriction endonuclease from H. influenzae (Hin). Figure 3 shows the results of a polyacrylamide gel electrophoresis of the resultant fragments. It is evident that the R6 band contains the largest amount of the ^{3}H-viral label. However, there is also a significant amount of the ^{3}H-label distributed in all the other fragments.

This background incorporation into all fragments was expected since as reported earlier (Razin et al., 1970) the [^{3}H-methyl]methionine does not exclusively label the methylated base. Under the in vivo labeling conditions described in Materials and Methods only 25-35% of the ^{3}H-viral DNA label is found in the 5-methylcytosine spot by base composition analysis. The remainder of the ^{3}H-label is mainly found in thymine and guanine. The data of Figure 3 suggest that this non-specific label is randomly distributed among the fragments.

The small peak trapped in the 20% gel cushion may consist of R10 and small nucleotides, possibly derived from slight contamination of the enzyme with an exonuclease activity. The amount of ^{3}H-label found in this peak is less than 2% of the total.

FIGURE 3. Polyacrylamide gel electrophoresis of DNA fragments produced by H. influenzae restriction endonuclease from methyl-labeled ϕX in vitro RF. The reaction mixture contained 40 μg RF DNA, 7 mM each of Tris (pH 7.4), β-mercaptoethanol, and $MgCl_2$, 50 mM NaCl and 30 μl of H. influenzae restriction enzyme, in a total volume of 90 μl. Incubation was at 37°C for 5 hr. The reaction was stopped by adding EDTA to 10 mM. Sucrose and sodium dodecyl sulfate were added to final concentrations of 15% and 1% respectively. The mixture was layered on a 1 cm x 19.5 cm 5% polyacrylamide gel (bottom 1 cm is 20% gel). Electrophoresis was carried out at a constant voltage of 60 V for 18 hr. ●—●, ^{3}H-methyl labeled viral DNA; -o—o-, ^{32}P cpm incorporated into the complementary strand using the methyl-labeled viral DNA as template, in presence of [α-^{32}P]dTTP and polymerase I.

Insert: The integrated ^{3}H-counts in each band were plotted against mobilities (band position in mm). Band R6 has been shown to contain three tracts very similar in size, and would be expected if the label were randomly distributed to contain three times (n = 3) the number of counts as those bands containing a single fragment (n = 1).


^{3}H cpm
^{32}P cpm x 10^{-3}
Fraction no.
20% gel
R1
R2
R3
R4
R5
R6
R7a
R7b
R8
R9
[^{3}H] cpm/Band x 10^{-1}
Band Position (mm)
n=6
n=3
n=1

It is interesting to note that the [α-^{32}P]dTTP label in the complementary strand is not uniformly distributed as is evident in the R5 and R7 peaks, which contain 50% more ^{32}P-counts than expected. This result suggests that some specific regions of the φX174 viral DNA are biased in their adenine composition.

The total ^{3}H-counts under each band were summed and plotted against their relative mobilities (Fig. 3, insert). A linear relationship is observed between the logarithm of the integrated counts and their electrophoretic mobility for every band except R6. Since band R6 has been shown to contain three distinct tracts very similar in size, it might be expected to contain three times the amount of counts as other bands. Instead, it contains about 30% of the total ^{3}H-viral label, twice as much the expected value. This result leads us to conclude that the [^{3}H-methyl]-viral label was preferentially located in the R6 band.

IV. Digestion of In Vitro [^{3}H-Methyl]RF with Hemophilus aegyptius Restriction Endonuclease

The restriction enzyme from H. aegyptius cleaves φX RF into 11 specific fragments (Z1 to Z10); Z6 consists of two tracts of similar size (Middleton et al., 1972; Lee and Sinsheimer, 1974b). This set of fragments is distinct from those produced from the H. influenzae. In order to resolve which of the three R6 fragments contains the methylated base, the in vitro RF containing [^{3}H-methyl]-viral strand and a [α-^{32}P] dCTP labeled complementary strand was digested with the H. aegyptius restriction enzyme. The distribution of the ^{3}H-viral label in the resultant fragments was analyzed by polyacrylamide gel electrophoresis.

In Figure 4, it is clearly demonstrated that the Z2 fragment contains the largest amount of the ^{3}H-label in the viral strand. When the ^{3}H-label under each band is summed and plotted against its relative mobility (Fig. 4, insert), Z2 is found to contain 36% of the total ^{3}H-counts recovered from the gel, 2.5 times more ^{3}H-label than otherwise expected. The ^{3}H-count in Z5 is also enhanced; this fragment contains about twice the expected label (9% of the total ^{3}H-radioactivity). Z6 has been shown to contain 2 tracts and thus has a normal content of ^{3}H-radioactivity with respect to other fragments. The peak trapped inside the 20% gel at the end of the gel may consist of Z10 and other nucleotides resulting from enzyme digestion and contains less than 2% of the total ^{3}H-label.

The [α-^{32}P]dCTP labeled complementary strand, in this case, does not show any bias in its labeling pattern in any of the Z fragments. There is a linear relationship between the logarithm of the integrated ^{32}P-counts in each peak and the electrophoretic mobility.

In summary of the above data, it can be concluded that the [^{3}H-methyl]-label in the viral DNA is primarily located in the Z2 fragment and (very likely) one of the R6 fragments.

Discussion

The data from these experiments are consistent with the restriction fragment map for ϕX174 (Fig. 5). That is, one of the R6 fragments (R6 contains the largest amount of ^{3}H-methyl label) overlaps with the Z2 fragment (which also contains the largest amount of ^{3}H-label).

FIGURE 4. Polyacrylamide gel electrophoresis of H. aegyptius restriction endonuclease fragments produced from methyl-labeled ϕX in vitro RF. The reaction mixture contained 30 µg of RF DNA, 7 mM each of Tris (pH = 7.4), β-mercaptoethanol, and $MgCl_2$, 20 mM NaCl and 15 µl of H. aegyptius restriction enzyme, in a total volume of 70 µl. Incubation was at 37°C for 4 hr. The resultant fragments were separated on a 1 cm x 19.5 cm 4% polyacrylamide gel (bottom 1 cm is 20% gel). Electrophoresis was at constant voltage of 60 V for 15 hr. -●—●-, ^{3}H-methyl labeled viral DNA; -o—o-, [α-^{32}P]dCTP label in complementary strand.

Insert: The integrated ^{3}H-counts found in each band were plotted against mobilities. Band Z6 has been shown to contain 2 tracts very similar in size and therefore contains 2 times (n = 2) the number of counts as those bands containing a single fragment.

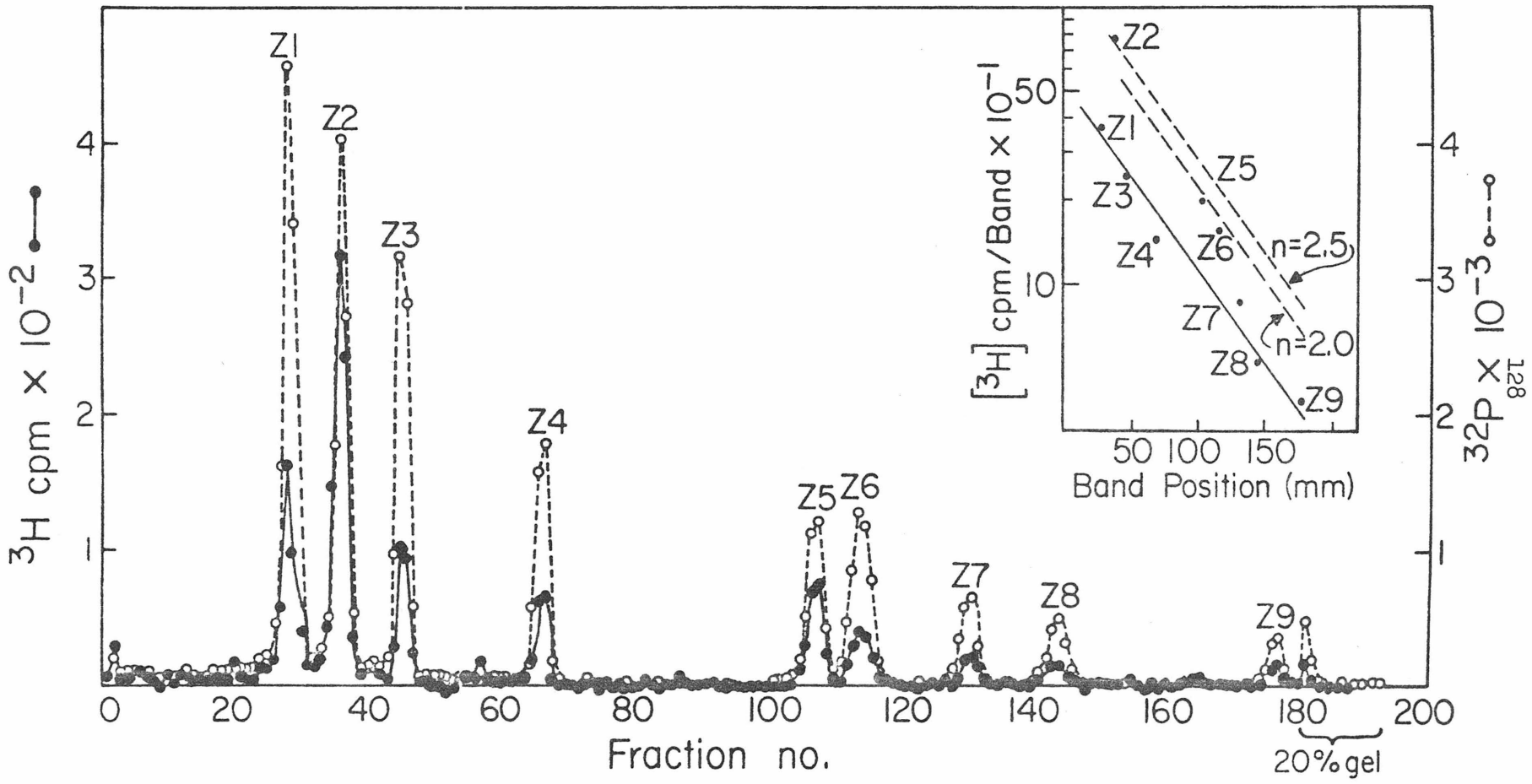
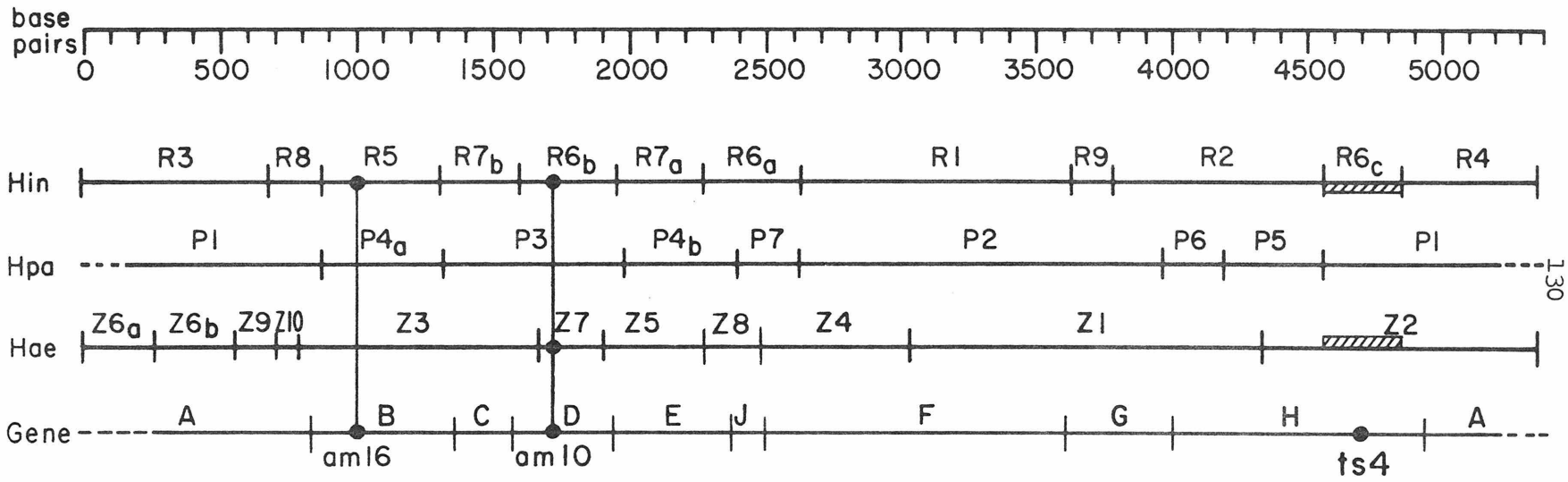

Z1
Z2
Z3
Z4
Z5
Z6
Z7
Z8
Z9
^{3}H cpm × 10^{-2}
^{32}P × 10^{-3}
Fraction no.
20% gel
[^{3}H] cpm/Band × 10^{-1}
Band Position (mm)
n=2.5
n=2.0

FIGURE 5. Restriction enzyme fragment map of ϕX174. The molecular size estimates and physical order of the ϕX DNA fragments produced by cleavage with the H. influenzae (Hin), H. parainfluenzae (Hpa) and H. aegyptius (Hae) has been described (Lee and Sinsheimer, 1974b). The order and the size of the ϕX genes shown in the map is derived from the results of Benbow et al. (1974). Using a modification of the genetic assays for ϕX fragments previously described (Weisbeek and Van de Pol, 1970; Edgell et al., 1972) R5 has been found to contain the am16 locus in gene B, and R6b and Z7 to contain am10 in gene D. The correlation of other fragments with the ϕX genetic map can be inferred but can only be considered as tentative. The location of the ^{3}H-methyl label in R6c and Z2 is indicated by the hatched area.


base pairs
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
Hin
R3
R8
R5
R7b
R6b
R7a
R6a
R1
R9
R2
R6c
R4
Hpa
P1
P4a
P3
P4b
P7
P2
P6
P5
P1
Hae
Z6a
Z6b
Z9
Z10
Z3
Z7
Z5
Z8
Z4
Z1
Z2
Gene
A
B
C
D
E
J
F
G
H
A
am16
am10
ts4
130

Preliminary genetic data correlating the restriction enzyme fragments with the ϕX genetic map indicate that R6c and Z2 are located at gene H (Fig. 5), in agreement with the findings of Hutchison et al. (1972) that both Z2 and R6c fragments contain the mutant ts4 locus of gene H.

From the in vivo methylation studies of ϕX reported earlier (Razin et al., 1973), it was concluded that replicating intermediates with single-strand tails are the substrates for methylation. In addition, it was found that the methyl-label sedimented as linear single-stranded DNA of almost double the length of the phage ϕX genome. This observation suggested that methylation occurs at a final stage of phage maturation, and that methylation in the phage ϕX system might serve as a signal for termination of a round of replication.

Considering the continuous mode of phage ϕX DNA synthesis suggested by the rolling circle model (Gilbert and Dressler, 1968) and the finding that single-strand DNA synthesis is initiated from a unique position, namely the restriction enzyme fragment R3 in gene A, and proceeds clockwise around the ϕX174 genetic map (genes A → H) (Johnson and Sinsheimer, 1974), the location of the unique methylated base in gene H accords well with the earlier suggestion that methylation occurs at a final stage of phage maturation and may serve as a signal for termination.

The excess ^{3}H-label found in Z5 (4-5% of total) is difficult to explain; it occurred in both preparations of methyl-labeled DNA with the different labeling conditions. Z5 overlaps in part with R6a and with R7a in the restriction enzyme fragment map. One possible explanation

is that the part of Z5 which overlaps with R6a is highly biased in its base composition since considerable ^{3}H-label was also incorporated, preferentially into thymine and guanine. Alternatively, it cannot be ruled out that a minor population of the ϕX DNA has a different (additional?) methylation site than that in gene H. Unfortunately, the preparation of highly radioactive methyl-labeled DNA, with exclusive labeling in the methylated base is so difficult that the obvious experiments to analyze this problem further are impractical at this time. Because of the limited radioactivity it is impossible to recover enough ^{3}H-label from the restriction enzyme fragments for further analysis.

References

Arber, W., and S. Linn (1969) DNA modification and restriction. Ann. Rev. Biochem. 38:467-500.

Benbow, R. M., A. J. Zuccarelli, G. C. Davis, and R. L. Sinsheimer (1974) Genetic recombination in bacteriophage ϕX174. J. Virol. 13:898-907.

Billen, D. (1968) Methylation of the bacterial chromosome: An event at the "Replication Point?" J. Mol. Biol. 31:477-486.

Dumas, L. B., G. Darby, and R. L. Sinsheimer (1971) The replication of bacteriophage X174 DNA *in vitro* temperature effects on repair synthesis and displacement synthesis. Biochim. Biophys. Acta 228:407-422.

Edgell, M. H., C. A. Hutchison, and M. Sclair (1972) Specific endonuclease R fragments of bacteriophage ϕX174 deoxyribonucleic acid. J. Virol. 9:574-582.

Gilbert, W., and D. Dressler (1968) DNA replication: The rolling circle model. Cold Spring Harbor Symp. Quant. Biol. 33:473-484.

Hutchison, C. A., J. H. Middleton, and M. H. Edgell (1972) Mapping specific fragments of ϕX174 replicative form DNA. Biophys. J. 12 (Abs.):31a.

Johnson, P. H., A. S. Lee, and R. L. Sinsheimer (1973) Production of specific fragments of ϕX174 replicative form DNA by a restriction enzyme from *Hemophilus parainfluenzae*, endonuclease HP. J. Virol. 11:596-599.

Johnson, P. H., and R. L. Sinsheimer (1974) Structure of an intermediate in the replication of bacteriophage ϕX174 deoxyribonucleic acid: The initiation site for DNA replication. J. Mol. Biol. 83:47-61.

Kelly, T. J., and H. O. Smith (1970) A restriction enzyme from Hemophilus influenzae. II. Base sequence of the recognition site. J. Mol. Biol. 51:393-409.

Lark, C. (1968a) Studies on the in vivo methylation of DNA in Escherichia coli 15 T^-. J. Mol. Biol. 31:389-399.

Lark, C. (1968b) Effect of the methionine analogs, ethionine and norleucine on DNA synthesis in E. coli 15 T^-. J. Mol. Biol. 31:401-414.

Lee, A. S., and R. L. Sinsheimer (1974a) A continuous electro-elution method for the recovery of DNA enzyme fragments. Anal. Biochem. 60: 640-644.

Lee, A. S., and R. L. Sinsheimer (1974b) A cleavage map of bacteriophage ϕX174 genome. Proc. Nat. Acad. Sci. U.S.A. 71:2882-2886.

Middleton, J. H., M. H. Edgell, and C. A. Hutchison (1972) Specific fragments of ϕX174 deoxyribonucleic acid produced by a restriction enzyme from Hemophilus aegyptius, endonuclease Z. J. Virol. 10:42-50.

Razin, A., J. Sedat, and R. L. Sinsheimer (1970) Structure of the DNA of bacteriophage ϕX174. VII. Methylation. J. Mol. Biol. 53:251-259.

Razin, A., J. Sedat, and R. L. Sinsheimer (1973) In vivo methylation of replicating bacteriophage ϕX174 DNA. J. Mol. Biol. 78: 417-425.

Scarano, E., M. Iaccarino, P. Grippo, and E. Parisi (1967) The heterogeneity of thymine methyl group origin in DNA pyrimidine isostichs of developing sea urchin embryos. Proc. Nat. Acad. Sci. U.S.A. 57:1394-1400.

Sinsheimer, R. L., B. Starman, C. Nagler, and S. Guthrie (1962) The Process of infection with bacteriophage ϕX174. I. Evidence for a "Replicative Form." J. Mol. Biol. 4:142-160.

Smith, H. O., and K. W. Wilcox (1970) A restriction enzyme from Hemophilus influenzae. I. Purification and general properties. J. Mol. Biol. 51:379-391.

Weisbeek, P. J., and J. H. Van de Pol (1970) Biological activity of ϕX174 replicative form DNA fragments. Biochim. Biophys. Acta 224:328-338.