RESTRICTION ENDONUCLEASES

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INTRODUCTION

Deoxyribonucleic acid is one of the most interesting and also the most complex of all biological macromolecules. Paradoxically, this complexity arises from the simplicity of its basic subunit structure. A large eukaryotic chromosome probably contains a single chain of DNA with a molecular weight in excess of 10^{11} daltons and is composed of a linear permutation of the four basic deoxyribonucleotides. Until recently, this fact posed considerable problems for the biochemist interested in isolating specific fragments of chromosomes as the methods available were nonspecific in nature. This is no longer so; the discovery of site specific endodeoxyribonucleases (restriction endonucleases) has opened new routes to the analysis of DNA structure and function and promises a revolution in molecular biology. A new field of genetic engineering is already being pioneered, and significant advances in many areas have been facilitated by the availability of these enzymes.

It was in the early 1950s that Luria and his colleagues first presented their genetic analysis of bacteriophage-cell interactions and described the phenomenon of variation.[1-3] They discovered that bacteriophages grown on one strain of bacteria displayed a wide variation in their ability to plate on other cells. They showed that this was a property of the bacterial cells rather than the bacteriophages and postulated the existence of some cellular mechanism to account for this phenomenon. Later studies by Arber and Dussoix[4,5] on *Escherichia coli* strains B and K led them to propose a model of restriction and modification to account for these observations. They proposed that the bacterial cell contains two distinct enzymes, one of which is an endonuclease that recognizes a specific sequence of bases within the DNA and then makes a double-stranded cleavage of that DNA. The second enzyme is a modification enzyme which recognizes that same base sequence and modifies it in such a way that it is no longer a substrate for the restriction enzyme. The DNA of the bacterial cell would always be modified and thus would be protected from digestion by the restriction enzyme. Because unmodified DNA would be destroyed by the restriction enzyme, the system would serve to protect the bacterial cell from invasion by foreign DNA, be it in a phage particle or as naked DNA. Many restriction modification systems have now been identified genetically and have been reviewed extensively.[6-12]

Study of the biochemistry of restriction modification systems began in 1968, when Meselson and Yuan[13] and Linn and Arber[14] reported the isolation of the first restriction endonucleases from strains of *Escherichia coli.* Unfortunately, these enzymes were not useful as analytical tools, being Class I enzymes* (see below), and it was not until 1970 that a major breakthrough occurred when Smith and his colleagues[15,16] described the first Class II* enzyme from *Haemophilus influenzae* serotype d. The discovery of this enzyme and its use by Danna and Nathans[17] to fragment SV40 DNA led to an exponential burst of papers applying this enzyme and others to the analysis of DNA from most imaginable sources. In the intervening years, the number of known restriction endonucleases has risen in excess of 80 (see Table 2), and their uses have ranged from DNA sequence analysis to the construction of new genomes. It is the intent of this review to describe in detail the occurrence, isolation, and properties of the Class II restriction endonucleases and to describe briefly the application of these enzymes to genome mapping, DNA sequence analysis, gene isolation, and genetic engineering.

PROPERTIES OF RESTRICTION ENDONUCLEASES

The term restriction enzyme originated from genetic observations and was defined as an enzyme involved in a genetic restriction-modification system. Consequently, the rigorous classification of a site-specific endodeoxyribonuclease as a restriction enzyme should also include the genetic characterization of the restriction-modification system. For the majority of the enzymes described in this review, such a correlation does not exist. In part, this is because many of these enzymes have been isolated from little-studied bacteria for which no formal genetics exists, but primarily because the inherent interest in these enzymes lies not in their biological properties, but in their usefulness as tools for the analysis of DNA. It seems likely that a cell containing a site-specific endonuclease will have some means of protecting its own DNA from destruction by that enzyme and three formal possibilities exist.

1. The site does not exist on the DNA.
2. The site is modified in some way, e.g., methylation or glucosylation.
3. The cell possesses some antagonist which inhibits the restriction enzyme.

The latter mechanism seems to operate in cells infected with bacteriophage T7,[18] which codes for a function capable of overcoming restriction by the *E. coli* B and K enzymes. Although there is some evidence[44,45] implicating Class II enzymes in a genetically defined restriction-modification system, the possibility remains that they have some other function in vivo. One such function might be site-specific recombination, and should this prove to be the case, the antagonist mechanism might be the more effective. For convenience, the terms restriction endonuclease, and specific endonuclease will be used interchangeably during the course of this presentation. The justification for this lies in popular usage rather than in strict scientific fact.

Although it is believed that restriction enzymes recognize specific nucleotide sequences, not all of them cleave DNA at specific sites. Those that are nonspecific in their cleavage have been called Class I enzymes[8,11] and include the first restriction enzymes characterized biochemically from *Escherichia coli* K and *Escherichia coli* B.[13,14] The *E. coli* B and K restriction enzymes and their companion modification enzymes have been characterized[13,19-21] and require ATP, Mg^{++}, and S-adenosylmethionine as cofactors for activity. They recognize specific sites on the DNA;[22-24] however, the points of cleavage appear to be random.[22,25-27] The mechanism of cleavage has been examined,[28,29] and the products characterized.[13,20,30] Two more features are unique to the Class I enzymes. The 5′-terminal nucleotides of the cleavage fragments appear to be modified in some way,[20,22] and following cleavage, the restriction endonuclease is converted into an efficient ATPase.[31,32] The bacteriophage PI restriction-modification system is usually placed in Class I because the products of cleavage are heterogeneous;[33] however, it differs in several ways from the two described above. The restriction endonuclease[13,21,34] shows an absolute requirement for Mg^{++} and ATP but not for S-adenosylmethionine.

*The Class I enzymes recognize a specific nucleotide sequence but cut at nonspecific sites away from the recognition sequence. The Class II enzymes both recognize and cleave a specific nucleotide sequence.

The latter does however stimulate the rate of cleavage. Unlike Eco B* and Eco K, there is no hydrolysis of ATP. The PI modification enzyme has been purified,[35,36] and its recognition sequence deduced.[37]

The most striking difference between the Class I and Class II enzymes lies in the products of digestion. The Class I enzymes lead only to heterogeneous products, and no specific fragments can be detected by gel electrophoresis. This fact makes the detection of these enzymes difficult and also lessens their usefulness for biochemical analysis. A model to acccount for this heterogeneous cleavage has been proposed,[39] and more detailed discussions of these enzymes may be found in other reviews.[6-11]

The rest of this review will focus on the Class II enzymes which both recognize and cleave specific sequences of DNA and have proved of such extraordinary value to the molecular biologist.

Occurrence

Following the isolation of the first restriction endonuclease, Hind II, from *Haemophilus influenzae* serotype d,[15] a number of other *Haemophilus* strains were examined for the presence of Class II restriction endonucleases. Thus, Hutchinson and his collaborators discovered enzymes in *Haemophilus parahaemolyticus*,[40] *Haemophilus aegyptius*,[41] and *Haemophilus influenzae* serotypes b and f,[40] while other enzymes were discovered in *Haemophilus parainfluenzae*.[42,43] *The Haemophilus* genus has been the most productive of all genera so far examined in that 22 enzymes have now been isolated from a total of 29 strains (Table 1). The closely related genus, *Moraxella*, has also proved productive; however, there are unfortunately few characterized strains in this genus. In other laboratories, enzymes were isolated from strains of *Escherichia coli* containing drug resistance plasmids[44] and also from *Bacillus subtilis*.[45] This latter finding of an enzyme from a Gram-positive organism was most encouraging as it pointed to the widespread distribution of these enzymes throughout the bacterial kingdom. This hope has, in fact, been justified as is evident from Table 1. Each group of bacteria so far examined has produced at least one genus from which a restriction endonuclease has been isolated and, of all strains so far examined, one in three contains a detectable restriction endonuclease. The quantities vary considerably. In some cases, these enzymes have only been detected by the presence of specific fragmentation patterns on bacteriophage lambda DNA (see below), and it has proved impossible to isolate sufficient enzyme for proper characterization. On the other hand, some bacteria produce immense quantities; for instance, from 10 g of *Haemophilus aegyptius* cells, enough of the enzyme (Hae III) can be isolated to completely digest 10 g of bacteriophage lambda DNA.

Attempts to find common features among bacterial strains or genera possessing restriction endonucleases have been disappointing. No clear pattern emerges from the information shown in Tables 1 and 2, mainly due to the absence of genetic and biochemical characterization of most of the strains from which these enzymes have been isolated. The enzymes Eco RI and Eco RII are coded by plasmids,[47-49] while BsuR I, although coded on the chromosome,[50] may be part of a cryptic prophage.[51] Perhaps many of these enzymes are coded by plasmids or phages, which use them to shuffle genetic information and create new recombinant genomes in the same way that has recently been accomplished in vitro (see section on genetic engineering).

What of higher organisms? Do they too contain restriction enzymes? The answer at the present time is that we do not know. Restriction-like enzymes might be expected to occur in *Tetrahymena* for the excision of ribosomal DNA,[52] in *Stylonichia*,[53] and the hypotrichous ciliates[54] to account for the dramatic change from micronucleus to macronucleus. Our own laboratory has

*The nomenclature used throughout this review is detailed in Reference 38. Restriction endonucleases bear a three-letter system name which abbreviates the genus and species of the organism from which they were isolated. Where necessary, a fourth letter is added to designate the strain. Roman numerals following the system name are assigned to differentiate multiple enzymes from the same source. Where only one enzyme has been isolated, the Roman numeral I is used to avoid later confusion if a second enzyme should be discovered. The prefix endo R (endonuclease R) is omitted to conserve space, and also because in most cases, the endonucleases have not been shown to form part of a genetic restriction-modification system. Examples are Hind II – one of multiple restriction enzymes from *H*aemophilus *in*fluenzae serotype *d*; Hinf I – an enzyme from *H*aemophilus *in*fluenzae serotope *f*; Alu I – an enzyme from *A*rthrobacter *lu*teus; Hph I – an enzyme from *H*aemophilus *para*haemolyticus (in this case, Hpa was already used for enzymes from *H*aemophilus *para*influenzae).

TABLE 1

Occurrence of Restriction Enzymes

Group[a]	Genus	Number of strains screened[b]	Number of restriction endonucleases[c]
1. Phototrophic bacteria	*Anabaena*	3	4
2. Gliding bacteria	*Myxococcus*	2	2
7. Gram-negative aerobic rods and cocci	*Alcaligenes*	1	–
	Achromobacter	1	1
	Azotomonas	1	–
	Bordetella	1	1
	Gluconobacter	2	–
	Pseudomonas	10	1
	Thermus	1	1
	Xanthomonas	5	6
	Zoogloea	1	–
8. Gram-negative facultatively anaerobic rods	*Chromobacterium*	1	–
	Citrobacter	1	–
	Erwinea	1	–
	Escherichia	5	5
	Flavobacterium	1	–
	Haemophilus	29	22
	Levinea	1	–
	Klebsiella	1	1
	Proteus	1	–
	Providencia	2	2
	Serratia	12	2
	Vibrio	1	–
10. Gram-negative cocci and coccobacilli	*Acinetobacter*	1	–
	Diplococcus	2	2
	Moraxella	9	7
	Neisseria	1	1
14. Gram-positive cocci	*Micrococcus*	3	–
	Sarcina	1	–
	Staphylococcus	4	–
	Streptococcus	1	1
15. Endospore-forming rods and cocci	*Bacillus*	10	9
17. Actinomycetes and related organisms	*Actinomyces*	1	–
	Arthrobacter	9	2
	Corynebacterium	3	2
	Nocardia	2	–
	Streptomyces	10	8
	Streptoverticillium	1	–

[a]Related organisms are grouped and numbered according to Reference 46.
[b]These numbers are the total of strains shown to produce enzymes (Table 2) plus strains unsuccessfully screened in the author's laboratory.
[c]Full references may be found in Table 2.

TABLE 2

Restriction and Modification Enzymes and their Recognition Sequences

Microorganism	Source	Enzyme	Sequence[a]	R/M[b]	Number of cleavage sites: λ	Ad2	SV40	References[c]
Achromobacter immobilis	ATCC 15934	Aim I[d]	?	R	?	?	?	55
Anabaena catanula	CCAP 1403/1	Aca I	?	R	?	?	?	70
Anabaena variabilis	Murray	Ava I	CGA$^{\downarrow}$CCG[e]	R	8	?	?	71
		Ava II	?[f]	R	?	?	?	71
Anabaena subcylindrica	Murray	AsuI	?	R	?	?	?	70
Arthrobacter luteus	ATCC 21606	Alu I	AG$^{\downarrow}$CT	R	>50	>50	32	72, 73
Bacillus amyloliquefaciens H	Young	Bam I	G$^{\downarrow}$GATCC	R	5	3	1	75, 76
Bacillus brevis S	Zarubina	BbvS I	GC $\binom{A}{T}$GC	M	–	–	–	77
Bacillus brevis	ATCC 9999	Bbv I	?	R	>30	?	?	78
Bacillus globiggi	Wilson	Bgl I	?	R	22	12	1	79
		Bgl II	A$^{\downarrow}$GATCT	R	5	10	0	79, 80
Bacillus stearothermophilus 1503-4R	Welker	Bst I	GGATCC	R	5	3	1	81
Bacillus subtilis strain N	Ando	BsuN I	GGATCC	R	5	3	1	82
		BsuN II	?	R	?	?	?	83
		BsuN III	?	R	?	?	?	83

[a]Recognition sequences are written from 5′ → 3′; one strand only is given and the site of cleavage, where known, is indicated by an arrow. For example, G$^{\downarrow}$GATCC is the abbreviation for $\begin{matrix}5'\ G^{\downarrow}GATCC\ 3' \\ 3'\ CCTAG_{\uparrow}G\ 5'\end{matrix}$ and GC$\binom{A}{T}$GC for $\begin{matrix}5'\ GCAGC\ 3' \\ 3'\ CGTCG\ 5'\end{matrix}$. In some cases, the recognition sequence has been inferred from a comparison of digestion patterns, e.g., Bst I and Bam I give identical patterns on all DNAs tested and a double digest with both enzymes gives no further cleavage. In these cases, the exact site of cleavage within the recognition sequence has not been determined and the arrow is omitted. For Hph I and Mbo II, the site of cleavage is eight base pairs from the 3′-nucleotide of the recognition sequence. The base modified by the methylase is indicated by an asterisk. N-6-methyladenosine is found in the case of Eco B, Eco K, Eco PI, Eco RI, Hind I, Hind II, Hind III, Hind IV, and Eco T2; 5-methylcytosine in the case of Eco RII and BbvS I.

[b]R/M–Enzymes designated R have been identified as specific endonucleases (restriction enzymes), whereas those designated M have been identified as specific methylases (modification enzymes).

[c]Where more than one reference is given, the first contains the purification procedure for the restriction endonuclease. A † indicates the reference giving the purification procedure for the methylase.

[d]The amounts of these enzymes are too low to warrant further investigation.

[e]Only one strand of the recognition sequence is indicated for convenience. The full duplex structure is $\begin{matrix}5'\ CGG^{\downarrow}TCG\ 3' \\ 3'\ GCC_{\uparrow}AGC\ 5'\end{matrix}$.

[f]5′-terminal trinucleotide sequences (GAC and GTC) have been found;[7†] however, the exact nature of the site recognized is unknown.

[g]The following methylated oligonucleotides have been isolated from Eco B modified DNA: TG$\overset{*}{A}$, C$\overset{*}{A}$C, AG$\overset{*}{A}$C, $\overset{*}{A}$AT; (A,G,C) $\overset{*}{A}$. $\overset{*}{A}$ is N-6-methyladenosine.

[h]Pst I cleaves ØX174 DNA at a single site, CTGCA$^{\downarrow}$G; however, it may recognize some other sequence(s) also.

TABLE 2 (continued)

Restriction and Modification Enzymes and their Recognition Sequences

Microorganism	Source	Enzyme	Sequence[a]	R/M[b]	Number of cleavage sites λ	Ad2	SV40	References[c]
Bacillus subtilis strain R	Trautner	BsuR I	GG↓CC	R	>50	>50	18	45, 84
Bordetella bronchiseptica	ATCC 19395	Bbr I	AAGCTT	R	6	11	6	68
Brevibacterium albidum	ATCC 15831	Bal I	TGG↓CCA	R	15	17	0	85
Brevibacterium luteum	ATCC 15830	Blu I	?	R	1	7	0	78
		Blu II[d]	GGCC	R	>50	>50	18	86
Brevibacterium umbra	Roberts	Bum I	?	R	15	22	3	87
Chromobacterium violaceum	ATCC 12472	Cvi I[d]	?	R	?	?	?	55
Corynebacterium humiferum	ATCC 21108	Chu I	AAGCTT	R	6	11	6	55
		Chu II	GTPyPuAC	R	34	20	7	55
Diplococcus pneumoniae	Lacks	Dpn I	GATC	R	?	?	?	88
Diplococcus pneumoniae	Lacks	Dpn II	GATC	R	>50	>50	6	88, 89
Escherichia coli RY13	Yoshimori	Eco RI	G↓AA^{m}TTC	R, M	5	5	1	64, 44†, 90–96
		Eco RI′	PuPuA↓TPyPy	R	>10	>10	?	97
Escherichia coli R245	Yoshimori	Eco RII	↓CC^{m}(A/T)GG	R, M	>35	>35	16	44†, 98–100
Escherichia coli B	Arber	Eco B	?[g]	R, M	?	?	?	20, 19†, 24, 101
Escherichia coli K	Meselson	Eco K	?	R, M	?	?	?	13, 21†
Escherichia coli (PI)	Murray	Eco PI	AGATCT	R, M	?	?	?	34, 13, 35†–37
Escherichia coli (T2)	VanOrmondt	Eco T2	G^{h}ATC	M	–	–	–	102
Haemophilus aegyptius	ATCC 11116	Hae I	(A/T)GG↓CC(T/A)	R	?	?	?	103
		Hae II	PuGCGC↓Py	R	>30	>30	1	104, 105
		Hae III	GG↓CC	R	>50	>50	18	41, 100, 106, 233
Haemophilus aphrophilus	ATCC 19415	Hap I	?	R	>30	?	?	68
		Hap II	C↓CGG	R	>50	>50	1	107–109
Haemophilus gallinarum	ATCC 14385	Hga I	?	R	>30	>30	0	107, 108
Haemophilus haemoglobinophilus	ATCC 19416	Hhg I	GGCC	R	>50	>50	18	68
Haemophilus haemolyticus	ATCC 10014	Hha I	GCG↓C	R	>50	>50	2	110
Haemophilus influenzae 1056	Stuy	Hin1056 I	?	R	>30	>30	?	111
Haemophilus influenzae serotype b, 1076	Stuy	Hinb III	AAGCTT	R	6	11	6	111
Haemophilus influenzae R_b	Hutchison	Hinb III	AAGCTT	R	6	11	6	68, 40
Haemophilus influenzae serotype c, 1160	Stuy	Hinc II	GTPyPuAC	R	34	20	7	111
Haemophilus influenzae serotype c, 1161	Stuy	Hinc II	GTPyPuAC	R	34	20	7	111
Haemophilus influenzae R_c	Landy Leidy	Hinc II	GTPyPuAC	R	34	20	7	112

TABLE 2 (continued)

Restriction and Modification Enzymes and their Recognition Sequences

Microorganism	Source	Enzyme	Sequence[a]	R/M[b]	Number of cleavage sites λ	Ad2	SV40	References[c]
Haemophilus influenzae R_d	Goodgal	Hind I	C$\overset{*}{A}$C	M	–	–	–	113, 114
	(exo– mutant)	Hind II	GTPy$^{\downarrow}$Pu$\overset{*}{A}$C	R, M	34	20	7	15, 16, 62, 112, 113†, 114
		Hind III	$\overset{*}{A}^{\downarrow}$AGCTT	R, M	6	11	6	15, 62, 63, 113†, 114
		Hind IV	G$\overset{*}{A}$T	M	–	–	–	113, 114
Haemophilus influenzae R_f	Hutchison	Hinf I	G$^{\downarrow}$ANTC	R	>50	>50	10	115, 40, 116–118
Haemophilus influenzae H-1	Takanami	HinH 1	PuGCGCPy	R	>30	>30	1	107, 108, 119
Haemophilus parahaemolyticus	Hutchison	Hph I	GGTGA → 8 bp	R	>50	>50	4	120, 40
Haemophilus parainfluenzae	Setlow	Hpa I	GTT$^{\downarrow}$AAC	R	11	6	4	43, 42, 62, 121, 122
		Hpa II	C$^{\downarrow}$CGG	R	>50	>50	1	43, 42, 62, 122, 123
Haemophilus suis	ATCC 19417	Hsu I	AAGCTT	R	6	11	6	68
Klebsiella pneumoniae OK8	Davies	Kpn I	?	R	2	8	1	124
Moraxella bovis	ATCC 10900	Mbo I	$^{\downarrow}$GATC	R	>50	>50	6	125, 126
		Mbo II	GAAGA → 8 bp	R	>50	>50	6	125, 127
Moraxella nonliquefaciens	ATCC 19975	Mno I	C$^{\downarrow}$CGG	R	>50	>50	1	129
Moraxella nonliquefaciens	ATCC 17995	Mnl I	?	R	>50	>50	>10	130
Moraxella osloensis	ATCC 19976	Mos I	GATC	R	>50	>50	6	125
Moraxella glueidi LG1	Davies	Mgl I[d]	?	R	?	?	?	124
Moraxella glueidi LG2	Davies	Mgl II[d]	?	R	?	?	?	124
Myxococcus virescens V2	Reichenbach	Mvi I	?	R	1	?	?	131
		Mvi II	?	R	?	?	?	131
Neisseria gonorrhoea	Wilson	Ngo I	PuGCGCPy	R	>30	>30	1	79
Providencia alcalifaciens	ATCC 9886	Pal I	GGCC	R	>50	>50	18	122, 125
Providencia stuartii 164	Davies	Pst I	CTGCA$^{\downarrow}$G[h]	R	18	25	2	124, 132
Pseudomonas facilis	VanMontagu	Pfa I	?	R	?	?	?	111
Serratia marcescens S_b	Mulder	Sma I	CCC$^{\downarrow}$GGG	R	3	12	0	74, 55
Serratia spp. SAI	Torheim	Ssp I	?	R	?	?	?	133
Streptococcus faecalis var. zymogenes	Wu	Sfa I	GGCC	R	>50	>50	18	134
Streptomyces achromogenes	ATCC 12767	Sac I	?	R	2	>7	0	135
		Sac II	?	R	3	>15	0	135
		Sac III	?	R	>30	>30	?	135
Streptomyces albus G	Ghuysen	Sal I	?	R	2	3	0	135
		Sal II	?	R	>30	?	?	135
Streptomyces griseus	ATCC 23345	Sgr I[d]	?	R	?	?	?	135

TABLE 2 (continued)

Restriction and Modification Enzymes and their Recognition Sequences

Microorganism	Source	Enzyme	Sequence[a]	R/M[b]	Number of cleavage sites			References[c]
					λ	Ad2	SV40	
Streptomyces stanford	Goff	Sst I	?	R	2	>7	0	136
	Rambach	Sst II	?	R	3	>15	0	136
		Sst III	?	R	>30	>30	?	136
Thermus aquaticus YTI	Brock	Taq I	T↓CGA	R	>50	>50	1	137
Unidentified bacterium	Roberts	Uba I	?	R	7	8	1	78
Xanthomonas amaranthicola	ATCC 11645	Xam I	?	R	2	3	0	135
Xanthomonas badrii	ATCC 11672	Xba I	?	R	1	4	0	80
Xanthomonas holicola	ATCC 13461	Xho I	?	R	1	7	0	111
		Xho II	?	R	>20	>20	?	111
Xanthomonas malvacearum	ATCC 9924	Xma I	C↓CCGGG	R	3	12	0	55
		Xma II	?	R	>15	>20	2	55

looked for restriction-like enzymes in *Tetrahymena, Oxytricha*, and several other lower eukaryotes, including *Saccharomyces cerevisiae* and *Aspergillus nidulans* without success.[55] Nevertheless, our failure to detect these endonucleases does not permit us to conclude that they are absent. They may have peculiar cofactor requirements or be present in such low amounts as to render their detection difficult when starting with small amounts of cells. One interesting nuclease has recently been detected in *Chlamydomonas*[56] and has some of the properties to be expected from a restriction endonuclease, namely that, upon cleavage of adenovirus-2 DNA, it produces a discrete and specific fragmentation pattern. Nevertheless, a complete digest with this endonuclease is more complicated than can be accounted for by equimolar yields of a single set of fragments. Furthermore, studies of the sequences present at the sites of cleavage indicate considerably less specificity than has been found for most restriction enzymes.[56] Presently, we must conclude that this enzyme is not a Class II restriction enzyme, leaving the bacterial kingdom as the only source of these enzymes.

Assay Procedures

The first restriction enzymes, Eco B and Eco K, were detected by their ability to degrade unmodified DNA, as compared to modified DNA which was resistant to their action. Degradation was monitored by testing biological activity[14] or by measuring changes in sedimentation velocity[13] of the substrate DNA. Both procedures are quite time consuming. Simpler procedures[15,41,57] take advantage of the endonucleolytic nature of restriction enzymes and measure changes in viscosity as a result of degradation. Alternative assay procedures based upon filter binding have been described,[58,59] and a new approach may also be suitable[60] based upon the kinetics of release of oligonucleotides from radioactively labeled DNA bound to a solid matrix. Assays of this sort are the only ones currently available for the Class I enzymes because of the absence of specific degradation products.

These assays are also suitable for Class II enzymes, and indeed the first such enzymes to be isolated were detected using the viscosity assay,[15,41] but they have now been superceded almost completely by the gel assay first introduced by Sharp et al.[43] This assay is based upon the specific nature of the fragments produced after digestion of a small DNA molecule with a Class II restriction enzyme. These fragments may be fractionated by agarose gel electrophoresis in the presence of ethidium bromide and visualized by their fluorescence upon UV irradiation of the gel.[43,61] Using slab gels with multiple slots, numerous assays can be performed simultaneously, thus permitting the direct visualization of the results of a chromatographic separation. Many of the advantages of this simple and inexpensive procedure are apparent from the photograph in Figure 1. First, the discrete banding pattern is dependent upon the specificity of the enzyme, so that if two different restriction enzymes occur within the same strain (this happens for instance with *H. influenzae* serotype d[62,63] or *H. parainfluenzae*[43]), their existence is immediately apparent. Furthermore, the presence of exonucleases often does not obscure this banding pattern, and the specific endonuclease can be detected in the presence of quite high concentrations of nonspecific nucleases. This illustrates another advantage of this kind of assay. In addition to detecting the required specific endonuclease, the contaminating nonspecific nucleases are located. A knowledge of how such contaminants fractionate with respect to the desired enzyme usually allows a judicious choice of fractions for further purification.

Purification Procedures

Detailed purification procedures have been reported for many of the Class II restriction endonucleases (references in Table 2). With the exception of Eco RI (64), no attempt has been made to estimate the purity of the final enzyme preparations because interest in them stems from their use as tools of the molecular biologist rather than in their inherent properties as proteins. Homogeneous enzyme preparations are not required; rather, they need be free only of contaminating nonspecific nucleases. The presence of these nonspecific nucleases makes it difficult to measure the amount of specific endonuclease present in the crude extract and hence to estimate overall recoveries. For this reason, yields are usually given as the amount of enzyme recovered after the final stage of purification, and this is conveniently expressed in arbitrary units defined such that one unit of a restriction enzyme is the amount required to digest completely 1 μg of bacteriophage lambda DNA in 1 hr at 37°.

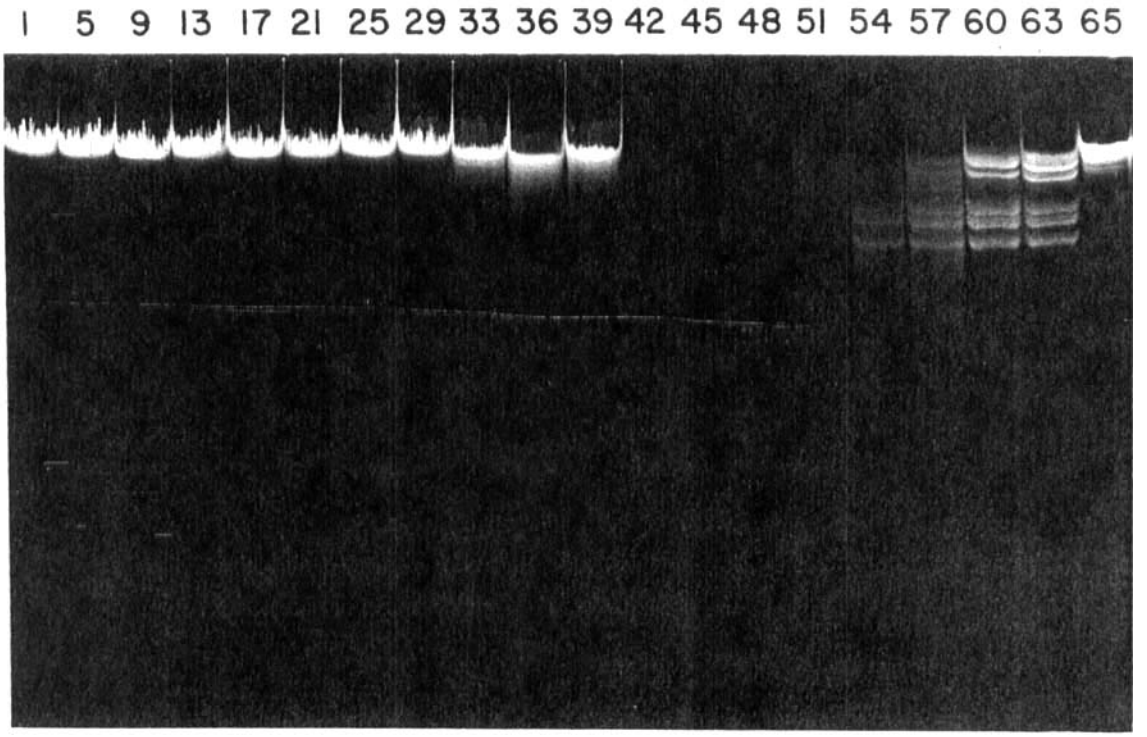

FIGURE 1. Typical assay profile using a 1.4% agarose slab gel. Ethidium bromide (0.5 μg/ml) was included in both the gel and the running buffer. The gel was photographed under UV illumination immediately after electrophoresis. Undigested lambda DNA is present in tubes 1 through 29; a nonspecific nuclease activity is present in samples 42 through 51, and the banding pattern typical of that produced by a restriction endonuclease can be seen in samples 54 through 65. One microgram of lambda DNA was used for each assay. (From Sugden, B., De Troy, B., Roberts, R. J., and Sambrook, J., *Anal. Biochem.*, 68, 36, 1975. With permission.)

One of the key factors responsible for the success in purifying these enzymes has been their quite remarkable stability. For instance, many of the enzymes, although purified only partially by means of two or three chromatographic steps, will continue to digest DNA in a linear fashion for periods in excess of 12 hr. This clearly reflects not only the inherent stability of the enzymes themselves, but also the small amounts of contaminating proteases present as a result of the purification procedures employed. Many of the purification procedures described in the literature represent minor variations of the procedure originally described[15] for the purification of the enzymes from *Haemophilus influenzae* serotype d. Biogel chromatography is useful for the removal of nucleic acids; however, streptomycin sulfate precipitation[64] and hydroxyapatite[65] may also be used. Further purification involves DEAE-cellulose and phosphocellulose chromatography, and in many cases, these procedures yield high quality enzymes. Certainly, if the enzymes are to be used for mapping or in studies where the loss of one or two nucleotides from the termini of the products is not critical, then further purification is unnecessary. However, for DNA sequence analysis, in particular for the characterization of the recognition sequences of the enzymes, it is often necessary to engage in a more extensive purification. Recently, two new types of columns have been introduced for the purification of restriction endonucleases. The first of these are the ω-aminoalkyl sepharose derivatives first used for the purification of glycogen synthetase.[66] ω-Aminopentyl sepharose has been extremely helpful for the purification of Hpa I,[67] while other members of this series with three to seven carbon atoms in the alkyl chain have proved of great use for the purification of other specific endonucleases.[68] A second procedure, which is of great utility, involves chromatography on single-stranded DNA agarose[68] prepared by mixing hot agarose with

denatured calf thymus DNA, allowing it to gel, and forming beads by mechanical disruption.[69] The rationale for using such a column was based upon the expectation that restriction enzymes that recognize double-stranded DNA might not bind to the column, whereas nonspecific nucleases that frequently do bind and attack single-stranded DNA might be retained by the column. For several of the restriction enzymes, this expectation is fulfilled,[68] and a dramatic purification ensues. Unexpectedly, a number of restriction enzymes bind extremely tightly to the column and can be eluted only with a sodium chloride gradient, often giving extremely sharp peaks. In most cases tested, a significant purification away from nonspecific nucleases has been observed on such a column,[68] and this finding points to the possibility of using other affinity columns for the purification of these enzymes. The use of double-stranded DNA agarose columns, in which the DNA is composed of a repeating copolymer of the recognition sequence, would be an obvious first choice; however, conditions would be required that did not result in digestion of the immobilized DNA.

SPECIFIC RESTRICTION ENZYMES

Presently, 86 specific endonucleases have been discovered (Table 2), recognizing more than 40 different specific sequences, 22 of which have been determined (see below). In the case of enzymes with an unknown recognition sequence, their digestion patterns on adenovirus-2 and lambda DNA clearly indicate new specificities. One striking observation is that many enzymes have been discovered from different sources which recognize the same nucleotide sequence. I have proposed the term "isoschizomers" for such enzymes, and a list of the known isoschizomers is presented in Table 3.

Although it can be shown by double digestion that these isoschizomers recognize the same sequence, in most cases, it has not been proved that the site of cleavage lies at the same position within the recognition sequence. One exception is the group Hap II, Hpa II, and Mno I, where it has been rigorously shown[109,122,129] that the site of cleavage within the recognition sequence C↓CGG

TABLE 3

Isoschizomers

Sequence recognized[a]	Specific endonuclease[b]
AAGCTT	Bbr I, Chu I, Hinb III (2), Hind III†, Hsu I
GTPyPuAC	Chu II, Hinc II (3), Hind II†
GGCC	Blu II, BsuR I, Hae III†, Hhg I, Pal I, Sfa I
CCGG	Hap II, Hpa II, Mno I†
GATC	Dpn I, Dpn II†, Mbo I, Mos I
PuGCGCPy	Hae II†, HinH I, Ngo I
GTTAAC	Apo I, Hpa I†
GGATCC	Bam I†, Bst I, BsuN I
CCCGGG[c]	Sma I, Xma I
?	Sal I†, Xam I
?	Blu I, Xho I†
?	Sac I†, Sst I
?	Sac II†, Sst II
?	Sac III†, Sst III

[a]The site of cleavage within the sequence, where known, may be found in Table 2.

[b]The strain which is considered the best source of each isoschizomer is marked †. Although *H. influenzae* Rd is still probably the highest yielding source of enzymes recognizing AAGCTT and GTPyPuAC. *H. suis* and *H. influenzae* Rc 1160 are good alternative sources which alleviate problems of cross contamination.

[c]Sma I and Xma I cleave at different sites within the recognition sequence.[55] The purification of Xma I is easier than Sma I; however, less enzyme is obtained per gram of cells.

lies as indicated. Similarly, Hae III and BsuRI also cleave at the same site within the recognition sequence GG↓CC.[84] One interesting result concerns Xma I and Sma I. In the case of Xma I, the site of cleavage is C↓CCGGG, giving fragments with a 5′-terminal tetranucleotide extension. Preliminary evidence[55] indicates that Sma I cleaves at a different site within this same sequence CCC↓GGG. This is the first example of isoschizomers recognizing the same sequence but cleaving at different sites within it.

Aside from the diverse nature of the organisms containing isoschizomers, one interesting observation lies in the co-occurrence of Hind II and Hind III in *Haemophilus influenzae* serotype d[15,63] and of their isoschizomers Chu I and Chu II in the Gram-positive organism, *Corynebacterium humiferum*.[55] It should also be noted that strains of *Haemophilus influenzae* serotype b contain only the Hind III-like activity,[40,111] whereas strains of *Haemophilus influenzae* serotype c contain only the Hind II-like activity.[111,112] As mentioned previously, the *Haemophilus* genus has been a particularly prolific source of restriction enzymes. Although several species have produced isoschizomers, nevertheless 13 different specific endonucleases have been isolated from species of this genus.

One point should be made when considering the screening of new bacteria for restriction enzymes. The presence of so many isoschizomers points to the likelihood that any given restriction enzyme will occur in several different bacterial strains. For this reason, it is possible to avoid working with highly pathogenic bacteria, as it is likely that any enzyme found in such a bacterium will in fact occur elsewhere. Furthermore, should a strain prove to be a particularly poor source of a given enzyme, it is again likely that an alternative and more productive source will exist. This is best exemplified by the Hae III isoschizomer group (Table 3); there are many orders of magnitude difference in the amount of enzyme that can be recovered from the highest producing strain (*Haemophilus aegyptius*) and the poorest producing strain (*Brevibacterium luteum*). Many of the enzymes listed in Table 2 do occur in rather small quantities or are unstable during the usual isolation procedure, and their purification is not worthwhile. For the most part, no attempts have been made to completely characterize these enzymes for this reason.

The enzymes Dpn I and Dpn II from strains of *Diplococcus pneumoniae* are of unusual interest. Dpn I is the only specific endonuclease known to cleave DNA only when it is methylated.[88] The recognition sequence for both of these enzymes was deduced from the observation[89] that Dpn II and Mbo I are isoschizomers, recognizing the tetranucleotide GATC and that Dpn I cleaves in vitro methylated T7 DNA to generate fragments identical with those formed after digestion of unmethylated T7 DNA with Dpn II.[88] The methylated base responsible for Dpn I cleavage is probably N-6-methyladenine.[89] Although the function of this enzyme is unknown, it seems unlikely that it forms part of a restriction-modification system. It should be noted that bacteriophage lambda DNA, grown in most *E. coli* strains, is susceptible to the action of Dpn I and is extensively modified against the action of Dpn II, Mbo I, and Mos I. Thus, *E. coli* must contain a methylase that has overlapping specificity for the sequence GATC. An enzyme coded by bacteriophage T2 also methylates this sequence,[102,138,139] and an enzyme from *H. influenzae* Rd[113,114] methylates the partial sequence GAT, leading in both cases to the formation of N-6-methyladenine.[102,114] Surprisingly, T7 DNA contains only five sites for Dpn II,[88] while ØX174 replicative form DNA is not cut at all.[140] The latter is probably due to the absence of the recognition sequence rather than modification, as this DNA contains only one modified base 5-methylcytosine[141] located at a specific site on the genome.[142] These unusual findings raise the possibility that this sequence, GATC, may form part of some control element in several different bacterial DNAs, which is either selected against (in bacteriophages T7 and ØX174) or defended against (in bacteriophage T2).

It might be anticipated that incubation of bacteriophage lambda DNA with a mixture of Dpn I and Dpn II would lead to complete digestion. This is not the case and is best explained by assuming that at any given site where modification may occur, it does not necessarily occur on both strands of the DNA. Some sites may have one strand modified and one strand unmodified. Such a partially methylated site would not be expected to be a substrate for either endonuclease by comparison with the Class I enzymes which fail to cleave DNA containing a modification on only one strand at the recognition site.[13,143] For a bacte-

rium containing a restriction-modification system, the newly replicated DNA will have one strand modified (the parent strand) and one strand unmodified (the daughter strand). If this DNA is to survive the effects of the restriction enzyme, then it must be a substrate for the modification system but resistant to the restriction system. Furthermore, it should be the preferred substrate for the modification system, and for the Eco B enzyme, this is indeed the case.[144]

In addition to being partially resistant to the Dpn II group of isoschizomers, bacteriophage lambda DNA grown on *E. coli* K 12 is also partially resistant to the action of Eco RII,[145] which recognizes the complementary oligonucleotides C$\overset{*}{C}$AGG and C$\overset{*}{C}$TGG.[98,99] The Eco RII modification enzyme methylates the cytosine residues indicated by the asterisks.[99] A mutant of *E. coli* K12 deficient in a cytosine specific DNA methylase (*E. coli* K mec$^-$) gives rise to a phage which is now sensitive to Eco RII, both in vitro[145] and in vivo.[146] This methylase therefore has overlapping specificity with the Eco RII recognition sequence, and analysis of pyrimidine tracts containing 5-methylcytosine in the DNA of bacteriophages grown in methylating hosts is consistent with this conclusion.[147]

In addition to the modifications introduced by methylation, many bacteriophage DNAs possess other modifications such as glucosylated hydroxymethylcytosine in phage T4[148] and hydroxymethyluracil in several phages of *Bacillus subtilis*.[149] Fully glucosylated T4 DNA is resistant to the action of Eco RI; however, the nonglucosylated hydroxymethylcytosine-containing DNA is susceptible with the apparent formation of a limit digest.[150,151] By contrast, the enzymes Eco RII, Hind II, and Hind III are without action on either DNA.[151] In each case, cytosine is present in the recognition sequence, albeit at different positions with respect to the cleavage site. A similar phenomenon has been observed for the DNA of *B. subtilis* phages SPO1, Ø*e*, and Ø82 which contain hydroxymethyluracil and are resistant to digestion by Hpa I. These DNAs are digested by Eco RI, Hind III, and Hpa II, although at a slower rate than thymine-containing DNA.[152]

Several of the enzymes in Table 2 share the property of apparently cleaving single-stranded DNA.[140,153,154] Treatment of single-stranded fl DNA with Hae III generates fragments that are identical with those produced by the action of this enzyme on the double-stranded replicative form DNA;[153] however, the rate of cleavage is much slower for the single-stranded DNA. Two possible explanations for the recognition process involved have been proposed. One suggests that the secondary structure within the single-stranded DNA generates small duplex regions containing the site which is then cleaved in the usual way,[154] whereas the other proposes direct recognition of a single-stranded site.[153]

In view of the large number of specific endonucleases now characterized, it is reasonable to consider how many might exist. For enzymes recognizing tetranucleotide palindromes such as Hae III, there are 16 different sequences possible, and presumably enzymes will be found which cleave each one. Similarly, for enzymes recognizing hexanucleotide palindromes, 64 possible sequences can be written. Thus, a total of 80 different enzymes might exist which recognize these simple palindromic sequences. This is far from an upper limit. If Hph I and Mbo II (which recognize unique pentanucleotides) are prototypes for a new family, then another $4^5 = 1024$ enzymes must be added to the potential list. Other families of recognition patterns are also found including sequences recognized by Hinf I, Eco RII, Ava I, Hae I, and Hind II. Many of these sequences overlap, and undoubtedly further variations will be discovered. So far, no enzyme has been characterized that recognizes only sequences containing A and T, and it is possible (although I believe unlikely) that such sequences cannot provide acceptable recognition sites. Even with this reservation, the potential list is enormous, and we must anticipate the discovery of many more specific endonucleases in the coming years.

Characterization

Enzyme characterization usually involves a detailed analysis of the kinetic parameters and mechanistic features of the reaction catalyzed, together with some description of the protein, its molecular weight, and subunit composition. For the majority of the Class II restriction enzymes, this information is not available because interest in them has centered around their uses as biochemical reagents rather than their inherent properties as nucleases. One exception is Eco RI which has been purified to near homogeneity (as assessed by SDS-polyacrylamide gel electrophoresis).[64] It has a native molecular weight of 59,000 and a subunit

molecular weight of 29,500.[155,156] K_m values and turnover numbers have been obtained for the enzyme acting on both SV40 DNA and a synthetic oligonucleotide TGAATTCA.[157] This oligonucleotide is also a substrate for the modification methylase.[157] Although the recognition sequence GAATTC[92,158] is necessary and sufficient for cleavage to occur, sequences outside this hexanucleotide do influence the rate of cleavage.[159] Furthermore, the specificity for this hexanucleotide can be relaxed by changing the pH and ionic conditions of the reaction[96] or by digesting in the presence of antibiotics.[160] It has also been shown for Hpa II and Mno I that factors other than the presence of the recognition sequence influence cleavage.[129]

A purification to homogeneity has not been reported for any other restriction enzyme. It has been noted that most enzymes show a broad pH optimum, a broad magnesium optimum, and are inhibited by high concentrations of sodium chloride.[107] Characterization has usually involved a description of the cleavage patterns on different DNA samples and the determination of the recognition sequence. For most practical purposes, it is the recognition sequence that is the fundamental property that needs to be known about a restriction endonuclease.

Before embarking upon a detailed sequence analysis of the recognition site, it is often useful to obtain some preliminary information by performing double digests between the new restriction enzyme and ones of known specificity. This will establish that the enzyme is indeed recognizing a new sequence as judged by the appearance of more bands in a double digest between the new enzyme and a known enzyme if the two are really dissimilar. In some instances, this kind of analysis has provided a useful, quick indication of the sequence specificity of the new enzyme. Some examples of this are shown in Figure 2. A comparison of the enzyme Alu I with the enzyme Hind III shows that although the primary digests are quite dissimilar, the double digest of the two enzymes cannot be distinguished from the single digest of Alu I alone. This arises because the enzyme Alu I recognizes AGCT,[72] which is the central tetranucleotide of the sequence recognized by Hind III. Thus, the Hind III recognition sequence is a subset of all sequences recognized by Alu I. Similarly, double digests between Hae III and Bal I show that the double digest is identical with the digest produced by Hae III alone (Figure 2). Care must be exercised in interpreting this kind of data because usually one of the enzymes will make relatively few cuts on DNA, whereas the second enzyme makes many more cuts. If, by chance, the enzyme making relatively few cuts were to cleave only very small fragments produced by the second enzyme, differences might be overlooked. One final example demonstrates the usefulness of synthetic polynucleotides. The enzyme Hha I recognizes the sequence GCGC[110] and upon cleavage of the synthetic polynucleotide poly d(G-C), a rather heterogeneous starting material is converted into a more homogeneous and smaller product by the action of this enzyme (Figure 2). Based upon the large number of cuts made by this enzyme on viral DNAs, it seems likely that it recognized a tetranucleotide, most probably GCGC or CGCG. This former sequence was confirmed by a more rigorous determination of the recognition sequence.[110]

The first restriction enzyme recognition sequence to be determined was that of Hind II,[16] and the methods employed are still those most widely used.[161] The most straightforward way to obtain sequence information about the recognition site uses polynucleotide kinase[162] to label the 5′-end of the DNA fragments produced by restriction. After labeling, complete digestion with a mixture of pancreatic deoxyribonuclease and venom phosphodiesterase gives a labeled 5′-deoxynucleoside monophosphate, allowing the identification of the 5′-terminal nucleotide present after cleavage by the restriction enzyme. Similarly, complete digestion with exonuclease I[163] gives a 5′-terminal dinucleotide, while partial digestion with pancreatic DNase leads to a range of products including di, tri, tetranucleotides, etc.[164] From analysis of the latter, the sequence common to the 5′-end of each restriction fragment can be deduced together with the point at which that sequence becomes degenerate. This is illustrated in Figure 3 for the analysis of pancreatic DNase digestion products of fragments generated by the restriction endonuclease Bam I. In this case, the restriction enzyme produces fragments bearing a 5′-terminal tetranucleotide extension after recognizing the hexanucleotide palindrome, GGATCC.[76] By virtue of the position of the cleavage site within this sequence, the resulting fragments contain the unique pentanucleotide GATCC sequence at their 5′-end, and in this case, the determination of the

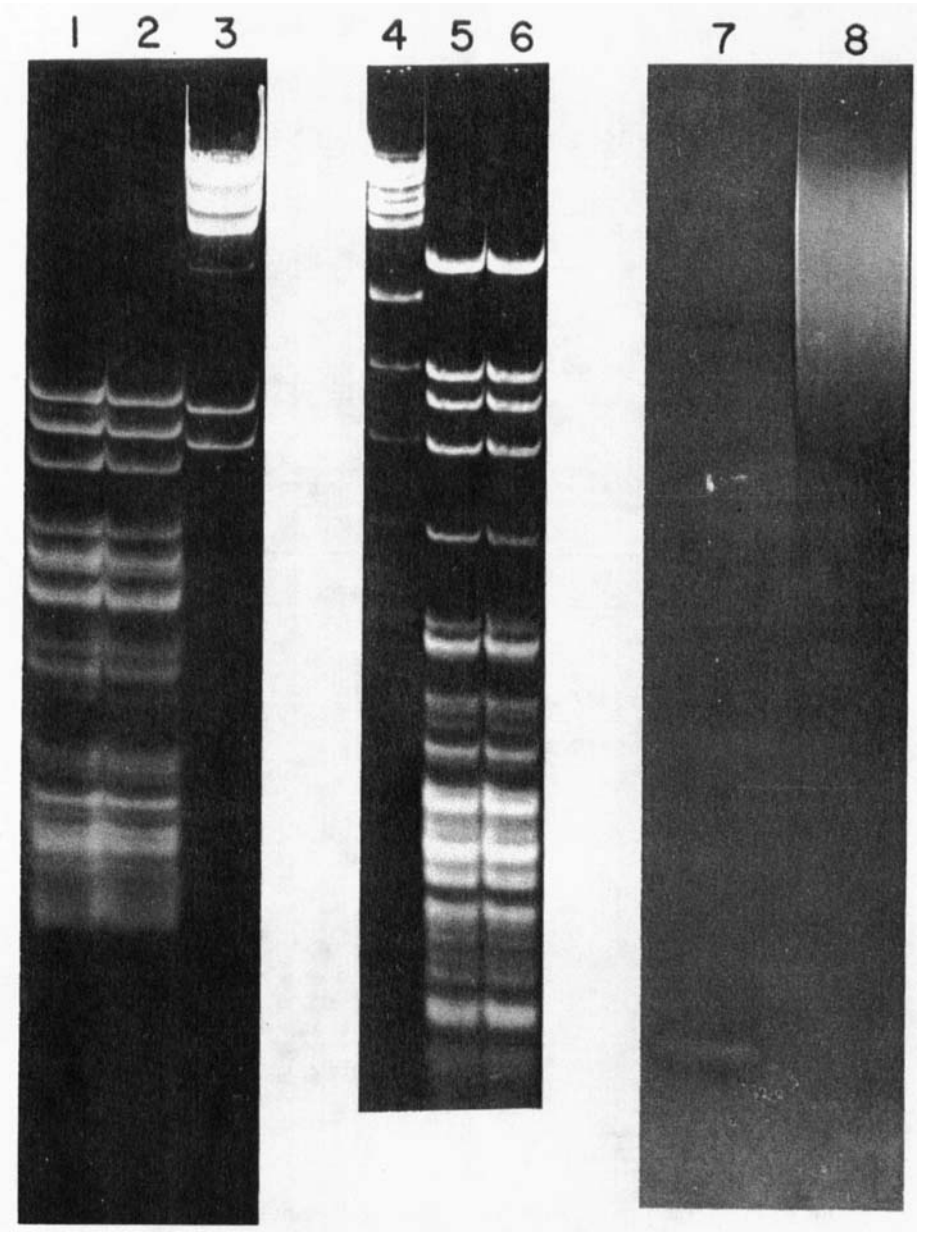

FIGURE 2. Restriction endonuclease digests. Channels 1 through 6: agarose slab gel analysis of digest of bacteriophage lambda DNA with (1) Alu I; (2) Alu I + Hind III; (3) Hind III; (4) Bal I; (5) Bal I + Hae III; and (6) Hae III. Channels 7, 8: Polyacrylamide slab gel analysis of the products of digestion of the alternating copolymer poly d(G-C) with Hha I. (7) Poly d(G-C) + Hha I; the lower band is the tetranucleotide CGCG. (8) Intact poly d(G-C). (From Roberts, R. J., Myers, P. A., Morrison, A., and Murray, K., *J. Mol. Biol.*, 102, 157, 1976. With permission.)

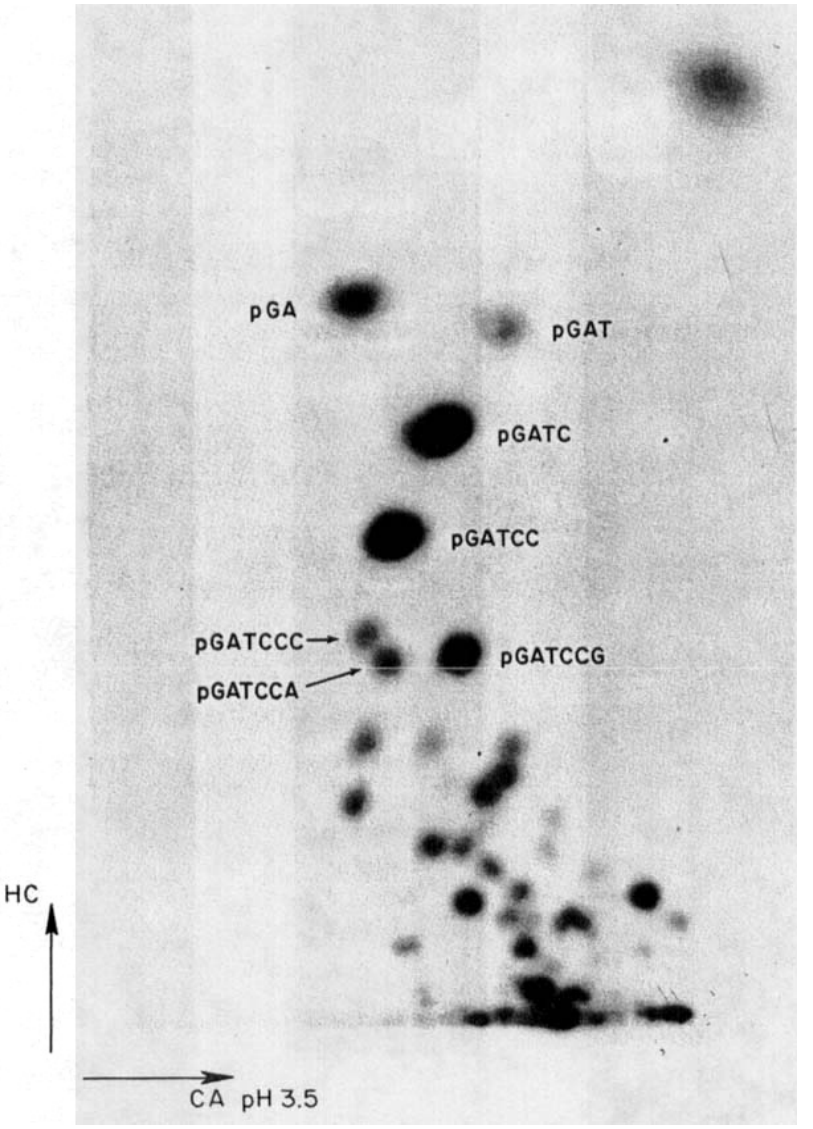

FIGURE 3. Pancreatic DNase fingerprint of 5′-^{32}P-labeled Bam I fragments. Adenovirus-2 DNA was digested with Bam I, deophosphorylated with alkaline phosphatase, and a 5′-terminal ^{32}P-phosphate introduced with polynucleotide kinase and γ-^{32}P-ATP. The labeled fragments were then cleaved with pancreatic DNase and the products analyzed by electrophoresis on cellulose acetate at pH 3.5 in the first dimension and homochromatography in the second dimension. Labeled oligonucleotides were visualized by autoradiography. pGA was identified by elution and coelectrophoresis with a standard. Sequences for the other products were deduced from mobility shifts.[165] The sequence becomes degenerate at the hexanucleotide level as judged from the presence of three of the four possible hexanucleotides. Because Bam I makes only three cuts on adenovirus-2 DNA, a maximum of six different 5′-sequences can be present in this fingerprint.

5′-terminal sequence leads to an unambiguous assignment for the recognition site. The sequence deduced from the data in Figure 3 depends upon assignment of mobility shifts[165] for the various partial products and was confirmed by the method of repair synthesis (see below).

For restriction enzymes that leave a 5′-terminal extension or produce flush-ended fragments (such as Hae III), the above analysis usually allows the recognition sequence to be determined unambiguously. In the case of flush-ended fragments, however, some analysis of 3′-terminal sequences is desirable, and in the case of fragments containing a 3′-oligonucleotide extension (e.g., Hha I), it is obligatory. This has usually been accomplished using micrococcal nuclease to digest uniformly ^{32}P-labeled restricted fragments. This is possible because all known Class II restriction endonucleases generate products with a 5′-terminal phosphate, 3′-terminal hydroxyl residue. Micrococcal nuclease is an endonuclease that gives dinucleotides bearing a 3′-phosphate from all internal positions, but generates a dinucleoside monophosphate from the 3′-terminal position of fragments containing a 3′-terminal hydroxyl group.[166] Fractionation systems[167,168] exist which allow the separation of dinucleoside monophosphates and dinucleotides, thus allowing the identification of the 3′-terminal dinucleotide. The 3′-terminal nucleotide itself can be labeled using terminal deoxynucleotidyl transferase;[169] however, this has not yet been used in the analysis of recognition sequences.

In the case of fragments terminating with a 5′-terminal oligonucleotide extension or with flush ends, two additional experimental approaches are possible, both based upon repair reactions. The first of these is applicable to 5′-terminal extensions and involves repair of the 3′-strand using a DNA polymerase and α-^{32}P-labeled deoxyribonucleoside triphosphates, thus permitting analysis of the bases incorporated and also their nearest neighbors. This approach has been used successfully to confirm the recognition sequences of Eco RI and Eco RII.[92,99] In this method, the 3′-terminal nucleotide of the fragment can be determined by nearest-neighbor transfer from the first incorporated nucleotide.

A second method is particularly useful for flush-ended fragments and involves the use of bacteriophage T4 DNA polymerase[170] for the analysis of 3′-terminal sequences. T4 polymerase contains both a DNA polymerizing activity and a 3′ → 5′ exonucleolytic activity.[171] In the presence of just one deoxyribonucleoside triphosphate, the 3′ → 5′ exonuclease digests the DNA until it exposes a base that can be used as a template by the one triphosphate present in the reaction mixture. The polymerizing activity then incorporates that deoxyribonucleoside triphosphate. Because of the absence of the other deoxyribonucleoside triphosphates, the polymerase is unable to extend the chain further, and the exonuclease functions again, resulting in the removal of the newly incorporated base. The result is an equilibrium situation in which exchange of the base corresponding to the one deoxyribonucleoside triphosphate added takes place. This labeling procedure can be used to great effect to generate nearest-neighbor information.[170]

It may be seen that restriction enzymes generating fragments with 5′-terminal extensions or flush ends present no difficulties when analyzing the sequences recognized. However, enzymes generating fragments with 3′-terminal extensions or more complicated situations (see below) are much less straightforward to analyze. To date, only one sequence containing a 3′-terminal extension has been deduced using methods described above, the Hha I recognition sequence.[110] Fortunately, none of the Class II restriction enzymes presently characterized produces 5′-termini that are resistant to the action of polynucleotide kinase, as is the case for the Class I enzymes.[20,22] However, at least two enzymes are known for which no specificity can be demonstrated at the site of cleavage. These enzymes are Hph I and Mbo II. Nonetheless, they do produce specific fragments, and recognition sequences have been deduced for these enzymes. The first of these, Hph I, was found to make a number of cuts in bacteriophage lambda DNA in the region of the operators,[120,172] and because of the extensive knowledge of DNA sequence in this region of the lambda genome, the sites of these cleavages could be accurately determined. Upon comparing a number of these sites, no common features were discovered at the site of cleavage itself. Instead, a common feature was observed[120] some eight nucleotides distant from the site of cleavage. It was postulated that this was the recognition site, and this enzyme, unlike other Class II enzymes, actually cleaved at a site remote from the recogni-

tion sequence. This was strikingly confirmed with the discovery that two of the operator constitutive mutants of bacteriophage lambda were lacking one of the Hph I cleavage sites. Both mutations affected the same base pair,[120,173] which was shown to coincide exactly with one of the bases within the postulated recognition sequence, leading to the conclusion that Hph I was indeed recognizing a specific oligonucleotide sequence but cleaving some eight nucleotides away from that sequence. This approach to the determination of recognition sequences, namely by locating cleavage sites within known DNA sequences, is proving extremely useful for the rapid determination of recognition sites. The great progress in DNA sequence analysis over the last year[174] has provided many thousands of nucleotides worth of sequence, which when correlated with mapping information can quickly provide potential restriction enzyme recognition sequences. Perhaps the most striking examples of this lie in ØX174, which is the most extensively sequenced of all small DNA molecules and from which the recognition sequences for Mbo II,[127] Taq I,[137] Hae II,[105] and Pst I[132] have been deduced. Mbo II appears to fall in the same class as Hph I; a common feature, GAAGA, can be found eight nucleotides 5′ to the site of cleavage at all positions mapped in ØX174 DNA. While this kind of analysis is not completely rigorous in proving a recognition site, in the case of enzymes like Hph I and Mbo II, it would appear to be the only way at present by which the preliminary identification of the sequence can be made. Clearly, it can pave the way for future experiments designed to confirm unambiguously the proposed sequence.

As a result of the large number of restriction enzyme recognition sequences now known, it is interesting to note the extreme variation in the kind of cleavage observed. Examples of fragments with 5′-extensions of five (Eco RII), four (Eco RI), three (Hinf I), and two (Hpa II) nucleotides; flush ends (Hae III); and 3′-extensions of two (Hha I) and four (Pst I) nucleotides have all been observed, in addition to the specificity of the sort shown by Hph I and Mbo II. This almost certainly points to a varied evolution of these enzymes, and it will not be too surprising if yet more variations are found. This diversity in the site of cleavage should prove of great mechanistic interest for the protein chemist interested in the nature of protein-nucleic acid interactions, an aspect of the restriction enzymes to which relatively little attention has been paid thus far. From this point of view, the enzyme Hind II shows a particularly interesting recognition pattern. The sequence recognized is GTPyPuAC[16] and for some time it was speculated that this enzyme recognized the two hexanucleotide palindromes, GTTAAC and GTCGAC. This proved to be an incorrect assumption, with the discovery that within the lambda operators the sequence GTTGAC (and hence GTCAAC)[172,175] was a substrate for the Hind II restriction enzyme. Presently, two[175,176] of the three possible recognition sequences* have been found in sequenced DNA; however, there is no reason to believe that the third, GTCGAC, is not recognized. Thus, Hind II is only able to distinguish purines from pyrimidines within the central dinucleotide of the recognition sequence. A more bizarre finding[117] is that Hinf I actually has no specificity at the central position of its recognition sequence. Here the sequence recognized is G↓ANTC where N can be any one of the four nucleotides. Again, a case that should be of more than usual interest for the connoisseur of protein-nucleic acid interactions.

USES OF RESTRICTION ENDONUCLEASES

Six years have elapsed since the isolation of Hind II from *Haemophilus influenzae* serotype d,[15] and only 3 years have passed since the first

*It should be noted that despite four possible sequences that can be written from the general sequence GTPyPuAC, only three duplex structures can be formed. These are:

5′ GTTAAC 3′	5′ GTCAAC 5′	5′ GTCGAC 3′
3′ CAATTG 5′	3′ CAGTTG 3′	3′ CAGCTG 5′

The second of these sequences would be expected to occur twice as frequently as the other two in a random DNA, because it does not have the polarity restrictions imposed by the palindromic nature of the other two. It should be compared with the Ava I sequence (Table 2), which will also occur at twice the frequency to be expected for a unique hexanucleotide palindrome.

physical map of the SV40 viral genome was available.[62] Since then, the number of experiments involving Hind II and other restriction enzymes has risen to such proportions that it is beyond the scope of this review to provide a comprehensive account of their uses. Rather, I shall concentrate upon practical aspects of their use, especially physical mapping, which serves as a prelude for many further applications. The uses of map information, together with the uses of restriction enzymes for gene isolation and genetic engineering will be outlined briefly with references to recent papers that provide a source to earlier material.

Practical Aspects

The use of agarose gel electrophoresis[43,61] for the fractionation of restriction enzyme digests has already been mentioned in relation to the assays of these enzymes during purification. These gels provide a simple, convenient, and inexpensive way to fractionate restriction enzyme digests, either analytically or preparatively.[177] By varying the percentage of agarose used in these gels, it is possible to fractionate DNA molecules ranging up to 5×10^7 daltons molecular weight.[178,179] These gels are not without their limitations, however, and band broadening due to diffusion can sometimes be a major problem. Fragments of similar molecular weight are frequently not adequately resolved on these gels, and either pure acrylamide or mixed agarose-acrylamide gels may be more suitable. Both of these systems, however, have molecular weight restrictions and cannot resolve fragments in excess of 5×10^6 daltons molecular weight. One apparent exception to this involves polyacrylamide gels with covalently incorporated ethidium bromide;[180] however, this system has not been exploited. One useful extension of acrylamide gel techniques has been the use of slab gels containing a linear gradient of polyacrylamide.[181] Such gels allow a good separation of DNA fragments in a wide molecular weight range, and also result in extraordinary sharpness of the bands.[123] The method chosen for fractionation of a particular digest depends upon the complexity of the digest and upon the purpose for which it is to be used. It is important to note that none of the gel systems mentioned so far are devoid of problems associated with base composition. In polyacrylamide gels, high molecular weight fragments with a high GC content migrate considerably faster than fragments with a low GC content,[182] and it is important that this phenomenon be taken into account when attempting to relate molecular weight and mobility. This is not necessarily the case for low molecular weight fragments where both sequence and structural parameters may be important.[183] While agarose gels seem to suffer fewer problems in this regard, nevertheless, some fragments do seem to behave anomalously in this system.[159] With rather small restriction fragments, many of these problems associated with base composition can be overcome by the use of denaturing gels.[183,184] However, the problem of molecular weight determination for restriction fragments is not trivial and will be referred to again when the determination of physical maps is discussed.

Many of the applications of restriction enzymes involve the preparation and isolation of a single restriction fragment from within a more complicated digest. Two extreme cases may be considered; the first case involves problems associated with the isolation of a single restriction fragment from a digest containing many thousands of fragments, which might occur following digestion of chromosomal DNA. These kinds of problems present great technical difficulties and will be considered under Analysis of Complex Genomes. The other extreme involves the task of isolating one restriction fragment from say a mixture of ten, which might be produced after cleavage of a low molecular weight viral DNA. In this case, the problems of fractionation on a gel are straightforward, and usually a system can be found such that the desired band may be separated from all contaminating bands; consequently, the problem becomes the recovery of the DNA fragment from the gel matrix. Several solutions to this problem have been described, some techniques being applicable to both agarose and acrylamide gels, while other techniques are more suitable for one or the other.

One technique, electrophoretic elution, has been used extensively for both DNA and RNA and can be performed either on bands excised from the gel[90] or may be used as a continuous procedure.[185-188] At first sight, this may seem the mildest of all treatments and one less prone to contaminating material; however, this is not necessarily the case. Usually, the fragments as they elute from the gel are collected in a dialysis bag, and

problems can arise as a result of the physical interaction of the DNA with the dialysis bag, sometimes resulting in low recovery. Fragments of high molecular weight move out of the gel extremely slowly, and recoveries decrease with increase of molecular weight. Furthermore, small DNA fragments frequently pass through the pores of the dialysis bag despite the fact that their molecular weight would be expected to prevent this.[78] Presumably, in the electric field, the DNA fragments behave not as random coils but rather as linear rods, and they are able to penetrate the pores in an end-on configuration. Often, contaminants from the gel matrix are also eluted electrophoretically, and it becomes necessary to free the resulting DNA from these by phenol extraction or some other procedure. Recoveries of between 50 and 80% of the theoretical yield might be expected from these kinds of procedures. Attempts have been made to design continuous elution devices that would allow samples to electrophorese onto the gel, separate during passage in the gel, and be collected directly from the bottom of the gel.[185-188] Generally, these devices suffer from problems of sample dilution during collection from the bottom of the gel and also from the amount of time required for even a medium molecular weight fragment to migrate through the gel. They have found little practical application for the preparation of restriction endonuclease fragments.

A second method simply involves diffusion from the gel.[100] In this technique, the gel slice (either acrylamide or agarose) is ground into very small pieces using some type of tissue grinder, suspended in buffer, and left for a sufficient period of time for the DNA to diffuse out of the gel and into the surrounding solution. Provided the dispersal of the gel into its surrounding buffer is done under mild conditions, this method results in little damage to the DNA and can give fairly high yields. One problem associated with this procedure is frequent solubilization of some gel matrix in the buffer, which must be removed from the DNA before it can be used further. Again, extraction with phenol is the method most commonly employed for this purpose. One useful technique for the removal of impurities from formamide gels involves soaking the gel slice in water prior to extraction of the DNA.[189]

Certain methods may be exclusively applied to agarose gels, and perhaps the most promising of these involves the use of chaotropic agents to melt the agarose gel containing the DNA without the concomitant melting of the DNA itself. Salts such as sodium perchlorate[190] and potassium iodide[191] have been used for this purpose. Several methods have then been tried to remove the gel matrix from the DNA, including hydroxyapatite chromatography[192] (which selectively retains the DNA) and gradient centrifugation[191] (which takes advantage of the density difference between agarose and DNA). In general, recoveries through these procedures are rather high; however, problems are sometimes encountered because of contaminants present in either the agarose or the chaotropic agents themselves, which behave like DNA during the purification procedure and inhibit the subsequent manipulation of the DNA.[193] It should be mentioned that this is one rather serious drawback of agarose. There is considerable variation from batch to batch with respect to contaminating material which seems to fractionate like DNA. Recently, a procedure has been described[194] which removes carbohydrate contaminants from DNA and might be useful as a final step to clean-up fragments from agarose gels. An alternative method, termed the "freeze-squeeze" procedure,[195] has also been described for the purification of DNA from agarose gels.

Among the variety of procedures described above, there is no one procedure which has been universally accepted. Indeed, it is fair to say that the ultimate procedure is yet to be found. The use of chaotropic agents to completely solubilize the gel matrix would appear to offer the most satisfactory first step, as this avoids all problems of trapping within the solid matrix encountered during other procedures without damaging the DNA. Although a cross-linker for acrylamide gels has been described[196] which allows them to be solubilized after use, the method for solubilization at the present time is too harsh to permit its use with DNA fragments.

In addition to gel electrophoretic methods for separating DNA fragments, some workers[197] have used isopycnic centrifugation as a means of separating fragments in specialized cases. Sucrose velocity gradients should also allow a coarse separation of say one very large fragment from a mixture of small fragments or vice versa. In this case, the final recovery of the DNA from the gradient would present no problems, and recovery should be extremely high. Furthermore, the prob-

lems of contaminants from gel matrices would be avoided completely. Unfortunately, this method cannot easily be scaled up for use with large quantities of DNA. It is to be expected that other techniques will be developed along these lines. For instance, gel filtration chromatography should be applicable to these problems, and in the case of small restriction fragments, even ion exchange chromatography may be used. Differential precipitation techniques[198] and hydroxyapatite chromatography[199] might also be exploited. Indeed, for large-scale work, it will be essential that alternative methods be developed because of the rather small capacity of even the largest preparative gels presently available.

The detection of DNA in gels is easily accomplished using autoradiography in the case of radioactive DNA or ethidium bromide as a fluorescent stain for unlabeled DNA.[43,61] The sensitivity of ethidium bromide upon excitation with shortwave length ultraviolet light is such that a few nanograms of DNA in a band can be detected.[43] Quantitation of the amount of DNA in bands is most easily accomplished by direct counting of radioactive samples. However, densitometer tracings of films obtained from both radioactive DNA and from ethidium bromide stained DNA are possible.[200] Methylene blue,[123] toluidine blue,[201] and Stains-all®[201,202] have been used to stain DNA, and these are all suitable for polyacrylamide gels, being only a little less sensitive than ethidium bromide. However, for agarose gels, there are problems of de-staining, and the backgrounds caused by these dyes are frequently too high to allow detection of bands present in small quantities.

Physical Maps

One of the very important uses of restriction enzymes has been the physical mapping of viral DNA genomes, and Table 4 contains a list of those genomes for which one or more complete physical maps is now available. The construction of these cleavage maps is no longer the arduous task that it once was for two main reasons. First, a large number of restriction enzymes is now available (Table 2), and it is usually possible to find at least one enzyme which makes relatively few cuts upon any given DNA. Clearly, it is easier to map a few fragments than a large number; consequently, a coarse map of just a few fragments can be constructed and used as a basis for more detailed mapping of either the whole genome or some selected part of it. The second reason why mapping is becoming easier lies in the recent technical advances mentioned below.

The first step in constructing a map is to identify the number and sizes of fragments produced by the restriction enzyme. Although the determination of the number of fragments would appear a simple matter, i.e., merely to count the number of bands found upon the gel, this has proved somewhat troublesome. Very small fragments are frequently missed, while others may not be resolved in the gel system used to fractionate the digest. Thus, the first map of SV40 using the Hind II + III restriction enzymes failed to account for two very small fragments that were not detected in the digests.[232] These two small fragments either ran off the gels used to fractionate the digests or ran as very broad bands in low percentage gels and therefore were considered as contaminants. This problem is best circumvented by using polynucleotide kinase[162] to label the 5′-ends of each DNA fragment. Analysis of such a digest, in which each individual fragment is labeled to approximately the same extent, immediately reveals the presence of small fragments, particularly when the digest is analyzed on both low and high percentage polyacrylamide gels.

The problem of determining the size of the fragment is much more difficult. Electron microscopy has been used routinely for length measurements of very large fragments,[159] and the determination of fragment yield after digestion of uniformly radioactively labeled DNA is used widely.[17] These methods are usually supplemented with analysis of electrophoretic mobilities of the fragments in agarose or polyacrylamide gels. As mentioned above, anomalous mobilities have been observed on both types of gels, and these problems become particularly severe for very short restricted fragments. For these reasons, even the comparison of an unknown fragment with standards, for which sequences have been determined, does not necessarily provide a reliable criterion for the determination of chain length. If the analysis is performed on denaturing gels, then one can be more certain of correct molecular weight determinations.[183] For fragments of high molecular weight, similar problems occur. An estimate of plus or minus 5% for a fragment of chain length 2,000 nucleotides means an error of plus or minus 100 base pairs. While this causes no concern for

TABLE 4

Viral DNA Restriction Enzyme Maps

DNA	Maps and references
φX 174 RF	Hind II, Hae III (203, 204) Hpa I (204) Hpa II (204, 205) Hae II (205) Alu I (206) Pst I, Ava I, Xho I (140)
G4 Rf	Eco RI, Hind II, Hae III, Hpa II (207) Pst I, Kpn I (140)
S13 RF	Hind II + III, Hae III (208) Ava I, Mbo I, Pst I (140)
fl	Hpa I, Hind II (209) Hae II, Hae III (209, 210) Hap II (210, 211) Eco RII (212)
fd	Hga I (213), Hin H-I (210, 213) Hap II (210-213) Alu I (210) Hind II (108, 210, 213)
M13	Hap II (211, 214) Hae III (214) Hind II (214, 215) Hae II, Alu I (210)
ZJ/2	Hap II, Hae II, Hae III, Hind II, Alu I (210)
T5	Eco RI (216, 217) Sal I, Sma I, Hind III (216)
Lambda	Eco RI (90, 159) Hind II (218) Bam I (219, 220) Pst I (124) Hind III (342) Sma RI (352, 353)
Lambda dv plasmids	Eco RI, Hind III, Hind II, Hpa I, Bsu R-I (221)
P4	Eco RI (222)
φ15	Eco RI, Hpa I (223)
φ29	Eco RI, Hpa I (223)
Col E2	Eco RI (224)
Col E3	Eco RI (224)
Adenovirus-2	Eco RI, Hpa I (225) Bam I, Hind III, Sma I (quoted in 226)
Adenovirus-5	Eco RI (225), Hpa I (225, 227) Bam I, Hind III, Sma I (quoted in 226)
Adeno-associated virus	Eco RI (228) Hind II, Hind III (229)
Human papilloma virus	Eco RI, Hind II, Hind III, Hpa I (230)
SV40	Eco RI (93, 94) Hpa I (43, 231) Hpa II (43, 62) Hind II, Hind III (62, 232) Hae III (100, 106, 233) Eco RII (100) Alu I (73) Hae II (104) Hinf I, Hha I (118) Kpn I (234) Bgl I, Hph I, Pst I, Taq I, Uba I (80)
Bk virus	Eco RI, Hind II + III, Hpa II (235)
Polyoma	Eco RI (236) Hind II (237, 238) Hind III (236, 237) Hpa II (236) Hae III (239) Kpn I, Pst I (234) Hga I (240) Bam I, Bum I, Hha I, Hae II (128)
Yeast mitochondrial DNA	Hind III, Eco RI, Hind II + III (241)
Human mitochondrial DNA	Eco RI, Hind III (242)
Mouse mitochondrial DNA	Eco RI, Hind III (242)
Monkey mitochondrial DNA	Eco RI, Hind III (242)

the determination of the primary map, it can cause severe problems when trying to fit other restriction enzyme maps to the first. For this reason, the mapping of genomes becomes an evolutionary process. As more enzymes are used, the map positions established for previous enzymes have to be changed so a self-consistent picture may emerge. From a practical point of view, therefore, it is not the sizes of the fragments themselves that are critical, but rather the relative order of the cuts by the various restriction enzymes together with a good estimate for the size of the fragment produced between any pair of individual cuts.

When constructing the primary map of a genome, it is necessary to have some point of reference. In the case of linear genomes, this is provided by the ends of the molecules themselves. However, in the case of circular genomes such as SV40, it is useful if one has an enzyme available that cuts such a genome just once, thus rendering

it linear. Having produced a linear DNA molecule, the terminal fragments produced by digesting with another restriction enzyme are most easily identified using an end-labeling procedure. Methods that have been employed include labeling the 5′-terminal phosphate with polynucleotide kinase[228] and labeling the 3′-terminal strand by repair synthesis with a DNA polymerase following exonuclease III digestion.[243] This latter method can also be used merely by removing bases from intact linear DNA, which can often cause a significant alteration in the electrophoretic mobility of the terminal fragments, thus allowing them to be identified. Both lambda exonuclease[244-246] and exonuclease III[55,247] can be used for this purpose. Yet another method for labeling the 3′-end involves the addition of labeled nucleotides using the enzyme terminal deoxynucleotidyltransferase.[248]

Having identified the terminal fragments, the order of the internal fragments must be deduced, and a variety of methods have been used for this purpose. Partial digestions have been used with great success in many systems, particularly for the analysis of small viral genomes.[62] Electron microscopic techniques such as heteroduplex analysis[249] and partial denaturation mapping[225-250] have been used, and these techniques are valuable when the fragments to be analyzed are of high molecular weight. Such fragments are difficult to map using partial digestion because of the problems of fractionating DNA molecules of high molecular weight.

In the case of bacteriophage lambda, another approach to mapping was possible because of the detailed genetic map available for this phage and the availability of a large number of physically mapped deletion mutants. In this case, comparison of restriction enzyme digestion products from both wild-type lambda DNA and a large number of deletion mutants allowed a detailed fragment map to be derived.[218] Of course, this kind of analysis allows the physical map to be related immediately to the genetic map, which is always a final goal.

Another specialized method has been developed for the analysis of the double stranded replicative form DNAs of certain single-stranded DNA phages.[211,251] In this method, the replicative form DNA was digested with a restriction enzyme; the fragments separated, and each in turn hybridized to the single-stranded DNA, setting up a template primer situation. Using DNA-polymerase I and α-^{32}P-deoxyribonucleoside triphosphates, a short stretch of labeled nucleotides was added to the 3′-end of the primer. An excess of cold triphosphates was then added, and polymerization allowed to continue around the single-stranded genome. Redigestion of the product with the restriction enzyme originally used to generate the primer fragment now gave a single labeled fragment which was the nearest neighbor of the fragment used to prime the reaction. In this way, it was possible to deduce nearest neighbors for each of the original restriction enzyme fragments and thus produce a map of the entire genome.

The methods described above are particularly useful for preparing the first cleavage map of a DNA genome. Once such a map is available, the mapping of fragments produced by other enzymes is greatly facilitated, and several techniques are available for the determination of successive maps. One of these techniques involves preparing a digest of the DNA with one enzyme, isolating the individual fragments, and then redigesting each in turn with the second enzyme.[210] From the sizes of the fragments produced during this process, it is frequently possible to construct an unambiguous map for most of the new fragments. If ambiguities still remain, then partial digestion may be necessary to complete the map. Alternatively, repeating the process with a third enzyme may resolve the difficulties and allow a map for all three sets of fragments to be constructed.

One of the most widely used techniques for comparing DNA sequences has been nucleic acid hybridization. This too may be applied to the ordering of restriction enzyme fragments. Recently, an elegant technique has been described[252] for transferring DNA fragments from an agarose gel onto a nitrocellulose filter. This procedure involves the denaturation of the fragments while still in the gel, followed by their elution from the gel, and subsequent entrapment on a nitrocellulose filter membrane. In this way, an exact replica of the gel is obtained upon the filter, which may then be used in a standard filter hybridization scheme. One way in which this technique might be used for mapping would be to hybridize, in separate experiments, individual ^{32}P-labeled fragments from a digest by a new enzyme against fragments of known order immobilized on the filters. If the map for only one enzyme is known, this would produce relatively little mapping information for the amount of work

involved. However, if maps for a number of enzymes are known, fragments produced by digestion with each of these enzymes could be immobilized on the same filter and hybridization carried out simultaneously with each of these sets of fragments. Thus, a great deal of information could be obtained from a single experiment. Recently, Hutchison[253] has used an ingenious modification of this procedure which does not require the prior purification of each individual fragment from the new digest. In this procedure, a set of unlabeled fragments of known map order are fractionated in one dimension on an agarose slab gel and then transferred to a nitrocellulose filter as described by Southern.[252] A second slab gel is now run containing ^{32}P-labeled fragments from the enzyme to be mapped. The second gel is then placed at 90° to the first gel, and the fragments transferred onto the same nitrocellulose filter which had been previously impregnated with the cold fragments from the first digest. Elution from the second gel onto the first nitrocellulose filter is accomplished under conditions such that the DNA fragments eluted from the gel do not bind nonspecifically to the filter but bind only at positions where they have hybridized to the cold DNA fragments. The hybridized fragments may now be detected by autoradiography and thus visualized as a radioactive spot present at the intersection of the bands that hybridize. This method may also be used for the analysis of partial digests, using an unlabeled partial digest to form the first filter followed by hybridization with a ^{32}P-labeled complete digest. It has also proved possible to hybridize DNA fragments directly within agarose gels,[254] but a direct comparison of the hybridization efficiency of the two methods has not yet been recorded.

USES OF MAP INFORMATION

The construction of a physical map is the first step in a more detailed study of the genome. The cleavage sites provide a convenient set of markers along the chromosome by which transcripts and their translation products may be ordered and positions assigned. The aim of this kind of approach is a direct correlation of physical and genetic maps ultimately taken to the nucleotide level. The techniques currently available for DNA sequence analysis rely heavily upon detailed physical maps, and the strategy employed often depends upon the nature of cleavage sites around the area of interest.

Comparison of Related DNAs

Just as tryptic peptide analysis has allowed the comparison of proteins and T_1-oligonucleotide maps of RNAs have proved of extraordinary value when comparing related molecules, so restriction enzyme fragmentation patterns have been used extensively for the analysis of related DNAs. This approach offers an alternative to electron microscopic heteroduplex analysis or to direct nucleic acid hybridization studies. The kind of differences between DNAs that may be detected by this approach depends upon the nature of the changes between the two DNAs. Thus, a single base change in a restriction enzyme recognition site would be undetectable either by electron microscopy or by hybridization, but leads to an immediate change in the restriction enzyme fragmentation pattern. Examples of this kind have been found during the structural analysis of the bacteriophage lambda operators,[120] where two operator constitutive mutations led to altered Hph I recognition sites. Similarly, promoter mutations in bacteriophage lambda[255,256] resulted in the abolition of Hind II sites. Nevertheless, there are many changes that can be completely undetected by the use of a single restriction enzyme. For instance, extensive differences in sequences occurring in an equivalent DNA segment spanned by two restriction enzyme targets might prove readily detectable by hybridization but could remain undetected when comparing fragmentation patterns. This is a limitation of this kind of approach; however, the use of a number of different restriction enzymes to analyze the DNA clearly increases the chance of recognizing differences. Recent examples include the demonstration that papovaviruses isolated from patients with progressive multifocal leukoencephalopathy are not related to SV40[257] or to the evolutionary variants of SV40 obtained during serial passage.[258,259] The latter,[259] which contain multiple initiation sites for DNA replication, bear a close structural resemblance to the defective polyoma DNAs.[260] Mitochondrial DNAs from a variety of eukaryotic sources[261-263] have also been compared, and a biochemical demonstration of maternal inheritance in the mule and hinny has been possible.[261] Among repeated eukaryotic structural genes, comparison of adjacent units

within clusters have been made for histone genes[264-266] and for the oocyte-type 5S genes of *Xenopus laevis*.[267,268] Similarly, the structural organization of amplified ribosomal DNA in two species of *Xenopus* has been described.[269] Studies of the histone genes[265] and the 5S genes[268] utilized fragments of the genome cloned in *E. coli* and emphasized the potential of this approach (see below). Herpes virus DNAs have also been compared.[270-272] The analysis of these DNAs is complicated considerably by the presence of inverted duplications, both internally and terminally, leading to four possible configurations for any one genome.[246,273] A final example of this approach has been the analysis of adenovirus recombinants,[226,275] which has led to the alignment of physical and genetic maps and allowed both *mutant*[226] and *wild-type*[275] genes to be mapped.

Mapping Chromosomal Functions

An earlier review[12] covered this area in some detail, and many of the methods employed at that time have since been applied to a large number of different systems. Thus, the termini and origins of replication for adenovirus-2 DNA have been shown to lie at the ends of this linear DNA molecule,[276-278] using an analysis of pulse labeled DNA as was first used for SV40.[279] A similar approach has located the origin of replication for ØX174,[280] while nascent ØX174 RFII DNA has been shown to contain a nonrandom location of gaps.[281]

RNA transcripts from many different viruses have been mapped by hybridizing RNA from infected cells to restriction enzyme fragments of the viral DNA. In the case of eukaryotic viruses, this kind of analysis has led to detailed transcription maps for SV40,[282-284] polyoma,[285] adenovirus,[286,287] and adeno-associated virus.[228] Both nuclear and cytoplasmic transcripts have been examined in this way, and one ingenious use of this technique has been to show the presence of very large nuclear transcripts from the adenovirus-2 genome.[288] The direction of transcription of viral chromosomes has also been established, taking advantage of restriction endonucleases to establish the $5' \rightarrow 3'$ orientation of each DNA strand relative to the cleavage map.[283] While much of the transcriptional mapping that has taken place has been at a somewhat coarse level, for certain systems such as SV40[289] and some bacteriophages,[290,291] mapping at the start and end of transcripts has been pursued to the nucleotide level.

The use of unfractionated RNA for the production of transcription maps only gives information pertinent to blocks of transcription and says little about the size of the viral messenger RNA transcribed from any given region of the genome. Information of this sort may be obtained using RNA fractionated on polyacrylamide gels or on sucrose gradients for the hybridization analysis.[292] Hybridization of RNA to DNA fragments has also been used as a means of obtaining RNA to program in vitro protein synthesis. Thus, mRNA obtained from both SV40[293] and adenoviruses[294] has been purified by hybridization to restricted fragments and then successfully translated in vitro. This has enabled the placement of gene products upon the viral genome. The use of a coupled transcription/translation system has also proved useful for the localization of genes.[295,296] These kinds of techniques should prove extremely useful for the analysis of cloned segments of eukaryotic chromosomes.

One of the early methods employed to relate the physical and genetic maps involves a technique known as "marker rescue." In this technique which was first used for ØX174,[297] a mutant single-stranded circle was annealed to a denatured restriction fragment of the replicative form DNA and the resulting partial heteroduplex molecule used to infect *E. coli*. By selecting for wild-type phage, growth will occur only if the fragment used to make the heteroduplex spans the portion of the genome containing the mutation, thus allowing it to be corrected. This technique has also been applied to the analysis of SV40 temperature-sensitive mutants[298] and amber mutants of fd, f1, and M13 bacteriophages.[211]

The preparation of biologically active fragments of DNA provides another route by which genes can be mapped. Thus, molecular cloning, in which restricted fragments of DNA are linked to bacterial plasmids and the recombinant molecules used to infect bacteria (see below), can also provide information on the map position of genes.[299] Fragments of SV40 have been introduced into monkey cells by microinjection and are shown to induce viral T-antigen synthesis.[300] One important advance has been the finding that viral DNA or fragments of viral DNA may be used directly to transform animal cells; this has been used to

localize the genes required for transformation by adenoviruses and SV40.[301,302]

One other kind of chromosomal function, amenable to restriction endonuclease analysis, involves the interaction of proteins (polymerases, repressors, etc.) with specific sites on the chromosome. Such sites necessarily include those responsible for the control of transcription and replication. Thus, extensive analysis of bacteriophage lambda operator-repressor interactions has been described,[175] and the catabolite gene activator protein (CAP) has been shown to bind to DNA containing the *lac* promoter.[303] Binding of RNA polymerase to various promoters can also be demonstrated.[211] Two experimental approaches have proved fruitful to these studies. One takes advantage of filter binding to selectively retain the fragment(s) which binds the protein from a mixture of many fragments,[304] while the other involves the protection of certain sites against restriction enzyme digestion by the prior binding of protein.[305]

The restriction enzymes themselves bind to DNA prior to cleavage and in the case of the *E. coli* K restriction enzyme (a Class I enzyme), a specific complex has been isolated between the enzyme and its substrate DNA.[28] Such complexes must also exist for the Class II enzymes and could, for instance, be used to map the cleavage sites by electron microscopy. Preliminary data for Eco RI indicates that this is indeed the case.[306] The availability of cloned segments of eukaryotic chromosomal DNA should encourage the search for specific DNA binding proteins in these systems. Similarly, the possibility of defining replication origins by specific binding of DNA-polymerases is eagerly awaited.

Nucleotide Sequence Analysis of DNA

Until recently, there were great problems associated with the direct determination of DNA sequences. One of the principal reasons for this was the absence of small, well-defined pieces of DNA that could be used for the development of techniques appropriate to DNA sequence analysis. DNA fragments comparable to the transfer RNAs and small RNAs of bacteria, which had proved invaluable for the development of RNA sequencing techniques, were not available. The advent of restriction enzymes changed this situation, and a wide variety of small DNA fragments became available and were used for the development of DNA sequencing techniques. Within a remarkably short time, novel approaches were developed, and DNA sequence analysis now seems likely to be faster and easier than RNA sequence analysis. Detailed descriptions of some of the methods used for the sequence analysis of DNA are available in recent reviews.[161,307,308] However, progress has been so rapid that two now widely-used methods[309,310] had not been developed at that time.

All methods presently in use depend upon the availability of fairly closely spaced restriction enzyme sites, and one of the limiting features arises when a segment of the genome is found which is not cleaved by one of the presently available restriction endonucleases. As new enzymes are discovered, this situation will change, and one of the goals of my laboratory has been directed toward the search for the 16 enzymes predicted to exist that will recognize the 16 possible tetranucleotide palindromes. So far, six of these enzymes have been conclusively identified (Table 5), and three more candidates exist based upon the high frequency with which they cut most DNAs. This set of enzymes is presently complemented by enzymes such as Hph I, Mbo II, and Hinf I, and as further variations are found, it seems unlikely that long stretches of nonrepetitive DNA will occur which cannot be cleaved into small segments. The new DNA sequencing tech-

TABLE 5

The 16 Tetranucleotide Palindromes

AATT	(Eco RI*)	CATG	
ACGT		CCGG	Hpa II
AGCT	Alu I	CGCG	
ATAT		CTAG	
GATC	Mbo I (Dpn I)	TATA	
GCGC	Hha I	TCGA	Taq I
GGCC	Hae III	TGCA	
GTAC		TTAA	

Note: References to the origins and characterization of these enzymes are given in Table 2. Eco RI* refers to the ability of Eco RI to cleave more frequently than usual when the reaction conditions are changed. It is not known whether conditions can be found that will allow cleavage at all AATT sites, so this activity is not yet a bonafide member of this set. Three enzymes – Mnl I, Pfa I, and Hin 1056 I – may also be members of this set; however, their recognition sequences have not yet been determined.

niques[309,310] are applicable to fragments of 50 or 60 nucleotides in length and may well be extended to include fragments up to 100 or more nucleotides.

Several different approaches have been used for DNA sequence analysis. Small fragments have been used for hybridization to obtain small segments of ^{32}P-labeled RNA which have then been sequenced by standard techniques.[173] Alternatively, these fragments have been transcribed in vitro with *E. coli* RNA polymerase either from natural[311] or adventitious[312,313] promoters and the ensuing transcripts used for RNA sequence analysis. Recently, small restriction enzyme fragments have been used for direct DNA sequence analysis using in vitro labeling. One method of labeling these fragments involves the use of T4-polynucleotide kinase to introduce a ^{32}P-labeled phosphate group onto the 5′-end of each strand.[162] The strands may then be separated either physically by the use of a gel[286,314] or in a practical way by recutting the fragment with a second restriction enzyme[172] as illustrated in Figure 4. Because the label is present only at the 5′-end of each strand of the starting fragment, the two smaller fragments generated by recutting will thus bear one labeled strand and one unlabeled strand. The polarity of each strand can then be correlated with the polarity of the starting DNA from the physical map of the restriction enzyme sites. Sequences may be derived from DNA labeled in this way by partial exonuclease digestion although the length of sequence that can be determined rarely exceeds 25 nucleotides.[172] More recently, Gilbert and Maxam have devised techniques that use chemically-induced cleavage at specific base residues to generate fragments from which sequences can be determined.[310] This method uses denaturing polyacrylamide gels for the analysis of intermediates and has the advantage that sequences in excess of 50 or 60 nucleotides may be determined in a single experiment. Fractionation procedures used in this case are similar to those used by Sanger and his colleagues

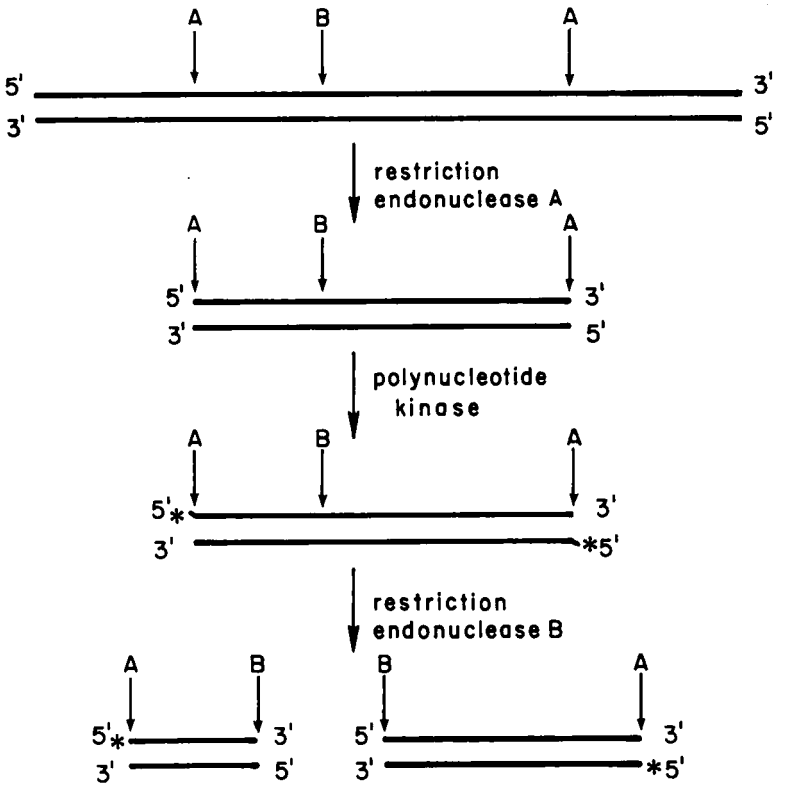

FIGURE 4. A scheme for labeling unique strands of restriction enzyme fragments. The asterisk represents a ^{32}P-phosphate group introduced at the 5′-terminus of a DNA fragment using polynucleotide kinase.

for the analysis of similar fragments generated by primer extension.[309] In this procedure, a primer (usually a restriction enzyme fragment) is denatured and hybridized to a template (the viral single-strand of ØX174 DNA) followed by partial extension with a DNA polymerase. Conditions are chosen so that four series of extended products are obtained. One series contains intermediates terminating in A residues, one in C residues, etc. Following cleavage by the restriction enzyme to generate the newly synthesized DNA, the extended products are analysed on denaturing polyacrylamide gels. While this latter procedure has had striking success in the analysis of ØX174 DNA[315] where a single-stranded template is readily available, it is not immediately applicable to most double-stranded DNA fragments. However, the general strategy and potential for DNA sequence analysis now seem to be clearly defined and promise an explosion of DNA sequence determinations in the next few years to a point where sequences will become available at a rate faster than their biological implications can be assessed.

ANALYSIS OF COMPLEX GENOMES

The foregoing uses of restriction enzymes have dealt almost exclusively with applications involving viral genomes. These genomes are small, and their restriction enzyme digests are usually simple in that many, if not all, fragments can be completely separated from one another. Recently, restriction enzymes have been applied to the analysis of more complicated genomes. Restriction enzyme fragmentation patterns for the chromosomes of *Bacillus subtilis*,[316] yeast,[317] and *Dictyostelium discoideum*[318] have been published. The resulting patterns are complex; however, distinctive features do emerge, and it seems likely that individual fragments could be purified from the mixture by techniques currently available for the fractionation of DNA. This is not the case for the more complex genomes. Restriction enzyme digests of *Drosophila* DNA[319] or of mouse DNA[320] appear as a continuous smear across the gel, because of the very large number of fragments produced. At any one point in the gel, there may be 10, 20, or 100 fragments running one on top of the other, and the problems associated with the purification of just one of these fragments are immense, but not hopeless. One solution,[321] using two restriction endonucleases and a two-dimensional fractionation scheme, may have some application for bacterial genomes but seems less appropriate for very complex genomes. A more general approach involves molecular cloning in which fragments of DNA from a complex digest are joined to the DNA of a bacterial plasmid or phage, and the resulting recombinants are purified as a bacterial clone (see below).

One characteristic of eukaryotic chromosomes is their content of repeated sequences; thus, they contain multiple copies of the genes for the ribosomal RNAs and the histones. In addition, most eukaryotes possess reiterated simple sequences known as satellite DNAs. Often these satellite DNAs and the ribosomal DNAs may be purified from main band DNA by ultracentrifugation and studied individually with regard to their restriction enzyme fragmentation patterns and their overall structure.[322] The satellite DNAs from a number of species have been studied extensively,[192,323-327] and because their biological function is unknown, studies have aimed at defining the organization of the sequences within the overall structure. These studies have provided evidence for higher order structures within the satellite DNAs and lead to the conclusion that one important mechanism for the evolution of these sequences involves unequal crossing over.[192,327] This same mechanism is invoked to account for the arrangement of the 5S ribosomal genes and spacers in *Xenopus laevis*.[268]

Studies of unique sequences within restriction enzyme digests of eukaryotic DNAs are hampered by the complexity of the digests. However, these difficulties may be overcome at the analytical level in several ways. One approach is the analysis of the renaturation kinetics of labeled probes in the presence of cellular DNA. Integrated genomes of adenovirus-2 have been studied in this way, and it has been shown that partial genomes are sufficient to maintain the transformed state.[274,351] Alternatively, the "blotting" method of Southern[252] (described above) has found elegant use for the analysis of integrated SV40 sequences.[328,350] The availability of DNA probes of very high specific activity, labeled in vitro by nick translation,[172,329] means that this latter approach is of great importance as it can be used to analyze the most complicated eukaryotic chromosomes.

The ability to produce mutations and to create new genomes by conjugation, transduction, or transformation has been essential to our efforts to understand the nature of bacteria and their viruses. Some progress has already been made in the genetic analysis of higher eukaryotes but many of the resources of the bacterial geneticist are not yet available for these systems. One limitation of classical genetics lies in the fact that manipulation is only possible between organisms that ordinarily exchange genetic information. With the help of restriction enzymes, it is now possible to overcome this limitation. Any two restriction fragments, no matter what their source, may now be joined using one of the two general procedures detailed below.*

One method for the preparation of recombinant DNAs uses terminal deoxynucleotidyl transferase for the addition of complementary homopolymers to the 3′-ends of the fragments to be joined.[330,331] For instance, oligo dT may be added to the 3′-end of one fragment and oligo dA to the 3′-end of the second fragment. Base pairing between the complementary 3′-termini leads to mixed dimers which can be covalently closed by the action of DNA polymerase I and DNA ligase.[330,331] A second procedure takes advantage of the fact that certain restriction enzymes (see Table 6) create fragments which have cohesive termini – that is, either a 5′- or a 3′-terminal oligonucleotide extension. Thus, Eco RI fragments contain AATT as a 5′-terminal extension[92] and upon incubation with DNA ligase may be circularized or dimerized.[332] Clearly, if a mixed population of fragments is present initially then a variety of products, both homo-dimers and hetero-dimers, are possible. While this latter method lacks the selectivity of the terminal transferase method, it has a number of advantages in terms of simplicity. The enzymes required are commercially available, and the reactions are straightforward and easily monitored.[333] DNA ligase is also able to join fragments with flush ended termini[334] so that cohesive termini are not a stringent requirement for this procedure. Although the isolation of the recombinant DNA molecules from the reaction mix may be carried out by using standard fractionation procedures on gels, in most cases, a biological purification scheme is preferable and desired. One of the fragments (the vector) involved in the joining is chosen to be either a bacteriophage such as lambda or a plasmid such as Col EI, both having the important capability for autonomous replication. The second DNA is chosen to contain some interesting region (a gene or a controlling region), and the recombinant molecules are used to transfect *E. coli*. Individual clones are then selected and tested for the presence of recombinant molecules either by direct genetic selection or by some indirect biochemical property. For example, the tryptophan operon of *E. coli* has been successfully joined to both bacteriophage λ[341] and to the plasmid Col EI[335] in vitro and the recombinant molecules selected by virtue of their ability to grow in the absence of added tryptophan. Similarly, the *E. coli* DNA ligase gene has been introduced into bacteriophage lambda.[336] In one important screening procedure, bacterial colonies are immobilized on nitrocellulose membranes, lysed to release the DNA, and then tested for their content of desired sequences by *in situ* hybridization.[337] A similar procedure has been developed for screening bacteriophage plaques.[356] Examples of this approach include the isolation of plasmids containing the gene for rabbit β-globin.[189] Vectors for cloning have the following basic properties: they must be cleaved by a restriction enzyme so as to leave

TABLE 6

Restriction Endonucleases Producing Cohesive Ends

Bam HI	G↓GATCC	Mbo I	GATC
Bgl II	A↓GATCT		
Eco RI	G↓AATTC	Eco RI*	AATT ?
Eco RII	↓CCAGG, ↓CCTGG		
Hae II	PuGCGC↓Py		
Hha I	GCG↓C		
Hind III	A↓AGCTT		
Hinf I	G↓ANTC		
Hpa II	C↓CGG		
Pst I	CTGCA↓G		
Taq I	T↓CGA		
Xma I	C↓CCGGG		
Sal I	?		

Note: References to these endonucleases and their recognition sites may be found in Table 2. Sal I has been shown to produce cohesive termini;[339] however, its recognition sequence is not yet known.

*A more detailed review of this field will appear in Reference 355.

intact a stretch of DNA containing the origin of replication and preferably some selectable marker. Plasmids have been described that are suitable for either the terminal transferase method or the direct ligation method of creating recombinant DNAs.[338,339] Similarly, several different bacteriophage lambda vectors have been described,[340,341] in which nonessential regions of the genome are deleted and can be replaced by the new DNA when forming the recombinant lambda phage. The terminal transferase reaction has not been used for bacteriophage lambda DNA because of complications arising from the nicks present at the cohesive ends. Vectors for Eco RI and Hind III fragments are now available,[340-342] and the present status of this field has been reviewed.[343]

Most studies reported to date involving genetic engineering have centered around the use of Eco RI and Hind III. The availability of many other restriction enzymes with similar properties will have important implications for the field. Three of the enzymes listed in Table 6 are worthy of special attention. The enzymes Bam I, Bgl II, and Mbo I all produce fragments bearing a 5′-terminal tetranucleotide extension, GATC. Thus, recombinant molecules may be produced between all three of these fragment sets, and by enzymatic manipulation, it should be possible to make Bgl I/Bam I heterodimers selectively in vitro. Ligation of mixed fragments, followed by recleavage with Bam I and Bgl II, would leave only heterodimers as recombinant molecules. Furthermore, a cloning vector for Bam I fragments[219,339] can also be used to clone Bgl II or Mbo I fragments. The latter enzyme should also be useful to reduce the size of a cloned Bgl II or Bam I fragment (containing a gene of interest) by partial digestion and repurification of the cloned fragment. Presumably, other sets of enzymes will be found displaying similar properties and should prove useful in the selection of recombinants prior to cloning. Of course, one such set comprises all restriction enzymes producing flush-ended fragments! The enzyme Hinf I gives fragments containing a 5′-terminal trinucleotide extension (ANT), in which the central base can be any of the four nucleotides. Thus, in vitro ligation of Hinf fragments should lead not to a complete mixture of all possible ligated fragments, but rather to some specific subset selected by the ability of the degenerate base to participate in base pairing. These techniques for making recombinant DNAs have also been used for the preparation of deletion and insertion mutants of SV40.[344-347] The resulting site-specific mutations have helped to localize essential and nonessential regions of the genome, and the methods employed will be of general utility.

With regard to the construction and cloning of these artificial recombinant DNA molecules, the reader's attention must be drawn to possible biohazards involved in their creation.[348] This whole area is still the center of heated controversy,[349] and it is generally agreed that there are certain kinds of experiments which should be postponed until the possible biohazards can be better evaluated.

Specific endodeoxyribonucleases are of widespread occurrence throughout the bacterial kingdom, and the 45 different specificities now recognized may be only the tip of the iceberg. Already their use has led to significant advances in the understanding of viral structure and function, and they have facilitated DNA sequence analysis to a point where the complete sequences of several small DNA viruses will soon be available. Despite their familiar place in the biochemist's refrigerator, little is known about their mechanism of action, and many other questions remain unresolved. What is the role of these enzymes in vivo? Are they really only involved in classical restriction-modification systems, or could they perhaps engage in the engineering feats which we have just discovered in vitro? Are similar enzymes present in higher organisms? Will it be possible to isolate mutant enzymes with altered specificities? Answers to these questions and many more will surely come in the next few years.

Just as organic chemistry moved from a degradative period of structure determination to the feats of synthesis of recent years, so we are fast moving into a new and exciting era of synthetic biology. The restriction endonucleases were a key element in this transformation and will continue to provide scope for the imagination of the molecular biologist.

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