Recombinant DNA Products: Insulin, Interferon and Growth Hormone

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PREFACE

The ability to isolate human genes, insert them into a microorganism which then produces a human protein, thereby serving as a biological factory, has already revolutionized biomedical research and purports to be the foundation for a new, highly lucrative industry. Of course, genetic engineering is not new, the novelty of recombinant DNA technology is the precision and efficiency with which scientists can manipulate genes. This book reviews advances made in recombinant DNA technology as it relates to the techniques employed, and the production and testing of potentially important products such as human interferon, insulin, and growth hormone.

The pioneering work of Paul Berg, Herbert Boyer, and Stanley Cohen, approximately 12 years ago, set the foundation for the present technology and represents one of the best testaments to the value of basic research. A prediction then that the analysis of bacterial restriction-modification would revolutionize cancer research, which at that time was receiving considerable priority, would have been considered at best a biased optimism on the part of the individuals working in that area. It is only in retrospect that certain decisions dominate. In prospect, such decisions are as influenced by temperament as by any scientific logistics. One obvious lesson to be learned from the development of recombinant DNA technology is that any attempt to minimize the commitment to basic studies will probably confer an enormous economic cost on our society.

Arthur P. Bollon, Ph.D.
Dallas, Texas
August 1, 1983

THE EDITOR

Arthur P. Bollon, Ph.D., is Director of Genetic Engineering and Chairman of the Department of Molecular Genetics at the Wadley Institutes of Molecular Medicine in Dallas, Texas.

Dr. Bollon received his B.A. degree in Biology from C.W. Post College in 1965, followed by a Fellowship to work on Microbial Genetics with Dr. Milaslav Demerec. In 1970, he received his Ph.D. in Microbiology at the Waksman Institutes of Microbiology at Rutgers University working on bacterial gene expression under Dr. Henry J. Vogel. Following a postdoctoral fellowship working on yeast genetics in the Department of Microbiology at Yale University, he joined the faculty of The University of Texas Health Science Center at Dallas in the Department of Biochemistry. Dr. Bollon presently maintains two adjunct faculty positions at North Texas State University and The University of Texas Health Science Center.

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Chapter 1

RECOMBINANT DNA TECHNIQUES: ISOLATION, CLONING, AND EXPRESSION OF GENES

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I. INTRODUCTION

The cloning and expression of foreign genes using recombinant DNA technology has permitted access to complex biological mechanisms such as eucaryotic RNA splicing, oncogene dynamics, and developmental systems such as antibody diversity. In addition, the technology has been the foundation for a new bio-technology industry. This chapter contains an analysis of some of the recombinant DNA techniques that have been employed for the manipulation of foreign genes in microorganisms resulting in protein production as diagrammed in Figure 1. Subsequent chapters will address the expression, clinical trials and production of genetically engineered human interferon, insulin, and growth hormone.

II. ENZYMES

The isolation and cloning of genes involves a series of linked enzymatic steps. Experience with two-enzyme coupled reactions is enough to sensitize the researcher to the complexity of linking five or more enzyme reactions, that are characteristic of the steps involved in the synthesis and cloning of cDNA. Considering that the substrates as well as the catalysts are biological macromolecules, it is not surprising that there are various opinions as to the most efficient protocols. The purity and correct storage procedures for the substrate and enzymes are clearly very critical. Selected restriction enzymes and other enzymes which are commonly used for cloning procedures will be discussed with emphasis on some of their characteristics and utility.

A. Restriction Endonucleases

Restriction endonucleases are enzymes which have been identified in prokaryotic organisms and recognize specific DNA sequences for their endonucleolytic activity. This mechanism permits organisms to prevent foreign DNA from integrating into their genome, which could jeopardize the genetic integrity of the species. Since bacterial sexuality involves direct movement of DNA between organisms by transformation, conjugation, or transduction, it is not surprising that some mechanism evolved to protect against undesirable DNA (*restriction*) as well as to protect native endogenous DNA (*modification*).

Three types of restriction endonucleases have been characterized as indicated in Table 1.

Type II enzymes have been most useful for DNA cloning due to the separation of the endonuclease and methylation activities into separate enzymes and the sequence specificity of the endonucleolytic action. A key feature of many restriction endonucleases is their asymmetric cleavage generating single-stranded ends. For example, the commonly used enzyme EcoRI recognizes and cleaves the following sequence at the arrows.

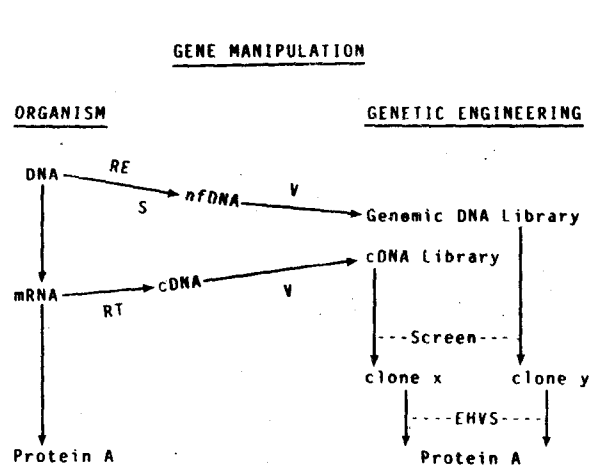

FIGURE 1. cDNA, copy DNA; EHVS, expression host-vector system; nf DNA, natural fragmented DNA; RE, restriction endonuclease; S, sheared; V, vector. Protein A is a protein made either naturally by the organism or by genetic engineering. Clone X is a single clone identified from a cDNA library containing Protein A cDNA. Clone Y is a single clone identified from a genomic library containing a Protein A natural gene.

Table 1
RESTRICTION ENZYME TYPES

Restriction Enzyme Characteristics	Restriction Enzyme Classes* Type I	Type II	Type III
Enzyme contains *both* modification (methylase) and endonuclease activity	+	–	+
Enzyme contains only endonuclease activity and a separate enzyme is involved in methylation	–	+	–
Recognize unmethylated sequences for endonuclease cleavage	+	+	+
Sequence specific cleavage	–	+	+
Class most useful for DNA cloning	–	+	–

* An enzyme type exhibiting the specific characteristic is indicated by a +.

$$\text{———}\overset{5'}{\text{}}\overset{\downarrow}{\text{}}\text{GAATTC}\overset{3'}{\text{———}}$$

$$\text{———}\underset{3'}{\text{}}\text{CTTAAG}\underset{\uparrow\ 5'}{\text{———}}$$

Such a cleavage generates two fragments containing single-stranded ends.

$$\text{———}\overset{5'}{\text{}}\text{G} \qquad \text{AATTC}\overset{3'}{\text{———}}$$

$$+$$

$$\text{———}\underset{3'}{\text{}}\text{CTTAA} \qquad \text{G}\underset{5'}{\text{———}}$$

Since the enzyme cuts between the GA and the recognition sequence is an inverted repeat, complementary single-stranded ends are generated. These fragments can either be religated or new DNA combinations can be generated by mixing DNA fragments containing complementary single-stranded tails. The restriction endonucleases can generate either single-stranded 5′ ends such as above, 3′ ends, or no single-stranded tails if the cleavage is at the center of the recognition sequence.

Although most of the restriction enzymes recognize different sequences, there is sequence overlap for some enzymes. Such enzymes which recognize similar sequences are called *isoschizomers*. As can be seen in Table 2, several enzymes such as XmaI and SmaI are isoschizomers, and several enzymes share compatible cohesive ends.

The interplay of enzyme recognition sites and the length of the recognition sequence bear on the efficiency of cloning and retrieval of specific genes from vectors. For example, MboI digested DNA can be cloned into a vector cleaved with BamHI but would not be retrievable by BamHI digestion. Another example is the useful capability of cloning a BglII digested DNA fragment into a vector cut with BamHI, such as pBR322. In this case the generated sequence AGATCC is not cut by either BamHI or BglII. Hence, the use of different restriction enzymes for cleavage at homologous sites or for ligation of cleaved fragments should be tempered with anticipation of the resulting sequences generated.

For restriction enzyme analysis of a cloned DNA fragment, the status of the host bacterial methylation system is important. *E. coli* contains the *dam* and *dcm* methylase systems which have different specificities. For cleavage of DNA by BclI, MboI, ClaI, XbaI, TaqI, MboII, and HphI, the DNA should be prepared from strains of *E. coli* which is *dam*$^-$. For cleavage of DNA by HhaI, HpaII, MspI, SalI, and XhoI the DNA should be generated from strains of *E. coli* that are *dcm*$^-$ (Roberts et al., 1980).[1]

B. Other Enzymes Used in Gene Isolation and Manipulation

1. DNA Polymerase I

E. coli DNA polymerase I is a 109,000 dalton protein containing three activities: (1) 5′→3′ polymerase activity using a 3′-OH primer and a single-stranded template, (2) 5′→3′ exonuclease activity which degrades double-stranded DNA at free 5′ ends, (3) 3′→5′ exonuclease activity which degrades ss DNA and ds DNA from 3′-OH ends. The 5′→3′ polymerase activity blocks the 3′→5′ exonuclease activity.

One of the major uses of the DNA polymerase I is *nick-translation* labeling of DNA fragments. This is possible due to the polymerase and exonuclease activities. The enzyme can remove bases from the 5′ side of the nick of treated DNA due to its 5′→3′ exonuclease activity and add labeled bases to the 3′ side due to its polymerase activity (Kelly et al., 1970).[2] DNA probes can be prepared for hybridization with specific activities in excess of 10^8 cpm/μg (Maniatis et al., 1975).[3] The high specific activity depends on the specific activity of the labeled bases and incorporation of about 30% of the [α-^{32}P] dNTPs into the DNA being nick-translated (Maniatis et al., 1982).[4]

2. Klenow Fragment of E. coli DNA Polymerase I

The Klenow fragment is a peptide of 76,000 daltons generated by cleavage of DNA polymerase I with subtilisin (Jacobsen et al., 1974).[5] Enzymatic activities include the 5′→3′ polymerase activity and the 3′→5′ exonuclease activity of the DNA polymer but not the 5′→3′ exonuclease activity.

This enzyme is used for sequencing DNA using the Sanger dideoxy system (Sanger et al., 1977),[6] filling the 3′ recessed termini of restriction enzyme treated DNA, labeling the termini of DNA fragments and second-strand cDNA synthesis involved in cDNA procedures. Labeling of DNA termini containing 3′ extensions is more efficient when T4 DNA polymerase is utilized (Maniatis et al., 1982).[4]

Table 2
RESTRICTION ENZYMES CHARACTERISTICS

Enzyme	Microorganism	Recognition Sequence	Common Isoschizomers[a]	Compatible Cohesive Sites	Number of Cleavage Sites		
					Lambda	SV40	pBR322
AluI	*Arthrobacter luteus*	AG↓CT		Blunt	>50	35	16
ApyI	*Arthrobacter pyridinolis*	CC↓(A/T)GG	AtuI, EcoRII		>35	16	6
AsuI	*Anabaena subcylindrica*	G↓GNCC	Sau961		>30	11	15
AsuII	*Anabaena subcylindrica*	TT↓CGAA	M1aI	AccI, AcyI, ClaI, HpaII, TaqI	7	0	0
AtuII	*Agrobacterium tumefaciens* ID 135	CC↓(A/T)GG	EcoRII		>35	16	6
AvaI	*Anabaena variabilis*	G↓PyCGPuG		SalI,XhoI,XmaI	8	0	1
AvaII	*Anabaena variabilis*	G↓G(A/T)CC	AflI, BamN,I	Sau961	>17	6	8
AvrII	*Anabaena variabilis*	CCTAGG			2	2	0
BalI	*Brevibacterium albidum*	TGG↓CCA		Blunt	15	0	1
BamHI	*Bacillus amyloliquefaciens* H	G↓GATCC	BstI	BclI, BglII, MboI, Sau3A, XhoII	5	1	1
BbvI	*Bacillus brevis*	GC(T/A)GC			>30	23	21
BclI	*Bacillus caldolyticus*	T↓GATCA		BamHI, BGlII, MboI, Sau3A, XhoII	7	1	0
BglI	*Bacillus globigii*	GCCNNNN↓NGGC			22	1	3
BglII	*Bacillus globigii*	A↓GATCT		BamHI, BclI, MboI, Sau3A, XhoII	6	0	0
BstEII	*Bacillus stearothermophilus* ET	G↓GTNACC	AspAI, BsPI		11	0	0
BstNI	*Bacillus stearothermophilus*	CC↓(A/T)GG	EcoRII		>35	16	6
ClaI	*Caryophanon latum* L.	AT↓CGAT		AccI, AcyI, AsyII, HpaII, TaqI	15	0	1
DdeI	*Beaulfovibrio desulfuricana* Norway strain	C↓TNAG			>50	19	8
DpnI	*Diplococcus pneumoniae*	G^{Me}A↓TC	Sau3A	Blunt	only cleaves methylated DNA		
EcoRI	*Escherichia coli* RY13	G↓AATTC			5	1	1
EcoB	*Escherichia coli* B	TGANN8			Type 1	1	0

Table 2 (continued)
RESTRICTION ENZYMES CHARACTERISTICS

Enzyme	Microorganism	Recognition Sequence	Common Isoschizomers[a]	Compatible Cohesive Sites	Number of Cleavage Sites		
					Lambda	SV40	pBR322
EcoK	*Escherichia coli* K	AACN6			Type I	0	2
EcoPI	*Escherichia coli* (PI)	AGACC			Type III	4	4
EcoRI'	*Escherichia coli* RY13	(A/G)(A/G)A ↓ T(C/T)(C/T)		Blunt	>10	24	15
EcoRII	*Escherichia coli* R245	↓ CC(A/T)GG	AtuI, ApyI		>35	16	6
Fnu4HI	*Fusobacterium nucleatum* 4H	GC ↓ NGC			>50	25	42
FnuDII	*Fusobacterium nucleatum* D	CG ↓ CG	ThaI	Blunt	>50	0	23
HaeI	*Haemophilus aegyptius*	(A/T)GG ↓ CC(A/T)		Blunt	?	11	7
HaeII	*Haemophilus aegyptius*	PuGCGC ↓ Py			>30	1	11
HaeIII	*Haemophilus aegyptius*	GG ↓ CC	BspRI, BsuRI	Blunt	>50	19	22
HgaI	*Haemophilus gallinarum*	GACGCN5 ↓ CTGCGN10			>50	0	11
HhaI	*Haemophilus haemolyticus*	GCG ↓ C	FnuDIII, HinP$_1$I		>50	2	31
HincII	*Haemophilus influenzae* R$_c$	GTPy ↓ PuAC	HindII	Blunt	34	7	2
HindII	*Haemophilus influenzae* R$_d$	GTPy ↓ PuAC	HincII, HinJCI	Blunt	34	7	2
HindIII	*Haemophilus influenzae*	A ↓ AGCTT	HsuI		6	6	1
HinfI	*Haemophilus influenzae* R$_f$	G ↓ ANTC	FnuAI		>50	10	10
HpaI	*Haemophilus parainfluenzae*	GTT ↓ AAC		Blunt	13	4	0
HpaII	*Haemophilus parainfluenzae*	C ↓ CGG	HapII, MnoI	AccI, AcyI, AsuIIClaI, TaqI	>50	1	26
HphI	*Haemophilus parahaemolyticus*	GGTGAN6 ↓ CCACTN7			>50	4	12
KpnI	*Klebsiella pneumoniae* OK8	GGTAC ↓ C		BamHI, BclI, BglII, XhoII	2	1	0
MboI	*Moraxella bovis*	↓ GATC	DpnI, Sau3AI		>50	8	22
MboII	*Moraxella bovis*	GAAGAN8 ↓ CTTCTN7			>50	16	11
MnlI	*Moraxella nonliquefaciens*	CCTC			>50	51	26
MspI	*Moraxella* species	C ↓ CGG	HpaII		>50	1	26
MstI	*Micrococleus* species	TGCGCA	AosI, FdiII		>10	0	4
PstI	*Providencia stuartii* 164	CTCGA ↓ G	SalPI, SfiI		18	2	1

PvuI	*Proteus vulgaris*	CGATCG	NblI, RshI		3	0	1
PvuII	*Proteus vulgaris*	CAG↓CTG		Blunt	15	3	1
SacI	*Streptomyces achromogenes*	GAGCT↓C	SstI		2	0	0
SacII	*Streptomyces achromogenes*	CCGC↓GG	CscI, SstII		>25	0	0
SacIII	*Streptomyces achromogenes*	ACGT			>100	?	?
SalI	*Streptomyces albus* subspecies *pathocidicus*	G↓TCGAC	HgiCIII, HgiDII	AvaI, XhoI	1	0	0
Sau3A	*Staphylococcus aureus* 3A	↓GATC	MboI	BamHI, BclI, BglII, MboI, XhoII	>50	8	22
Sau96I	*Staphylococcus aureus* PS96	G↓GNCC	AsuI		>30	11	15
SmaI	*Serratia marcescens* S_b	CCC↓GGG	XmaI	Blunt	3	0	0
SphI	*Streptomyces phaeochromogenes*	GCATG↓C			4	2	1
SstI	*Streptomyces* Stanford	GAGCT↓C	SacI		2	0	0
SstII	*Streptomyces* Stanford	CCGC↓GG	SacII		4	0	0
SstIII	*Streptomyces* Stanford	ACGT	SacIII		>100	?	?
TaqI	*Thermus aquaticus* YTI	T↓CGA		AccI, AcyI, AsuII, ClaI, HpaII	>50	1	7
ThaI	*Thermoplasma acidophilum*	CG↓CG	FnuDII	Blunt	>50	0	23
XbaI	*Xanthomonas badrii*	T↓CTAGA			1	0	0
XhoI	*Xanthomonas holcicola*	C↓TCGAG	BluI, PaeR7I	AvaI, SalI	1	0	0
XhoII	*Xanthomonas holcicola*	(A/G)↓GATC(T/C)		BamHI, BclI, BglII, MboI, Sau3A	>20	3	8
XmaI	*Xanthomonas malvacearum*	C↓CCGGG	SmaI	AvaI	3	0	0
XmaIII	*Xanthomonas malvacearum*	C↓GGCCG			2	0	1

[a] Presented are representative isoschizomers. A complete list can be obtained in the New England Biolabs' catalogue, 1982—1983.