

Digestion of Deoxyribonucleic Acids from Bacteriophage T7, λ , and ϕ 80h with Site-Specific Nucleases from *Hemophilus influenzae* Strain Rc and Strain Rd†

Arthur Landy,* Elizabeth Ruedisueli,‡ Linda Robinson,§ Carl Foeller, and Wilma Ross

ABSTRACT: The digestion profiles of DNA from bacteriophage T7, λ , and ϕ 80h by the site-specific nucleases from *Hemophilus influenzae* strain Rc and strain Rd have been analyzed. The DNA fragments range in molecular weight from approximately 2×10^4 to 3×10^6 and are resolved by polyacrylamide-agarose gel electrophoresis. The digestion profiles thus produced are unique and characteristic for both the DNA and the nuclease. The T7 DNA digestion products produced by the Rc and Rd nucleases are identical. With ϕ 80h and λ DNAs only 87 and 71 %, respectively, of the total number of fragments produced by the Rd nuclease are common to both nuclease digestions, the remaining DNA fragments being unique to either the Rc or the Rd digestions. Strain Rc possesses a site-specific nuclease activity which is identical to an activity found in strain Rd. In addition strain Rd carries a second activity which is not found in strain Rc and which elutes at slightly higher salt on phosphocellulose. Protection experiments with individual modification methylases of strain

Rd (Roy, P. H., and Smith, H. O. (1974), *J. Mol. Biol.* (in press)) establish that the nuclease which is common to both Rd and Rc is associated with methylase II. This nuclease in strain Rd is called *Hind*II and in strain Rc is called *Hinc*II according to the nomenclature proposed by Smith and Nathans (Smith, H. O., and Nathans, D. (1973), *J. Mol. Biol.* 81, 419–423). The additional activity which elutes second from phosphocellulose is *Hind*III. The *Hinc*II–*Hind*II activity (*i.e.*, *Hinc*II or *Hind*II) is best prepared from strain Rc. It makes 34 cuts in λ DNA, 43 cuts in ϕ 80h DNA, and 51 cuts in T7 DNA. The *Hind*III nuclease makes no cuts in T7 DNA and 6 and 4 cuts in λ and ϕ 80h DNA, respectively. A nomenclature system for the DNA digestion products is proposed and all the DNA fragments produced by Rc and Rd digestions of the three related phages, ϕ 80h amb1, ϕ 80h $\text{psu}_{\text{III}}^{+}$, and ϕ 80h $\text{psu}_{\text{III}}^{+}$ are identified and their molecular weights are presented.

The phenomenon of host restriction–modification, whereby a cell is able to recognize and degrade foreign DNA (see review by Arber and Linn, 1969), has provided a powerful tool for the generation and analysis of specific genomic fragments in several different systems (Danna and Nathans, 1971; Edgell *et al.*, 1972; Morrow and Berg, 1972; Allet *et al.*, 1973; Landy *et al.*, 1974; Mowbray and Landy, 1974). Each restriction–modification system is composed of two enzymes which act on DNA. The modifying enzyme methylates the genome at a small number of sites which are defined by a specific sequence of bases characteristic for each system (the recognition sequence). The restricting enzyme is a nuclease which will cleave any DNA that contains the recognition sequence in an unmodified form.

The restriction enzymes can be divided into two categories (see reviews by Boyer, 1971, and Meselson *et al.*, 1972). The cleavage of DNA by the type I enzymes, such as those found in *Escherichia coli* K or *E. coli* B, requires *S*-adenosylmethionine and ATP (Meselson and Yuan, 1968; Linn and Arber, 1968; Roulland-Dussoix and Boyer, 1969), but is not confined to the recognition sequence, and does not result in a molar yield of specific fragments (Horiuchi and Zinder, 1972; Morrow and Berg, 1972). In contrast, the cleavage of DNA by the type II enzymes, such as those isolated from *Hemo-*

philus influenzae Rd (Smith and Wilcox, 1970) and *E. coli* harboring certain R factors (Yoshimori, 1971), does not require *S*-adenosylmethionine or ATP, does take place at the recognition sequence (Kelly and Smith, 1970; Hedgpeth *et al.*, 1972; Bigger *et al.*, 1973; Boyer *et al.*, 1973), and does result in a molar yield of specific fragments (Danna and Nathans, 1971). The genus *Hemophilus*, *e.g.*, *H. influenzae* Rd (Smith and Wilcox, 1970), *Hemophilus aegyptius* (Middleton *et al.*, 1972), and *Hemophilus parainfluenzae* (Gromkova and Goodgal, 1972; Sharp *et al.*, 1973), has been a particularly useful source of different site-specific (type II) deoxyribonucleases.

In this report we examine the individual digestion products of T7, ϕ 80, and λ DNAs and compare the *S*-adenosylmethionine–ATP-independent site-specific DNAases of *H. influenzae* strain Rd and strain Rc.

Nomenclature

We have adopted that portion of the nomenclature system proposed by Smith and Nathans (1973) which describes the restriction–modification enzymes. The enzymes, which have been isolated from either *H. influenzae* strain Rc or *H. influenzae* strain Rd, are formally designated *Hinc* and *Hind*, respectively. The different restriction–modification systems within each organism are distinguished by roman numerals.

It has, however, been necessary to introduce a different nomenclature system for the DNA fragments produced by the restriction endonucleases. The adopted system was dictated by the complexity of the digestion profiles and the frequent occurrence of similar but nonidentical digestion profiles. The latter arise in one, or a combination, of three ways: (a) when using DNAs of related bacteriophage, such as a family

† From the Division of Biological and Medical Sciences, Section of Microbiology and Molecular Biology, Brown University, Providence, Rhode Island 02912. Received December 10, 1973. Supported by Grant CA11208 from the National Cancer Institute, U. S. Public Health Service, and Grant NP-118B from the American Cancer Society.

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of deletion mutants or transducing derivatives (e.g., see Figure 3); (b) when using nuclease preparations with overlapping site specificities (e.g., see Figure 4); (c) when varying the parameters of gel electrophoresis (in preparation).

The digestion profiles are divided into a number of regions on the basis of groupings which appear in the distribution of bands across the gel. Each "group" of fragments is assigned a capital letter starting at the top of the gel and the fragments within each group are numbered consecutively from the top, A1, A2, ..., B1, B2, ..., etc. A canonical set of conditions has been adopted to which all other related digestion profiles are referred.

In the work reported here the canonical nuclease was chosen to be the unresolved nuclease from *H. influenzae* strain Rd as prepared by Smith and Wilcox (1970). The standard gel condition was chosen as that which gives the best resolution of the largest number of fragments (see Methods section). For each bacteriophage a particular strain was chosen as the source of the canonical DNA. For λ the strain CI857S7, obtained from William Dove, was chosen for the canonical DNA because of its ease of preparation. For ϕ 80 the strain ϕ 80h amb1, from the Cambridge collection, was chosen to be the canonical strain because of its immediate antecedence to the tRNA^{Tyr} transducing phages (Russell *et al.*, 1970). For T7 a wild-type Studier strain obtained from William Summers was used.

When DNA digestions are analyzed under a variety of electrophoresis conditions the profiles (distribution of fragments) can vary considerably from the canonical profile. Each fragment retains its canonical name irrespective of its position in the noncanonical gel.

In digestion profiles made with related DNAs and/or related nucleases, those fragments which also occur in the canonical profile retain their original labels. Each "new" or "unique" fragment derives its label from the closest canonical fragment and a small letter suffix. Each label for a non-canonical fragment is used only once. For example, in a particular DNA B2a and B2b are two "new" fragments lying close to the position of the canonical fragment B2 (B2 need not be actually present in this particular DNA). B2a will generally be closer to B2 than B2b under the standard electrophoresis conditions. If in another related DNA there is a unique fragment in this region which is different from B2a or B2b, it will receive the name B2c; however, in this case (since it is a different DNA) the suffix does not connote position relative to B2a and B2b. In those cases where a purified DNA fragment is eluted from a gel and subjected to a secondary digestion with a different nuclease, the resulting fragments are assigned the name of the parent fragment followed by a letter identifying the second nuclease and an arabic number. For example, endo R-*Hind* fragment B10a digested with endo R-*Hae*, (*H. aegyptius*), will give rise to fragments called *Hind* B10a-Z1, Z2, ..., etc.

The application of this nomenclature system to the six digestion profiles generated from three related ϕ 80h phages with two related nucleases is illustrated in Figure 3. This system has also proven workable during construction of a λ cleavage map requiring the analysis of a rather large number of related DNAs under a range of different digestion and gel conditions (in preparation).

Materials and Methods

Materials. DNase I (DP) was purchased from Worthington Biochemical Corp. Lysozyme (grade 1), bovine albumin (fraction V), Dowex 50W (hydrogen form, 4% cross-linked,

dry mesh 100–200), and ethidium bromide were obtained from Sigma. Optical grade CsCl was obtained from the Harshaw Chemical Co. Reagents for electrophoresis: acrylamide, Bis (*N,N'*-methylenebis(acrylamide)), Temed,¹ and ammonium persulfate were from Bio-Rad Laboratories and "Seakem" Agarose was from Marine Colloids, Inc. "Stains-all" (1-ethyl-2-[3-(1-ethylnaphtho[1,2d]thiazolin-2-ylidene)-2-methylpropenyl]naphtho[1,2d]thiazolium bromide) was obtained from Eastman. Omnifluor and Aquasol were purchased from New England Nuclear.

Preparation of Bacteriophage. Bacteriophage λ was prepared from the heat-inducible lysogen CI90/ λ CI857S7. Cells were grown at 31° to an OD₅₅₀ of 1.0 in YT medium (5 g of NaCl, 8 g of tryptone, and 5 g of yeast extract per l.). An equal volume of YT medium at 55° was added and the culture was incubated at 42° for 15 min, then at 37° for 3–4 hr. Cells were harvested and resuspended in 1/10th volume of 10 mM Tris-Cl (pH 7.4), 5 mM MgSO₄, 0.1 M NaCl, and 0.05% gelatin, lysed by freezing at –20°, thawing to room temperature, and passing the suspension through a small bore pipet. The lysate was incubated at 37° for 30 min with 2 μ g/ml of DNase I and 200 μ g/ml of lysozyme, after which cell debris was pelleted. Phage were purified by banding in two successive CsCl step gradients with steps of 1.3 (initially containing phage), 1.4, 1.5, and 1.7 g per cm³. ϕ 80h amb1 and its derivative transducing phages were grown on *E. coli* strain CA275 in LB medium (Landy *et al.*, 1967) and purified on CsCl step gradients.

Preparation of DNA. Phage were dialyzed to remove CsCl, diluted to an OD₂₆₀ of less than 15, brought to 10 mM Tris-Cl (pH 7.9), 10 mM EDTA, 0.3 M NaCl, 0.2% sodium dodecyl sulfate, and 1 M NaClO₄, and extracted twice with phenol saturated with 0.1 M Tris-Cl (pH 7.9). The DNA was dialyzed against 10 mM Tris-Cl (pH 7.9), 1 mM EDTA, and finally against 10 mM Tris-Cl (pH 7.9). Two preparations of T7 DNA were used. One was a gift from Hamilton Smith, the other was prepared as described above from a purified lysate of T7 (Studier strain) received from William Summers. ³H-Labeled and ³²P-labeled SV40 DNA, form I, were gifts from Daniel Nathans and Malcolm Martin, respectively.

Purification of Site-Specific Nucleases. Restriction endonucleases were prepared from *H. influenzae* strain Rd (obtained from Paul Roy) and strain Rc (obtained from Grace Leidy) according to the method of Smith and Wilcox (1970) with a modification in the final purification step. Phosphocellulose (Whatman P11) was prepared by treating successively with 0.5 M HCl for 30 min, distilled water to pH 5.0, 0.5 M KOH for 30 min, distilled water to pH 8.5, 4 M KCl–50 mM potassium phosphate buffer (pH 7.4) overnight, and finally washing and storing in 50 mM potassium phosphate buffer (pH 7.4).

Phosphocellulose columns (1.6 \times 25 cm) were equilibrated with column buffer (20 mM potassium phosphate (pH 7.4)). A portion of the 50–70% ammonium sulfate fraction, equivalent to the extract from approximately 7 g of cells (OD₂₆₀ approximately 2.0; OD₂₈₀ approximately 1.8), was diluted tenfold with column buffer just prior to loading on the column. Protein was eluted from the column at a flow rate of 20 ml/hr, first with a 300-ml linear gradient of 0–0.28 M KCl in column buffer, followed by 50-ml steps of 0.28, 0.30, and 0.36 M KCl in column buffer. Exonuclease activity, assayed by measuring the appearance of acid-soluble radioactivity from sonicated ³²P-labeled λ DNA, eluted at approximately 0.20–0.23 M KCl. The site-specific nucleases, quantitated and characterized using

¹ Abbreviation used is: Temed, *N,N,N',N'*-tetramethylethylenediamine.

digestion profiles of a standard λ DNA preparation (see below), eluted at approximately 0.28–0.32 M KCl. In preparations from strain Rd, the site-specific nuclease activity separated into two broad overlapping peaks. For unresolved Rd nuclease, all fractions showing activity were pooled together. To study the individual resolved nucleases only the fractions from the extreme ends of the activity profile were used. In preparations from strain Rc there was no evidence of two peaks, and all fractions containing activity were pooled.

Pooled fractions were concentrated by precipitation in 75% saturated ammonium sulfate and resuspension in 0.2 M NaCl–20 mM Tris-Cl (pH 7.4). Bovine albumin was added to concentrated samples to 3 mg/ml and the enzyme was stored at 0° where it is stable for several months.

Digestion of DNA with Site-Specific Nucleases. All digestions were carried out for 4.5 hr at 37° in 10 mM Tris-Cl (pH 7.9), 6.6 mM MgCl₂, 6 mM mercaptoethanol, and 60 mM NaCl with DNA at 200 μ g/ml. Endonuclease was added in three aliquots, at 1.5-hr intervals. Reactions were terminated by chilling and the addition of $1/10$ th volume of 0.2 M EDTA. In instances where the combined effect of two separate enzyme activities was studied on the same DNA sample, the enzymes were added simultaneously in quantities of each sufficient to yield complete profiles.

Gel Electrophoresis Conditions. Several variations in gel composition, time of electrophoresis, temperature, voltage, and length of gel have been utilized to gather more precise information about specific groups of DNA fragments. However, unless specified otherwise all of the gels illustrated here were of the “standard” composition used in determining canonical nomenclature (see Nomenclature): 2% acrylamide (0.1% methylenebis(acrylamide))–0.5% agarose, 0.05% Temed, and 0.03% ammonium persulfate. Gels were prepared and run in Tris-borate buffer (pH 8.3) according to the procedure of Peacock and Dingman (1968). Before loading, the digested DNA samples (approximately 10 μ g) were mixed with $1/10$ th volume loading solution (80% sucrose, 1% Bromophenol Blue, and 0.1 M EDTA). Electrophoresis was carried out at 200 V, 30 mA, 0° for 5–5.5 hr in an EC Model 470 slab gel cell. Gels were stained in “Stains-all” (50% formaldehyde, 0.005% “Stains-all”) (Dahlberg *et al.*, 1969).

Preparative scale gels, double the thickness of analytical gels and containing a single 7.5-cm length slot, were used to electrophorese 1-mg samples of digested DNA. These gels were stained for 2 hr in a 10- μ g/ml solution of ethidium bromide in distilled water, and fragments were visualized with a short-wave ultraviolet lamp. Gel strips containing individual fragments were cut from the gels and the DNA electrophoretically eluted in a chamber specifically designed for this purpose. Eluted samples were dialyzed against 0.8 M NaCl, 50 mM Tris-Cl (pH 7.9), 10 mM EDTA plus 200 g of Dowex 50W per l., and then against 10 mM Tris-Cl (pH 7.9).

Determination of Molecular Weights of Endonuclease Fragments. Molecular weights of ϕ 80h DNA fragments were determined by two methods. The molecular weights of the larger fragments (A1–C1a) were determined by contour length measurement in the electron microscope. Electron micrographs of purified ϕ 80h Rd fragments were prepared by Nancy Brimlow in the laboratory of David Freifelder. ϕ X174 RFII molecules were included as internal standards. Negatives were enlarged 15–20-fold, traced, and lengths measured with a Keuffel and Esser Model 62-0300 map measurer. The molecular weight of ϕ X174 RFII is taken to be 3.4×10^6 (Sinsheimer, 1959). Selected fragments were measured and their log molecular weights were plotted as a function of migration

distance in a 2%, 45-cm gel. The curve drawn through these points was used to assign the molecular weights of other fragments in this size range.

Molecular weights of the smaller fragments were determined by electrophoretic mobility relative to an Rd digest of SV40 DNA. Rd digests of unlabeled ϕ 80h DNA and ³H- or ³²P-labeled SV40 DNA were electrophoresed in the same lane on acrylamide–agarose gels which were then stained with “Stains-all” to visualize ϕ 80h fragments. Lanes were then cut into 1-mm slices and slices were counted in Omnifluor (³²P label) or were first digested in 0.3 ml of 30% hydrogen peroxide then counted in Aquasol (³H label). Using the molecular weights of the SV40 DNA fragments as determined by Danna and Nathans (1971), standard curves of log molecular weight *vs.* migration distance were constructed; from these ϕ 80h fragment molecular weights were derived for each experiment. Average molecular weight values for each fragment were determined from several different experiments and using several gel conditions (2, 3, and 5%). The standard deviation of the values determined in separate experiments was $\pm 3\%$ and those molecular weights determined both by electrophoretic mobility and by contour length measurement were within this same range.

The molecular weights of the T7 and λ DNA fragments were obtained by comparison of their electrophoretic mobilities with those of the ϕ 80h DNA fragments.

Results

Digestion Profiles of T7 and λ DNA with Enzyme from Rc and Rd. When DNA molecules of the appropriate size and/or complexity are treated with restriction enzyme and fractionated by polyacrylamide–agarose gel electrophoresis, the resulting “digestion profile” of DNA fragments is unique and characteristic for both the substrate DNA and the particular restriction enzyme (see Figures 1 and 3). A digestion profile is probably the most sensitive means of comparing the site specificities of several different nuclease preparations because every cleavage site can be identified, even if it occurs only once in a molecule. Once an appropriate substrate DNA has been found, the limits of detection of differences in nuclease site specificities between two preparations are governed only by the degree to which every DNA fragment can be resolved or identified on the gel.

In working with nucleases which make a rather large number of cuts and with phage DNAs in the molecular weight range of 30×10^6 or larger, it is desirable and often necessary to vary one or more parameters of the electrophoresis conditions in order to obtain optimal resolution in different portions of the digestion profile (in preparation). Although in this report only a single electrophoresis condition is presented for most of the digestions, the conclusions discussed are always based upon several different electrophoresis conditions, each chosen to maximize the resolution of a particular group of fragments.

The nucleases of *H. influenzae* strain Rc and strain Rd were examined in the hope of finding some useful and/or informative differences between the two enzyme preparations. Figure 1 shows a comparison of the digestion profiles generated with enzyme from strain Rc and strain Rd on both T7 DNA and λ DNA.

The Rd and Rc digestion profiles of T7 DNA are apparently identical to one another, suggesting that there is no difference in the nuclease preparations from the two strains. No differences have been observed between the two digestion

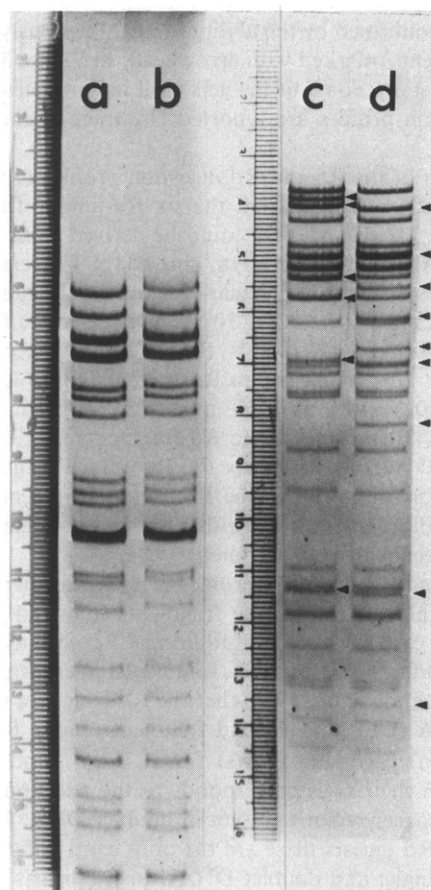


FIGURE 1: Rc and Rd digestion profiles of T₇ and λ DNA. Conditions of digestion and gel electrophoresis were as described in the Methods section. T₇ DNA was digested with Rc enzyme (a) or Rd enzyme (b) and electrophoresed for 5.5 hr. λ DNA was digested with Rc enzyme (c) or Rd enzyme (d) and electrophoresed for 5 hr. Arrowheads to the right of the λ DNA profiles indicate fragments unique to each digest (some of the unique fragments are not resolved in this gel and appear as bands of increased intensity, see Methods).

profiles with any of the several different electrophoresis conditions utilized to optimize resolution of the different size classes of fragments. The T₇ DNA is cleaved by either enzyme into 52 fragments which range in molecular weight from approximately 2×10^4 to 1.4×10^6 .

A similar size range of fragments is obtained when λ DNA is digested with the two nuclease preparations (Figure 1). However, the digestion profiles of λ DNA generated with each of the enzyme preparations are congruent for only 71% of the total number of Rd fragments. Analysis of the digestion products using several different electrophoresis conditions establishes that the nuclease from strain Rc cuts λ DNA into 35 fragments and the nuclease from strain Rd cuts λ DNA into 41 fragments. There are six fragments which are unique to the Rc digestion and twelve fragments which are unique to the Rd digestion.

To test the possible effect of ionic conditions on site specificity, a series of digestions of λ DNA was carried out under a range of conditions of pH and NaCl concentration, and with DNA that had been pretreated by heating followed by quick cooling or slow annealing. None of the variations tried had the effect of abolishing or reducing the difference in the digestion profiles of λ DNA produced by the two enzyme preparations.

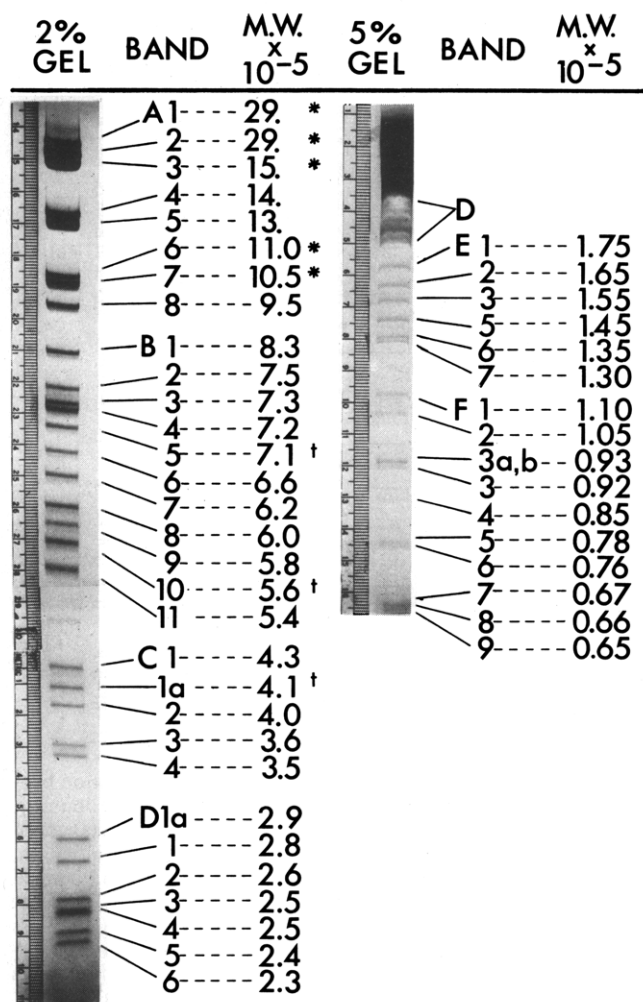


FIGURE 2: Gel profiles and molecular weights of Rd fragments of $\phi 80h$ psu_{III}⁺ DNA. Rd digests of singlet DNA were electrophoresed either on a 2%, 45-cm gel at 500 V for 12 hr or on a 5% 17-cm gel at 300 V for 4 hr. The assigned name, as described in Nomenclature, and the estimated molecular weights are given for each fragment. Molecular weights were determined by contour length measurements in the electron microscope and also by relative electrophoretic mobility (see Methods section). (*) Molecules measured by electron microscopy; (+) molecules measured by electron microscopy which are within the molecular weight range covered by the standards used for relative electrophoretic mobility determinations. The agreement of the two types of determinations was better than 5%.

Another interpretation of the results was based upon the observation that although the Rc-unique fragments have approximately the same aggregate molecular weight as the Rd-unique fragments, the former is composed of a smaller number of larger fragments than the latter. This is suggestive of a precursor-product relationship, as if the Rd nuclease contains two activities, only one of which is present in strain Rc. This can be seen more clearly in a comparison of the digestion profiles of three closely related strains of bacteriophage $\phi 80$.

Digestion Profiles of DNAs from Three Related $\phi 80$ Phages. The phage $\phi 80h$ psu_{III}⁺ is a nondefective transducing phage carrying a duplication of the tRNA_I^{Tyr} gene. (This will be referred to as double copy phage or doublet.) The phage $\phi 80h$ psu_{III}⁺ (hereafter referred to as single copy phage or singlet) is a phage derived from the doublet phage by an unequal recombination event between homologous sequences, such that

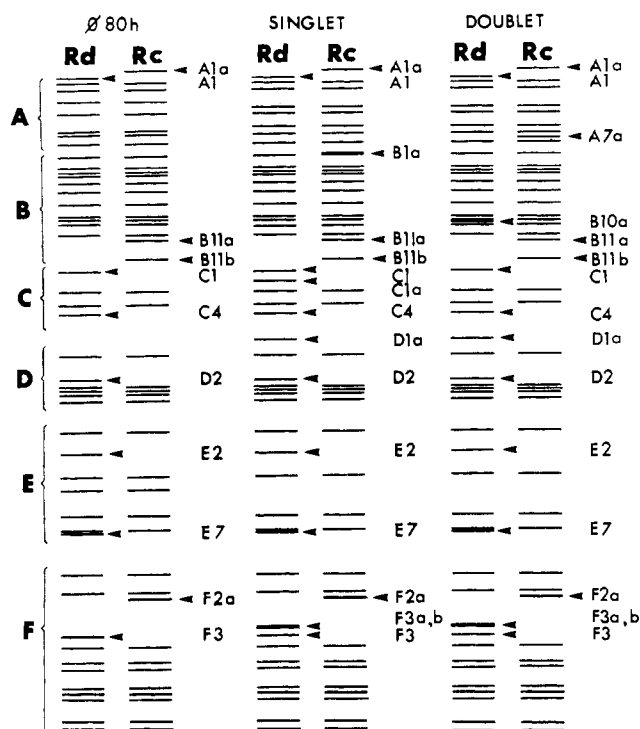


FIGURE 3: Schematic diagram comparing Rd and Rc digestion profiles of $\phi 80h$, $\phi 80h$ psu_{III} (singlet), and $\phi 80h$ psu_{III} (doublet) DNAs. Profiles drawn are based on relative migration distances of fragments on a 2% acrylamide-0.5% agarose gel, 45 cm in length, electrophoresed for 8 hr at 500 V, 4°; and on information obtained from analyzing the digestion products under several different electrophoresis conditions (see text and Methods section). Letters A-F indicate names of groups of fragments (see section on Nomenclature). Arrows to the right of each profile point out fragments unique to either the Rd or the Rc digest of a given DNA type. Names for the unique fragments of both digests are given to the right of each set of profiles. The following five categories of unique fragments can be described: (1) unique to Rc, found in all three phages: A1a, B11a, B11b, and F2a; (2) unique to Rd, found in all three phages: A1, C1, C4, D2, E2, E7, and F3; (3) unique to Rd, found only in singlet and doublet: D1a, F3a and F3b; (4) unique to Rc, found only in singlet: B1a; unique to Rc, found only in doublet: A7a; (5) unique to Rd, found only in singlet: C1a; unique to Rd, found only in doublet: B10a.

one of the two tRNA genes has been eliminated. The parental phage from which the transducing phage were derived is $\phi 80h$ amb1, hereafter referred to as $\phi 80h$ (Russell *et al.*, 1970).

Figure 2 shows the Rd digestion profile of $\phi 80h$ psu_{III} (singlet) DNA on 2 and 5% polyacrylamide-agarose gels along with the names and molecular weights of the individual fragments. It should be noted that the electrophoretic mobility of a DNA fragment is not completely independent of its nucleotide sequence, since under certain conditions some fragments have anomalous R_F values (unpublished results). The sum of the molecular weights of the individual fragments is 25.1×10^6 whereas the value obtained from electron microscopy of the intact molecule is 28.7×10^6 (Miller *et al.*, 1971). This difference could be accounted for statistically solely on the basis of the standard deviation ($\pm 3\%$) of our individual measurements.

Figure 3 is a composite schematic diagram of the Rd and Rc digestion profiles of the three different $\phi 80h$ DNAs. Information obtained under several different electrophoresis conditions has been combined and referred to the canonical gel condition. The different gels always contain overlapping regions of the digestion profiles and proper matching of the

overlaps is confirmed by the ability to unambiguously identify those fragments (marked with arrowheads in Figure 3) unique to each digestion. Some of the gels used in constructing these total digestion profiles are reported elsewhere (Landy *et al.*, 1974).

Inspection of the Rc and Rd digestion profiles of the wild-type $\phi 80h$ DNA suggests that the six Rd-unique fragments, A1, C1, C4, D2, E2, and E7 could be derived from the three Rc-unique fragments A1a, B11a, and B11b. For example, a model consistent with molecular weights would predict one nuclease cut in Rc A1a (32×10^5) giving rise to Rd fragment A1 (29×10^5) and D2 (2.6×10^5), one nuclease cut in Rc B11a (5.3×10^5) giving rise to Rd fragments C1 (4.3×10^5) and E7 (1.30×10^5), and one nuclease cut in Rc fragment B11b (4.9×10^5) giving rise to Rd fragments C4 (3.6×10^5) and E2 (1.65×10^5). Similarly the Rc-unique fragment F2a most probably gives rise to the Rd-unique fragment F3 plus another small fragment which either runs off our gels or is too small to detect by these procedures.

The Rc and Rd digestion profiles of the transducing phage DNAs contain all the unique fragments found in the $\phi 80h$ digests, and each contains one additional Rc-unique fragment and four additional Rd-unique fragments. We have reported elsewhere (Landy *et al.*, 1974) that the Rd fragment unique to singlet DNA (C1a) and the Rd fragment unique to doublet DNA (B10a) carry the gene(s) for $tRNA_{I}^{Tyr}$ and that the difference in their sizes corresponds to the additional tRNA gene (plus spacer region) present in doublet DNA. In a comparison of Rd digests these are the only band differences between the singlet and doublet DNAs. In Rc digests of singlet and doublet DNA, the fragments C1a and B10a are missing from their respective profiles and a larger singlet-unique fragment (B1a) and a larger doublet-unique fragment (A7a) are found (see Figure 3). Since A7a in doublet DNA (10.5×10^5) and B1a in singlet DNA (8.3×10^5) are the only fragments which are unique to the transducing phages as well as being unique to the Rc digestion, they are the only possible "precursors" to those fragments which are unique to both the transducing phages and the Rd digestion, *i.e.*, fragments C1a (4.1×10^5), D1a (2.9×10^5), F3a and F3b (0.93×10^5 each) in singlet DNA and fragments B10a (5.6×10^5), D1a, F3a, and F3b in doublet DNA. The relative molecular weights of the individual fragments involved are also consistent with these conclusions.

Digestion of Rc-Unique Fragments with Rd Enzyme. The above comparisons of the Rc and Rd digests of λ and $\phi 80h$ DNA strongly suggest the presence of a nuclease activity in strain Rd which is absent in strain Rc and which is responsible for the conversion of Rc-unique fragments into Rd-unique fragments. The following experiment confirms the relation between the Rc-unique and the Rd-unique fragments.

A preparative scale gel of an Rc digest of λ DNA was run and the top three bands, including the two largest Rc-unique bands A1a and A1b (see Figure 1), were eluted together from the gel. Aliquots of the eluted fragments were either digested with sufficient Rc or Rd enzyme for complete profiles or left untreated. The three aliquots were then electrophoresed along side a complete Rd and a complete Rc digest of λ DNA (Figure 4). The untreated aliquot and the aliquot redigested with Rc enzyme are identical and show the same three unaltered bands expected from comparison with the total Rc digest. In the aliquot treated with Rd enzyme, the two Rc-unique bands A1a and A1b disappear and give rise to four Rd-unique bands: A2, B4, B8, and D1. The approximate molecular weights of these fragments are compatible with the frag-



FIGURE 4: Digestion of purified Rc-unique λ DNA fragments A1a and A1b with Rd enzyme. The three top fragments from an Rc digest of λ DNA, which include the two Rc-unique fragments A1a and A1b, were eluted from a preparative gel (see Methods) and divided into three aliquots. Lane: (a) purified fragments digested with Rd enzyme; (b) purified fragments digested with Rc enzyme; (c) purified fragments with no further digestion. Reference digestions are in lane (d), λ DNA digested with Rd enzyme, and lane (e), λ DNA digested with Rc enzyme. Arrowheads in (d) mark the Rd-unique fragments corresponding to those seen in (a). In (e) the arrowheads mark the fragments corresponding to those eluted from the preparative gel.

ments D1 and A2 having been derived by a single nuclease cut in A1a, and the fragments B4 and B8 having been derived by a single nuclease cut in A1b. Similar results have always been obtained when other Rc-unique fragments from λ or $\phi 80h$ DNA have been eluted and digested with Rd enzyme.

Although the purpose of this particular experiment was to establish the relationship between the nucleases of strain Rc and strain Rd it also serves to illustrate a useful procedure in the purification of individual DNA fragments which can often be applied using appropriate combinations of the several available site-specific nucleases.

For example, by this procedure the preparation of pure fragment B4 or fragment B8, which even on analytical gels are very poorly resolved from their neighbors, can be accomplished quite readily with large quantities on preparative scale gels. The secondary digestion profile generated from a purified fragment can also be used analytically as a fingerprint of the parent fragment. This has proven especially useful when it is necessary to establish whether two DNA fragments with the same electrophoretic mobility (*e.g.*, in digests of two related DNAs) have different sequences.

The Fractionation on Phosphocellulose of Two Different Site-Specific Nuclease Activities from H. influenzae Rd. Extracts of strain Rd can be resolved on phosphocellulose

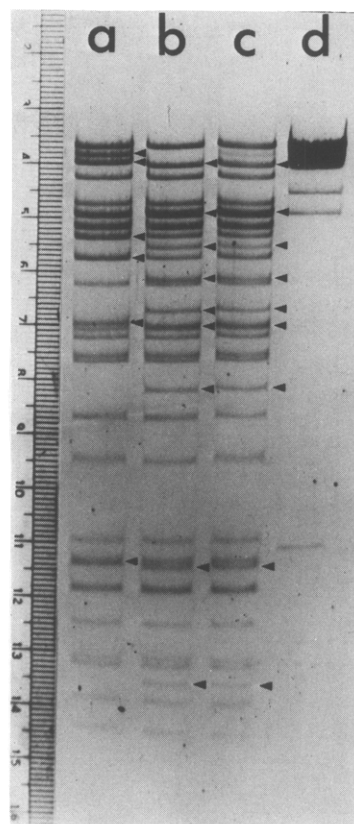


FIGURE 5: Digestion profiles of λ DNA produced by the second-eluting Rd phosphocellulose fraction. Conditions of digestion and electrophoresis were as described in Methods. Digestion profiles were generated by: (a) Rc enzyme; (b) Rd enzyme; (c) Rc enzyme plus the second-eluting phosphocellulose fraction; (d) the second-eluting phosphocellulose fraction alone. Arrowheads indicate the following: (a) Rc-unique fragments; (b) Rd-unique fragments; (c) Rd-unique fragments appearing in simultaneous digest with Rc enzyme and the second-eluting phosphocellulose fraction.

columns (see Materials and Methods) into two nuclease activities which, on the basis of λ or $\phi 80h$ DNA digestion profiles, have distinctly different substrate site specificities. When the two nucleases are mixed together in a reconstruction experiment the digestion profiles characteristic of the unresolved Rd enzyme are generated.

Extracts prepared from strain Rc, on the other hand, contain only the nuclease activity corresponding to the first-eluting Rd nuclease, *i.e.*, they produce identical DNA digestion profiles. The Rc enzyme is also equivalent to the first-eluting Rd nuclease in its ability, when mixed with the second-eluting Rd enzyme, to produce a λ DNA digestion profile identical to that of unresolved Rd enzyme (see Figure 4). A mixture of the first-eluting Rd nuclease and the Rc nuclease produces digestion profiles identical with either one alone. It is concluded that the enzyme found in strain Rc is identical with (has the same nucleotide recognition site specificity as) the Rd enzyme which elutes first from phosphocellulose.

The activity of the second-eluting Rd enzyme on the three phage DNAs is consistent with the conclusion that its absence in Rc constitutes the observed difference between Rd and Rc digestion profiles. On λ DNA or $\phi 80h$ DNA the second-eluting Rd nuclease, in contrast to the first-eluting Rd nuclease and the Rc enzyme, generates a small number of large fragments (see Figure 5). These fragments occur in molar yield and are not a subset of the fragments generated by a total Rd or Rc digestion. By summing the band differences between the

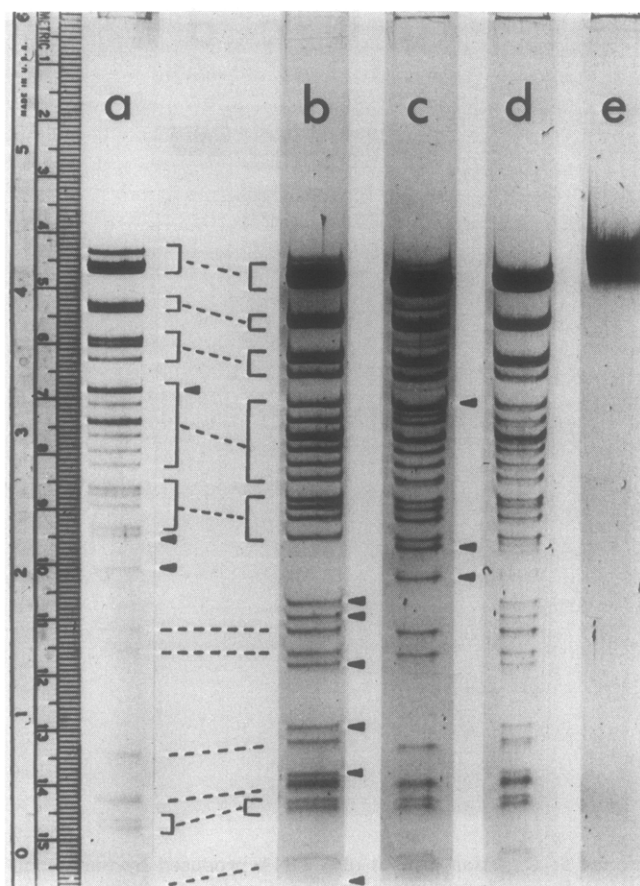


FIGURE 6: The effect of pretreatment with individual Rd methylases on Rd digestion profiles. Aliquots of ϕ 80h singlet DNA were methylated by Paul Roy with the methylases purified from strain Rd (Roy and Smith, 1974). Methylated DNAs were phenol extracted and dialyzed into 10 mM Tris-Cl (pH 7.4). Endonuclease digestion and electrophoresis conditions were as described in Methods. Lanes (b)–(e) are from the same gel. Lane (a) was electrophoresed under slightly different conditions of voltage and temperature. Correspondence of fragments in this profile with those in the Rd control (b) is indicated by brackets. Lane: (a) unmethylated control, Rc digest; (b) unmethylated control, Rd digest; (c) methylated with *HindIII*, then Rd digested; (d) methylated with *HindIV* then Rd digested; (e) methylated with *HindII* then Rd digested. The faint partial digestion products seen in (c) are due to slight contamination of methylase dIII with methylase dII. Arrowheads in lanes (a) and (c) mark the Rc-unique fragments, and in lane (b) they mark the Rd-unique fragments which are not found in (a) or (c).

Rc and Rd digestion profiles (*e.g.*, see Figures 1 and 3) it is seen that the second-eluting Rd nuclease makes six cuts in λ DNA and four cuts in ϕ 80h DNA. On T7 DNA the second-eluting Rd nuclease has no detectable activity, thus explaining why the Rd and Rc digestion profiles of this DNA are identical.

Correlation of the Nuclease Activities with the Site-Specific DNA Methylases of *H. influenzae* Strain Rd. Four site-specific adenine methylases (modification enzymes) from *H. influenzae* Rd have been purified and characterized by Roy and Smith (1974). They have shown that methylase *HindI* corresponds to the *S*-adenosylmethionine-ATP-dependent restriction enzyme (Gromkova *et al.*, 1973) first described in the genetic studies of Glover and Piekawicz (1972). In order to determine the relationship of the Rd methylases *HindII*, *HindIII*, and methylase IV to the nuclease activities described above, aliquots of ϕ 80h singlet DNA were each treated with one of the purified methylases, and then digested with the nuclease from strain Rd. The digestion profiles are shown in Figure 6

along with unmethylated control digestions with either Rc or Rd nuclease. (Identical results and conclusions are obtained from λ DNA treated in the same way.)

Pretreatment of the DNA with the methylase *HindII* results in abolishing the majority of the Rd digestion cuts, indicating that this methylase corresponds in site specificity to both the nuclease activity found in *H. influenzae* strain Rc and also the Rd activity which elutes first from a gradient phosphocellulose column. The DNA aliquots which have been pretreated with methylase *HindIII* show an Rd digestion profile which is identical with the Rc digestion profile. We conclude that methylase *HindIII* protects those loci on the genome which are responsible for the difference between Rc and Rd digestion profiles, *i.e.*, those sites which are cut by the nuclease which is absent in strain Rc and which elutes second from the phosphocellulose column. DNA which has been protected by methylase IV does not show a difference in the Rd digestion profile from untreated DNA under these electrophoresis conditions suggesting that if there is a site-specific nuclease corresponding to methylase IV, it is most probably not present in this phosphocellulose fraction of Rd enzyme.

Discussion

The results presented above indicate that *H. influenzae* strain Rc lacks one of the two ATP-*S*-adenosylmethionine-independent site-specific deoxyribonucleases identified in *H. influenzae* strain Rd. The two different nuclease activities in strain Rd have also been identified by H. Smith (personal communication). Although the differential loss or inactivation of one of the two nucleases during the work up of Rc extracts cannot be definitely ruled out, this interpretation is not favored for several reasons. The same difference between Rc and Rd DNA digestion profiles has been observed in several different enzyme preparations, in several different batches of cells and even prior to the phosphocellulose chromatography step when characteristic digestion profiles can first be distinguished. Furthermore, in an unresolved Rd preparation it is the site-specific nuclease which is missing from strain Rc which is the more stable of the two nucleases.

Genetic studies bearing on the above nucleases have not been carried out. However, the kind of unstable expression observed for the ATP-*S*-adenosylmethionine-dependent restriction enzyme *HindI* (in which both positive and negative cultures continuously segregate several per cent of the alternative phenotype (Glover and Piekawicz, 1972)) would not appear to account for the apparently stable and consistent difference in nucleases between strains Rc and Rd.

The *S*-adenosylmethionine-ATP-independent nuclease fraction from strain Rd is resolved on phosphocellulose into two activities with different site specificities. The nuclease of strain Rc elutes as a single activity on phosphocellulose and has the same substrate recognition site as the nuclease from strain Rd which elutes first from phosphocellulose.

On the basis of the Rd nuclease digestion profiles of λ DNA and ϕ 80h singlet DNA protected with the individual Rd site-specific methylases, it is concluded that methylase *HindII* corresponds in site specificity to the nuclease which elutes first in the phosphocellulose fractionation of Rd preparations and which is present in strain Rc. This is the nuclease for which Kelly and Smith (1970) determined the nucleotide recognition sequence using T7 DNA as a substrate and which is called *HindII* (Smith and Nathans, 1973).

Using the same system of nomenclature the nuclease from strain Rc has been called *HincII*. Although this is the only

nuclease studied thus far in strain Rc this roman numeral assignment seems the least confusing in light of the DNA digestion profiles and methylase data discussed above, *i.e.*, since *HincII* and *HindII* have the same substrate recognition sites. Until the nucleotide sequence analysis of the termini produced by Rc enzyme is completed it cannot be determined whether *HincII* cleaves the recognition site in the same manner as *HindII* (an even break), or whether it makes a staggered break.

The nuclease which is absent from strain Rc and elutes second on phosphocellulose chromatography of Rd preparations corresponds to methylase *HindIII*. This nuclease, *HindIII*, evidently does not attack T7 DNA since the Rc and Rd digestion profiles of T7 DNA are identical. It is for this reason that Kelly and Smith (1970), using T7 DNA as a substrate, obtained only a single recognition sequence with an unresolved Rd (*HindII* plus *HindIII*) nuclease preparation. There is at present no evidence to indicate whether the refractivity of T7 DNA is due to the absence of *HindIII* recognition sequences or whether there is a T7 methylation system which "covers" potential *HindIII*-sensitive nucleotide sequences.

The *HincII-HindII* nuclease (*i.e.*, *HincII* or *HindII* nucleases) makes 34 cuts in λ DNA, 43 cuts in ϕ 80h DNA, and 51 cuts in T7 DNA. The fragments generated range in size from 2×10^4 to 3×10^6 . The *HindIII* nuclease, which is absent from strain Rc makes only six and four cuts in λ and ϕ 80h DNA, respectively.

All of the phage DNAs studied contain approximately 45,000 base pairs. For each of them the number of cuts made by the *HincII-HindII* nuclease, which has in one strand the nucleotide recognition sequence 5'...N-G-T-Py-Pu-A-C-N... (Kelly and Smith, 1970) is rather close to the 44 cuts expected in a random nucleotide sequence of 45,000 base pairs. The number of cuts expected in a random sequence is independent of whether the *HincII-HindII* nuclease is a single enzyme or whether it is a mixture of two or more enzymes, each with the appropriate (less degenerate) nucleotide recognition sequence.

HindIII, which has in one strand the nucleotide recognition sequence 5'...N-A-A-G-C-T-T-N... (Old, Roizes, and Murray, personal communication), should make approximately 11 cuts in a random DNA sequence of 45,000 base pairs. On ϕ 80h DNA and λ DNA *HindIII* cuts with approximately one-third and one-half (55%) of this frequency, respectively. The "low frequency" of *HindIII* cuts may be simply a consequence of the small numbers involved and the obvious fact that genomic sequences are not random. However, it is also possible that the *HindIII* cleavage site has one or more elements of specificity in addition to the known six base-pair sequence.² Experiments bearing on this point are currently in progress.

The *HindIII* activity accounts for a minority (approximately 10%) of the cuts made in λ DNA or ϕ 80h DNA by unresolved nuclease from strain Rd. However, under certain, as yet undefined, conditions the *HindII* activity decays (or is "pre-empted") leaving *HindIII* as the predominant nuclease. This normally problematical phenomenon may have worked to advantage in the determination of the *HindIII* nucleotide recognition sequence on λ DNA (Old, Roizes, and Murray, personal communication). Even in purified preparations of

HindII trace amounts of *HindIII* can become the predominant activity (unpublished results; Hamilton Smith, personal communication). For this reason the *HincII-HindII* nuclease activity is best purified from strain Rc.

In preparing the *HindIII* nuclease from strain Rd the phosphocellulose fraction should be further purified. This can be accomplished most simply by passage through a DEAE-cellulose column in the absence of salt. Under these conditions (10 mM Tris-Cl, pH 7.4) the *HindIII* nuclease passes through the column and *HindII* is retained until the column is eluted with 0.1 M NaCl (Hamilton Smith, personal communication).

All of the fragments produced from λ DNA or from ϕ 80h DNA by a combined *HindII* plus *HindIII* digest (*i.e.*, unresolved nuclease from strain Rd) can be resolved by varying one or more of the parameters of the acrylamide-agarose gel electrophoresis, and maps describing the genomic location and relationship of individual DNA fragments have been constructed for selected regions of interest in the λ and ϕ 80 genomes (in preparation). In this respect the application of enzymes with different site specificities in secondary digestions (*e.g.*, see Figure 4) and tertiary digestions (*e.g.*, see Landy *et al.*, 1974) is extremely useful at the analytical as well as the preparative level.

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² An eight-base recognition sequence, in which the 5'- and 3'-terminal positions on one strand could be satisfied by any base as long as both were purines or both were pyrimidines, would by the usual procedures yield an apparent nondegenerate six base-pair sequence but would produce a frequency of 5-6 cuts/45,000 random base-pair sequence.

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Physical Studies of *N*-Acetoxy-*N*-2-acetylaminofluorene-Modified Deoxyribonucleic Acid†

Chiang-Tung Chang, Stephen J. Miller, and James G. Wetmur*

ABSTRACT: Denatured T4 phage DNA was reacted with *N*-acetoxy-*N*-2-acetylaminofluorene. The rate of reaction was determined using the change in the melting temperature of renatured DNA as an indicator of the percentage of modification. The renaturation rates of a series of modified DNAs were investigated. This rate is reduced by a factor of 2 when the melting temperature is lowered 15°. The absorption at 305 nm, the negative circular dichroism band around 300 nm, the melting temperature change, and the buoyant density shift

due to modification were investigated. From these data, we conclude that the melting temperature change due to 1% modified base pairs is 0.9°. Electric dichroism of modified DNA was studied in an alternating field. From the dichroism, we calculate the angle between the aminoacetylfluorene residue 305-nm transition moment and the DNA helix axis to be $60 \pm 4^\circ$ under conditions where the 305-nm band is optically active. The aminoacetylfluorene residue must be located in an asymmetric potential field outside of the DNA helix.

Previous studies have shown that *N*-2-acetylaminofluorene a carcinogen, when administered *in vivo*, is converted in rat liver to the reactive carcinogenic ester, *N*-2-acetylaminofluorene *N*-sulfate. N-AcO-AAF¹ is used as an analog for *N*-sulfate-*N*-2-acetylaminofluorene as the analog reacts slower with nucleophiles, hydrolyzes slower, but forms the same products (Cramer *et al.*, 1960; Miller *et al.*, 1961; Marroquin and Farber, 1962, 1965; Henshaw and Hiatt, 1963; Willard and Irving, 1964; Irving *et al.*, 1967; Kriek, 1968; Agarwal and Weinstein, 1970). The N-AcO-AAF reaction site on DNA has been demonstrated to be the C-8 position of guanine with AAF present in the product (Kriek *et al.*, 1967). Recent work has shown that adenine nucleotides and N-AcO-AAF may

react to some extent, although with lower reactivity than guanine nucleotides (Miller and Miller, 1969; Marroquin and Coyote, 1970; Kapuler and Michelson, 1971; Kriek and Reitsema, 1971; Michelson *et al.*, 1972).

The effects of various mismatched base pairs and chemically modified bases on the thermal stability of nucleic acids have been investigated (Kotaka and Baldwin, 1964; Bautz and Bautz, 1969; Laird *et al.*, 1969; Uhlenbeck *et al.*, 1971; Fink and Crothers, 1972; Lee and Wetmur, 1973; Hutton and Wetmur, 1973; Gralla and Crothers, 1973). The effect of N-AcO-AAF modification of DNA on the melting temperature has been studied by Troll *et al.* (1969) and Fuchs and Daune (1971, 1972). Because of the methods used to separate the hydrolysis products of N-AcO-AAF from the modified DNA, the melting temperature change resulting from modification was substantially underestimated. Recently, Fuchs and Daune (1972) interpreted their results on the optical properties of AAF-DNA to indicate that the AAF residue was inserted into the DNA helix.

In this work, we present data on the time course of the reaction of N-AcO-AAF with denatured DNA, measurements of

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¹ Abbreviations used are: N-AcO-AAF, *N*-acetoxy-*N*-2-acetylaminofluorene; AAF, the *N*-2-acetylaminofluorene residue; AAF-DNA, the *N*-2-acetylaminofluorene residue covalently linked to DNA bases; CD, circular dichroism; ED, electric dichroism.