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A simple method for DNA restriction site mapping

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ABSTRACT

When a DNA molecule, enzymatically labelled with ^{32}P at one end, is partially digested with a restriction enzyme labelled DNA fragments are obtained which form an overlapping series of molecules, all with a common labelled terminus. A restriction map can then be constructed from an analysis of the size distribution of these molecules. This technique has been used for the restriction site mapping of cloned histone DNA (h22) where as many as 35 cleavage sites may be accurately determined in a single experiment.

INTRODUCTION

Development of a restriction enzyme cleavage map for a given DNA molecule is often a prerequisite for the detailed study of its genetic organization and for the determination of its base sequence. Most of the commonly used mapping procedures involve identification of the cleavage fragments in a complete restriction enzyme digest and the subsequent laborious ordering of these fragments by various methods, most commonly by analysis of a subset of fragments contained in each of several overlapping partial digestion products (1). We describe here a simple and rapid procedure for direct mapping of the restriction sites relative to the termini of the DNA molecule. The method is similar in concept to that utilized by Gilbert and Maxam for DNA sequencing (2) and we suggest that our restriction mapping technique will be most useful in connection with their sequencing protocol.

The principle of the site mapping procedure is as follows: The DNA in question is labelled at the 5' termini with ^{32}P-phosphoryl groups using polynucleotide kinase and (γ-^{32}P) ATP. The labelled DNA is then cleaved assymmetrically with a suitable

restriction enzyme into two fragments that are separable by gel electrophoresis. Each DNA segment, labelled at only one end, is recovered and digested with the chosen restriction enzyme so as to produce a partial digest. A large spectrum of partial digestion products may be produced, but the labelled fragments form a simple overlapping series, all with a common labelled terminus. These are fractionated according to molecular weight by gel electrophoresis and detected by autoradiography. The relative mobility of each labelled fragment allows determination of its molecular weight by comparison with a set of DNA molecular weight standards and in turn locates the distance, in base pairs, of the respective restriction sites from the labelled terminus. The order of the fragments and their lengths thus correspond directly to the order of restriction sites along the DNA molecule. Partial DNA digests using several different restriction enzymes can be analyzed simultaneously in adjacent slots of a slab gel, allowing the relative positions of all the restriction sites to be read directly from the autoradiogram.

To illustrate the general method we describe its application to mapping of a number of restriction sites in a cloned 6 kb repeat unit of the sea urchin Psammechinus miliaris histone DNA.

MATERIALS AND METHODS

Enzymes. EcoRI was a gift from Ch. Weissmann, Zürich, Hae III enzyme (activity) was obtained from Miles Laboratories. Hpa II (2.5u/µl) was prepared according to DeFilippes (3) HindII and HindIII (10u/µl) were prepared by an adaptation of the DeFilippes procedure (4). The preparation of Alu I (1u/µl) by a similar protocol will be described elsewhere (4). In all cases, one unit yields a complete digest of 1µg DNA under standard reaction conditions in 1 h at 37°C. T4 polynucleotide kinase (Miles Laboratories) was supplied at 3u/µl in 50% glycerol. Bacterial alkaline phosphatase (Worthington, BAPC 20u/ml) was supplied as a 70% ammonium sulfate suspension. The enzyme was pelleted at 12000 x g for 5 min, redissolved in 10mM Tris, 10mM $MgCl_2$, pH 7.6 at 60 u/ml and heated at 95° for 10 min to inactivate any contaminating nucleases.

DNAs. Calf thymus DNA was dissolved in TE buffer (10mM Tris-Cl, 1mM EDTA, pH 7.6) at 2.5 mg/ml, sonicated for 1 min at maximum intensity on a Branson sonicator, extracted with phenol, ethanol precipitated and redissolved in TE buffer at 10mg/ml. Wild-type phage λDNA was obtained by phenol extraction of CsCl purified λ Sam 7 phage (5).

For the recovery of the 6 kb h22 DNA, the recombinant λ histone DNA was first cleaved with HindIII restriction enzyme. The reaction mixture contained 1 mg λh22 DNA in 1 ml TE buffer, 10µl 1M $MgCl_2$, 20µl 1M Tris-Cl, pH 7.6, 0.1ml HindIII enzyme and was incubated 2.5 h at 37°. The reaction was terminated by addition of 0.1 ml 0.5M EDTA, pH 7.6; 2ml of 0.2M glycine, 15mM NaOH buffer; 10% (final) glycerol, and 0.1ml ethidium bromide 2mg/ml.

Isolation of the 6 kb histone DNA by preparative gel electrophoresis. A 5 cm 0.6% agarose gel was prepared in a 6 cm x 20 cm glass tube supported at the bottom by a dialysis membrane held in place with a rubber band. A large tube was clamped vertically and suspended in a 2 litre beaker filled with electrophoresis buffer (0.2M glycine; 0.015M NaOH; pH 8.3). The glass column was filled with buffer to the same height as in the beaker. Circular platinum electrodes were placed around the outside and on the inside of the glass tube. The entire DNA digest was loaded onto the gel and electrophoresis begun at approximately 60V and 50mamps. The DNA was easily visible with a longwave UV lamp. As soon as it had entered the gel, the buffer compartments were circulated by pump. After 3 h voltage was decreased to 25V and continued for 16 h at which time the 6 kb histone DNA fragment was about 3 cm into the gel and the 7 x 10^6 and 19 x 10^6 dalton (right and left arm of the phage) were approximately 1.5 and 0.5 cm into the gel. Electrophoresis was continued at 80-90V under surveillance until the histone fragment was just about to exit. At this point the gel was slipped out of the tube and inverted so that the band was ready to emerge upward. The buffer was replaced and a 2cm layer of 20% glycerol in electrophoresis bu-fer was layered over the gel. Electrophoresis was continued at 90V with reversed polarity until the histone DNA was completely

into the glycerol layer. This was then removed by pipetting.

The DNA was loaded onto a DE52 column of 1.4ml bed volume equilibrated with 50mM Tris-Cl, 10mM EDTA, pH 7.6, washed with 50ml of equilibrium buffer, 25ml 0.15M NaCl, 20mM Tris-Cl, 10mM EDTA, pH 7.6, and then eluted with 4ml 1M NaCl into this buffer. Two volumes of ethanol were added, the tube was stored at -20° overnight, and centrifuged at 15,000 rpm for 30 min. The precipitate was rinsed with 95% ethanol, dried and dissolved in 1 ml of TE buffer. The overall yield was 46µg of histone DNA from 1mg of recombinant DNA.

5'-^{32}P labelling of the 5.1 kb fragment. For labelling, the isolated 6 kb cloned histone DNA h22 was treated with phosphatase to remove 5'-phosphoryl groups. The termini were then labelled using (γ -^{32}P) ATP and T4 polynucleotide kinase. The reaction mixture contained 100µl h22 DNA (4.6µg), 1µl of $MgCl_2$ 1M, 0.5µl of 1M dithiothreitol, and 5µl of bacterial alkaline phosphatase. Incubation was for 1 h at 37°. Phosphatase was inactivated at pH 2 by addition of 10µl of 0.1M HCl and incubated at 23° for 10 min. The pH was readjusted by addition of 5µl of 1M Tris-Cl, pH 8. Then 1.2µl of 5M NaCl, 10µl (γ -^{32}P) ATP (400 Ci/mmole, 10µM), and 2µl of kinase (3000 u/ml) were added and incubation continued at 37° for 30 min. The labelling reaction was terminated by addition of 2µl of 5 mM ATP and immediately raising the temperature to 75° for 5 min. The terminally labelled h22 DNA was then cleaved into two fragments of 5.1 kb and 0.9 kb (1) by incubation with 5µl of EcoRI enzyme at 37° for 1 h. The reaction was terminated by addition of 30µl of a 0.12% bromphenol blue, 2% sarkosyl, 25% glycerol, 0.125M EDTA (glycerol-dye mixture) and heating at 65° for 5 min.

The reaction mixture was divided into 3-50µl aliquots and electrophoresed on a 1% agarose cylindrical gel (0.9 x 12cm) at 65V until the dye marker had progressed 6 cm. The lower half containing unincorporated radioactivity was cut off and discarded. The upper portion was stained for 10 min in 0.5µg/ml ethidium bromide. The larger and the smaller bands were visualized by long wave UV light and excised. The agar slices containing the large and the small fragments were separately pooled

and dissolved in 3ml of 4M KI, 14mM 2-mercaptoethanol at 45°. One µl of calf thymus DNA (sonicated, 10mg/ml) was added to each tube as carrier. The DNA fragments were adsorbed onto small hydroxyapatite columns (0.3 ml), washed with several volumes of 4M KI, 14mM 2-mercaptoethanol followed by several volumes of 10mM sodium phosphate, pH 6.7, and then eluted with about 1 ml of 0.4M phosphate buffer. The phosphate was removed by chromatography on a 8-9 ml G50 Sephadex column in TE buffer. The DNAs contained in about 2 ml were removed in the region of the void column. The DNA was precipitated by addition of sodium acetate to 0.3M and magnesium acetate to 10 mM, and 2 volumes of ethanol. After 2 h at -20° the DNA was pelleted in a SW50 rotor at 40,000 rpm for 30 min. Each labelled fragment was dissolved in 50µl of TE buffer. The large and small fragment preparations contained about 1200 cpm/µl and 4000 cpm/µl respectively.

Electrophoresis and autoradiography for restriction site mapping. Labelled digestion products (in 5-12µl) containing 0.2 volume of glycerol-dye mixture (see above) were electrophoresed on 1-2% agarose slab gels (0.2 cm x 14 cm x 40 cm or 0.3 cm x 16 cm x 18 cm) in a 0.2M glycine, 0.015M NaOH buffer (pH 8.3) at approximately 5V/cm. At completion of the run, the gel was removed onto a sheet of Saranwrap on a glass plate, covered with a sheet of Whatman DE81 paper, 4-6 sheets of 3MM paper and another glass plate. The 3MM paper was changed every 5-10 min as it became wet. 1% gels were reduced to a thin skin-like film in 30 min and 2% gels in about 1 h. The Saranwrap-covered gel still adhering to the DE81 paper was covered with a sheet of Kodak XR-5 X-Omat film, sandwiched between 2 glass plates, and exposed 1-3 days at room temperature. In some cases, to decrease exposure time by a factor of 2-3, a sheet of Fast Tungstate (Ilford) was placed on top of the film sheet and exposure was at -70° for 1-3 days. Film was developed using Kodak DX-80 developer and FX-40 fixer.

RESULTS

Location of Hpa II restriction sites in H22 DNA. A partial digestion in which all of the partial products are adequately

represented is essential for the site mapping procedure. The conditions for this are best determined by analyzing a number of early time points in a reaction so constituted as to reach completion in 1-2 h. An example of this approach is shown in Fig.1.

Sea urchin histone DNA (h22) for restriction site mapping was obtained from λred⁻Sam7 Pm hist22, i.e. a phage λred⁻Sam7 hybrid carrying one 6 kb repeat unit of Psammechinus histone DNA inserted at a single HindIII site in the cI gene (3). The phage was grown on 803 $r_k^- m_k^-$ rec A⁻supII Met⁻ (a gift of N. Murray, Edinburgh). A 1.8 litre lysate was purified by two consecutive CsCl gradients. After dialysis against TE buffer (see Methods) the phage was extracted twice with phenol and the DNA precipitated with ethanol, washed with 94% ethanol and finally dissolved in 5ml of TE buffer at a concentration of 1mg/ml.

The 6 kb histone DNA (h22 DNA) was excised from the recombinant DNA with HindIII restriction enzyme. The digest yielded the 7 x 10^6 dalton and 19 x 10^6 dalton right and left arms of λ in addition to the excised 4 x 10^6 dalton h22 DNA. The histone DNA was separated and recovered electrophoretically (see Methods). The 6 kb h22 DNA was treated with phosphatase, labelled with ^{32}P-ATP by means of T_4 polynucleotide kinase and cleaved with EcoRI into the 5.1 and the 0.9 kb DNA fragments. Both kinds of molecules were isolated and subjected to further analysis.

For restriction site mapping analysis the 5.1 kb EcoRI fragment now labelled at only one of its 5' termini was digested with Hpa II enzyme and samples removed for electrophoretic analysis at 2, 4, 10, 20, and 40 min. The 2 min sample still contained a large portion of undigested DNA but 4 partials can be seen at increasing intensities with time of incubation (Figure 1). The 4 and 10 min samples reveal nearly optimal intensities for each fragment band while at 20 and 40 min the reaction is nearing completion. The number of base pairs (bp) of each partial fragment was determined graphically from a semi-logarithmical plot of standard DNA fragment sized in kb units versus relative mobility in cm. Reading from the labelled terminus there are four Hpa II sites at 975, 2400, 3150, and 3920 bp. The RI site

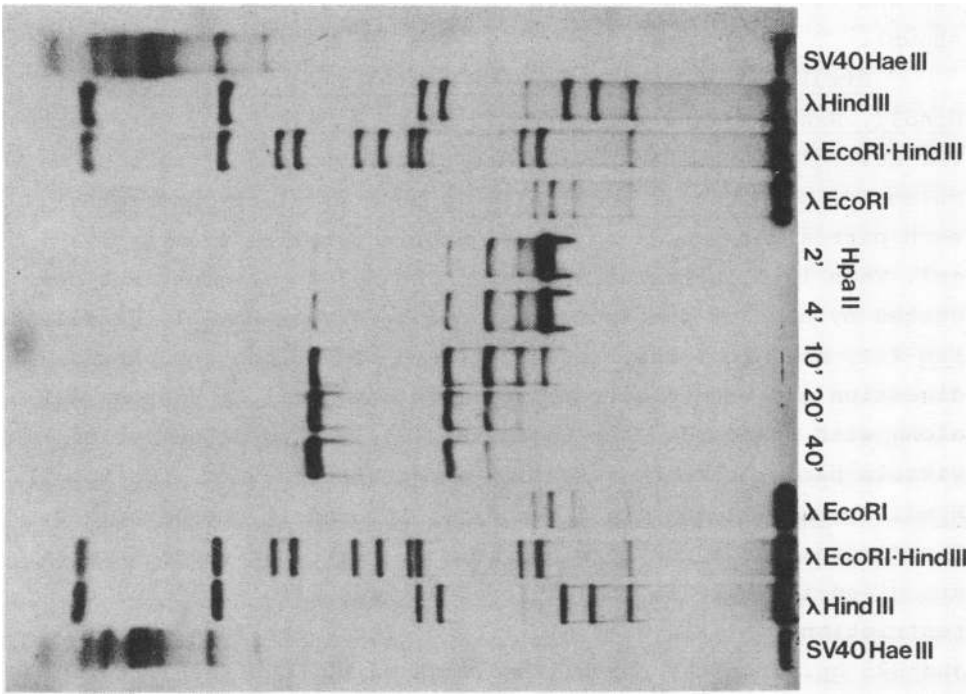


Fig. 1: Mapping of Hpa II restriction sites in the 5.1 kb EcoRI fragment of h22 DNA. For restriction site mapping of the 5.1 kb unit the DNA was treated with Hpa II enzyme as follows: The reaction mixture (50µl) contained 7500 cpm of h22, 5.1 kb EcoRI fragments, 10mM MgCl pH 7.6, 0.5mM EDTA, 14mM 2-mercapto-ethanol, and 1µl of Hpa II enzyme. Incubation was at 37°. Aliquots of 10µl were removed at 2, 4, 10, 20, and 40 min in 2.5µl of dye mixture and heated at 65° for 5 min prior to loading onto a 1% agarose slab gel (0.3 x 16 x 18 cm) and electrophoresis at 120V for 2 h (bromophenol blue marker at 11.3 cm). The gel was dried onto DE81 paper as described in the Methods section and a 3 day autoradiographic exposure was made. Molecular weight markers of terminally labelled total digest fragments were included as indicated in the Figure. These included HindIII, EcoRI and a double digest with EcoRI and HindIII of λDNA. Molecular weights for the fragments were as given by Murray & Murray (12). A labelled Hae III digest of SV40 DNA (2.5µg) was similarly prepared (3400 cpm/µl). Molecular weights of the fragments as given by Yang et al. (13).

marking the extreme end of the 5.1 kb fragment is at a distance of 5050 bp. Four very light bands can be seen above and below the smallest fragment in the 20 and 40 min digests. These might be due to contamination with a trace of an as yet undescribed Hpa enzyme or conceivably to a relaxed specificity of Hpa II, similar to EcoRI* (6). The 0.9 kb EcoRI fragment was analyzed in a similar way. Two restriction sites were discovered at

125 and 250 from the labelled terminus at 930 bp (data not shown).

Simultaneous mapping of the restriction sites for Hpa II, HindII, Hae III, and Alu I in h22 DNA. In all cases where sites for several different enzymes are closely spaced along the DNA molecule, ordering is most reliably done by electrophoresing each partial enzyme digest in adjacent, parallel tracts of a gel. This is illustrated in Fig.2. The 5.1 kb fragment was digested by each of the four restriction enzymes Hpa II, HindII, Hae III, and Alu I under predetermined conditions for partial digestion and then electrophoresed on a 40 cm 1.5% agarose gel along with standards (see Legend Fig.2). The total number of visible bands, each representing a restriction site, for Hpa II, HindIII, Hae III and Alu I are 4, 4, 16, and 11, respectively. The distance from the terminus in base pairs for these enzymes are given in Table 1. The 0.9 fragment was also subjected to restriction analysis with Alu I. Two sites were found at $\sim$ 100 and 825 bp. From the unpublished work of W. Schaffner (Zürich) it is known that Hae III cleaves the same DNA segment three times. These sites have not as yet been mapped.

Distribution of restriction sites in h22 DNA. The histone DNA of Psammechinus miliaris (h22) comprises AT-rich spacer and GC-rich cistronic DNA in about equal proportions (5). The distribution and sites of these distinct kinds of DNA sequences in the 6 kb DNA unit have been determined by partial denaturation mapping in the electron-microscope (7), and are depicted schematically in Fig.3. Hpa II and Hae III restriction enzymes recognize and cleave the sequences CCGG (8) and GGCC (9), respectively and would therefore be expected to cut spacer DNA with a GC content of 36% (1,7), on a random basis, every 950 bp. As anticipated, the number of restriction sites in the AT-rich spacer DNA is low for these two enzymes. Alu I, with the restriction site AGCT (10) should cleave every 330 bp, on a random basis, but only few sites are detected experimentally. Spacer DNA, then, clearly has a strong base sequence bias which excludes recognition sites for Alu I. However, tight cluster of nine Alu sites are found in the cistronic region coding for the H1 mRNA, and

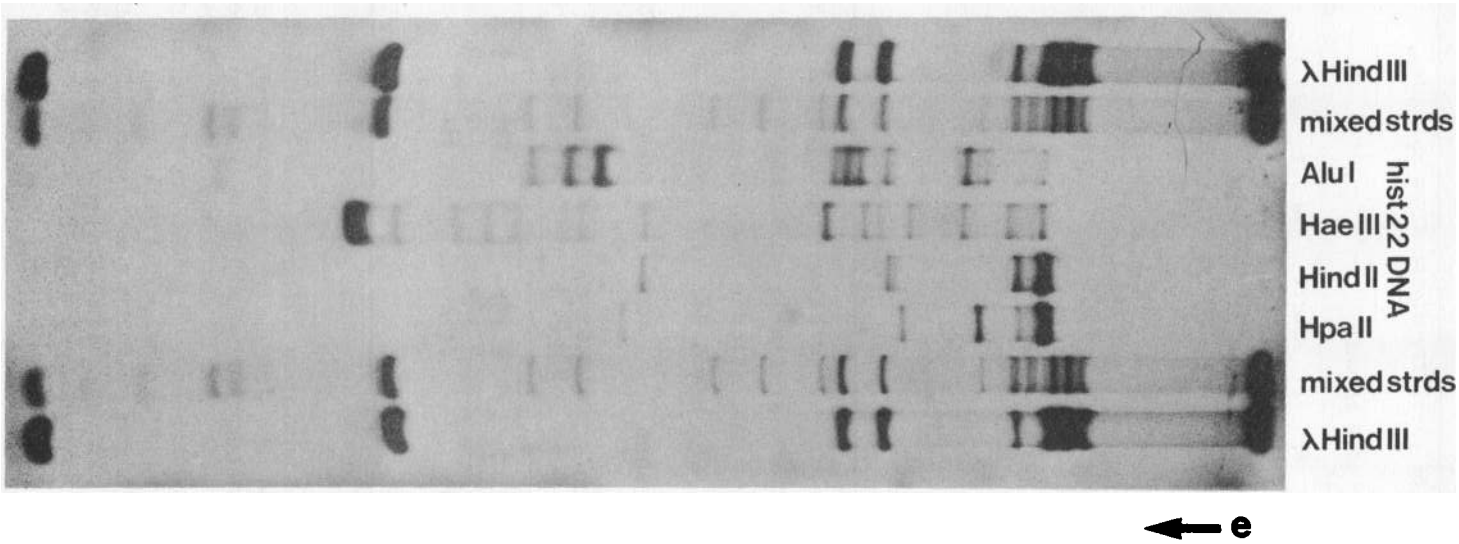
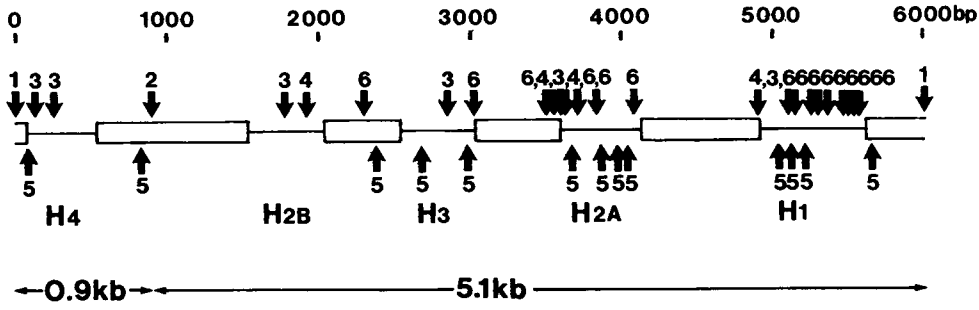


Fig. 2: Simultaneous mapping of the Hpa II, HindII, Hae III and Alu I restriction sites in the 5.1 kb EcoRI fragment of the h22 DNA. All reaction mixtures contained 10mM Tris-Cl pH 7.6, 10mM $MgCl_2$, 14mM 2-mercaptoethanol and 500 cpm/μl of the 5.1 kb EcoRI fragment of H22 DNA. The Hpa II reaction mixture (5μl)contained 0.1μl of enzyme and was incubated at 37° for 15 min. The Hind II reaction mixture (5μl) contained 0.1μl of enzyme, 50mM NaCl, and was incubated for 10 min at 37°. The Hae III mixture (10μl) contained 1μl enzyme and was incubated for 60 min at 37°. The Alu I mixture (10μl) contained 1.2μl enzyme and was incubated for 3 min at 37°. The reaction was terminated with 0.2 volumes of dye mixture and heating to 65° for 5 min. Each reaction mixture was layered onto a slot of a 1.5% agarose slab gel (0.2 x 14 x 40 cm) and electrophoresed at 200V for 6.5 h (dye marker at 25 cm) at room temperature. The gel was dried onto DE81 paper (See Methods). Autoradiographic exposure was 60 h at - 70° using a Fast Tungstate intensifier screen. Molecular weight standards were included as indicated in the Figure. "Mixed standards" consisted of λ digested with EcoRI, with HindIII, with EcoRI and HindIII together and SV40 cleaved with Hae III (12, 13).

Table 1: Lengths (in base pairs) of partially digested RNA molecules obtained from the

5.1 kb unit				*0.9 kb unit*			
Hpa II	*HindII*	*Hae III*	*Alu I*	*Hpa II*	*HindII*	*Hae III*	*Alu I*
4210	*4080*	*(3700)*	*3620*	*250*	*none*	*three*	*825*
3200	*2430*	*(2980)*	*3320*	*~125*		*n.d.*	*~100*
2950	*2390*	*2480*	*3020*				
1020	*1150*	*2290*	*2330*				
		2170	*2130*				
		1910	*2030*				
		1090	*1960*				
		950	*960*				
		860	*880*				
		760	*790*				
		710	*345*				
		655					
		555					
		515					
		505					
		490					

an additional four in or near the H2A cistronic DNA segment. The restriction sites for Hae III are similarly clustered in the cistronic regions H2A and H1 of the histone DNA. Therefore, restriction of histone DNA by Alu I and Hae III provides a



Legend to Figure 3: Distribution of some restriction cleavage sites in histone DNA (h22). The positions of the restriction sites along the histone DNA 5.1 and 0.9 kb fragments were combined with the partial denaturation map of the same DNA molecule. The spacer DNAs are represented as double lines, the cistronic DNA as single lines. The Hae III sites have not as yet been mapped in the 0.9 kb unit, nor is it certain that all restriction sites have been discovered in the distal part of the 5.1 kb unit (see Discussion). There is a relative lack of restriction sites in spacer DNA while the cistronic regions, especially those containing the H2A and the H1 coding sequences, are cleaved many times. Restriction cleavage sites: 1 HindIII; 2 EcoRI; 3 HpaII; 4 HindII; 5 Alu I; 6 Hae III

sufficient number of defined DNA segments to facilitate rapid sequencing of those two genes by the Maxam and Gilbert procedure (2).

DISCUSSION

The experiments of Fig.2 illustrate the power of the terminal labelling - partial digestion procedure for the ordering of multiple restriction sites. All sites in the region of 0-3 kb have been accurately mapped from this single gel (see Table 1). This work has led to the discovery of a new HindII site in the interval 2390 - 2430, yielding a DNA fragment with only 40 nucleotides which had previously escaped our notice (1). Certain partial fragments band at lesser or greater intensity than others but this is no point for concern so long as the restriction enzymes are reasonably pure. This finding suggests that not all sites are equivalent substrates.

In the region of 3-5.1 kb the bands are not sufficiently well spaced out in the gel to distinguish sites that are within 0.1 - 0.2 kb. For this reason we cannot for the moment exclude that there may be additional Hae III and Alu I sites in this region of histone DNA. There are several possible methods for detecting all possible sites. One could resort to polyacrylamide gradient gels of the sort described by Jeppesen (11). Alternatively, a lower percentage agarose gel could be used to expand the high molecular weight region while sacrificing the resolution of the smaller fragments. Perhaps the best method would be to obtain a different assymmetric fragment labelled from the opposite end to complete the fine structure mapping of the distal 3-5.1 kb region.

The limitations of the gel electrophoresis for the analysis of DNA molecules more than approximately 10×10^6 daltons, make our procedure of most value for relatively small molecules. A large viral genome would need to be mapped in segments. However, because of the rapidity and ease of the procedure we believe it will be useful in such cases.

Another major advantage of our procedure is the ability to

detect and accurately order sites that are only 20-50 bases apart; in other procedures, very small fragments often go undetected in electropherograms of complete digests where band intensities by staining or uniform labelling depend on fragment mass. Since partials are identical in sequence except for the terminal regions, mobility artefacts due to sequence composition are minimized.

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