DNA Cloning and the Analysis of Plasmid Structure and Function

Article *in* Progress in Molecular and Subcellular Biology · January 1978

DOI: 10.1007/978-3-642-66856-2_1

CITATIONS
33

READS
1,880

3 authors, including:

Felipe Cabello
New York Medical College
246 PUBLICATIONS **10,988** CITATIONS

SEE PROFILE

DNA Cloning and the Analysis of Plasmid Structure and Function

K. N. Timmis, S. N. Cohen, and F. C. Cabello

A. Introduction

Genetic recombination is the fundamental biological process of exchange of genetic information between different chromosomes. Its constant activity during evolution has enabled the large number of spontaneously occuring genetic changes that improve the fitness of individuals for their particular environments to accumulate in the most successful members of each biological species. Recombination has provided much of the foundation for the science of genetics; it has been exploited to great social benefit by plant and animal breeders and to great scientific benefit by classical and molecular geneticists. Although there is evidence that recombination between DNA segments that have little or no ancestral relationship can occur under some circumstances (Cohen, 1976; Starlinger and Saedler, 1976), "ordinary" or "generalized" recombinational events commonly involve the reciprocal exchange of genetic material and require DNA sequence homology in the region of exchange. Thus, recombination in the laboratory between unrelated species of organisms having little DNA sequence homology is ordinarily not feasible. However, it has long been apparent that great benefits could be derived from intergeneric, as well as intrageneric, genetic manipulations.

Genetic recombination consists essentially of the breakage and joining of DNA molecules. Recent developments now permit DNA obtained from a wide variety of prokaryotic and eukaryotic sources to be cut in vitro at precisely defined locations and the DNA fragments thus generated to be coupled enzymatically to a self-replicating genetic element, known as a cloning vector or vehicle (either a plasmid or bacterial virus genome). Hybrid molecules generated in this fashion are introduced into *Escherichia coli*, where they are perpetuated and can be studied (Cohen, 1975). The host *E. coli* cells containing a hybrid molecule thus can serve as "cellular factories" for producing large amounts of the cloned DNA (and in some instances, the gene products specified by the cloned DNA) and, in addition, can serve as a well defined genetic background against which to study the expression of the cloned DNA fragment. The technologies that have been developed to permit the in vitro cloning of individual fragments of foreign DNA are collectively termed "DNA cloning", "molecular cloning", "gene cloning", "gene manipulation", and "genetic engineering".

The potential applications in the biochemical sciences to obtain basic information about fundamental biological processes, and in the applied sciences to obtain a variety of biological products that are of medical, agricultural, and commercial importance, and that are otherwise expensive or unobtainable in large quantity, indicate that DNA cloning methods represent a tool of extraordinary usefulness (Ashby Report, 1975; Curtiss III, 1976; Cohen, 1977).

The spectacular advances in the investigation of the structure and function of prokaryotic genes and operons, for example in the bacterio-

F. E. Hahn et al. (eds.), *Progress in Molecular and Subcellular Biology*

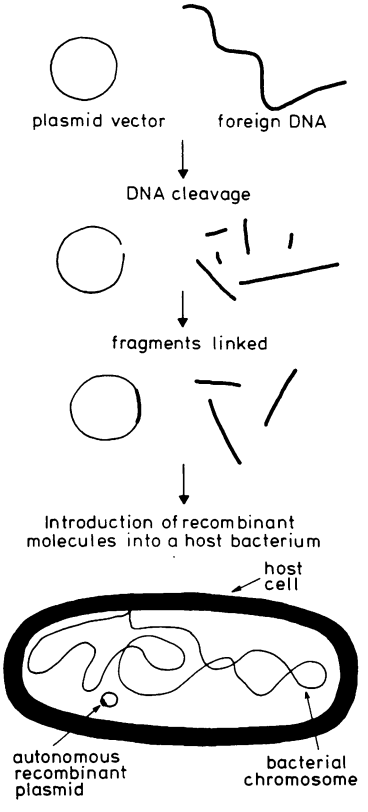


Fig. 1. The DNA cloning procedure

phage *lambda* and the *lac* operon, occurred largely because the DNA segments containing the genes under investigation were either part of a small autonomous replicon (*lambda*) or were easily sequestered on to such replicons (e.g., lambdoid phages and the sex factor F). In this state of reduced genetic complexity, genes could be investigated in a variety of ways in systems greatly depressed in non-specific genetic and biochemical background activity (see various articles in "The Lactose Operon", eds. Beckwith and Zipser, 1970, and "The Bacteriophage Lambda", ed. Hershey, 1971). The enormous genetic complexity of eukaryotic cells (some of which are five orders of magnitude more complex than the bacteriophage λ) is thus the main obstacle to a molecular analysis of gene structure, function, and regulation in higher organisms. Hence the development of methods to sequester defined eukaryotic DNA sequences on to small prokaryotic or eukaryotic autonomous extrachromosomal elements represents a major breakthrough in eukaryote molecular biology. As a consequence of this and other newly developed technologies, an understanding of the basic regulatory mechanisms controlling eukaryotic gene expression is expected to be greatly facilitated. Such an understanding is an absolute requirement for the elucidation of the biochemical bases of a number of pathological conditions, including tumorogenesis.

Cloned DNA sequences from prokaryotic and eukaryotic sources may be easily isolated in quantity in a degree of purity that was previously unattainable and that is suitable for structural studies, such as physical mapping and DNA sequencing. Furthermore, the gene products coded

by cloned fragments, at least those from many prokaryotic sources, may be manufactured in large amounts by the host cell. This latter aspect of molecular cloning has obvious significance for the production of biological products important in medicine, agriculture, and industry. A few selected examples of potential benefits of gene cloning experiments are given below:

1. The use of non-pathogenic host bacteria containing genes for antigens characteristic of specific agents of disease should greatly improve the effectiveness and safety of vaccine production.

2. Pollution of the environment by oil wastes may be combated with bacteria specifically armed with DNA sequences enabling them to utilize these products as sole carbon sources for growth. Indeed single cell protein might be manufactured from such wastes.

3. Provided that problems of transcription and translation of some types of foreign DNA in *E.coli* are overcome, the exciting possibility of manufacturing medically important products like insulin and other hormones, interferon, enzymes, and antibiotics, in convenient bacterial systems may soon be realized.

4. Food plants and animals, or the microbes that associate with them, may be genetically manipulated in order to increase the yield or the quality of the food material produced. Currently receiving great attention is the possibility that nitrogen fixation genes might be introduced into bacteria that can colonize the roots of nonlegumenous plants.

It would not be appropriate for us to discuss the potential benefits of gene cloning methods without mentioning the concerns that have been raised about possible hazards of some experiments that use these methods (see Cohen, 1977). Organisms appear to have evolved biological mechanisms that originally limited genetic exchange with unrelated species. Although the significance of these mechanisms is not yet elucidated, their functions are assumed to be of some evolutionary importance. DNA cloning in vitro permits the investigator to construct molecular chimeras by the fusion of DNA segments derived from organisms that are not known to be otherwise capable of exchanging genetic information. Since the properties of certain gene combinations made in this way may not be entirely predictable, the investigator is now required by national research organizations to carefully evaluate potential biohazards of projected molecular cloning experiments and subsequently to perform such experiments under appropriate laboratory conditions of containment similar to those used for work with organisms known to be hazardous (NIH Guidelines, 1976; Williams Report, 1976).

In this review we will focus our discussion on how the molecular cloning technology can be fruitfully exploited to investigate plasmid DNA structure and function. Because the problems of transcription/translation of cloned plasmid DNA segments are minor compared with those associated with cloned eukaryotic DNA segments, significant advances promoted by the use of the cloning technology have already taken place in the plasmid field in the four years since its first description in the literature (Cohen et al., 1973). However, most of the cloning strategies that have been employed for investigation of plasmid DNA structure and function are appropriate for the study of larger, more complex replicons such as chromosomes. We therefore feel that this is an appropriate point in time at which to review recent advances in the plasmid field resulting from molecular cloning experiments and to underscore those experimental approaches that may find a wider use in the study of more complex genetic systems. Although it will be neces-

sary for us to discuss relevant aspects of restriction enzymes, plasmids, and the cloning technology, these topics will not be covered exhaustively here. For other pertinent reviews of these subjects the order is: Arber, 1974 (restriction and modification); Nathans and Smith, 1974; Roberts, 1976; Roberts, 1977 (restriction enzymes); Helinski, 1973; Falkow, 1975 (plasmids); Cohen, 1975; Murray, 1976; and Collins, in press 1977 (gene cloning).

B. Restriction Endonucleases

Restriction endonucleases are site-specific endodeoxyribonucleases that cleave double-stranded and in some instances single-stranded DNA. Although the first site-specific endodeoxyribonucleases to be characterized were known to function as enzymatic blocks or restriction barriers for the prevention of invasion of the host cell by foreign DNA, i.e., were components of restriction-modification systems and hence were called restriction endonucleases, there is no evidence that many of the more recently described endonucleases are also bona fide components of restriction-modification systems. Nevertheless, for convenience we will continue to refer to site-specific endodeoxyribonucleases as restriction endonucleases, as suggested by Roberts (1976). All restriction endonucleases *recognize* specific DNA sequences; some, but not all, may also *cleave* at specific sequences. Class II enzymes are of the former type and generate specific DNA fragments usually, but not always, by cleavage at a sequence within the endonuclease recognition sequence. Class I enzymes are of the latter type and appear to cleave DNA randomly, there by generating heterogeneous DNA products. The ability of class II enzymes to cleave long complex molecules of DNA at precise locations and the availability of a wide range of enzymes having different recognition sequences has revolutionized the investigation of the structure and function of genetic material.

Table 1 lists the recognition sequences and cleavage sites of a selection of currently available and widely used restriction endonucleases. It may be noted that endonucleases differ from one another not only in the composition and sequence of bases within the recognition sequence, but also in the length of the sequence and in the type of cleavage effected. In general, an enzyme that recognizes a tetranucleotide sequence will cleave a given DNA molecule more frequently than will an enzyme that recognizes a hexanucleotide sequence. However, the relative frequency of different restriction site sequences and their distribution within a DNA molecule varies from one DNA species to another (contrast the number of cleavage sites in SV40 DNA for the enzymes Alu1 and TaqI, enzymes which recognize mirror image sequences). Thus, the number and location of restriction endonuclease cleavage sites on any given DNA molecule depends upon the sequence of the nucleotides recognized by the enzyme, the length of the sequence, and its frequency and distribution in the substrate DNA molecule.

Some restriction endonucleases cleave both DNA strands at a single site on the molecule (i.e., cleave both phosphodiester linkages between two nucleotide pairs), thus generating DNA fragments having "flush" or "blunt ends", whereas others cleave one DNA strand several nucleotides away from the cleavage on the opposing strand, thereby generating "cohesive" ends. Restriction endonucleases that generate fragments with cohesive ends can produce 5' extensions (EcoRI) or 3' extensions (PstI) composed of a dinucleotide (TaqI), a trinucleotide (HinfI), a tetranucleotide (HindIII), or a pentanucleotide (EcoRII). As can be seen in Table 1, in some cases the same DNA sequence can be

Table 1. Selected restriction endonucleases: recognition sequences and sites of cleavage[a]

	Endonucleases that generate fragments with cohesive termini			Endonucleases that generate fragments with flush termini		
Hexanucleotide	Bam HI	G↓GATCC	(5;1)			
recognition	Bgl II	A↓GATCT	(5;0)	Hpa I	GTT↓AAC	(11;5)
	Eco RI	G↓AATTC	(5;1)	Sma I	CCC↓GGG	(3;0)
	Hind III	A↓AGCTT	(6;6)			
	Pst I	CTGCA↓G	(18;2)			
	Xma I	C↓CCGGG	(3;0)			
	Hae II	PuGCGC↓Py	(>30;1)			
Tetranucleotide	Hha I	GCG↓C	(>50;2)	Alu I	AG↓CT	(>50;32)
recognition	Hpa II	C↓CGG	(>50;1)	Hae III	GG↓CC	(>50;18)
	Mbo I	↓GATC	(>50;8)			
	Taq I	T↓CGA	(>50;1)			

[a]Compiled from the review by Roberts, 1976. For convenience, only one strand of the recognition sequence is shown. The left hand end of each sequence is the 5' end. The arrow indicates the site of cleavage of the phosphodiester linkage. The two numbers shown in brackets after each recognition sequence represent the numbers of cleavage sites present in lambda and SV40 DNAs respectively.

recognized by two or more enzymes that generate DNA fragments with different termini (e.g., SmaI and XmaI), whereas in other cases cleavage at different recognition sequences can generate DNA fragments with identical termini (i.e., BamH1 and BglII; Roberts, 1976a).

Because restriction endonuclease-generated DNA fragments with identical cohesive ends readily anneal with one another they can be efficiently joined together by the action of DNA ligase. This particular property of DNA fragments generated by the EcoRI restriction endonuclease greatly facilitated the early DNA cloning experiments.

C. DNA Cloning

I. In Vitro Recombination

1. Generation of DNA Fragments

To permit insertion of foreign DNA at an appropriate location in the vector, the vector is cleaved at a unique location that is not in or near functions that are essential for its effective use (see below). Multiple cleavages are sometimes made to remove non-essential segments of vector DNA in order to permit packaging of the maximum amount of foreign DNA in bacteriophage vectors (Murray and Murray, 1974; Thomas et al., 1974). However, because of the specificity of cleavage required, vector cleavage is always accomplished by means of a restric-

tion endonuclease. It is usually convenient to generate vector DNA having cohesive termini that can subsequently anneal with similar termini on foreign DNA fragments that are to be cloned.

Cleavage of foreign DNA to be cloned is also most conveniently accomplished by the action of one or more restriction endonucleases. Fragments thus generated may possess either cohesive ends or flush ends, or one of each type, according to the enzyme (s) used. Ordinarily, enzymatic digestion of the DNA is allowed to proceed to completion, i.e., all sites on the DNA molecule that are susceptible to cleavage by the enzyme (s) are cleaved. However, in those instances where all available enzymes cleave within the required DNA fragment, or where the cloning of a series of overlapping fragments of DNA is desired, it is necessary to obtain partial endonuclease digestion products of the DNA (Skurray et al., 1976), or to generate random fragments, for example, by mechanical shearing (Clarke and Carbon, 1975; see, however, Backman et al., 1976).

If a fragment of DNA to be cloned has been identified, it may be possible to purify this fragment prior to cloning, if it has a physical property (e.g., size or buoyant density) that distinguishes it from other fragments. For example, it was possible to purify an EcoRI endonuclease-generated fragment of DNA coding for ampicillin (Ap) resistance derived from the plasmid pSC122 by equilibrium centrifugation in caesium chloride (Timmis et al., 1975). This Ap DNA fragment banded in the gradient according to its buoyant density of 1.692 g/cc, whereas the other DNA fragment of the pSC122 plasmid banded at a more dense part of the gradient owing to its buoyant density of 1.710 g/cc. Similarly, DNA fragments of different sizes may be purified by electrophoresis through agarose gels (Lovett and Helinski, 1976). The recent description of a method (R loop formation) to form stable hybrids of RNA and duplex DNA should facilitate the purification of DNA fragments containing sequences for which a purified complementary RNA probe is available (Thomas et al., 1976). Prior purification of the fragment to be cloned greatly facilitates the subsequent cloning procedures.

2. Joining of DNA Fragments

The in vitro covalent linkage of fragments of foreign DNA to a cloning vector is achieved by treatment with *E.coli* or T4 DNA ligase to form phosphodiester linkages between the DNA fragments. The most convenient method currently employed is to ligate foreign DNA and vector fragments having identical cohesive ends (Mertz and Davis, 1972; Sgaramella, 1972; see Fig. 2). Under appropriate reaction conditions, the cohesive ends anneal and permit efficient covalent linkage by DNA ligase. While cohesive termini are convenient for some experiments, they are not absolutely required for the linkage of DNA fragments. Although the termini of flush ended DNA fragments cannot anneal they nevertheless can be joined by the action of T4 DNA ligase if the ligase and DNA fragment ends are present at high concentration in the ligation reaction (Sgaramella et al., 1970; Sgaramella and Khorana, 1972).

The recovery of a cloned DNA fragment from a hybrid molecule, subsequent to construction, is an important consideration in determining the strategy of a cloning experiment. Fragment recovery is always possible if the cloning vector and the foreign DNA fragment have been generated by the same endonuclease, since ligation of two ends produced by a single enzyme will regenerate the original endonuclease recognition sequence. If the cloning vector and foreign DNA are cleaved by different restriction enzymes and subsequently ligated together, in most in-

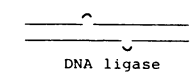
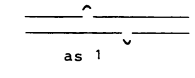
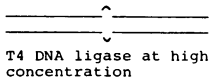
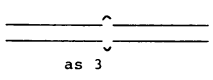
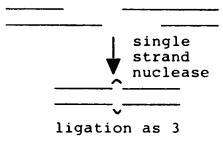
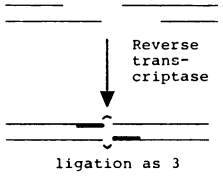
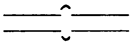
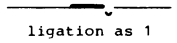
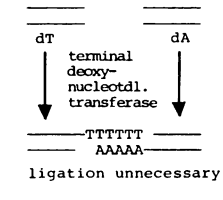
	Recognition Sequence	Type of Ends	Joining	Recovery of Cloned Fragment
1.	Identical	Identical, cohesive	 DNA ligase	Yes; with original cloning enzyme (e.g. EcoRI)
2.	Different	Identical, cohesive	 as 1	Not with cloning enzymes. Hybrid recognition site generated (e.g. BamH1, BglII)
3.	Identical	Identical, flush	 T4 DNA ligase at high concentration	Yes; with original cloning enzyme (e.g. SmaI)
4.	Different	Identical, or different, flush	 as 3	Not with cloning enzymes. Hybrid recognition site generated (e.g. AluI, SmaI)
5.	Different	Different, cohesive	a)  single strand nuclease ligation as 3	Not usually possible; no specific sequence at joint
			b)  Reverse trans-criptase ligation as 3	Yes, if one of the original recognition sequences is regenerated (e.g. EcoRI, XmaI); with one cloning enzyme
6.	Identical, or different	Different, one cohesive, one flush	Convert cohesive ends to flush as in 5, ligation as 3 	as 5
7.	Use of *adaptor fragments*		Ligation to *adaptor fragments*  ligation as 1	Yes, with enzyme that cleaves *adaptor fragment*
8.	Homopolymer tailing		 dT dA terminal deoxy-nucleotdl. transferase TTTTTT AAAAA ligation unnecessary	Yes, if one of the original recognition sequences is regenerated (e.g. if SmaI or PstI cleaved DNA is tailed with dG)

Fig. 2. Methods of joining DNA fragments

stances a hybrid recognition sequence will be formed that cannot be recognized by either of the original endonucleases. Unless an enzyme is available that recognizes the hybrid sequence, precise recovery of the cloned fragment will not be possible.

Occasionally, cleavage of the cloning vector and foreign DNA with different enzymes will generate DNA fragment with two different types of cohesive ends or with one type of cohesive end and one type of blunt end. In such situations, prior to ligation it is necessary either to generate appropriate cohesive ends in both types of DNA fragment or to convert both types of fragment to the flush-ended form.

The synthesis of *universal* cohesive ends on DNA fragments having any type of end (flush, or with 5' or 3' terminal extensions) is readily accomplished by means of the enzyme terminal deoxynucleotidyl transferase (Bollum, 1974). In the presence of cobalt ions this enzyme can add long homopolymer blocks composed of any one of the four deoxyribonucleotides to the 3' terminus of DNA fragments (Roychoudhury et al., 1976). Thus, a series of identical deoxyribonucleotides (e.g., dA) is added to the 3' ends of the cloning vector and a series of complementary deoxyribonucleotides (e.g., dT) is added to the 3' ends of the DNA fragments to be cloned (so-called *homopolymer tailing*; Jackson et al., 1972; Lobban and Kaiser, 1973). The two types of fragment are then mixed, allowed to anneal, and introduced into a bacterial host. Because it is not possible to synthesize homopolymer blocks of precisely defined length, DNA fragments annealed at their homopolymer tails will contain single-stranded regions at these joints. Such single-stranded regions may be repaired and covalent linkage of the fragments effected in vitro by the action of exonuclease III, DNA polymerase I and DNA ligase or in vivo by cellular repair enzymes (Jackson et al., 1972; Lobban and Kaiser, 1973; Wensink et al., 1974; Clarke and Carbon, 1975).

This particular method has certain advantages over other methods of joining DNA fragments. Firstly, circularization of single fragments, i.e., *intra*molecular joining, is absolutely precluded because any particular fragment has identical terminal homopolymer tracts. Thus, regeneration of the original cloning vehicle with the resultant elevation of "background" *non-hybrid* molecules is obviated. Secondly, because association of the long annealed homopolymer tails is quite stable, in vitro ligation of the cloning vehicle and the fragment to be cloned is unnecessary. This specific advantage is partly offset by the fact that precise cleavage of hybrid molecules at the vector:cloned fragment junction is rarely possible. If the cloned fragment can be recovered, due to the fortuitous location of appropriate restriction enzyme cleavage sites on the vector or fragment side of the homopolymer tract, either it will contain the homopolymer tract or it will be missing some of its terminal sequences. Recently, the use of single-stranded endonucleases to attack partially denatured dA-T rich segments has provided a simple method of separation of fragments that have been joined together by dA-T homopolymer tailing (Hofstetter et al., 1976). In some instances, judicious choice of the nucleotide of the homopolymer block to be added to the fragments of foreign DNA can regenerate the recognition sequence of the enzyme orginally used to obtain these DNA fragments (for instance, the addition of poly dG tails to SmaI-generated DNA fragments), and can thus permit recovery of the cloned fragment.

Conversion of DNA fragments containing 5' terminal extensions to the flush-ended form is accomplished either by removal of the extension with a single strand-specific exonuclease or by synthesis of a complementary sequence to the 5' termini (*filling-in*) by extension of the 3'

prime termini with reverse transcriptase or DNA polymerase I. Joining of blunt-ended DNA fragments thus generated may then be achieved by the T4 DNA ligase blunt-end ligation procedure. Whereas removal of a cohesive end destroys the original endonuclease recognition sequence and prevents subsequent recovery of the cloned fragment, filling-in of the single-stranded terminal region and subsequent blunt-end joining to another fragment in some instances may regenerate the original endonuclease recognition sequence, and thereby facilitate fragment recovery from the hybrid molecule (Backman et al., 1976).

A more general method to enable recovery of cloned DNA fragments is the linkage of so-called *adapter* or *linker fragments* to the ends of either the cloning vector, or the DNA fragments to be cloned, or both. Adapter fragments are small DNA fragments that contain one or more recognition sequences for restriction endonucleases. There are two types of adapter fragment. One type is a chemically synthesized short stretch of duplex DNA. Such a DNA fragment, an octanucleotide containing the EcoRI recognition site and having the sequence 5'-TGAATTCA-3', has been synthesized by Greene et al., 1975; see also Bahl et al., 1976). The other type of adapter fragment is a DNA fragment that can be excised from a naturally occurring DNA molecule and that contains a cluster of restriction endonuclease cleavage sites. Because the pSC101 plasmid contains within a continuous sequence of about 700 nucleotides in the region of the Tc resistance gene a cluster of recognition sequences for restriction endonucleases that are commonly used for cloning, it is a convenient source of a series of adapter fragments. The complete adapter fragment sequence is terminated on one side by the unique EcoRI cleavage site and on the other by the unique SalI site of the pSC101 plasmid, and therefore can be excised from the plasmid by treatment with these two endonucleases (Fig. 3). The adapter fragment thus generated has the following cleavage sites in the order given: EcoRI, AluI, HindIII/AluI, EcoRII, HaeIII, HaeII, BamHI, HaeII, SalI/HincII (Cohen et al., 1977a; Bolivar et al., 1977b). If required, the purified primary adapter fragment may subsequently be cleaved with additional enzymes to generate derivative adapter fragments with different cohesive (HindIII, EcoRII, HaeII, BamH1) or flush (AluI, HaeIII) termini. Alternatively, the EcoRI and SalI cohesive ends of the primary adapter fragment may be filled in with reverse transcriptase or DNA polymerase I to form a blunt-ended fragment containing all the original cleavage sites. Adapter fragments with either cohesive or flush ends, or one of each type, can be linked to the cloning vector or to the DNA fragments to be cloned by the action of T4 DNA ligase.

Adapter fragments are useful for the conversion of a DNA fragment terminus generated by one restriction endonuclease into a terminus generated by a different enzyme, thus allowing considerable flexibility in the types of restriction endonuclease that can be used in cloning experiments (e.g., see Heyneker et al., 1976; Bahl et al., 1976). More importantly, use of the adapter fragment technology readily permits excision of inserted DNA pieces from hybrid molecules.

Finally, it should be noted that in vitro ligation of DNA fragments is not an absolute requirement for the construction of hybrid molecules, although it greatly increases the yield of such molecules. In some instances, in vivo ligation of DNA fragments after transformation has generated the required DNA hybrids. This is especially true of constructions involving the homopolymer tailing technique with the ColE1 vector (Clarke and Carbon, 1975), but in vivo ligation has also been used for the cloning of some DNA fragments coding for *selectable* properties, for example, kanamycin resistance (Cohen et al., 1973; Collins et al., 1976). However, in vivo ligation of DNA fragments to EcoRI-

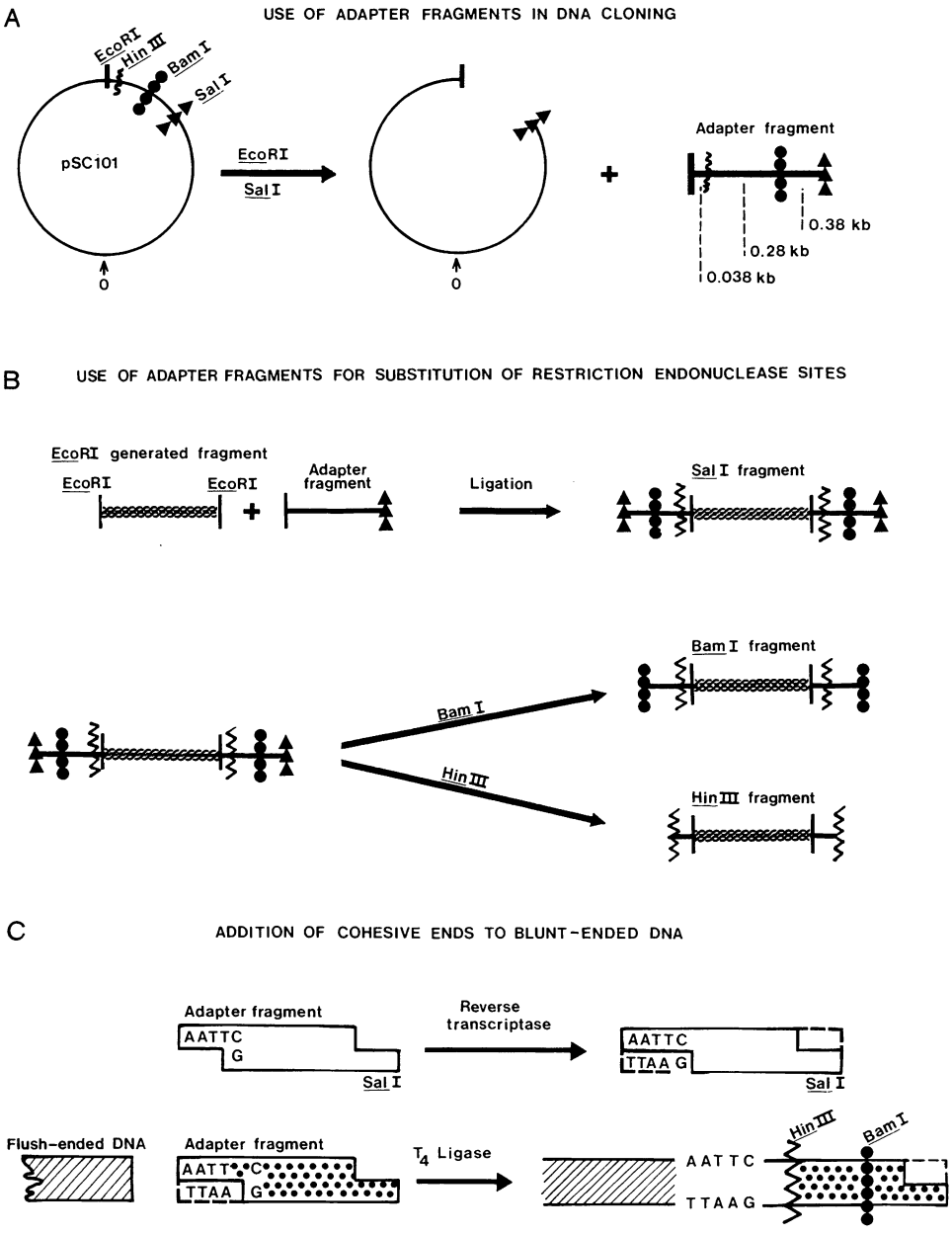


Fig. 3A-C. Use of adapter fragments in DNA cloning. The pSC101 plasmid contains EcoRI, HindIII, BamI, and SalI restriction endonuclease cleavage sites in close proximity to each other. The location of these sites is shown in relation to the pSC101 origin of replication (O). Treatment of pSC101 DNA with both the EcoRI and SalI enzymes leads to formation of the structures shown: the adapter fragment resulting from cleavage of the plasmid by the two enzymes can be used to convert a terminus produced by one restriction endonuclease into one that can be joined to a cleavage site generated by another enzyme. Use of adapter fragments for substitution of restriction endonuclease sites and for the addition of cohesive termini to blunt-ended DNA fragments is illustrated

cleaved pSC101 plasmid DNA apparently occurs very rarely (Timmis and Cabello, unpublished experiments).

II. Cloning and Propagation of Recombinant Molecules

The DNA mixture used for the infection of bacteria in cloning experiments consists of a heterogeneous mixture of molecules that are covalently linked or hydrogen bonded at their termini. Only some of the molecules in the mixture will be of the desired type, i.e., consisting of the cloning vector and a fragment of foreign DNA that have been circularized together. The problem therefore is to select from the DNA mixture and to amplify and propagate individually just those required molecules. This process is usually performed in two stages. The crude DNA mixture is used to infect a host bacterium, and because only DNA molecules that can self-replicate are able to propagate in the host, those DNA molecules that do not contain the cloning vector are selected against at this stage. Subsequently, those bacteria that have been infected with DNA from the construction mixture are screened for the presence of hybrid molecules.

1. Introduction of DNA into a Host Bacterium

The process of the introduction of DNA into bacteria has been termed *transformation* when involving chromosomal or plasmid DNA, and *transfection* when involving donor bacteriophage DNA. For the transformation of *E.coli*, DNA fragments or molecules are mixed with bacterial cells that have been made permeable by treatment with calcium chloride or other divalent cations and have been kept at $0^{o}C$. The cells subsequently receive a heat shock during which they take up exogenous DNA and, after incubation for a period of time to allow expression of the newly acquired DNA, the bacteria are plated on to a medium that permits growth of only those bacteria that have been transformed for the required character; in cloning experiments this is usually a selectable function present on the cloning vector. The procedure used for transfection is similar to that used for transformation, except that the transfected cells do not usually survive and must be detected by plating with indicator bacteria (Mandel and Higa, 1970; Cohen et al., 1972; Cosloy and Oishi, 1973; Wackernagel, 1973; Taketo, 1975).

One feature of transformation that is of profound importance for the study of plasmids in general and for the DNA cloning technology in particular is the fact that from gross mixtures of DNA molecules, single molecules can be isolated in separate cells and individually propagated. This capability (Cohen et al., 1972) has made it possible to study plasmids in ways previously feasible only with infectious extracellular particles such as bacteriophages.

A difficulty in using transformation and transfection for some DNA cloning studies is that the procedures are rather inefficient: typical values for non-restricted plasmid or phage DNA molecules are 10^{6} transformants per μg of DNA or 10^{-5} transformants per molecule (Cohen et al., 1972; Hohn and Murray, 1977), compared with values approaching unity for *Pneumococcus* and *Haemophilus* (Hotchkiss and Gabor, 1970). The yield of transformants obtained from DNA construction mixtures is usually orders of magnitude below these values. Although such yields are adequate for certain experiments, they may be inadequate for others. Recently, methods have been described for the in vitro packaging in phage particles of DNA from construction experiments involving λ DNA as the cloning vector (Hohn and Murray, 1977; Sternberg et al., 1977). Compared with naked DNA, the phage particles are very efficient at in-

fection and the relative yield of clones containing recombinant DNA can be increased by 1 to 2 orders of magnitude.

2. Detection of Cloned Recombinant DNA Molecules

The objective of cloning experiments is usually to obtain hybrid DNA molecules that consist of the cloning vector and single fragments of foreign DNA. DNA ligase joins together random fragments of DNA so that a certain proportion of recombinant molecules containing multiple fragments of foreign or vector DNA is always generated (Dugaiczyk et al., 1975). Furthermore, except where the homopolymer tailing technique is used, ligated DNA mixtures often contain a high proportion of reconstituted cloning vector molecules that have equal or greater efficiency in transformation than have recombinant molecules. Thus, frequently less than 50% of transformant bacterial clones contain hybrid molecules, and of these only a proportion may be of the required type.

The only definitive method to determine which type of DNA fragment has been cloned is to isolate the transformed DNA and determine the specific sequences contained in it. If the cloning experiment has been designed to permit the recovery of the cloned DNA fragment by treatment with a restriction endonuclease, it will be possible to determine by agarose gel electrophoresis of endonuclease-digested plasmid DNA exactly which DNA fragment (s), if any, other than the cloning vehicle are present. However, the commonly used procedure for the isolation of plasmid DNA from 30 to 100 ml cultures of bacteria (Clewell and Helinski, 1969) is tedious and expensive if many bacterial clones must be analyzed. Therefore considerable effort has recently been directed toward the development of microscale preparation procedures for the isolation and characterization of plasmid DNA. As a result, there are now so-called "toothpick assays" in which single bacterial colonies grown on nutrient agar plates are removed with a toothpick and their plasmid DNA extracted and characterized (Barnes, 1977; Telford et al., 1977). Such procedures greatly facilitate the analysis of DNA from large numbers of transformant bacterial clones.

Because normally a high proportion of transformed bacteria receive only the DNA of the cloning vehicle, much effort can be spared if the bacteria carrying recombinant clones can be identified by their phenotype or if the proportion of bacteria carrying recombinant molecules can be increased subsequent to transformation. One obvious way a recombinant plasmid can be identified is if insertion of foreign DNA always leads to activation or inactivation of a particular phenotypic trait. The unique EcoRI cleavage site of plasmid ColE1 lies in the structural gene for colicin E1. Insertion of DNA at this cleavage site inactivates colicin E1 production, a phenotypic trait that is readily identified (Hershfield et al., 1974; Timmis et al., 1974a; see Fig. 4). This process, termed *insertional inactivation* (Timmis et al., 1974a), has been used to identify a variety of recombinant plasmids generated with several different types of cloning vectors (Timmis et al., 1977c), such as pML21 (insertion at the HindIII site causes inactivation of kanamycin resistance), and pWL7 (insertion at the PstI site causes inactivation of ampicillin resistance; W. Goebel, personal communication). The convenience of this particular method of identifying recombinant DNA molecules is such that almost all new cloning vehicles developed have the unique restriction endonuclease cleavage sites that are used for cloning within structural genes that code for readily identifiable properties (Bolivar et al., 1977a; Cohen et al., 1977a; see Table 2).

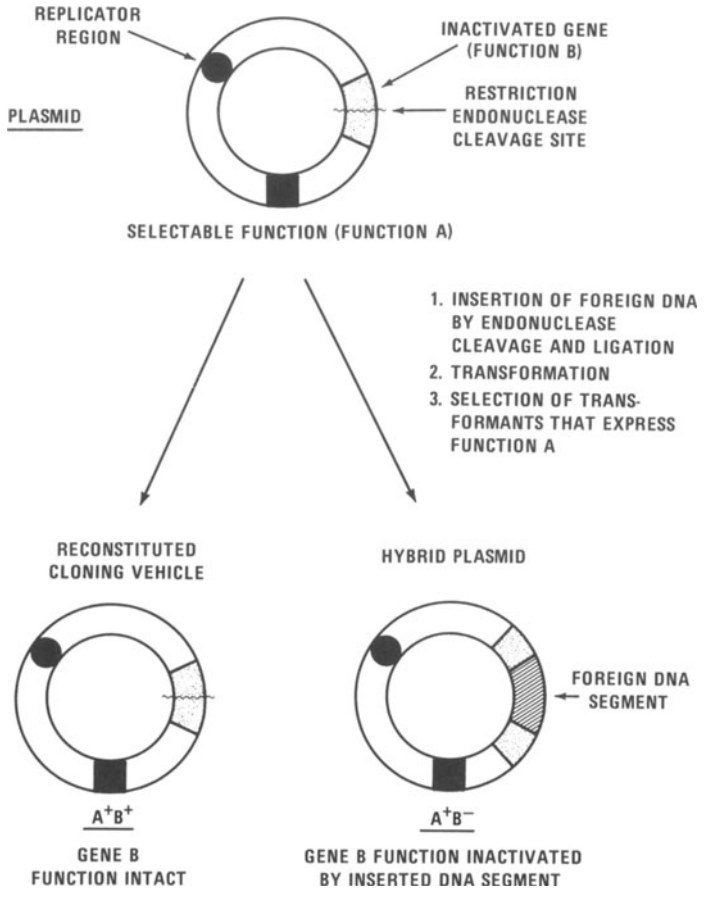


Fig. 4. Insertional inactivation. This procedure requires that a restriction endonuclease cleavage site be present in a gene coding for a readily identifiable function. Insertion of foreign DNA into this site leads to inactivation of the gene coding for function B, and loss of expression of that function. Use of the procedure is described in the text

One method designed for the enrichment of recombinant DNA molecules utilizes the increased size of such molecules over that of the cloning vector. Essentially the method involves the isolation of plasmid DNA from the whole population of transformed bacteria, the fractionation of this DNA on the basis of size [sucrose gradient centrifugation (Fig. 5), agarose gel electrophoresis] and retransformation with DNA having a greater molecular weight than that of the cloning vehicle (Cohen and Chang, 1974). Although one cycle of transformation, size fractionation and retransformation may be adequate, the procedure may be repeated several times to increase the degree of enrichment of recombinant DNA molecules prior to the isolation and analysis of individual transformant clones. (Because the transformation of bacteria with DNA from ligation mixtures is very inefficient due to the low concentration of *viable* plasmid DNA molecules and the high concentration of *non-viable* competing DNA, it is often an advantage to go through a cycle of this type, with or without a size fractionation, in order to amplify the viable molecules and to remove the competing non-viable molecules present in the original ligation mixture). Although this type of procedure greatly enriches the DNA construction mixture for recombinant molecules, its disadvantage is that it actually selects against recombinant molecules containing very small cloned fragments of DNA.

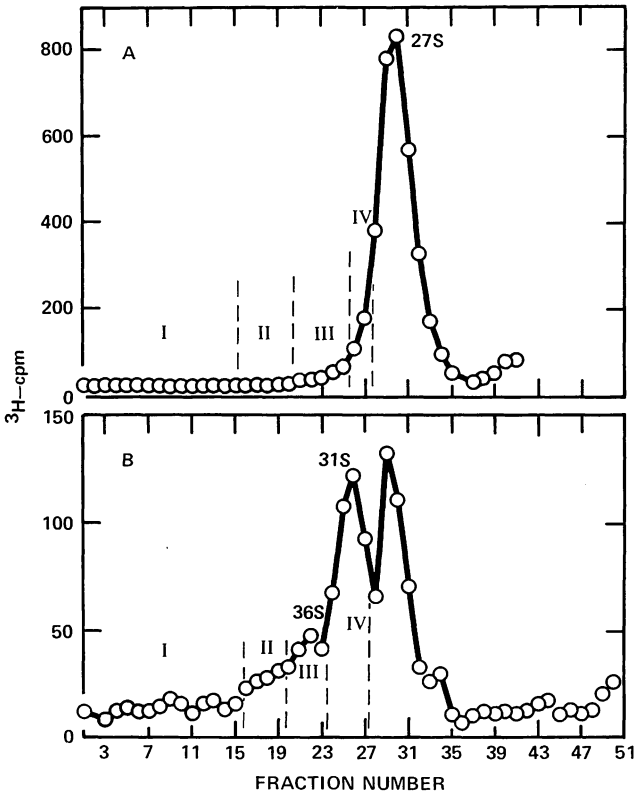


Fig. 5A and B. Enrichment of plasmid chimeras by sucrose gradient centrifugation and repeated transformation (from Cohen and Chang, 1974). In this experiment, a mixture of *Xenopus laevis* rDNA and pSC101 plasmid DNA was treated by EcoRI endonuclease, ligated, and used for transforming calcium chloride-treated bacteria. Primary selection of tetracycline resistant transformants was carried out. (A) Total CCC-DNA isolated from the heterogeneous population of tetracycline resistant transformants was fractionated by sucrose gradient centrifugation. The fractions comprising the lower portion of the gradient shown in the figure and the leading edge of the single 27S peak (which represents the recircularized cloning vehicle) were pooled, dialyzed, and used for another cycle of transformation. (B) Sucrose gradient centrifugation of DNA isolated from the population of Tc resistant cells transformed by fraction 1 from gradient shown in (A). As shown here, one cycle of centrifugation and transformation resulted in major enrichment of the desired population of chimeric plasmids

A second type of enrichment procedure utilizes the principle of *insertional inactivation* combined with a genetic procedure for mutant enrichment (e.g., see Davis, 1948; Curtiss et al., 1965) using antibiotics that inhibit cell wall biosynthesis. In this method, insertion of DNA into the unique endonuclease cleavage site used for cloning results in the inactivation of a gene that codes for a nutritional marker (for example, tryptophane biosynthesis), or for resistance to a bacteriostatic antibiotic such as tetracycline. Growth of transformant bacteria in the *absence* of the required nutrient, or in the *presence* of the bacteriostatic antibiotic, followed by ampicillin or cycloserine treatment, results in the lysis of bacteria carrying only the cloning vector and a substantial enrichment of recombinant DNA-carrying bacteria (Bolivar et al., 1977a).

It should be pointed out that during the usual transformation procedure little or no cell multiplication occurs until transformant bacteria are plated out on a selective medium for single colony isolation (Cohen et al., 1972). Thus, rarely do two or more primary transformant clones contain identical recombinant DNA molecules that are the progeny of a single DNA molecule from the original cloning mixture. A drawback of the enrichment procedures described above is that substantial multiplication of transformed bacteria occurs prior to single colony

isolation, which can result in the isolation of multiple clones of bacteria carrying identical recombinant plasmids.

Lastly, it should be mentioned that in some instances it is possible directly to select or at least to identify bacterial clones carrying specific recombinant plasmids. If the DNA fragment of interest codes for a selectable function, it is possible to select directly bacteria that have been transformed with this DNA, as was the case in the cloning of *Staphylococcus* plasmid DNA fragments coding for penicillin resistance (Chang and Cohen, 1974). The availability of a radioactive DNA or RNA probe with sequences complementary to some of those present on the DNA fragment to be cloned permits the direct identification of primary transformant clones or phage plaques containing the desired recombinant molecule. In these procedures, the primary colonies or plaques are grown on or are transferred to nitrocellulose filters, are replica plated, and then treated so that the DNA is released, denatured, and immobilized on the filter. Hybridization in situ with the radioactive probe then reveals the required colonies or phage plaques (Grunstein and Hogness, 1975; Sanzey et al., 1976; Skalka and Shapiro, 1976). The same principle was utilized in the subculture cloning selection procedure originally used for the cloning of sea urchin histone gene sequences (Kedes et al., 1975). However, whereas the number of transformant clones that can be screened by the in situ hybridization method is limited by the number of colonies that can be conveniently handled, the subculture cloning method is limited only by the sensitivity of the hybridization reaction.

The use of a radioactive DNA or RNA probe to detect specific cloned DNA sequences does not require that the cloned sequences are transcribed and translated, and consequently can identify those sequences that are not expressed. For identification of expressed sequences, special biochemical detection methods similar to those that have been developed for the genetic analysis of the *lac* operon or immunological probes are required. Recently, the identification of transformant clones that express genes present on cloned DNA by in situ immunoprecipitation reactions has been described (Sanzey et al., 1976; Skalka and Shapiro, 1976). It is expected that such analytical techniques will play a major role in the effort to obtain expression of eukaryotic and certain prokaryotic gene sequences in *E.coli*.

3. Stability of Recombinant DNA Molecules

From the early gene cloning experiments it was realized that while some recombinant molecules seemed perfectly stable, others were very unstable and rapidly underwent sequence rearrangements, including sequence deletion and insertion. Sequence rearrangements occurred less frequently in a recA host than in a recA$^+$ host, indicating that such rearrangements were promoted by, but not absolutely dependent on, the host recA gene product (Chang et al., 1975; Cohen et al., 1977a; Timmis et al., 1977c). One possible explanation of this phenomenon is that in vivo recombination events over many bacterial generations may have resulted in arrangements of DNA sequences that are optimal for their normal functioning. In vitro reassortment of DNA sequences in the course of cloning experiments may generate DNA arrangements that are unfavorable for optimal expression of certain sequences and, as a result, compensating in vivo rearrangements may be induced. Thus to minimize sequence rearrangements it is advisable to store bacterial clones containing recombinant DNA molecules in glycerol at -20°C after colony purification, rather than maintain the clones by serial subculture.

III. Characterization of Recombinant Molecules

1. Physical Analysis

Physical characterization of cloned DNA fragments is usually directed toward answering the following questions:

- Which of the sequences of the parent DNA have been cloned?
- In which orientation relative to the cloning vector has the cloned DNA fragment been inserted?
- What are the intrinsic physical properties of the cloned fragment?

For several types of physical analysis it is important to be able to separate the cloned fragment from the cloning vector by cleavage at the junction points. As indicated above, this can be easily achieved with recombinant DNA molecules constructed by means of a single restriction endonuclease. However, joined DNA fragments that were generated by different restriction endonucleases or by the homopolymer tailing technique can usually be separated only by the action of a third (or third and fourth) restriction endonuclease and are not usually cleaved precisely at the junction points. Fragments of cloned DNA recovered from this type of construction are likely either to be missing some of the terminal sequences of the original cloned fragment or to contain some of the cloning vector DNA. In the case of poly dA-dT joined DNA fragments it may be possible to selectively digest the junction DNA by treatment with single strand-specific endonuclease under mildly denaturing conditions (Hofstetter et al., 1976; Ksenzenko et al., 1976).

If the foreign DNA in the cloning experiment is a small molecule such as a plasmid or phage genome, direct identification of the cloned DNA fragment is relatively simple. The most common form of analysis is by electrophoresis of restriction endonuclease-cleaved recombinant and parent DNAs through agarose or acrylamide gels (see Fig. 8). Under conditions where all the restriction fragments of the parent DNA are separated, the cloned DNA fragment is identified as a specific fragment of the parent DNA molecule. If a restriction endonuclease cleavage map of the latter is available, the location of the cloned fragment on the physical map of the parent molecule can be immediately deduced. However, in some instances, cleavage of the parent DNA molecule with the restriction endonuclease used for cloning may generate several fragments with similar molecular sizes. Such fragments may appear after electrophoresis as a single DNA band of double or triple intensity (Fig. 8), and if the cloned DNA belongs to such a group of fragments, then it will not be possible to infer from the electrophoretic mobility its position on the physical map of the parent molecule.

A more direct method of physical mapping is the heteroduplex technique (Davis et al., 1971) that permits the electron microscope visualization of single strands of the cloned DNA fragment annealed to complementary single strands of the parent molecule at the region of sequence homology. If the parent DNA molecule contains sequence features (the termini in linear molecules, inverted repeats in circular molecules) that serve as internal reference markers for electron microscopy, then the region of homology can be accurately related to the physical map of the parent molecule (Fig. 8).

If the material for cloning consists of, or is derived from, a complex genome, exact sequence comparisons are normally impossible, and it is more appropriate to relate the cloned DNA fragment to a general region

of the parental molecule (this region may represent a long or short segment of DNA, depending upon the resolution capabilities of the available methods). In situ hybridization of radioactive cloned *Drosophila melanogaster* DNA fragments to squashed polytene chromosomes has been successfully employed in the physical mapping of *Drosophila* DNA fragments (Wensink et al., 1974).

It should be noted that whereas the gel electrophoresis analysis of recombinant molecules requires their cleavage at the cloned fragment vector junction, the two hybridization methods do not. It is nevertheless possible to use gel electrophoresis to identify a cloned DNA fragment that cannot be precisely excised from the recombinant molecule by combining gel electrophoresis with DNA-DNA hybridization. With this method, the parental DNA is digested by the restriction endonuclease used for cloning and the fragments generated are subjected to electrophoresis through an agarose or acrylamide gel. The resolved DNA fragments are denatured in situ and are then transferred to a nitrocellulose filter by the method described by Southern (1975). After immobilization of the DNA fragments the filter is used for hybridization with denatured radioactive recombinant DNA and subsequently autoradiographed to detect the DNA fragment of the parental DNA that is homologous with the cloned fragment.

After identification of the cloned DNA fragment it is often desirable to determine its orientation within the cloning vector, particularly if expression of the cloned fragment is required, and if such expression is dependent on the activity of an external promoter carried by the cloning vector. Orientation determination is normally accomplished by means of an endonuclease that cleaves the cloning vector and cloned DNA fragment at single sites that are located asymmetrically with respect to the fragment junctions. DNA fragment patterns obtained with this enzyme alone and in combination with the cloning enzyme reveal the orientation of the two DNA fragments. For example, the hybrid plasmid pSC135 was constructed from two EcoRI-generated DNA fragments. It was subsequently determined that HindIII endonuclease cleaves both EcoRI fragments at unique sites that are asymmetric with respect to the EcoRI termini. Appropriate endonuclease digestion of the hybrid plasmid revealed the specific orientation of the two component DNA fragments relative to each other (Fig. 6; Andres and Timmis, unpublished experiments).

The cloning of a particular fragment of DNA containing a function or DNA sequence of interest may permit a detailed genetic and functional analysis and therefore one of the first forms of characterization is usually the determination of a physical map based on the cleavage re-

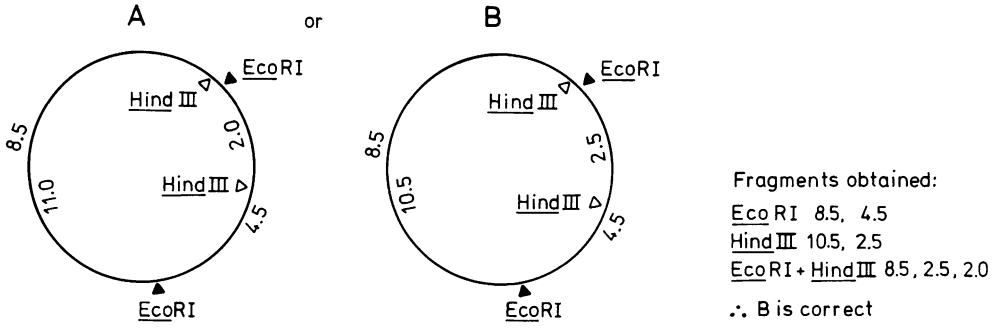


Fig. 6. Orientation of component fragments of the pSC135 plasmid

actions of one or more restriction endonucleases. Furthermore, a fine structure restriction endonuclease cleavage map is an absolute prerequisite for the sequencing of a particular region of a cloned fragment by the rapid DNA sequencing methods of Sanger and Coulson (1975) and Maxam and Gilbert (1977). Such a restriction map is frequently constructed by starting with enzymes that cleave the fragment at only a few sites and progressing to enzymes that cleave at many sites. If the cloned fragment can be cleanly excised from the cloning vehicle, it is usually prepared in quantity for subsequent analysis. If it cannot be excised cleanly then it may be necessary to construct a restriction endonuclease cleavage map of the complete recombinant molecule. In this case, it is necessary to have a map of the cloning vector in order to locate the cleavage sites specific to the cloned fragment.

Large amounts of cloned DNA fragment can be conveniently isolated by cleavage of the recombinant molecule by the cloning enzyme, followed by agarose gel electrophoresis to separate the vector from the cloned fragment. After electrophoresis the region of the gel containing the cloned fragment can be cut out, dissolved in 4 *M* potassium iodide, and the DNA fragment recovered by equilibrium centrifugation of the KI solution (Blin et al., 1975). During ultracentrifugation, KI solutions form density gradients, much like those of caesium chloride, and DNA focuses in such gradients at a position corresponding to its buoyant density. Alternatively, DNA may be recovered from the KI solution by adsorption on to a hydroxylapatite column equilibrated with a KI solution (Smith and Birnstiel, 1976).

Initial mapping of the cleavage sites of enzymes that cleave at only a few locations may be accomplished by several double-digestions involving different enzyme pairs. Orientation of the cleavage sites is usually accomplished by relating the sites to one unique asymmetric site on the molecule. Subsequent mapping with restriction enzymes that cleave the molecule at many sites is usually accomplished by one of two methods.

Method 1. Incomplete endonuclease digestions are carried out and the partial products separated by electrophoresis through an agarose gel. The fragments are then extracted as described above, digested to completion, and analyzed by a further agarose gel electrophoresis. In this way a series of overlapping fragments is analyzed and the overall fragment order may be deduced. While this method proved to be very useful in early mapping studies, the difficulty in separating large partially digested DNA fragments and isolating sufficient amounts for the second restriction endonuclease digestion make it rather cumbersome when compared with newer methods.

Method 2. Probably the simplest method for restriction mapping currently available is one described by Smith and Birnstiel (1976) that is conceptually based on the DNA sequencing method of Maxam and Gilbert (1977). This method relies on the fact that when a DNA molecule enzymatically labeled with ^{32}p at one end is partially digested with the restriction enzyme, labeled DNA fragments are obtained that form an overlapping series of molecules, all with a common labeled terminus. Analysis of the sizes of the labeled DNA fragments permits a restriction map to be constructed. A major convenience of this method is that restriction maps for a number of different restriction enzymes can be deduced simultaneously from a single gel if the labeled fragments are separately treated with several enzymes.

2. Functional Analysis

A functional analysis of a cloned DNA fragment involves the characterization of the gene products and the regulation of their synthesis. RNA species transcribed from the fragment may be analyzed and any operator-promoter sequences located within the fragment examined. Fine structure genetic analysis of the functions encoded by a recombinant molecule is most conveniently accomplished by means of complementation assays in a collection of bacterial strains that contain mutations in each step of a particular biochemical pathway. Gene products for which assays are available can be readily detected and their regulation studied. The assays may depend on the functioning of the gene product (e.g., enzyme assays), or on the structure of the product (e.g., immunodetection methods). If the host chromosome codes for a product that is similar or identical to that specified by the cloned fragment, it may be necessary to utilize host cells that contain mutations in, or delections of, the chromosomal determinants in order to be able to analyze the recombinant molecule. If no assays exist for the products coded by the cloned fragment, their detection in viable cells is rendered difficult, if not impossible, due to the high levels of synthesis of non-plasmid gene products. In such cases, it is necessary to eliminate the expression of chromosomal DNA. For this purpose, the minicell mutants of bacteria provide excellent in vivo systems for the idenfification of plasmid-coded products. Minicell mutants are defective in the cell division machinery such that the septum, instead of forming in the middle of the growing cell, thus partitioning the cell into two equal halves and resulting in equipartition of the chromosomes, forms close to one of the cell poles and results in the production of a chromosomeless minicell (Adler et al., 1967; Frazer and Curtiss, 1975). Minicells do not receive a copy of the host chromosome, but they do receive some of the plasmid copies present in the parent cell. Minicells are easily purified from cultures of minicell-producing bacteria and have been shown to be effective in vivo systems for the study of plasmid macromolecule synthesis (Roozen et al., 1971; Frazer and Curtiss, 1973; van Embden and Cohen, 1973). The use of minicells has made possible the detection of synthesis of RNA from cloned fragments of *Xenopus laevis* ribosomal RNA (Morrow et al., 1974) and from cloned mouse mitochondrial DNA (Chang et al., 1975). More recent studies have shown that some proteins can be made from cloned segments of *Drosophila* DNA in *E.coli* (Rambach and Hogness, 1977).

In vivo studies of DNA functions are best complemented by in vitro studies using purified RNA polymerase to examine transcription, and the DNA-driven in vitro protein synthesis system described by Zubay et al. (1970) to investigate translation of cloned DNA fragments. Although the purified fragment may be used for such studies, the supercoiled complete recombinant molecule seems to be a more effective substrate for transcription and translation. Current studies now in progress in a number of laboratories to characterize the transcripts and proteins determined by a number of cloning vectors will facilitate the interpretation of RNA and protein species that are obtained with recombinant DNA molecules. The availability of "minimum size" vectors that specify few or no large proteins (Inselberg, personal communication) should simplify this analysis.

The possession of a fine structure restriction map of a cloned DNA fragment can greatly simplify a functional analysis of the fragment. For example, it is possible to cleave the fragment into several subsegments with an appropriate restriction enzyme, to separate these by gel electrophoresis, and to use them to hybridize with purified radioactive RNA transcripts made from the recombinant plasmid in minicells or in vitro. Thus a transcriptional map of the cloned fragment may be

readily deduced. Furthermore, the isolated subsegments of the cloned fragment can be used to determine more precisely the locations of the genes coding for the proteins of the fragment. This may be done in two ways. Firstly, the subsegments may be used directly in an in vitro protein synthesis system. Using this approach only those fragments that contain internal promoters will be transcribed (Herrlich, personal communication). Such an experiment should therefore permit the mapping of certain genes and provide information as to the distribution of promoters within the cloned fragment (promoter distribution can be subsequently confirmed by a direct mapping analysis of the RNA polymerase binding sites). A second approach is to clone the subsegments in another cloning vector and examine the proteins made in vivo or in vitro.

One feature of DNA cloning is that specific regulation of a structural gene may be destroyed due to the separation of the gene from its normal promoter. This can sometimes provide information as to how regulation is effected. For example, certain chromosomal mutations that affect the functioning of transfer operon cistrons of the sex factor F must act on the regulation of transcription since these mutations have no effect on the same cistrons that have been cloned and are under the control of the cloning vector promoter, instead of their natural promoter (Achtman, personal communication).

IV. Cloning Systems

Although cloning systems based on host cells other than *E.coli* are currently receiving widespread attention, significant progress in the cloning technology has thus far been limited to the *E.coli* system. For this reason, most of the following discussion will focus on developments within this system.

1. Vector Requirements

Vectors suitable for cloning fragments of foreign DNA should be small, genetically and physically well defined autonomous replicons that can be easily purified in large quantities. They must code for a property that can be used to select bacteria which have taken up the DNA during transformation or transfection, and neither this selectable property nor the vector's replication functions must be inactivated when foreign DNA is inserted. The vector should contain unique cleavage sites for as many of the commonly used cloning enzymes as possible and should have properties that permit detection or selection of recombinant molecules. Preferably, the vector should contain one or more strong promoters that can be used for the transcription of inserted DNA. The following are some of the available plasmid and phage vectors analyzed according to how these different requirements are satisfied in the two types of system.

2. Plasmid Vectors

Plasmids are autonomous covalently closed circular extrachromosomal genetic elements that have been found in a wide variety of microorganisms (for reviews see Helinski, 1973; Falkow, 1975). They range in size from about 1 to 100 mega Daltons (mD) and are present in host bacteria at cellular levels of between 1 and 100 copies per chromosome. Plasmids code for a range of different properties (Table 4) that are

non-essential for normal survival of the host, although may confer certain selective advantages that contribute to the successful colonization of a particular ecological niche (e.g., plasmids that code for resistance to antibiotics and for factors that increase the overall pathogenicity of certain pathogenic organisms).

Many small plasmids are maintained at a high cellular copy number: e.g., ColE1 is normally maintained at about 20 copies per chromosome. This means that not only are they relatively easy to isolate in large quantities, but also via a gene dosage mechanism they may induce the synthesis of large amounts of plasmid-coded products (Hershfield et al., 1974; Cabello et al., 1976). Some small plasmids, unlike the host chromosome, replicate in the absence of protein synthesis such that, after incubation of plasmid-containing cells for several hours in the presence of chloramphenicol, the cellular plasmid level may reach several thousand copies per chromosome. This method for plasmid amplification is obviously very convenient, and most recently developed vehicles have this property.

As indicated above, cloning vectors must specify a property for which there is a powerful selection, and which is not inactivated as a result of DNA insertion during the cloning procedure. In addition, because it is desirable to analyze the behavior of many types of cloned fragments in a number of different genetic backgrounds, it is advantageous if the selectable function can be employed in a range of different mutant strains of the bacterial host used. It has been demonstrated that certain chromosomal biosynthesis genes (e.g., trp: Hershfield et al., 1974) can be used as selective markers for cloning vectors. Although such markers are adequate for some purposes, especially for the cloning of fragments that are not to be subjected to a genetic and functional analysis, they do restrict the use of the cloning vector to certain host bacteria. This is because the host must be chromosomally defective for the particular gene present on the vector in order for the selection procedure to function and, additionally, must either be recombination deficient or contain a deletion for the whole of the segment of chromosomal DNA contained on the plasmid, to prevent recombinational interactions of the plasmid and the chromosome.

One of the most convenient and powerful types of selection that can be used for transformation of bacteria with DNA is resistance to an antibiotic. Plasmid-specified antibiotic resistances are mediated largely by antibiotic-inactivating enzymes and are functionally distinct from those specified by the chromosome. In most cases no homology exists between chromosome and plasmid-carried antibiotic resistance genes. Cloning vectors containing antibiotic resistance markers, therefore, have greater flexibility than those containing chromosomal genes, and they may be used in almost all host strains of interest. On the other hand, because effective chemotherapy of bacterial infections is sometimes compromised by the presence of antibiotic-resistant bacteria, it is necessary to handle antibiotic-resistant laboratory strains of bacteria with more caution than is usually employed for antibiotic-sensitive strains.

Where possible, plasmid vectors should also code for a product that is easily assayed and that is inactivated by insertion of DNA fragments during cloning (i.e., that is subject to insertional inactivation). There are three levels of usefulness for insertional inactivation, according to whether inactivation permits the *detection*, the *enrichment*, or the direct *selection* of recombinant molecules. We have already described how cells carrying ColE1 molecules containing DNA fragments inserted at the EcoRI cleavage site, and pML21 molecules containing DNA fragments inserted at the HindIII cleavage site can be

Table 2. A selection of plasmid cloning vehicles[a]

Cloning site	Plasmid designation[b]	Primary selection	Insertional inactivation of	Enrichment by cycloserine or ampicillin[c]	Molecular weight x 10^{-6}	Copy number[d]	Amplification by chloramphenicol	References[f]
Eco RI	pSC101	Tc	-	-	6.0	6	-	1
	ColE1	E1-imm	E1 gene	-	4.2	18	+	2,3
	RSF2124 (ColE1-TnA)	Ap	E1 gene	-	7.4	10	+	4
	pAC181	Tc	Cm	(+, Cm)	4.2	(60)	(+)[e]	5,6
	pBR322	Ap/Tc	-	-	2.6	(60)	+	7
HindIII	pML21	E1-imm	Km	-	7.1	60	+	8
	pSC134 (ColE1-pSC101)	E1-imm	Tc	+, Tc	10.2	16	+	3,9
	pAC181	Cm	Tc	+, Tc	4.2	(60)	(+)[e]	5,6
	pBR322	Ap	Tc	+, Tc	2.6	(60)	+	7
Bam HI	pGM16 (pSC101-pML21)	Km	Tc	+, Tc	13.4	(10)	+	10
	pAC181	details as for HindIII cloning						
	pBR322	" " " " "						
Sal I	pGM706 (pSC101-RSF2124)	Ap	Tc	+, Tc	13.7	(10)	+	10
	pAC181	details as for HindIII cloning						
	pBR322	" " " " "						
Pst I	pWL7 (pML21-TnA)	Km	Ap	-	10.3	(60)	+	11
	pBR322	Tc	Ap	-	2.6	(60)	+	7

[a]Tc, tetracyline; Ap, ampicillin; Km, kanamycin; Cm, chloramphenicol; ColE1, the colicin E1 plasmid; E1, colicin E1; E1-imm, ColE1-specified immunity to colicin E1.

[b]For each enzyme, the original cloning vehicles are listed first and the newer (in most cases preferable) vectors last.

[c]The sign indicates whether or not enrichment is possible, and the symbol indicates the antibiotic required for treatment of transformant bacteria prior to the enrichment step.

[d]Values in brackets are inferred.

[e]Because the vector is resistant to chloramphenicol, amplification by this antibiotic may be ineffective. However, other methods of inhibiting protein synthesis such as amino acid starvation may be effective (e.g., valine-induced isoleucine starvation and serine hydroxamate treatment have been effective for inhibiting protein synthesis in studies of the different modes of DNA replication of filamentous phages: Timmis and Marvin, 1974; Timmis et al., 1974b).

[f]1, Cohen and Chang, 1973; 2, Hershfield et al., 1974; 3, Timmis et al., 1974a; 4, So et al., 1975; 5, Cohen et al., 1977a; 6, Chang and Cohen, in preparation; 7, Bolivar et al., 1977b; 8, Lovett and Helinski, 1976; 9, Cabello et al., 1976; 10, Hamer and Thomas, 1976; 11, W. Goebel, personal communication.

detected by their failure to produce colicin E1 and their sensitivity to kanamycin, respectively (Timmis et al., 1974a; Timmis et al., 1977a). Enrichment may be accomplished by ampicillin or cycloserine treatment if inactivation of the property leads to bacteriostasis (e.g., inactivation of the trp gene in bacteria grown without tryptophan, or inactivation of the Tc gene in bacteria grown in the presence of Tc: Bolivar et al., 1977a). The possibility of using insertional inactivation for the direct selection of recombinant molecules is currently being explored (Collins, 1977) and is based on the fact that chromosome-mediated resistance to certain antibiotics, such as nalidixic acid or rifampicin, is recessive to the sensitive allele. In cells diploid for such a gene, if the wild type allele is present on the cloning vector and the resistance allele is present on the chromosome, the cell will be antibiotic-sensitive. However, if the wild type allele on the cloning vector is inactivated by the insertion of DNA, the cell should become antibiotic-resistant. Thus in principle it should be possible to select directly bacteria carrying recombinant DNA molecules.

Are there any naturally occurring plasmids that have all the requirements of a cloning vector? Probably not. All the recently developed vectors have been constructed in vitro from segments of different plasmids. Table 2 lists some of the original and some of the more recently developed plasmid cloning vectors. The pSC101 plasmid was the first vector used (Cohen et al., 1973), specifies resistance to tetracycline, and has a single cleavage site for the EcoRI endonuclease. The use of ColE1 for EcoRI endonuclease cloning was described soon after (Hershfield et al., 1974), and whereas the selectable property of the vector, colicin E1 immunity, is not very effective due to the high frequency of generation of chromosomal mutations to colicin resistance, the high copy number of the ColE1 plasmid, and the fact that it can be amplified in chloramphenicol, are definite advantages over the pSC101 vector. However, there are some indications that, under normal conditions, cloned DNA segments which lack an internal promotor are expressed more efficiently in the pSC101 vector than in the ColE1 vector (Achtman, personal communication). This is almost certainly due to the fact that the tetracycline gene promoter located close to the EcoRI site of pSC101 is considerably more active than the corresponding colicin E1 promotor of ColE1. However, the activity of this latter promoter can be dramatically increased by mitomycin C induction, resulting in high levels of transcription/translation of cloned DNA. Such induction however does result in cell death (Selker et al., 1977).

Insertional inactivation of a vector gene by a cloned DNA fragment was first employed with the ColE1 plasmid (Timmis et al., 1974a). In this case, insertion of DNA into the EcoRI site of ColE1 prevents the synthesis of colicin E1, a property that can be readily scored. Subsequently, an ampicillin transposon was translocated to ColE1, creating RSF2124, to provide a more effective selection marker than E1 immunity (So et al., 1975). However, as noted previously, the instability of some types of recombinant molecule is a problem and several investigators have expressed doubts as to the advisability of having on cloning vectors DNA sequences that are known to be promiscuous in recombination.

The next development was to join various vectors together. Of particular importance was the fact that pSC101 has single cleavage sites for the HindIII, BamHI, and SalI restriction endonucleases close to or within the tetracycline resistance gene, whereas ColE1 is insusceptible to these enzymes. Linkage of ColE1 to pSC101 thus created a plasmid, termed pSC134 (Timmis et al., 1974a), that had all the advantages of ColE1 plus the ability to use insertional inactivation of Tc-resistance for cloning with HindIII, BamHI, and SalI. Because the pSC134

plasmid when used for cloning with these latter enzymes could only be selected on the basis of its immunity to colicin E1, recombinant plasmids were constructed from pSC101 and ColE1 derivatives that contained additional antibiotic resistances (e.g., pGM16 and pGM706; Hamer and Thomas, 1976).

Although ColE1 and pSC101 are small plasmids (4.2 and 6.0 mD respectively), the derivative vectors are all greater than 10 mD in size. Large cloning vectors are less convenient than smaller vectors because they are less efficient in ligation reactions and because their increased genetic content complicates structural and functional analysis of recombinant molecules. Subsequent efforts to improve vectors have therefore focused on size reduction. One of the latest vectors, pBR322, that is derived from ColE1 and pSC101 and an ampicillin transposition unit, has a molecular weight of 2.6 mD, a single EcoRI cleavage site, and single HindIII, BamHI, and SalI cleavage sites in or near the Tc gene derived from pSC101 that permit insertional inactivation enrichment of recombinants obtained by cloning with these last three enzymes (Bolivar et al., 1977b). In this vector, the ampicillin transposition unit has been partially deleted such that the unit no longer transposes. The vector has a high copy number and is amplifiable in chloramphenicol. In another new vector, termed pAC181, insertional inactivation can also be accomplished with EcoRI cloning (Chang and Cohen, in preparation; Cohen et al., 1977a). This vector contains the Cm resistance gene from R6-5 which had previously been shown to be cut with EcoRI (Timmis et al., 1977c).

Because the current cloning vectors already have most of the basic requirements, except perhaps the ability to permit direct selection of recombinant molecules, it is anticipated that the next generation of vehicles will not necessarily be more sophisticated but rather will be based on other plasmids that contain unique cleavage sites for enzymes less commonly used for cloning, which either do not cleave current vehicles or cleave them in essential regions. However, one recent development, the isolation or synthesis of so-called *adapter fragments* (see above), will endow a great deal of flexibility on a variety of available cloning vectors.

Having discussed the properties required for general plasmid vectors and examined some of those currently available, it is now appropriate to consider some of the particular properties required for special-purpose cloning vectors and probes (Table 3). As noted in the Introduction, certain types of cloning experiments are considered to be potentially biohazardous. Such experiments must be performed under laboratory conditions that provide a specific degree of *physical containment* (Williams Report, 1976; NIH Guidelines, 1976). In addition to physical containment, it is possible to arrange the cloning system so that it has a specific degree of inherent *biological containment*, i.e., if cells carrying recombinant plasmids were released into the environment, less than one in 10^8 recombinant-carrying cells would survive (the so-called EK2 cloning system: NIH Guidelines, 1976). Biological containment may be effected through modification of the vector or the bacterial host (see below), or both. Modifications of the vector that have been suggested are: temperature sensitivity of vector replication; host dependence of vector replication; vectors carrying restriction-modefication systems in which the modification genes are temperature-sensitive (so-called self-destroying vectors). To date, there have been no reports of substantial advances in the isolation of a biosafe plasmid cloning vector, although temperature-sensitive ColE1 plasmids have been described (Collins et al., 1977). One such plasmid is shown in Table 3.

Table 3. Special purpose plasmid vectors[a]

Purpose	Plasmid or fragment designation	Cloning site	Primary selection	Molecular weight x 10^{-6}	Copy number	Amplification by chloramphenicol	Maintenance conditions	References[b]
I. Biological containment								
	pJC307 ($ColE1_{ts}$)	Eco RI	E1-imm	4.2	10	+	32°C	1
II. Broad host range								
	RP4 (RK2)	Eco RI	Tc/Nm/Cb/Km	36	2	-	many Gm-ve bacteria	2,3
III. Low copy number								
	pKT020	Hind III	Ap	11.0	3	-	-	4
	pMF3	Eco RI Hind III	Ap	7.5	2	-	-	5
IV. High levels of transcription/translation								
	ColE1-λtrp48	Eco RI	trp	9.9	(16)	(+)	32°C	6
	pGBP120 (RSF2124-lac)	Eco RI	Ap	11.5	(16)	(+)	-	7
V. Detection of transcription initiation and termination sequences								
	pSC101 (initiation)	Hind III	Tc	6.0	6	-	-	8
	pMC81 (initiation and termination)	Hind III	Ap	16.0	(16)	+	-	9

VI. Cloning of self replication DNA fragments							
EcoRI-Ap (from pSC122)	Eco RI	Ap	4.5	-	-	-	10
EcoRI-Km (from pSC105)	Eco RI	Km	4.5	-	-	-	11
PstI-Km (from pKT029)	PstI	Km	6.5	-	-	-	12
SalI-Km (from pKT085)	SalI	Km	2.6	-	-	-	12
HaeII-Km (from pKT101)	HaeII	Km	1.0	-	-	-	12
Hind III-Tc (from pKT007)	Hind III	Tc	3.2	-	-	-	13
VII. Isolation of replication mutants of a second replicon							
ColE1	Eco RI	E1-imm	4.2	18	+	32°C in a $polA_{ts}$ host	14,15

[a]Nm, neomycin; Cb, carbenicillin; Su, sulfonamide; Sm, streptomycin; trp, tryptophane; lac, lactose; $polA_{ts}$, temperature-sensitive DNA polymerase I. Other abbreviations given in Table 2.

[b]1, Collins et al., 1977; 2, Jacob et al., 1976; 3, Meyer et al., 1975; 4, K. Timmis, unpublished experiments; 5, Manis and Kline, 1977; 6, Helinski et al., 1977; 7, Polisky et al., 1976; 8, A. Rambach, personal communication; 9, Cohen et al., 1977a; 10, Timmis et al., 1975; 11, Cohen et al., 1973; 12, Andres et al., 1978; 13, Timmis et al., 1977c; 14, Timmis et al., 1974a; 15, Cabello et al., 1976.

Although it is usually most convenient to clone DNA fragments using *E.coli* systems, it is often desired to study the function and expression of cloned DNA fragments in another host. For this purpose it is necessary that the cloning vector can propagate itself in the alternative host. This may be the case if the replication functions of the vector do not have a very limited host specificity, or if the vector is composed of two replicons, one of which can function in *E.coli* and the other of which can function in the alternate host. The latter situation is inherently the most flexible, and the first in vitro construction of a bifunctional replicon demonstrated its potential feasibility (Timmis et al., 1974a). In this study it was shown that a hybrid replicon composed of ColE1 and pSC101 could be propagated by either replication system according to the host functions that were available. More recently, plasmid replicons from less related hosts have been linked in vitro although, because of the lack of suitable transformation systems, replication in the alternative host has so far not been demonstrated (Chang et al., 1975; Hollenberg et al., 1976). Less flexible but nevertheless of considerable value are the naturally occurring broad host range plasmids, such as RP4 and RK2, that belong to the P incompatibility group (Jacob and Grinter, 1975). These plasmids can transfer among, and be propagated in, a wide range of gram-negative bacteria. They have high molecular weights (about 40 mD) however and are proving to be difficult to reduce in size (Figurski et al., 1976; Helinski et al., 1977). Such plasmids nevertheless have been used as cloning vectors (Meyer et al., 1975; Jacob et al., 1976) and more convenient derivatives are expected to be generated in the near future.

When large quantities of cloned DNA fragment are required, a plasmid that has a high copy number and that is amplifiable with chloramphenicol is the usual choice for a cloning vector. When large quantities of cloned DNA fragment product are required, it is advantageous to have both a high fragment cellular copy level (gene dosage) and a high frequency of fragment transcription. Recently considerable effort has been directed toward the construction of high copy number cloning vehicles that contain efficient promoters proximal to their cloning site (s). By inserting into the RSF2124 vector a fragment from lambda plac5 containing the lac operator, promoter, and betagalactosidase gene, Polisky et al., (1976) were able to derive a cloning vector, pGB120, in which the level of transcription of the cloned DNA fragment increased 9-fold after lac operon induction. A similar rationale is the basis for current attempts to construct a ColE1-λtrp vector that enables utilization of the λ leftward promoter plus the antiterminator function of the λ *N* gene product for inducible high level expression of cloned DNA (Helinski et al., 1977). Apparently transcription of DNA inserted at the EcoRI site of pSC101 is controlled by the promoter of the tetracycline resistance gene. Since tetracycline resistance of pSC101 is inducible (Cabello et al., 1976), it should be possible to increase transcription of cloned DNA fragments by induction with tetracycline (see however Tait et al., 1977).

For studies of function or regulation involving a gene product that is normally present to the extent of a few molecules per cell, and that may be lethal for the cell if synthesized in larger amounts, a cloning vector with a low copy number is required. Two such low copy vectors currently available, pKT020 and pMF3 (Manis and Kline, 1977; Timmis, unpublished experiments; see Table 3), contain the replication functions of the low copy large plasmids R6-5 and F'lac respectively, and were derived in a similar fashion from the mini R6-5 (pSC135) and mini F'lac (pSC138) constructed by Timmis et al. (1975).

Recently, it has become possible to test DNA fragments for the presence of transcription initiation and termination sequences. Because the

HindIII site is located within or close to the promoter of the tetracycline resistance gene of plasmid pSC101, insertion of DNA at this site ordinarily inactivates the expression of tetracycline resistance. However, if the cloned fragment contains a promoter sequence in the correct orientation, tetracycline resistance is restored (Rambach, personal communication). A more sophisticated vector than pSC101 is the pMC81 plasmid which contains the lac genes of *E.coli* under control of the ara operon promoter (Casadaban and Cohen, in preparation; Cohen et al., 1977a). Separating the ara promoter from the lac genes is a DNA fragment that contains a HindIII cleavage site. In this system, expression of lac requires induction with arabinose. Insertion at the HindIII site of this plasmid of a DNA fragment carrying a promoter region results in lac expression in the absence of arabinose induction. Alternatively, insertion of a DNA fragment with a transcription termination signal prevents arabinose-induced expression of lac. Thus far, the pMC81 plasmid has been employed for the isolation and study of promoter regions of complex plasmid replicons such as R6-5 (M. Casadaban, personal communication).

It was recently demonstrated for two large plasmids that essential plasmid-specific replication functions are clustered together in the region of the plasmid origin of replication (Timmis et al., 1975). The main difficulty associated with the cloning of DNA fragments that carry the replication functions of a particular replicon is the inability to select such fragments *directly*. These fragments could however be selected *indirectly* by means of a specific probe that contains a selectable property, resistance to ampicillin (Ap), and that is incapable of self-replication. In vitro linkage of the probe fragment to a DNA fragment capable of self-replication leads to the formation of a replicon that can be selected in transformation. The original probe fragment was an EcoRI-generated DNA fragment from a *Staphylococcus* penicillin resistance plasmid, but more recently a variety of probes from the antibiotic resistance plasmid R6 have been generated (Table 3; Timmis et al., 1977b; Andres et al., 1978).

When the cloning and properties of the ColE1-pSC101 hybrid replicon were described, it was pointed out that such a replicon would allow the isolation of mutants that are absolutely defective in replication (that is, non-conditional mutants having mutations in essential replication genes, or in DNA sequences essential for replication, for instance, the origin of replication) of one of the component plasmids (Cabello et al., 1976). This was due to two features of the recombinant replicon: firstly, the inability of one replicon to function would not prevent perpetuation of the plasmid, and secondly, the two component replicons are dissimilar and the replication of each one could be individually inhibited (low levels of DNA polymerase I prevent replication of ColE1; chloramphenicol treatment prevents the replication of pSC101). Thus, defective mutants of one component are detected by the inability of the plasmid to replicate under conditions where the second component is temporarily inhibited. ColE1 is a good candidate as one of the components of a bifunctional replicon because when this plasmid is resident in $polA_{ts}$ host cells its replication can be halted by a temperature shift (see Table 3). In principle, absolute defective replication mutants of a replicon that can replicate with low levels of DNA polymerase I can be generated after linkage to ColE1. Recently, absolute defective mutants (in this case, origin of replication mutants) of λdv have been isolated by this method (G. Hobom, personal communication).

3. Bacteriophage Vectors

Parallel to the development of a series of plasmid cloning vectors there was a development of a series of lambda phage cloning vectors. Because λ contains multiple target sites for the EcoRI and HindIII restriction endonucleases, some of these sites had to be removed by mutation or in vitro construction, before the λ genomes could be suitable as cloning vectors. Phages currently available for cloning have genomes that either are reduced in size and contain a single EcoRI site, or contain two EcoRI sites bracketing a replaceable DNA fragment (Murray and Murray, 1974; Murray and Murray, 1975; Rambach and Tiollais, 1974; Thomas et al., 1974). One feature of the lambda system is that only λ DNA molecules within a certain size range can be packaged into mature phage particles. This feature can be an advantage and a disadvantage of λ vectors. The disadvantage is that only DNA fragments within certain size ranges appropriate for the different λ vectors can be cloned. The advantage is that in the case of λ vectors containing two EcoRI cleavage sites, if the replaceable DNA fragment is removed prior to cloning, the remaining essential fragments cannot form viable lambda unless ligated to a foreign DNA fragment, such that the overall size of the genome is large enough for packaging into phage particles. This procedure provides a powerful selection for recombinant λ molecules.

Finally it should be mentioned that another type of phage vector for cloning in *E.coli* has recently been described. Filamentous phages are cylindrical phages without morphological heads or tails that contain single-stranded circular DNA. They have been extensively characterized genetically and biochemically. Filamentous phages frequently occur as dimers and trimers and thus apparently do not have precisely defined size limits for DNA that can be packaged into phage particles. Filamentous phages fd and M13 have been modified so that they now contain in non-essential regions of the genome, DNA sequences containing appropriate restriction endonuclease cleavage sites that can be used for cloning (H. Shaller, personal communication; P.H. Hofschneider, personal communication). Because DNA isolated from the virion is single-stranded, the vector DNA per se is the double-stranded circular RFI form that is isolated from phage-infected bacteria.

For the majority of cloning experiments involving EcoRI or HindIII-generated DNA fragments, plasmid or λ genomes are equally suitable as vectors and the decision to use one or other system is a matter of personal choice. However, in some cases, one particular cloning system may be more appropriate than the other and therefore we will try to identify salient characteristics of each system that may influence the decision. Firstly, the λ genome is a large molecule and contains a large number of targets for most restriction endonucleases (see review by Roberts, 1976a, and Table 1). The manipulation of these targets to produce suitable vector genomes is difficult for enzymes that are not of *E.coli* origin. Thus at present, the rapid development of new cloning vectors for a wide range of restriction enzymes is limited to plasmids. Secondly, it is sometimes necessary to clone a DNA fragment with a molecular weight in excess of 11 mD (e.g., cloning of the F factor genes required for pilus synthesis; Skurray et al., 1976), currently the upper limit of a DNA fragment to be cloned in λ. In principle, DNA fragments of a much larger size can be incorporated into plasmids, and fragments with molecular weights of 40 mD have been cloned in pSC101 (Timmis, unpublished experiments). Thirdly, λ is the most intensively investigated and best characterized of all genomes, and the extensive knowledge of its genetics and biochemistry can be very effectively utilized for a variety of purposes in the gene cloning technology. Although much simpler, the small plasmids are genetically and biochemically poorly understood. Probably one of the potential advantages of

λ that is immediately identified is the combination of the powerful leftward promoter P_L and the N gene antiterminator product. These two properties should in principle allow efficient transcription of cloned DNA sequences due to "read-through" from the P_L promoter. Another important advantage of extensive knowledge of λ genetics is the construction of "crippled" λ derivatives suitable as high containment vectors. Thus λ cloning vectors now exist that contain several amber-suppressible mutations in phage assembly genes, that are defective in lysogeny and integration, and that are lysis-defective (Enquist et al., 1976; Tiemeier et al., 1976; Donoghue and Sharp, in press, 1977; Blattner, personal communication). This last property improves phage yields and additionally facilitates the physical containment of the phage. In conclusion, it should be stressed that the use of adaptor fragments, as described above, will increase considerably the flexibility of current λ vectors, and the cloning of λ DNA sequences in plasmid vectors will introduce into plasmid vectors many of the advantages of the λ system. Indeed, the λ*dv* plasmid would appear to have many of the advantages of both systems (Matsubara et al., 1975; Mukai et al., 1976).

4. Bacterial Hosts

Because the initial cloning experiments were performed using *E.coli* plasmids as cloning vectors, the host organism employed was *E.coli*. For those experiments that involved the cloning of *E.coli* DNA, the host strain used was the K-12 derivative C600. The restriction/modification negative mutant of this strain was subsequently used for experiments involving the cloning of non-*E.coli* DNA. Currently a variety of strains are used, many of them being recombination-deficient in order to reduce recombinational activity of the cloned fragment. Recently certain crippled *E.coli* strains that require diamino pimelic acid and thymine and that can grow only under very stringent laboratory conditions have been developed for the biological containment of recombinant DNA molecules judged to be potentially biohazardous (Curtiss, 1976).

At present other bacterial systems potentially suitable for recombinant DNA experiments are receiving considerable attention, in particular *Bacillus subtilis* (Ehrlich et al., 1976; Lovett et al., 1976; Duncan et al., 1977; Graham et al., 1977) and some *Pseudomonas* strains (Chakrabarty, 1976).

D. Analysis of Plasmid Structure and Function

Plasmids are autonomous extrachromosomal elements that code for functions generally not essential for the survival of the host cell. They are very important to society in general and to molecular biologists in particular. Of clinical importance are plasmid-specified multiple resistance to antibiotics, enterotoxins, hemolysins, and other factors that contribute to the pathogenicity of pathogenic bacteria and that are appearing increasingly in clinical isolates. Certain plasmids are capable of transfer among bacteria by the process of conjugation. Plasmid-specified functions also appear to be essential for the pathogenicity of *Agrobacterium tumefaciens*, a bacterium that causes tumor formation in plants. On the other hand, some plasmids code for functions of potential benefit to society, for example, the ability to catabolize a variety of complex organic compounds such as toluene and camphor. It is already proposed that oil spills may be combated with bacteria containing such plasmids, and even that single cell protein might be made

from oil wastes (Chakrabarty, 1976). For the molecular biologist, plasmids serve as excellent model systems for the study of chromosomal behavior, in particular its duplication since, like the chromosome, their replication is tightly coupled to cell division but, unlike the chromosome, they are small and easily isolated intact for various forms of analysis. Plasmids have the advantage over phage model systems that they have an entirely intracellular life cycle and their behavior does not appear to be subject to the influence of any morphogenesis signals.

Table 4. Some plasmid-coded properties

Properties	Examples
Resistance to antibiotics	R1, R6
Resistance to heavy metals (Cd^{++}, Hg^{++})	pI258, R6
Enterotoxin	Ent
Virulence factors, hemolysin, K88 antigens	ColV, Hly
Fertility	F, R1, ColI
Bacteriocins	ColE1, CloDF13
Restriction/modification	R1 plasmid of RY13
Resistance to UV, irradiation	ColIb, R46
Tumorogenicity in plants	Ti
Metabolism of camphor, toluene, etc.	Cam, Tol

Given that plasmids are extremely important entities, it is at first sight surprizing that their basic functions, with the exception of fertility, are still poorly characterized genetically. The main problem in plasmid genetics is the difficulty of performing genetic complementation tests, which depend on creating cells diploid for the allele under investigation. This difficulty is due in part to the phenomenon of plasmid incompatibility, which prevents the coexistence in a single cell of two closely related plasmids.

Since the development of the DNA cloning technology there have been dramatic advances in the analysis of basic plasmid functions, such as replication and fertility. There are three main reasons for these advances. Firstly, DNA cloning makes possible the sequestering of genes derived from one plasmid on to another of different incompatibility type, thus permitting the construction of cells diploid for genes under investigation. Secondly, this technology has allowed the development of conceptually novel approaches to the analysis of DNA structure and function. Lastly, the specific use of plasmids as cloning vectors has focused interest on a wide range of plasmid properties.

I. Plasmid DNA Replication

Although plasmids consist of covalently closed circular molecules of DNA, in all cases thus far examined, replication is initiated at one (in some cases two) point on the molecule, the replication origin. Synthesis of progeny DNA strands upon the parental template strands proceeds unidirectionally or bi-directionally away from the origin causing

the parental strands progressively to unwind (Inselburg, 1974; Lovett et al., 1974; Tomizawa et al., 1974; Cabello et al., 1976; Crosa et al., 1976). When the parental strands have been unwound completely, the newly formed daughter molecules separate and are converted to covalently closed circles lacking superhelical turns. Superhelical turns are then introduced, probably in step-wise fashion, by the action of the recently described DNA gyrase enzyme (Crosa et al., 1976; Gellert et al., 1976; Timmis et al., 1976).

Although available data on plasmid DNA synthesis requirements are incomplete, they suggest that plasmid replication is largely dependent upon host-coded DNA synthesis functions. Thus far, the products of the dnaC (initiation of replication), and the dnaB and dnaG genes (progeny chain elongation) have been shown to be required for plasmid replication, as have DNA polymerase III, RNA polymerase, and the DNA gyrase enzyme (Clewell et al., 1972; Goebel, 1973; Collins et al., 1975; Goebel and Bonewald, 1975; Staudenbauer, 1975; Gellert et al., 1976a,b). The ColE1 plasmid, but not some larger plasmids, requires high cellular levels of DNA polymerase I (Kingsbury and Helinski, 1970) and may also be dependent on the initiation product coded by the dnaA gene (Goebel, 1973). On the other hand, some of the large plasmids are dependent on a chloramphenicol-sensitive protein that either is not required for ColE1 replication or is present in a more stable form in the ColE1 *replisome*. The role of several other host-specified DNA replication products in plasmid DNA synthesis has not been investigated in detail.

Although plasmids are dependent on a number of host functions for their replication, they do retain overall control of this process, as evidenced by the following observations. Firstly, each plasmid is maintained at a constant cellular level during exponential growth of host bacteria, which indicates the existence of a precise mechanism for controlling the frequency of initiation of plasmid replication. Plasmid mutants (copy number mutants) have been described in which this mechanism is disturbed, resulting in an alteration in the cellular plasmid concentration, i.e., the plasmid copy number (Nordström et al., 1972). Thus a specific plasmid function is responsible, at least in part, for the control of initiation of plasmid replication. Secondly, whereas two or more unrelated plasmids can stably coexist in the same cell, two different but closely related plasmids cannot: such plasmids are termed incompatible. One plausible explanation for this phenomenon is that the mechanism responsible for restricting the frequency of initiation of replication of one plasmid will also restrict the initiation of replication of an incompatible plasmid resident in the same cell. This would then tend to make coexistence of the two plasmids unstable. Supporting the notion that the plasmid function that regulates plasmid copy number is involved in the incompatibility phenomenon is the finding that some copy number mutants have altered incompatibility properties (Uhlin and Nordström, 1975). Thirdly, not only do many plasmids seem to be independent of the host dnaA initiation product, but also their presence in a dnaA-defective host bacterium can allow the host chromosome to replicate under certain circumstances (the phenomenon of suppressive integration: Nishimura et al., 1971). Thus, such plasmids possibly code for a dnaA-like initiation product or remove the need for the dnaA product. Fourthly, supercoiled covalently closed circular plasmid DNA may be isolated from plasmid-containing cells in two forms: as a protein-free molecule and as a protein-DNA complex (Clewell and Helinski, 1969). Because treatment of the protein-DNA complex with agents known to affect protein conformation, such as SDS, causes the supercoiled DNA to *relax* due to the introduction of a nick (subsequently shown to be near the origin of replication: Lovett et al., 1975), into a specific polynucleotide strand it was postulated that these so-called *relaxation complexes* are involved in the initia-

tion of plasmid DNA replication (Helinski and Clewell, 1971) Consistent with this suggestion was the finding that some plasmid mutants that are temperature-sensitive for replication have temperature-sensitive relaxation complexes (Collins et al., 1977). Whether these four different plasmid-specified phenotypes reflect the activity of four different plasmid genes or whether some phenotypes represent different aspects of a single gene, and whether other plasmid-specified replication functions exist, remains to be determined.

The initiation of chromosomal DNA replication is one of the most fundamental events in the bacterial life cycle. The frequency of initiation determines the overall rate of DNA synthesis, the amount of DNA per cell, and the frequency of cell division. Elucidation of the mechanism of regulation of initiation of DNA synthesis is therefore of paramount importance for an understanding of these basic cellular processes. Several models for the regulation of initiation have been proposed. Models for positive control suggested that the time of initiation could be determined by the position on the genome of a gene coding for an initiation protein, or that initiation occurs when an initiation protein is accumulated above a threshold concentration. Models of negative control of initiation proposed the involvement of an inhibitor of initiation. Such models envisage the initiation of DNA replication occurring when the inhibitor is diluted below a specific threshold concentration by the increase in cell volume during cellular growth. Because DNA replication begins by the synthesis of a primer RNA at the replication origin, regulation of initiation of replication may well take place at the level of synthesis of *origin* RNA. Messer has recently presented evidence that the synthesis of the *origin* RNA of the *E.coli* chromosome may indeed be negatively regulated (Messer et al., 1975). The difficulty of handling DNA molecules as large as the *E.coli* chromosome however has thus far prevented a definitive molecular analysis of this system. Even if the negative control of *origin* RNA synthesis is confirmed, this information alone does not demonstrate conclusively that regulation of DNA replication is effected by a negatively acting mechanism and it will still be necessary to characterise the mechanism that exerts overall control of the process of initiation.

Plasmids are convenient tools for the study of DNA mechanisms at the molecular level, and bacause their replication seems to be regulated in a fashion similar to that of the bacterial chromosome, they are ideal models for investigations of the control of DNA replication. Although DNA synthesis is usually initiated at single specific locations on a molecule, in the past few years there have been a number of reports describing replicons that contain and may utilize multiple origins of replication (for example plasmids RSF1040 and NR1, and *Drosophila* chromosomal DNA: (Kriegstein and Hogness, 1974; Crosa et al., 1976; Perlman and Rownd, 1976). The additional complexity of regulation of initiation of DNA synthesis in multiorigin replicons poses problems of great interest. Multiorigin plasmids therefore constitute attractive model systems for the study of regulation of origin usage.

DNA cloning methods have been and are currently being used to:

a) locate the replication origins of large multiorigin plasmids;
b) analyze the mode of replication of these large plasmids;
c) identify and analyze plasmid-specific replication genes;
d) analyze the spatial arrangement of replication genes in relation to the replication origin (s);
e) identify *silent* replication origins and functions not ordinarily used;
f) test certain aspects of models for the control or initiation of DNA replication;

g) obtain plasmid mutants that are absolutely defective in DNA replication.

1. In Vitro Reduction of Replicon Size

Large replicons are difficult to analyze genetically and to characterize physically as intact structures, and the deletion of DNA sequences not carrying replication functions greatly facilitates these types of analysis. Furthermore, in multiorigin replicons, the use of individual origins within the molecules often cannot be distinguished because of the paucity of information available on the replication properties of the individual components. A characterization of the component replication systems on a multiorigin plasmid is considerably simplified by their physical separation and their individual analysis. The deletion of DNA sequences not responsible for replication, and the separation of component replication systems of multiorigin replicons can, in principle, be conveniently accomplished by DNA cloning methods. Two types of cloning approach can be employed according to the two types of DNA fragment to be cloned. One type of fragment must contain a replication origin and all the essential plasmid replication genes; such a DNA fragment, if circularized, should be capable of autonomous replication. The other type of DNA fragment may contain a replication origin or some replication genes or both, but in any case lacks at least one essential replication component and hence is incapable of self-replication. An important consideration in the cloning of a DNA fragment that is capable of self-replication is the distribution of essential replication genes relative to the homologous replication origin. It has been shown that such genes are clustered about the origin in the case of the bacteriophage λ (Stevens et al., 1970), but that they are randomly distributed around the genome in the case of the *E.coli* chromosome (Gross, 1972).

In order to clone fragments of DNA containing individual replication origins from a multiorigin plasmid and to determine whether a replication origin and its essential plasmid-coded replication genes are clustered together on a small segment of DNA, it was considered whether it would be possible to clone in vitro from the large plasmids R6-5 (molecular weight 65 x 10^6) and F'lac (molecular weight 90 x 10^6) individual EcoRI restriction endonuclease-generated fragments capable of self-replication. To isolate such fragments, an EcoRI-generated DNA fragment that codes for ampicillin resistance but which is incapable of self-replication was employed as a selection probe (see Table 3). Ligation of this fragment to the unfractionated EcoRI fragments R6-5 and F'lac and subsequent transformation of the ligation mixture into *E.coli* permitted the isolation of Ap-resistant clones of *E.coli*. Because the Ap fragment is incapable of self-replication, Ap-resistant transformants can be obtained only if this fragment becomes a functional Ap-resistance replicon by linkage to EcoRI fragments of R6-5 of F'lac that are capable of self-replication (see Fig. 7). Figure 8 shows that by this method it was possible to isolate miniplasmids that are composed of the Ap-EcoRI fragment and either the second largest EcoRI fragment of R6-5 (molecular weight 8.5 x 10^6) or the sixth largest fragment of F'lac (molecular weight 6 x 10^6: Timmis et al., 1975). This result indicates that for at least one replication origin of R6-5 and one origin of F'lac, the essential homologous plasmid-specified replication genes are clustered in a very small region of the plasmid in the neighborhood of the replication origin. Such a sequence of DNA was termed a *replication region*. An identical replication region fragment from F'lac has also been isolated using as a cloning probe a kanamycin resistance DNA fragment (Lovett and Helinski, 1976; Skurray et al., 1976). Heteroduplex analysis of the miniplasmids (e.g., see Fig. 8)

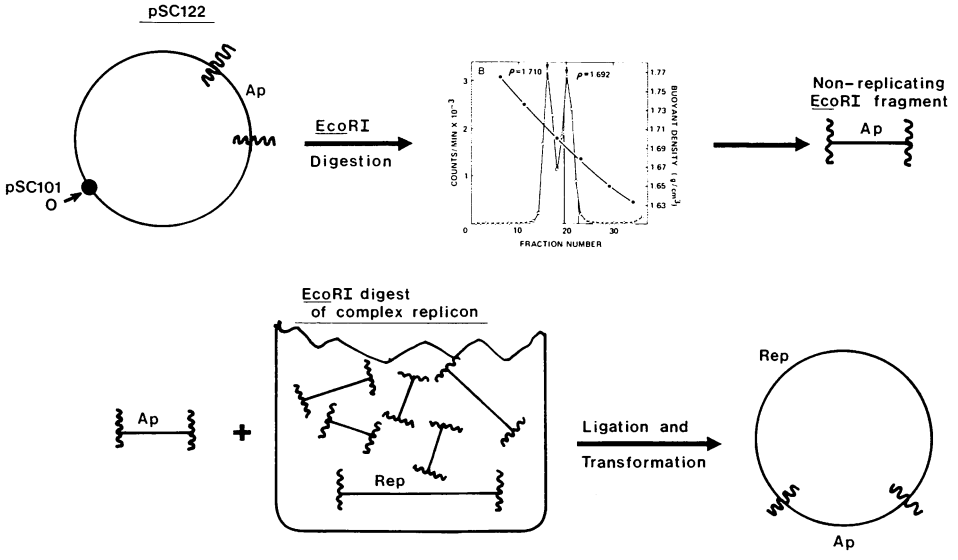


Fig. 7. Isolation of replication regions of complex plasmid genomes. In the experiment shown, the pSC122 plasmid, which carries a non-replicating Ap-resistance segment, was cleaved by the EcoRI restriction endonuclease, and the Ap resistance "probe" fragment was separated from its replication region. The probe was then added to a mixture of DNA fragments from an EcoRI cleaved complex replicon, and ligation and transformation were carried out. Since the probe fragment is incapable of replication its propagation in transformants requires linkage to a DNA segment carrying replication functions

has confirmed that the cloned DNA fragments are specifically derived from R6-5 and F'lac and furthermore locate the cloned fragment on the physical maps of the parent plasmids. The mini R6-5 contains the EcoRI fragment of R6-5, having EcoRI termini with coordinates 87 and 1.0 kb on the R6-5 physical map. This means that the replication origin and functions cloned in the mini R6-5 are those of the *resistance transfer factor* (RTF) component segment of the R6-5 plasmid (Cohen and Miller, 1970). The mini F'lac contains the EcoRI fragment of F that has termini with coordinates of 40.3 and 49.3 kb on the F physical map (Guyer et al., 1976). Table 5 compares the replication-related properties of the miniplasmids with their parent replicons. In all properties examined (copy number, incompatibility reactions, ability to suppress the host dnaA defect), the miniplasmids behaved in a manner identical to that of their parent plasmids. It was therefore concluded that the miniplasmids not only contain the genes for copy number regulation, incompatibility, and a dnaA-like activity, but also that the replication region isolated in each type of miniplasmid is the one that is functionally predomonant in determining the replication phenotype of the parent plasmid. Recently it was demonstrated that all functions essential for regulated autonomous replication of R6-5 are clustered in a 1.8×10^6 Dalton DNA segment within EcoRI fragment 2 (Andres et al., 1978).

The miniplasmids contain cloned fragments of DNA that are greater than one order of magnitude smaller than the parent plasmids, and are therefore very convenient molecules for the analysis of replication functions and origins of the parent plasmids. They are also convenient sources of DNA for the further cloning of individual plasmid replication genes.

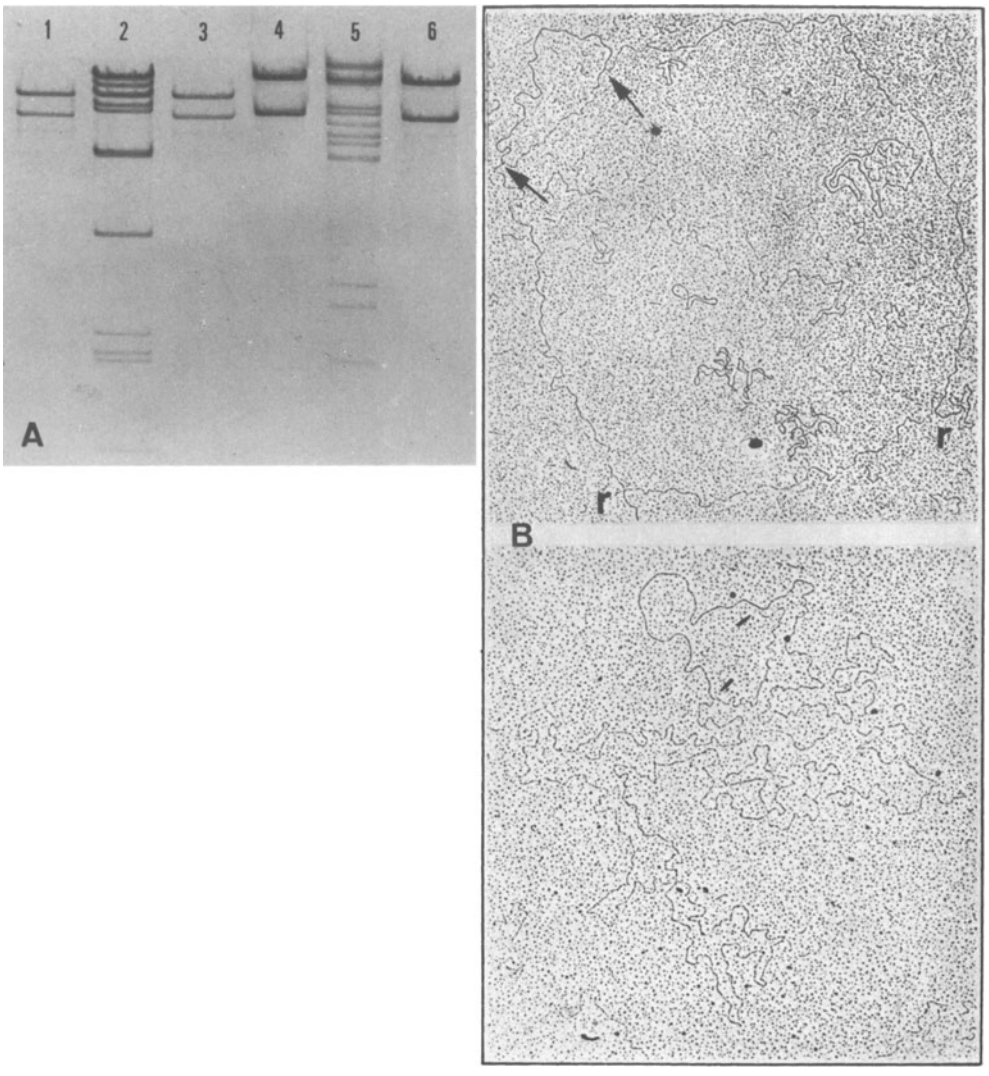


Fig. 8A and B. Electrophoretic and heteroduplex analysis of mini R6-5 and mini F'lac. (A) Covalently closed circular plasmid DNA preparations were cleaved to completion with EcoRI endonuclease and subjected to electrophoresis through a borate-buffered 0.8% agarose slab gel as described by Timmis et al. (1977a). 1 and 3 are two mini F'lac plasmids; 2 is the F'lac plasmid; 4 and 6 are two mini R6-5 plasmids; 5 is the R6-5 plasmid. (B) Heteroduplexes between R6-5 and the mini R6-5, pSC135 (upper heteroduplex), and F'lac and the mini F'lac, pSC138 (lower heteroduplex), were prepared as described by Davis et al. (1971). Regions of sequence homology are indicated by the arrows. Direct physical mapping of the DNA fragment cloned in pSC135 is possible from the R6-5/pSC135 heteroduplex due to the presence of reference features on the R6-5 molecule: the two inverted repeat structures indicated by the symbol r

The physical separation of replication genes will demonstrate which phenotypes derive from different genes and which derive from the same gene.

Table 5. Properties of mini plasmids cloned from R6-5 and F'lac

Plasmid	Molecular weight x 10^6	Copy number	Incompatibility type	Suppressive integration of $dnaA_{ts}$ mutants	Sensitivity to acridine orange
R6-5	65	2-3	F II	+	-
mini R6-5 (pSC135)	13	2-3	F II	+	-
F'lac	90	0.5-0.8	F I	+	+
mini F'lac (pSC138)	10.5	0.6-1.0	F I	+	+

The ability to clone miniplasmids from R6-5 and F'lac indicates, at least in these plasmids, that replication genes are clustered around a replication origin, as has been shown for the bacterial phage λ. A variety of probes for the EcoRI and HindIII cloning of self-replicating DNA fragments from R6-5 have been used (Timmis et al., 1977c). In all these experiments only one type of replication region fragment capable of autonomous replication could be isolated. Since R6-5, which is very closely related to plasmid NR1, is presumed to contain at least two *structural* replication origins, it may be that the replication genes essential for the *functioning* of a second origin are not entirely contained on the restriction endonuclease fragments carrying the second origin, or are not even clustered at all around this particular origin (Timmis et al., 1977b). In this connection it should be noted that an attempt to isolate a small DNA fragment capable of autonomous replication from the 40 mD RK2 plasmid was unsuccessful (Helinski et al., 1977) and a subsequent cloning analysis of the DNA replication functions and origin of this plasmid indicated a lack of clustering of essential replication genes and replication origin (Figurski et al., 1976). Furthermore, Chandler et al (1977) were able to generate in vivo miniplasmids that contain the R-determinant part of plasmid R100.1 from dnaA mutant bacteria integratively suppressed by this plasmid. The authors could not however observe replication of the miniplasmids in cells other than those in which they were generated, indicating that these plasmids lack essential replication functions specified by the parent plasmid.

In order to clone from plasmid R6-5 other restriction endonuclease-generated DNA fragments that carry replication functions or origins but that are incapable of self-replication, it was necessary to use a normal cloning vehicle that could replicate the inserted DNA fragments. For this purpose we used ColE1 for cloning EcoRI-generated DNA fragments and pML21 for cloning HindIII-generated fragments (see Table 2). Table 7 lists the DNA fragments that were cloned and their phenotypes. A specific advantage of the ColE1-based cloning vehicles is that when they are resident in a $polA_{ts}$ host their replication activity can be switched off by a temperature shift. Thus one method to test for the presence of replication functions on a DNA fragment cloned on a ColE1-type vector is to determine whether temperature-resistant replication of the hybrid molecule occurs in a $polA_{ts}$ host. By this method, EcoRI fragments 1 and 4 were shown to carry functions that permitted replication under such conditions (Timmis et al., 1977; Timmis et al., 1977c). The nature of these functions is not yet established, nor indeed is it known whether such functions simply suppress the temperature-sensitive polA mutation or whether they provide an alternate replication mecha-

nism. Studies are currently in progress to analyze the nature of the replication of these hybrid molecules in a polA$_{ts}$ host at high temperature to distinguish the above possibilities.

In vitro or in vivo deletion of DNA segments that are not required for replication of the plasmid can provide useful information on the essentiality of certain genes that have been implicated in plasmid replication. Although it has been suggested that plasmid DNA-relaxation complexes play a role in plasmid replication, it has been shown that ColE1 can replicate in cell-free extracts prepared from plasmid-negative bacteria, i.e., under conditions in which the plasmid-specified component of the relaxation complex is not available (Tomizawa et al., 1975), suggesting that if the complex is involved in plasmid replication, it is not an absolute requirement for such replication. This implication was confirmed by the isolation of mini ColE1 plasmids. Such plasmids have a molecular weight of about 2 mD, code for colicin E1 immunity but not colicin E1 synthesis, and are generated spontaneously in vivo from ColE1 recombinant molecules. Mini ColE1 plasmids cannot be isolated as relaxation complexes, even though they carry the DNA sequence at which the relaxation event occurs. However, loss of the relaxation complex is correlated with the loss of another plasmid-specific property: its ability to be conjugally transferred by a sex factor. Furthermore, these mini ColE1 plasmids have a very high copy number, indicating that the copy number control gene has also been deleted (Hershfield et al., 1976; Avni et al., 1977). In vitro manipulation of the ColE1 plasmid has resulted in a further reduction in its size to about 700 nucleotide pairs without loss of its ability to replicate (R.L. Rodriguez, personal communication; Inselberg, personal communication).

Miniplasmids have also been isolated in vivo from copy number mutants of the large antibiotic resistance plasmids R1 and NR1 (Goebel and Bonewald, 1975; Mickel and Bauer, 1976). With these derivatives, it also appears that the copy number regulating factor and the relaxation complex are dispensible. However, in addition, some of them fail to show incompatibility reactions with their parent plasmids, and hence it appears that the incompatibility function is dispensible (Boidol et al., 1977). Thus, the overall picture that emerges is that the minimal plasmid replication requirements are a DNA replication origin (but see below) and perhaps one gene product that has yet to be identified. However, it is apparent that several other products are necessary for the type of *controlled* DNA replication that is observed in vivo with non-mutant plasmids.

2. Characterization of Origins of Replication

The electron microscopic mapping of replication origins of large molecules has been hampered by their physical fragility and their lack of convenient reference points. Denaturation mapping has been used to examine replicating molecules of NR1 and R100 (Perlman and Rownd, 1976; L. Silver, M. Chandler, and L. Caro, personal communication) but the imprecision inherent in comparing denatured molecules restricts the accuracy and hence the usefulness of this particular method. Restriction endonuclease cleavage of replicating molecules of large plasmids has also been utilized for origin mapping (Warren et al., 1977), but the large number of DNA fragments thereby generated, the similarity in size of some fragments, and ambiguities in fragment orientation in the parent molecule, may in some instances lead to difficulties in the interpretation of origin data. In contrast, small molecules are readily analyzed because of their ease of handling and because there is normally a restriction endonuclease available that cleaves the molecule

at a unique location, thus providing a convenient reference point for electron microscopic analyses.

Miniplasmids constructed in vitro such as pSC135, are not only of a convenient size but also contain appropriate reference points, the two restriction endonuclease cleavage sites that were utilized in their construction. Replicating molecules isolated by conventional methods are mounted for electron microscopy prior to cleavage with a restriction endonuclease, in order to determine whether more than one replication origin per molecule is used, and after cleavage in order to locate the origin(s) relative to the endonuclease cleavage sites (for example, see Fig. 10). Using these methods, the replication origin of the mini F plasmid, pML31, was shown to be located 1.6 mD from one EcoRI terminus, or at 42.6 kb on the F physical map. The mode of replication of mini F was found to be bidirectional (Eichenlaub et al., 1977). Interestingly, the proposed location of the replication origin of mini F is 3-5 kb distance from the proposed location of the FI incompatibility gene (Palchaudhury and Maas, 1977). Recent data indicate that the origin of the mini R6-5 plasmid, pSC135, is located 1.5 mD from the EcoRI terminus that is proximal to the R-determinant segment of R6-5 (Timmis at al., in press, 1977d), and close to, but distinguishable from, the FII incompatibility gene (Andres et al., 1978; Timmis et al., 1978).

In the discussion above as to what constitute the essential plasmid-specified requirements for plasmid replication, we concluded that an origin of replication and perhaps a plasmid-encoded function are necessary. However, the considerable amount of origin mapping that has been performed in the past few years would indicate that the origin normally used in replication may also be dispensible, provided another DNA sequence that can function as an origin is present on the plasmid. Manis and Kline (1977) were able to delete from the mini F plasmid pML31 the small BamHI fragment that Eichenlaub et al. (1977) have shown to contain the replication origin normally used in the replication of pML31. The deletion derivative, pMF21, apparently replicates normally. That other DNA sequences, capable of acting as replication origins, are present in plasmids is suggested by the recent work of Warren et al. (1977), which shows that several different DNA sequences in plasmid NR1 are capable of acting as replication origins, under experimental conditions in which regulation of plasmid replication is probably disturbed. Whether these DNA sequences are bona fide replication origins that are not normally used (*silent origins*), or whether they represent sequences that have a different function, remains to be determined. Although DNA synthesis normally begins at a unique location on a DNA molecule, once replication has begun initiation of synthesis of the small Okazaki fragments occurs at a number of different sites on unpaired regions of the parental DNA strands. Perhaps such initiation sites can be used in the absence of the normal origin of replication. Alternatively, it has been suggested that DNA replication may be started with any appropriate RNA primer, and hence alternative DNA replication origins may simply be sites located close to functional promoters (Eichenlaub, personal communication).

3. Bifunctional Replicons

Whilst one approach to the study of the regulation of initiation of replication in a multiorigin replicon is first to characterize the component replicons and then to analyze the behavior of the complete replicon, an alternative approach is to synthesize a small model biorigin replicon from two different well-characterized plasmids. Such a

model replicon was constructed by in vitro linkage of the ColE1 and pSC101 plasmids at their unique EcoRI restriction endonuclease cleavage sites. ColE1, but not pSC101, can replicate in the absence of de novo protein synthesis whereas pSC101, but not ColE1, can replicate in mutant bacteria deficient in DNA polymerase I. Furthermore, the two plasmids are maintained at different copy levels in the cell: pSC101 is maintained at about 6 copies per chromosome DNA equivalent, whereas ColE1 is maintained at about 18 copies. The two plasmids are thus *functionally* distinct with respect to their replication properties. The component replicons of the hybrid are also *structurally* distinct and can be isolated as separate units following cleavage of the composite molecule by the EcoRI enzyme. The molecular weights of ColE1 and pSC101 are 4.2 mD and 6 mD respectively, and the origin of replication of ColE1 lies 18% of the molecule length from its unique EcoRI cleavage site, whereas that of pSC101 lies 50% of the molecule length from its EcoRI cleavage site. Thus replicating forms of the two molecules can be easily distinguished in the electron microscope (see Fig. 10). The composite plasmid, termed pSC134, was shown to be of considerable value in testing predictions of models for the control of initiation of DNA replication (Timmis et al., 1974a; Cabello et al., 1976).

The replicon model of Jacob et al. (1963) postulates that replication and maintenance of replicons takes place on membrane attachment sites. Replication is initiated when the sites are duplicated, and after DNA synthesis has been completed newly replicated progeny molcules are distributed among daughter bacteria at cell division. According to this model, compatible plasmids have different membrane attachment sites, whereas incompatible plasmids compete for the same sites; incompatible plasmids initially present in the same cell thus segregate when the cell divides. This model postulates that the initiation of

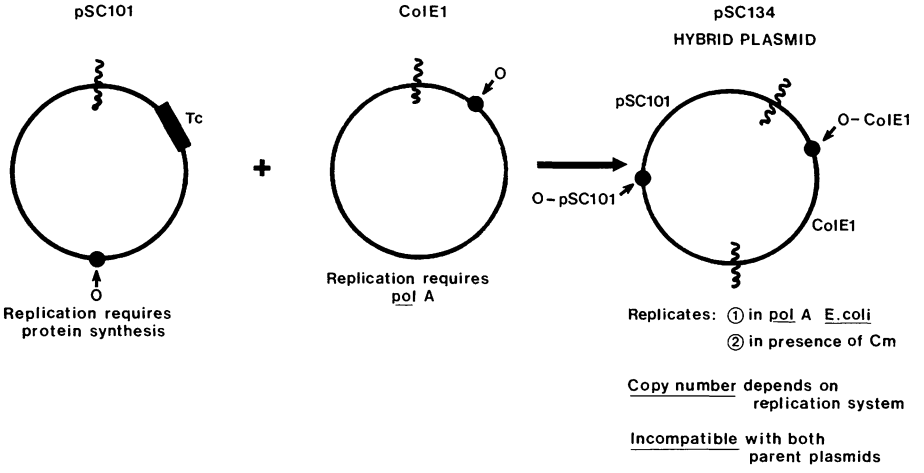


Fig. 9. Construction of a hybrid plasmid replicon. The pSC101 and ColE1 plasmids, which carry functionally distinguishable replication systems, are joined to each other at their EcoRI cleavage sites, and the hybrid plasmid is inserted into a bacterial cell by transformation. Since the pSC101 component does not replicate in the presence of chloramphenicol, and the ColE1 plasmid component requires the function of *E.coli* DNA polymerase I, it could be shown that either component of this hybrid replicon can accomplish propagation of the composite plasmid. Hybrid plasmids such as pSC134 have been useful for study of control of plasmid copy number and incompatability functions (see text)

DNA replication is controlled by a cis-dominant positively acting element and that this element is responsible for the phenomenon of incompatibility. The repressor dilution model of Pritchard et al. (1969) postulates that the initiation of DNA replication is inhibited by a trans-dominant freely diffusible repressor substance. The cellular *amount* of this element remains constant and therefore its cellular *concentration* falls during bacterial growth. When the cellular repressor concentration falls below a certain threshold level, initiation of replication occurs. Coupled with a new round of DNA synthesis is the synthesis of a burst of repressor such that further initiations are inhibited. Two replicons that specify similar repressor elements will not be able to coexist in a cell, i.e., will be incompatible, because the combined copy number of the two replicons cannot exceed the normal copy number of one of them when it exists alone in the cell.

This model predicts that a composite replicon composed of a high copy number plasmid and a low copy number plasmid will direct the synthesis of both types of repressor, and hence will be incompatible with both parent plasmids, but will be regulated only by the high copy number plasmid component. In contrast, the replicon model predicts that the composite replicon will utilize both types of attachment site, both sets of replication functions, and hence will have a copy number that is the sum of the copy numbers of both parent plasmids, and will be incompatible with both parents. Alternatively it predicts that the composite replicon will utilize only one type of attachment site, one component replication system, and hence will have a copy number typical of one parent, and will be compatible with the other parent.

We have been able to show that the composite plasmid can replicate either in chloramphenicol-treated cells or in mutant bacteria defective in DNA polymerase I, but not when these two restrictive conditions are combined. Thus, both component replicons of the pSC134 plasmid are completely functional and *either* can separately accomplish replication of the entire composite molecule (Fig. 9). However, when ColE1 and pSC101 exist as separate replicons in the same cell, ColE1 cannot provide *in trans* the chloramphenicol-sensitive product required for pSC101 replication, nor can pSC101 provide *in trans* a function that enables ColE1 to replicate under conditions of low DNA polymerase I activity.

Under non-restrictive conditions of growth it was found that the pSC134 composite plasmid was maintained at a level of about 16 copies per chromosome (Table 6), indicating that the ColE1 replication functions are used predominantly. This was confirmed by examining EcoRI endonuclease-cleaved replicative intermediates of pSC134 by electron microscopy. All EcoRI fragments containing a replication "eye" were the

Table 6. Replication properties of the pSC134 composite replicon and its parent plasmids

Host strain	Plasmid	Plasmid copy number	Replication ColE1	Origin used pSC101	Incompatible with
CR34 polA$^+$	pSC134	16	36	0	ColE1; pSC101
W3110 polA1	pSC134	6	0	14	-
CR341 polA$^+$	ColE1	18	-	-	ColE1
CR34 polA$^+$	pSC101	7	-	-	pSC101
CR34 polA$^+$	ColE1, pSC101	18,6	-	-	ColE1; pSC101

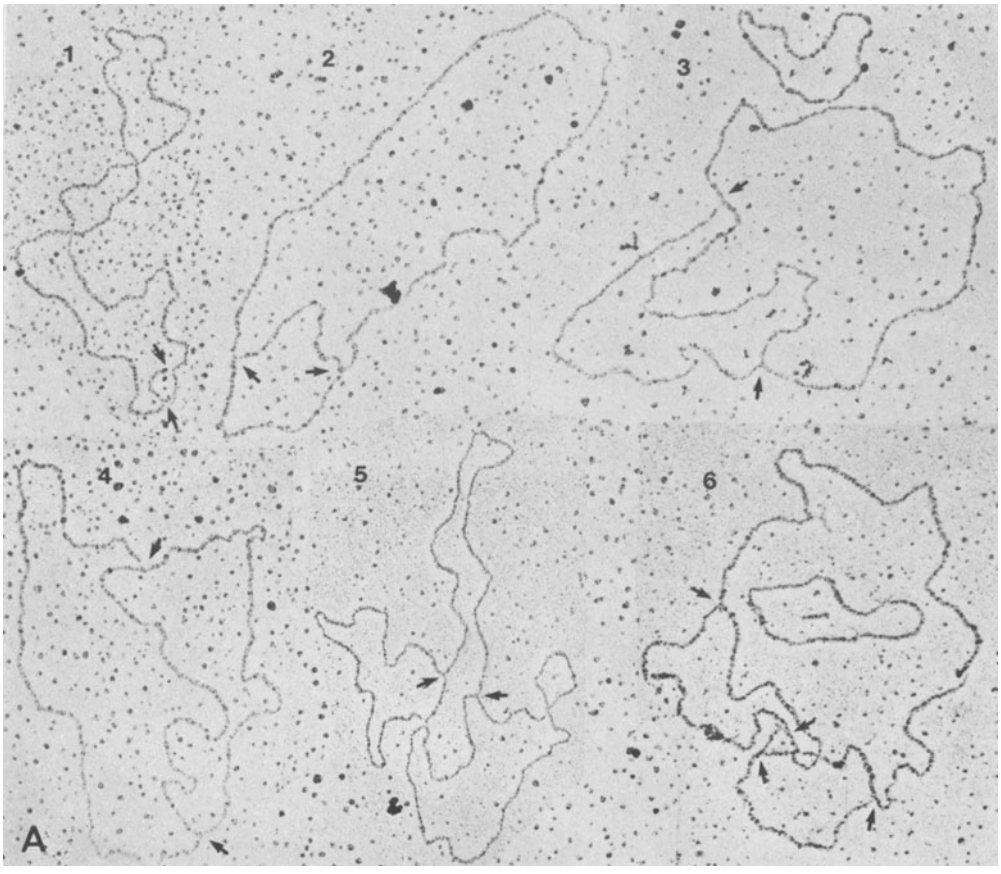
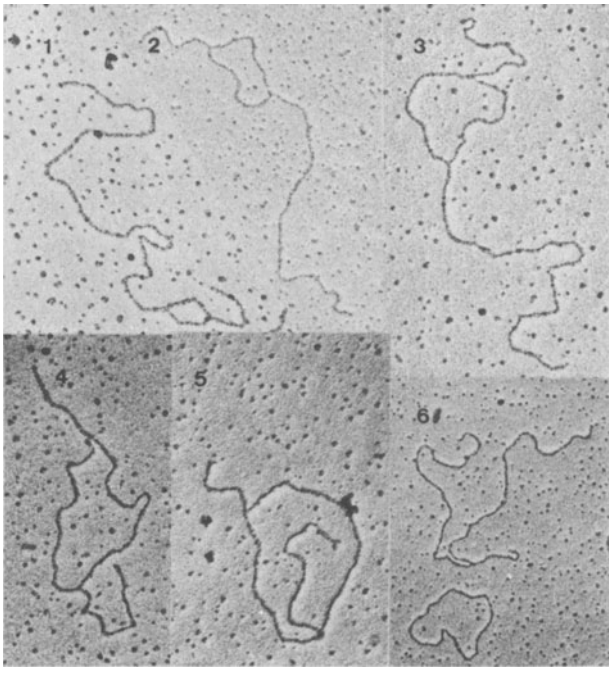


Fig. 10A-C. Replication origin usage by the pSC134 composite plasmid. Replicating molecules of the pSC134 plasmid were prepared from bacteria that contained normal (B) or low (C) levels of DNA polymerase I and either "nicked" with X-rays (A), to reveal the possible usage of two origins on individual molecules, or cleaved with EcoRI restriction endonuclease to genereate linear ColE1 and pSC101 component segments (B and C) prior to mounting for electron microscopy. EcoRI-cleaved molecules containing replication "eyes" or forks were photographed and measured and the fractional lengths of replicated and unreplicated segments calculated and displayed

size of ColE1 and contained replication forks that had originated from a position 18% of the molecule length from an EcoRI terminus. Thus, only the ColE1 replication origin and functions are ordinarily used for the replication of the pSC134 composite plasmid. The pSC101 origin is, however, functional in the pSC134 plasmid because it is used exclusively under conditions of low cellular levels of DNA polymerase I when the ColE1 origin functions cannot be used (Table 6).

Under normal conditions of growth, when the pSC134 plasmid is replicating exclusively by means of its ColE1 replication origin and functions, the composite plasmid nevertheless is incompatible with both ColE1 and pSC101 derivative plasmids. Expression of incompatibility between a pSC101 derivative and the pSC134 plasmid therefore does not require use of the pSC101 replication origin on the composite plasmid.



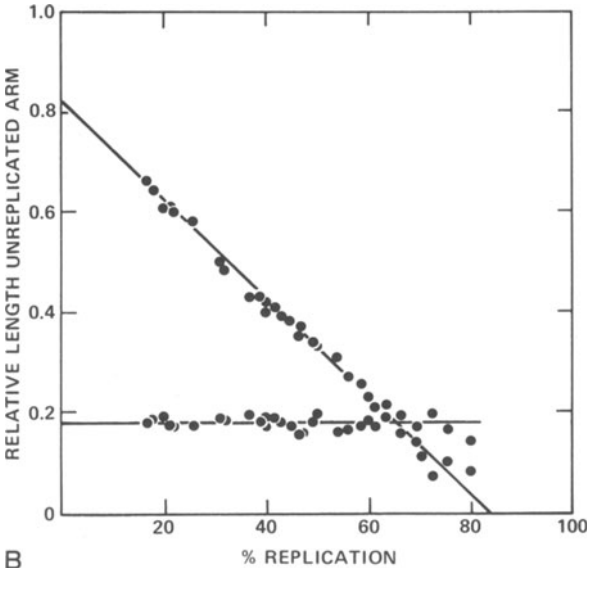
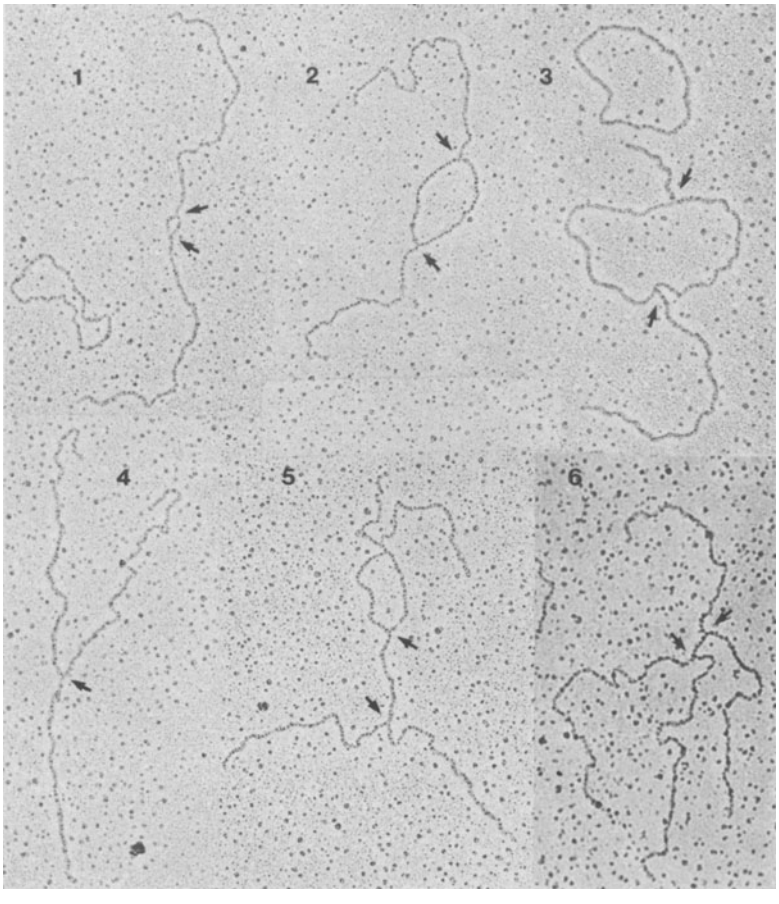


Fig. 10B (legend see p. 43)

Since the pS134 composite plasmid has the copy number of ColE1 alone, and since it replicates solely by means of the ColE1 origin and functions, according to the replicon model it is presumably maintained and controlled only at the ColE1 attachment site. Thus, the pSC101 membrane attachment site should be available for the replication of concurrently



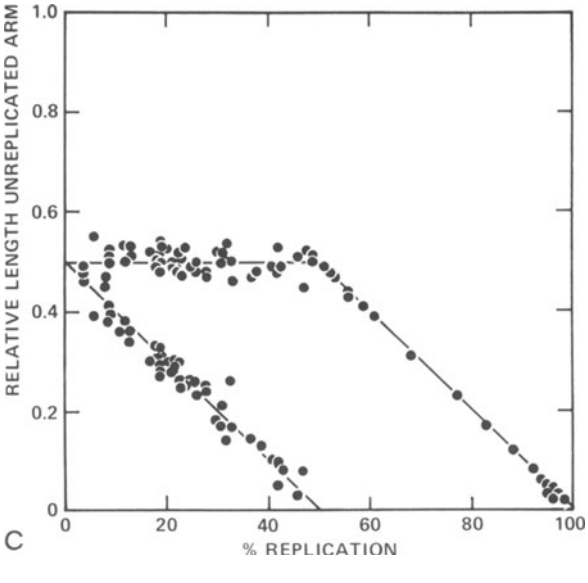


Fig. 10C (legend see p. 43)

present pSC101 molecules and hence pSC101 derivatives should be compatible with the pSC134 composite plasmid. The data obtained with the pSC134 plasmid are inconsistent with this prediction of the replicon model and are more consistent with predictions of the repressor dilution model. Therefore, although plasmid replication may well take place on defined membrane sites, the *control* of such replication is unlikely to be effected by a cis-acting cellular site or territory mechanism and is much more probably achieved by means of an origin-specific, freely diffusible, trans-dominant plasmid gene product. Whether or not this element effects a negative type of control, as seems likely from these and other experiments (K. Nordström, personal communication), has yet to be rigorously proved. Furthermore, recent experiments suggest the possibility that incompatibility may result from the activity of more than one gene product (Novick and Schwesinger, 1976). Experiments to isolate the plasmid replication controlling element and to study its activity in in vitro DNA synthesis systems are in progress.

The DNA cloning approaches for the study of plasmid DNA replication outlined above should facilitate the identification and mapping of DNA sequences and genes involved in plasmid replication and should further permit the isolation and characterization of DNA replication gene products. Small DNA segments containing origins of replication from large, low copy number plasmids may be cloned on to vectors that can be amplified in chloramphenicol in order to produce large quantities of origin DNA for sequence studies. Replication genes from plasmids of one incompatibility type will be cloned on to high copy number vectors that are of a different incompatibility type in order to isolate the gene products in quantity and in order that in vivo complementation studies may be performed (e.g. see Timmis et al., 1978)

II. Plasmid Fertility

Conjugation appears to be a major mechanism for the transfer of DNA from one bacterium to another, especially among Gram negative bacteria, and hence is of profound importance for the process of genetic recombination. This mechanism for DNA transfer is highly complex requiring, in the case of F factor promoted conjugation, the activity of probably more than 17 gene products and involving a cell surface appendage termed a sex pilus (for reviews see Willetts, 1972; Achtman, 1973) DNA transfer appears to require cell-cell contact and the sex pilus may play a role in promoting such contacts. Conjugation systems appear to be widely distributed among enteric bacteria and are almost always plasmid specified. Their presence in bacteria carrying plasmids that code for multiple drug resistance permits, under appropriate selective conditions, the epidemic spread of such plasmids among populations of bacteria and frequently compromises effective clinical therapy of bacterial infections.

Although the genetics of most plasmid-coded functions have not been well characterized, the genetics of fertility, as specified by the archtype sex factor F and some of the large antibiotic resistance plasmids like R100, have been extensively investigated. One reason for this is that such studies have not been greatly hindered by the problem of incompatibility that has restricted the study of other plasmid functions. Complementation tests involving fertility genes requires that the diploid state need only be established for a brief period of time (less than a cell doubling) during which incompatibility does present experimental difficulties. Fourteen of the transfer (tra) genes of F (tra A-tra I) are known to constitute a single operon, greater than 15 mD in size, that is under the positive control of tra J (Helmuth and Achtman, 1975). Nine of the genes of the tra operon, tra A, L, E, K, B, C, F, H, and G, are required for the formation of the sex

pilus which is the product of tra A. All these genes plus tra D, M, and I are necessary for transfer proficiency. A region within the tra operon, previously termed tra S, is required for the expression of surface exclusion, a function that reduces DNA transfer from one F plasmid-carrying cell to another. tra J, a regulatory gene, which exerts positive control of the tra operon, is itself part of a second operon and is subject to negative control by plasmids specifying the fin OP system of fertility inhibition (e.g., R100). These latter plasmids seem to have a similar transfer system in that they can complement most F tra mutants and have DNA sequences that are homologous with the tra region of F (Willetts, 1972; Sharp et al., 1973). However, unlike the F plasmid, most naturally occurring conjugative R plasmids are repressed for transfer.

The precise function of only a few tra gene products is known and no tra product, with the exception of the pilin protein (tra A product), has been isolated and characterized. Thus, the cloning of individual tra genes on high copy number vectors should greatly promote the biochemical analysis of conjugal transfer. Recently Skurray et al. (1976) reported the cloning of F DNA fragments generated by partial digestion with EcoRI restriction endonuclease, using the pSC101 plasmid vector. This study has made possible a more precise ordering of the tra genes on the F physical map and has provided convenient sources of tra operon DNA for the subsequent cloning of individual restriction endonuclease-generated fragments (Achtman et al., 1977) and ultimately individual genes. A similar cloning study with the plasmid R6-5, a plasmid closely related to R100, is currently in progress (Timmis and Achtman, unpublished experiments).

The extensive genetic knowledge of the tra region of F and the availability of *amber* mutations in most identified genes permits the introduction of such mutations into hybrid molecules containing cloned fragments of F. Comparisons of proteins made in vivo in minicells and in vitro from *wild type* cloned fragments with those proteins made from cloned fragments containing *amber* mutations in specific genes permit the identification of certain proteins with particular tra genes (Achtman and Herrlich, personal communication). The identification of individual gene products facilitates both their purification and studies to determine their cellular location. This is exemplified by recent progress relating to the phenomenon of surface exclusion. The cloning of the surface exclusion region of the tra operon in conjunction with genetic studies has resulted in the resolution of this region into two cistrons, tra S, and T, both of which are involved in surface exclusion, and the subdivision of the surface exclusion phenomenon into two distinct mechanisms. The product of the tra T gene has been shown to be involved in the inhibition of the formation of stable contacts between F-carrying cells and to be located in the bacterial outer membrane. Some pSC101 hybrid plasmids carrying tra T manifest a degree of surface exclusion greatly exceeding that exhibited by the parent F plasmid and concurrently an increased level of tra T protein in the outer membrane (Achtman, personal communication).

The availability of several hybrid plasmids that are compatible with F and carry DNA fragments that collectively span the tra operon, and the introduction of mutations into specific genes carried by these plasmids, has permitted the construction of a "tra operon mapping kit" that can be used for the mapping and identification of fertility functions on plasmids (Achtman et al., 1977).

III. Other Plasmid Coded Functions

A wide variety of other important plasmid coded functions has been and is being subjected to analysis using cloning methods for gene purification and amplification and gene product identification and characterization. Receiving particular interest are the tumor-inducing principle on the Ti plasmid of *Agrobacterium tumefaciens* that is involved in crown gall formation in plants, clinically important functions like antibiotic resistance, enterotoxins, and virulance factors, and the functions involved in the catabolism of organic compounds such as toluene, camphor etc.

In order to map the genes and identify the products of a number of R plasmid-specified functions, almost all the EcoRI and HindIII fragments of the multiple antibiotic resistance plasmid R6 and its tetracycline-sensitive derivative R6-5 have been cloned (Timmis et al., 1977a,c). Insertional inactivation was employed to detect hybrid molecules containing non-selectable functions (see Table 2). Table 7 lists the plasmid fragments cloned and the functions thus far identified. It may be seen that fragments coding for all of the known antibiotic resistances, namely Cm, Km, Tc, Sm, and Su, have been cloned, although only fragments containing resistance to Sm and Su were generated by both restriction enzymes. Resistances to Cm, Tc, Sm, Su, and mercury salts, were detected on HindIII fragments. Resistances to Km, Sm, and Su, were detected on EcoRI fragments. This finding allows a more accurate mapping of these resistances on the R6 and R6-5 physical maps since the Cm, Tc, and Hg resistance genes must contain, or must be located close to, EcoRI cleavage sites and the Km resistance gene must be close to a HindIII cleavage site. None of the fragments coding for antibiotic resistances that were cloned from R6 and R6-5 is capable of self-replication and therefore constitute specific probes for the in vitro cloning of EcoRI and HindIII-generated DNA fragments that are capable of self-replication (see Table 3).

The availability of a "bank" of high copy number hybrid plasmids that contain restriction endonuclease-generated fragments representing the whole of the R6/R6-5 plasmid genome will permit detailed studies of a variety of plasmid DNA sequences and functions, including the many plasmid segments that code for proteins to which no function has yet been ascribed. Although we and others have previously shown that several plasmid-specified functions may respond to gene dosage effects (e.g., bacteriocin production: Timmis and Winkler, 1973; ampicillin resistance: Nordström et al., 1972; tetracycline resistance: Cabello et al., 1976), such effects are dependent on the presence of the same promoter or one of equivalent activity. This is not always the situation for cloned DNA fragments that are separated from their normal promoter and in such cases the activity of the vector promoter involved in transcription of the cloned fragments may partly reinforce or cancel effects due to gene dosage (Tait et al., 1977; Timmis et al., 1977c).

The R6 and R6-5 plasmids have been shown to contain several DNA segments that are known to be aggressive in recombination: insertion sequences IS1, IS2, and IS3, and the sequences that flank the Km gene. How these DNA sequences insert into receptor DNA, which enzymes are involved in their insertion, and which sequences are present at their termini, are all questions that are currently receiving great attention. Because they can be amplified in chloramphenicol, the hybrid plasmids of R6 and R6-5 that contain these DNA sequences are good sources of such sequences. For similar reasons, the hybrid plasmids containing antibiotic resistance genes serve as convenient DNA hybridization probes for epidemiological studies of antibiotic resistance plasmids.

Table 7. Properties of cloned DNA fragments of plasmid R6-5

R6-5 EcoRI fragment	Molec. weight	Functions identified[b]	R6-5 HindIII fragment	Molec. weight[a]	Functions identified[b]
1	15.4	Rep B	1	22.2	Rep A, Fin O, Tra
2	8.7	Rep A, Fin O	2	16.1	Cm^r, Rep B
3	8.6	Tra	3	10.3	Tra
4	8.6	Rep C	4	7.2	Hg^r
5	5.2	Tra	5	3.7	
6	4.6	Km^r	6	n.c.	
7	4.0	Sfx	7	2.3	Sm^r
8	3.4	Sm^r Su^r	8	1.9	Su^r
9	n.c.		9	1.0	
10	2.7		10	0.2	
11	1.9				
12	1.1				
13	0.8				

[a]Expressed in megadaltons and determined by electron microscopy.

[b]*Rep:* replication functions; *Sfx:* surface exclusion; *FinO:* fertility inhibition; *Tra:* transfer functions; *Hg:* mercury; *n.c.:* not cloned.

Although not so extensively investigated as plasmid-specified antibiotic resistance, various plasmid-coded virulence factors such as enterotoxins, hemolysins, and the adhesion antigens like K88 and K99, are also of great clinical importance. However, studies of their genetics and mode of action have been greatly inhibited by the lack of means to manipulate the genes involved. The DNA cloning procedure will obviously simplify some of these problems, and recently the cloning of a 5.7 mD DNA fragment containing the heat-stable enterotoxin gene of a plasmid from an enterotoxigenic strain of *E.coli* from calves was reported (So et al., 1976). The increased levels of enterotoxin synthesized in bacteria carrying the cloned fragment should greatly aid the purification of the toxin and the determination of its mode of action. Although this particular toxin is non-antigenic, the cloning of the heat-labile enterotoxin genes and other genes for antigenic virulence factors should achieve the same ends and, in addition, may facilitate the development of specific vaccines in those instances where vaccines can be effective means of prophylaxis. Furthermore, as is the case with cloned antibiotic resistance genes, cloned virilance genes will provide useful DNA hybridization probes for epidemiological studies.

The cloning of genes for virulence factors may involve some hazard, and in such cases the use of appropriate physical or biological containment is warranted. In this connection, the availability of a self-destroying (biosafe) vector as discussed above would be very useful.

The recent findings that antibiotic-producing *Streptomyces* species contain plasmids (Schrempf et al., 1975), and that at least in two cases the antibiotic biosynthesis genes are plasmid-located (Kirby et al., 1975; Akagawa et al., 1975) indicate that the in vitro mani-

pulation of these very important genes for scientific and commercial purposes may be achieved in the near future.

Two experiments involving plasmids relevant to plants have been reported. A 10.9 mD fragment carrying the nitrogen fixation (nif) genes from *Klebsiella pneumoniae* was recently cloned on the pMB9 vector (Ausubel et al., 1978). This achievement is another major step forward toward the long-term goal of introducing the nif genes into a system that can achieve nitrogen fixation in or in association with non-legumenous plants. Secondly, DNA segments of the Ti plasmid that appear to be involved in tumor induction have been identified (Chilton et al., 1978; Schell et al., 1978). The cloning of such segments will greatly simplify the analysis of their sequences and of the plasmid functions involved in the transfer of these segments into plant chromosomes, which results in crown gall formation.

E. Concluding Remarks

The past few years have seen the development of an entirely new form of genetic analysis: an analysis that attempts to relate, and occasionally succeeds in relating, genotype and phenotype to the primary structure of DNA. Whereas this type of analysis had previously been restricted to a few special genetic systems the new developments now permit similar investigations of a variety of different systems. Crucial to this new-style genetic analysis has been the discovery of a wide range of restriction enzymes that allow detailed molecular analyses of large complex molecules of DNA, the development of rapid sequencing techniques for DNA, and the development of the DNA cloning technology. Because these methods are conceptually and technically simple compared with methods previously used for the same purpose, they will permit genetic and molecular analyses of complex chromosomes to proceed at a greatly accelerated pace. We have tried to present an overview of the current methods in use in the in vitro cloning of plasmid DNA fragments and their subsequent analysis. Although we have indicated the availability of a variety of experimental approaches, we have nevertheless attempted to stress the kinds of consideration that are important in deciding on an optimum strategy for particular cloning problems. Furthermore, we have emphasized novel approaches that have been developed to study a number of different aspects of plasmid structure and function, in the belief that such approaches are widely applicable.

Acknowledgments. We are indebted to all our colleagues who communicated published and unpublished material for inclusion in this review, to John Reeve for his critical reading of the manuscript, and to Irmgard Schallehn and Ingeborg Kreitmeyer for their superb typewriting.

References

Achtman, M.: Genetics of the F sex factor in *Enterobacteriaceae*. Current Topics in Microbiology and Immunology 60, 79-123 (1973)

Achtman, M., Skurray, R., Thompson, R., Helmuth, R., Beutin, L., Hall, S., Clark, A.J.: The assignment of tra cistrons to EcoRI fragments of F sex factor DNA. To be submitted (1977)

Adler, H.I., Fisher, W.D., Cohen, A., Hardigree, A.A.: Miniature *Escherichia coli* cells deficient in DNA. Proc. Nat. Acad. Sci. (Wash.) 57, 321-326 (1967)

Akagawa, H., Okanishi, M., Umezawa, H.: A plasmid involved in chloramphenicol production in *Streptomyces venezuelae*: evidence from genetic mapping. J. Gen. Microbiol. 90, 336-346 (1975)

Andres, I., Slocombe, P.M., Timmis, K.N.: Submitted for publication (1978)

Arber, W.: DNA modification and restriction. Prog. Nucleic Acid Res. Molec. Biol. 14, 1-37 (1974)

Ashby Report. Report of the Working Party on Experimental Manipulation of the Genetic Composition of Microorganisms. pp. 6-7. London: Her Majesty's Stationery Office 1975

Ausubel, F., Riedel, G., Cannon, F.: Cloning of *his* and *nif* genes from *Klebsiella pneumoniae*. In: Microbiology 1978. Washington, D.C.: ASM Publications, in press, 1978

Avni, H., Berg, P.E., Markovitz, A.: New mini-ColE1 as a molecular cloning vehicle. J. Bacteriol. 129, 358-366 (1977)

Backman, K., Ptashne, M., Gilbert, W.: Construction of plasmids carrying the cI gene of bacteriophage λ. Proc. Nat. Acad. Sci. (Wash.) 73, 4174-4178 (1976)

Bahl, C.P., Marians, K.J., Wu, R., Stawinsky, J., Narang, S.A.: A general method for inserting specific DNA sequences into cloning vehicles. Gene 1, 81-92 (1976)

Barnes, W.M.: Plasmid detection and sizing in single colony lysates. Science 195, 393-394 (1977)

Beckwith, J.R., Zipser, D.: The Lactose Operon. Cold Spring Harbor: Cold Spring Harbor Laboratory 1970

Blin, N., Gabain, A.V., Bujard, H.: Isolation of large molecular weight DNA from agarose gels for further digestion by restriction enzymes. FEBS Letters 53, 84-86 (1975)

Boidol, W., Siewert, G., Lindenmaier, W., Luibrand, G., Goebel, W.: Mol. Gen. Genet. 152, 231-237 (1977)

Bolivar, F., Rodriguez, R.L., Betlach, M.C., Boyer, H.W.: Construction and characterization of new cloning vehicles. Submitted for publication (1977a)

Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L., Boyer, H.W., Crosa, J., Falkow, S.: A new multipurpose cloning system. Construction and characterization. Gene (in press, 1977b)

Bollum, F.J.: Terminal deoxynucleotidyl transferase. In: The Enzymes (ed. P.D. Boyer). Vol. 10, pp. 145-171

Cabello, F., Timmis, K., Cohen, S.N.: Replication control in a composite plasmid constructed by in vitro linkage in two distinct replicons. Nature (London) 259, 285-290 (1976)

Chakrabarty, A.M.: Molecular cloning in *Pseudomonas*. In: Microbiology - 1976, pp. 579-582. Washington, D.C.: ASM Publications, 1976

Chandler, M., Silver, L., Frey, S., Caro, L.: Suppression of an *E.coli* dnaA mutation by the integrated R factor R100.1: Generation of small plasmids after integration. J. Bacteriol. 130, 303-311 (1977)

Chang, A.C.Y., Cohen, S.N.: Genome construction between bacterial species in vitro: replication and expression of *Staphylococcus* plasmid genes in *Escherichia coli*. Proc. Nat. Acad. Sci. (Wash.) 71, 1030-1034 (1974)

Chang, A.C.Y., Lansman, R.A., Clayton, D.A., Cohen, S.N.: Studies of mouse mitochondrial DNA in *Escherichia coli*: Structure and function of the eucaryotic-procaryotic chimeric plasmids. Cell 6, 231-244 (1975)

Chilton, M.D., Drummond, M.H., Gordon, M.P., Merlo, D.J., Montoya, A., Sciaky, D., Nester, E.W.: Foreign DNA of plasmid origin detected in crown gall tumor. In: Microbiology - 1978. Washington, D.C.: ASM Publications. In press, 1978

Clarke, L., Carbon, J.: Biochemical construction and selection of hybrid plasmids containing specific segments of the *Escherichia coli* genome. Proc. Nat. Acad. Sci. (Wash.) 72, 4361-4365 (1975)

Clewell, D.B., Evenchik, B., Cranston, J.W.: Direct inhibition of ColE1 plasmid DNA replication in *Escherichia coli* by rifampicin. Nature (London) 237, 29-30 (1972)
Clewell, D.B., Helinski, D.R.: Supercoiled circular DNA-protein complex in *E.coli*: purification and induced conversion to an open circular DNA form. Proc. Nat. Acad. Sci. (Wash.) 62, 1159-1166 (1969)
Cohen, S.N.: The manipulation of genes. Sci. Am. 233, 24-33 (1975)
Cohen, S.N.: Transposable elements and plasmid evolution. Nature 263, 731-738 (1976)
Cohen, S.N.: Recombinant DNA: Fact and fiction. Science 195, 654-657 (1977)
Cohen, S.N., Cabello, F., Casadaban, M., Chang, A.C.Y., Timmis, K.: DNA cloning and plasmid biology. Miami Winter Symposium: Molecular Cloning of Recombinant DNA, pp. 35-58. Academic Press, 1977a
Cohen, S.N., Cabello, F., Chang, A.C.Y., Timmis, K.: DNA cloning as a tool for the study of plasmid biology. Tenth Miles International Symposium: Recombinant Molecules: Impact on Science and Society (eds. R.F. Beers, Jr., E.G. Bassett), pp. 91-105. Raven Press, 1977b
Cohen, S.N., Chang, A.C.Y.: Recircularization and autonomous replication of a sheared R-factor DNA segment in *Escherichia coli* transformants. Proc. Nat. Acad. Sci. (Wash.) 70, 1293-1297 (1973)
Cohen, S.N., Chang, A.C.Y.: Selective cloning of eukaryotic DNA segments in *Escherichia coli* by repeated transformation. Mol. Gen. Genet. 134, 133-141 (1974)
Cohen, S.N., Chang, A.C.Y., Boyer, H.W., Helling, R.B.: Construction of biologically functional bacterial plasmids in vitro. Proc. Nat. Acad. Sci. (Wash.) 70, 3240-3244 (1973)
Cohen, S.N., Chang, A.C.Y., Hsu, L.: Non chromosomal antibiotic resistance in bacteria: Genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Nat. Acad. Sci. (Wash.) 69, 2110-2114 (1972)
Cohen, S.N., Miller, C.A.: Non chromosomal antibiotic resistance in bacteria. II Molecular nature of R-factors isolated from *Proteus mirabilis* and *Escherichia coli*. J. Mol. Biol. 50, 671-687 (1970)
Collins, J.: Gene cloning with small plasmids. Current Topics in Microbiology and Immunology (in press, 1977)
Collins, J., Fiil, N.P., Jørgensen, P., Friesen, J.D.: Cloning of genes involved in regulation of ribosome synthesis in *Escherichia coli*. In: Control of Ribosome Synthesis. IX Alfred Benzon Symposium, pp. 356-367. Cogenhagen: Munksgaard Press 1976
Collins, J., Williams, P., Helinski, D.R.: Plasmid ColE1 DNA replication in *Escherichia coli* strains temperature-sensitive for DNA replication. Mol. Gen. Genet. 136, 273-289 (1975)
Collins, J., Yanofsky, S., Helinski, D.R.: Involvement of the DNA protein relaxation complex in the replication of the plasmid ColE1. Submitted for publication (1977)
Cosloy, S.D., Oishi, M.: Genetic transformation in *Escherichia coli* K12. Proc. Nat. Acad. Sci. (Wash.) 70, 84-87 (1973)
Crosa, J.H., Luttropp, L.K., Falkow, S.: Covalently closed circular DNA molecules deficient in superhelical density as intermediates in plasmid life cycle. Nature (London) 261, 516-519 (1976)
Crosa, J.H., Luttropp, L.K., Heffron, F., Falkow, S.: Two replication initiation sites on R-plasmid DNA. Mol. Gen. Genet. 140, 39-50 (1975)
Curtiss III, R.: Genetic manipulation of microorganisms: Potential benefits and biohazards. Ann. Rev. Microbiol. 30, 507-533 (1976)
Curtiss III, R., L.J. Charamella, C.M. Berg and P.E. Harris: Kinetic and genetic analyses of D-cycloserine inhibition and resistance in *E.coli*. J. Bacteriol. 90, 1238 (1965)
Davis, B.D.: Isolation of biochemically deficient mutants of bacteria by penicillin. Amr. Chem. Soc. J. 70, 4267 (1948)
Davis, R.W., Simon, M., Davidson, N.: Electron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic

acids. In: Methods in Enzymology (eds. L. Grossman and K. Moldave) Vol. 21, pp. 413-428. New York: Academic Press 1971

Donoghue, D.J., Sharp, P.A.: An improved bacteriophage Lambda vector: Construction of model recombinants coding for kanamycin resistance. Gene 1, 209-227 (1977)

Dugaiczyk, A., Boyer, H.W., Goodman, H.M.: Ligation of EcoRI endonuclease-generated DNA fragments into linear and circular structures. J. Mol. Biol. 96, 171-184 (1975)

Duncan, C.H., Wilson, G.A., Young, F.E.: Transformation of *Bacillus subtilis* and *Escherichia coli* by a hybrid plasmid pCD1. Gene 1, 153-167 (1977)

Ehrlich, S.D., Bursztyn-Pettegrew, H., Stroynowski, I., Lederberg, J.: Expression of the thymidylate synthetase gene of the *Bacillus subtilis* bacteriophage Phi-3-T in *Escherichia coli*. Proc. Nat. Acad. Sci. (Wash.) 73, 4145-4149 (1976)

Eichenlaub, R., Figurski, D., Helinski, D.R.: Bidirectional replication from a unique origin in a mini-F plasmid. Proc. Nat. Acad. Sci. (Wash.) 74, 1138-1141 (1977)

Embden, J. van, Cohen, S.N.: Molecular and genetic studies of an R-factor system consisting of independent transfer and drug resistance plasmids. J. Bacteriol. 116, 699-709 (1973)

Enquist, L., Tiemeier, D., Leder, P., Weisberg, R., Sternberg, N.: Safer derivatives of bacteriophage λgt·λC for use in cloning of recombinant DNA molecules. Nature (London) 259, 596-598 (1976)

Falkow, S.: Infectious Multiple Drug Resistance. London: Pion 1975

Figurski, D., Meyer, R., Miller, D.S., Helinski, D.R.: Generation in vitro of deletions in the broad host range plasmid RK2 using phage Mu insertions and a restriction endonuclease. Gene 1, 107-119 (1976)

Frazer, A.C., Curtiss III, R.: Derepression of anthranilate synthetase in purified minicells of *Escherichia coli* containing the Col-trp plasmid. J. Bacteriol. 115, 615-622 (1973)

Frazer, A.C., Curtiss III, R.: Production, Properties and utility of bacterial minicells. Current Topics in Microbiology and Immunology 69, 1-84 (1975)

Gellert, M., Mizuuchi, K., O'Dea, M.H., Nash, H.A.: DNA gyrase: an enzyme that introduces superhelical turns into DNA. Proc. Nat. Acad. Sci. (Wash.) 73, 3872-3876 (1976)

Gellert, M., O'Dea, M.H., Itoh, T., Tomizawa, J.-I.: Novobiocin and coumermycin inhibit DNA supercoilling catalysed by DNA gyrase. Proc. Nat. Acad. Sci. (Wash.) 73, 4474-4478 (1976)

Goebel, W.: The influence of dnaA and dnaC mutations on the initiation of plasmid DNA replication. Biochem. Biophys. Res. Commun. 51, 1000-1007 (1973)

Goebel, W., Bonewald, R.: Class of small multicopy plasmids originating from the mutant antibiotic resistance factor R1drd-19B2. J. Bacteriol. 123, 658-665 (1975)

Graham, R.S., Young, F.E., Wilson, G.A.: Effect of site-specific endonuclease digestion on the *thy*P3 gene of bacteriophage ø3T and the *thy*P11 gene of bacteriophage *p*11. Gene 1, 169-180 (1977)

Greene, P.J., Poonian, M.S., Nussbaum, A.L., Tobias, L., Garfin, D.E., Boyer, H.W., Goodman, H.M.: Restriction and modification of a self-complementary octanucleotide containing the EcoRI substrate. J. Mol. Biol. 99, 237-261 (1975)

Gross, J.: DNA replication in bacteria. Current Topics in Microbiology and Immunology 57, 39-74 (1972)

Grunstein, M., Hogness, D.S.: Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. Proc. Nat. Acad. Sci. (Wash.) 72, 3961-3965 (1975)

Guyer, M.S., Figurski, D., Davidson, N.: Electron microscope study of a plasmid chimera containing the replication region of the *Escherichia coli* F plasmid. J. Bacteriol. 127, 988-997 (1976)

Hamer, D.H., Thomas, Jr., C.A.: Molecular cloning of DNA fragments produced by restriction endonucleases SalI and BamI. Proc. Nat. Acad. Sci. (Wash.) 73, 1537-1541 (1976)
Helinski, D.R.: Plasmid determined resistance to antibiotics: molecular properties of R factors. Ann. Rev. Microbiol. 27, 437-470 (1973)
Helinski, D.R., Clewell, D.B.: Circular DNA. Ann. Rev. Biochem. 40, 899-941 (1971)
Helinski, D.R., Hershfield, V., Figurski, D., Meyer, R.J.: Construction and properties of plasmid cloning vehicles. Tenth Miles International Symposium: Recombinant Molecules: Impact on Science and Society (eds. R.F. Beers, Jr., E.G. Bassett), pp. 151-165. Raven Press, 1977
Helmuth, R., Achtman, M.: Operon structure of DNA transfer cistrons on the F sex factor. Nature (London) 257, 652-656 (1975)
Hershey, A.D.: The Bacteriophage Lambda. Cold Spring Harbor: Cold Spring Harbor Laboratory , 1971
Hershfield, V., Boyer, H.W., Chow, L., Helinski, D.R.: Characterization of a mini ColE1 plasmid. J. Bacteriol. 126, 447-453 (1976)
Hershfield, V., Boyer, H.W., Yanofsky, C., Lovett, M.A., Helinski, D.R.: Plasmid ColE1 as a molecular vehicle for cloning and amplification of DNA. Proc. Nat. Acad. Sci. (Wash.) 71, 3455-3459 (1974)
Heyneker, H.L., Shine, J., Goodman, H.M., Boyer, H.W., Rosenberg, J., Dickerson, R.E., Narang, S.A., Itakura, K., Lin, S., Riggs, A.D.: Synthetic lac operator DNA is functional in vivo. Nature (London) 263, 748-752 (1976)
Hofstetter, H., Schamböck, A., van den Berg, J., Weissmann, C.: Specific excision of the inserted DNA segment from hybrid plasmids constructed by the Poly (dA)·Poly (dT) method. Biochim. Biophys. Acta 454, 587-591 (1976)
Hohn, B., Murray, K.: Packaging recombinant DNA molecules into bacteriophage particles in vitro. Proc. Nat. Acad. Sci. (Wash.) 74, 3259-3263 (1977)
Hollenberg, C.P., Degelmann, A., Kustermann-Kuhn, B., Royer, H.D.: Characterization of 2-µm DNA of *Saccharomyces cerevisiae* by restriction fragment analysis and integration in an *Escherichia coli* plasmid. Proc. Nat. Acad. Sci. (Wash.) 73, 2072-2076 (1976)
Hotchkiss, R.D., Gabor, M.: Bacterial transformation, with special reference to recombination process. Curr. Rev. Genet. 4, 193-224 (1970)
Inselburg, J.: Replication of colicin E1 plasmid DNA in minicells from a unique replication initiation site. Proc. Nat. Acad. Sci. (Wash.) 71, 2256-2259 (1974)
Jackson, D.A., Symons, R.H., Berg, P.: Biochemical method inserting new genetic information into DNA of simian virus 40: circular SV40 DNA molecules containing Lambda phage genes and the galactose operon of *Escherichia coli*. Proc. Nat. Acad. Sci. (Wash.) 69, 2904-2909 (1972)
Jacob, A.E., Cresswell, J.M., Hedges, R.W., Coetzee, J.N., Beringer, J.E.: Properties of plasmids constructed by the in vitro insertion of DNA from *Rhizobium leguminosarum* or *Proteus mirabilis* into RP4. Mol. Gen. Genet. 147, 315-323 (1976)
Jacob, A.E., Grinter, N.J.: Plasmid RP4 as a vector replicon in genetic engineering. Nature (London) 255, 504-506 (1975)
Jacob, F., Brenner, S., Cuzin, F.: On the regulation of DNA replication in bacteria. Cold Spring Harbor Symp. Quant. Biol. 28, 329-248 (1963)
Kedes, L.H., Chang, A.C.Y., Houseman, D., Cohen, S.N.: Isolation of histone genes from unfractionated sea urchin DNA by subculture cloning in *E.coli*. Nature (London) 255, 533-538 (1975)
Kingsbury, D.T., Helinski, D.R.: DNA polymerase as a requirement for the maintenance of the bacterial plasmid colicinogenic factor E1. Biochem. Biophys. Res. Commun. 41, 1538-1544 (1970)

Kirby, R., Wright, L.F., Hopwood, D.A.: Plasmid determined antibiotic synthesis and resistance in *Streptomyces coelicolor*. Nature (London) 254, 265-270 (1975)

Kriegstein, H.J., Hogness, D.S.: Mechanism of DNA replication in *Drosophila* chromosomes: structure of replication forks and evidence for bidirectionality. Proc. Nat. Acad. Sci. (Wash.) 71, 135-139 (1974)

Ksënzenko, V.N., Tikhomirova, L.P., Matvienko, N.I.: Specific fragmentation of DNA heteroduplex molecules of two bacteriophage λ mutants with endonuclease S1 from *Aspergillus oryzae*. Nucleic Acids Res. 3, 1081-1093 (1976)

Lobban, P.E., Kaiser, A.D.: Enzymatic end-to-end joining of DNA molecules. J. Mol. Biol. 78, 453-471 (1973)

Lovett, M.A., Helinski, D.R.: Method for the isolation of the replication region of a bacterial replicon: construction of a mini-F'*Km* plasmid. J. Bacteriol. 127, 982-987 (1976)

Lovett, M.A., Katz, L., Helinski, D.R.: Undirectional replication of plasmid ColE1 DNA. Nature (London) 251, 337-340 (1974)

Lovett, M.A., Sparks, R.B., Helinski, D.R.: Bidirectional replication of plasmid R6K DNA in *Escherichia coli*: correspondence between origin of replication and position of single strand break in relaxed complex. Proc. Nat. Acad. Sci. (Wash.) 72, 2905-2909 (1975)

Lovett, P.S., Duvall, E.J., Keggins, K.M.: *Bacillus pumilus* plasmid pPL10: properties and insertion into *Bacillus subtilis* 168 by transformation. J. Bacteriol. 127, 817-828 (1976)

Mandel, M., Higa, A.: Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. 53, 159-162 (1970)

Manis, J.J., Kline, B.C.: Restriction endonuclease mapping and mutagenesis of the F sex factor replication region. Mol. Gen. Genet. 152, 175-182 (1977)

Matsubara, K., Takagi, Y., Mukai, T.: In vitro construction of different oligomeric forms of λdv DNA and studies on their transforming activities. J. Virol. 16, 479-485 (1975)

Maxam, A.M., Gilbert, W.: A new method for sequencing DNA. Proc. Nat. Acad. Sci. (Wash.) 74, 560-564 (1977)

Mertz, J.E., Davis, R.W.: Cleavage of DNA by RI restriction endonuclease generates cohesive ends. Proc. Nat. Acad. Sci. (Wash.) 69, 3370-3374 (1972)

Messer, W., Dankwarth, L., Tippe-Schindler, R., Womack, J.E., Zahn, G.: Regulation of the initiation of DNA replication in *E.coli*. Isolation of I-RNA and the control of I-RNA synthesis. In: DNA Synthesis and its Regulation (eds. H.M. Goulian, P. Hanawalt, C.F. Fox). Mento Park, Calif.: Benjamin, 1975

Meyer, R., Figurski, D., Helinski, D.R.: Molecular vehicle properties of the broad host range plasmid RK2. Science 190, 1226-1228 (1975)

Mickel, S., Bauer, W.: Isolation by tetracycline selection, of small plasmids derived from R-factor R12 in *Escherichia coli* K-12. J. Bacteriol. 127, 644-655 (1976)

Morrow, J.F., Cohen, S.N., Chang, A.C.Y., Boyer, H.W., Goodman, H.M., Helling, R.B.: Replication and transcription of eukaryotic DNA in *Escherichia coli*. Proc. Nat. Acad. Sci. (Wash.) 71, 1743-1747 (1974)

Mukai, T., Matsubara, K., Takagi, Y.: Cloning bacterial genes with plasmid λdv. Mol. Gen. Genet. 146, 269-274 (1976)

Murray, K.: Biochemical manipulation of genes. Endeavour 126, 129-133 (1976)

Murray, K., Murray, N.E.: Phage lambda receptor chromosomes for DNA fragments made with restriction endonuclease III of *Haemophilus influenzae* and restriction endonuclease I of *E.coli*. J. Mol. Biol. 98, 551-564 (1975)

Murray, N.E., Murray, K.: Manipulation of restriction targets in phage λ to form receptor chromosomes for DNA fragments. Nature (London) 251, 476-481 (1974)

Nathans, D., Smith, H.O.: Restriction endonucleases in the analysis and restructuring of DNA molecules. Ann. Rev. Biochem. 44, 273-293 (1974)

NIH Guidelines: National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules. NIH, Bethesda 1976

Nishimura, Y., Caro, L., Berg, C.M., Hirota, Y.: Chromosome replication in *Escherichia coli*. IV Control of chromosome replication and cell division by an integrated episome. J. Mol. Biol. 55, 441-456 (1971)

Nordström, K., Ingram, L.C., Lundbäck, A.: Mutations in R factors of *Escherichia coli* causing an increased number of R-factor copies per chromosome. J. Bacteriol. 110, 562-569 (1972)

Novick, R.P., Schwesinger, M.: Independence of plasmid incompatibility and replication control functions in *Staphylococcus aureus*. Nature (London) 262, 623-626 (1976)

Palchaudhury, S., Maas, W.K.: Physical mapping of a DNA sequence common to plasmids of incompatibility group F I. Proc. Nat. Acad. Sci. (Wash.) 74, 1190-1194 (1977)

Perlman, D., Rownd, R.H.: Two origins of replication in composite R plasmid DNA. Nature 259, 281-284 (1976)

Polisky, B., Bishop, R.J., Gelfand, D.H.: A plasmid cloning vehicle allowing regulated expression of eukaryotic DNA in bacteria. Proc. Nat. Acad. Sci. (Wash.) 73, 3900-3904 (1976)

Pritchard, R.H., Barth, P.T., Collins, J.: Control of DNA synthesis in bacteria. Symp. Soc. Gen. Microbiol. 19, 263-297 (1969)

Rambach, A., Hogness, D.S.: Expression of *Drosophila* DNA segments propagated in *Escherichia coli*. Proc. Nat. Acad. Sci. (Wash.), in press (1977)

Rambach, A., Tiollais, P.: Bacteriophage λ having EcoRI endonuclease sites only in the nonessential region of the genome. Proc. Nat. Acad. Sci. (Wash.) 71, 3927-3930 (1974)

Roberts, R.J.: Restriction endonucleases. Critical Rev. Biochem. 4, 123-164 (1976)

Roberts, R.J.: The role of restriction endonucleases in genetic engineering. Tenth Miles International Symposium: The Recombinant Molecules: Impact on Science and Society (eds. R.F. Beers, Jr., E.G. Bassett), pp. 21-32. Raven Press, 1977

Roozen, K.J., Fenwick Jr., R.G., Curtiss III, R.: Synthesis of ribonucleic acid and protein in plasmid-containing minicells of *Escherichia coli* K-12. J. Bacteriol. 107, 21-33 (1971)

Roychoudhury, R., Jay, E., Wu, R.: Terminal lebeling and addition of homopolymer tracts to duplex DNA fragments by terminal deoxynucleotidyl transferase. Nucleic Acids Res. 3, 101-116 (1976)

Sanger, F., Coulson, A.R.: A rapid method for determining sequences of DNA by primed synthesis with DNA polymerase. J. Mol. Biol. 94, 441-448 (1975)

Sanzey, B., Mercereau, O., Ternynck, T., Kourilsky, P.: Methods for identification of recombinants of phage λ. Proc. Nat. Acad. Sci. (Wash.) 73, 3394-3397 (1976)

Schell, J., Montague, M. van, Picker, A. de, Engler, G., Genetello, C., Hernalsteens, J.P., Holsters, M.: Agrobacterium tumefaciens: what segment of the plasmid is responsible for the induction of crown gall tumors? In: Microbiology - 1978. Washington, D.C.: ASM Publications. In press, 1978

Schrempf, H., Bujard, H., Hopwood, D.A., Goebel, W.: Isolation of covalently closed circular DNA from *Streptomyces coelicolor* A3(2). J. Bacteriol. 121, 416-421 (1975)

Selker, E., Brown, K., Yanofsky, C.: Mitomycin C-induced expression of trpA of *Salmonella typhimurim* inserted into the plasmid ColE1. J. Bacteriol. 129, 388-394 (1977)

Sgaramella, V.: Enzymatic oligomerization of bacteriophage P22 DNA and of linear simian virus 40 DNA. Proc. Nat. Acad. Sci. (Wash.) 69, 3389-3393 (1972)

Sgaramella, V., Khorana, H.G.: Studies on polynucleotides. CXII Total synthesis of the structural gene for an alanine transfer RNA from yeast. Enzymatic joining of the chemically synthesized polydeoxynucleotides to form the DNA duplex representing nucleotide sequence 1 to 20. J. Mol. Biol. 72, 427-444 (1972)

Sgaramella, V., Sande, J.H. van, Khorana, H.G.: Studies on polynucleotides. C. A novel joining reaction catalyzed by the T4-polynucleotide ligase. Proc. Nat. Acad. Sci. (Wash.) 67, 1468-1475 (1970)

Sharp, P.A., Cohen, S.N., Davidson, N.: Electron microscope heteroduplex studies of sequence relations among plasmids of *Escherichia coli* II. Structure of drug resistance (R) factors and F factors. J. Mol. Biol. 75, 235-255 (1973)

Skalka, A., Shapiro, L.: *In situ* immunoassays for gene translation products in phage plaques and bacterial colonies. Gene 1, 65-79 (1976)

Skurray, R.A., Nagaishi, H., Clark, A.J.: Molecular cloning of DNA from F sex factor of *Escherichia coli* K-12. Proc. Nat. Acad. Sci. (Wash.) 73, 64-68 (1976)

Smith, H.O., Birnstiel, M.L.: A simple method for DNA restriction site mapping. Nucleic Acids Res. 3, 2387-2398 (1976)

So, M., Boyer, H.W., Betlach, M., Falkow, S.: Molecular cloning of an *Escherichia coli* plasmid determinant that encodes for the production of heat-stable enterotoxin. J. Bacteriol. 128, 463-471 (1976)

So, M., Gill, R., Falkow, S.: The generation of a ColE1-Ap^R cloning vehicle which allows detection of inserted DNA. Mol. Gen. Genet. 124, 239-249 (1975)

Southern, E.M.: Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503-517 (1975)

Starlinger, P., Saedler, H.: IS elements in microorganisms. Current Topics in Microbiol. and Immunol. 75, 111-152 (1976)

Staudenbauer, W.L.: Replication of colicinogenic factor E1 DNA in plasmolysed *E.coli* cells. Coupling of DNA replication and RNA synthesis. Europ. J. Biochem. 58, 303-313 (1975)

Sternberg, N., Tiemeier, D., Enquist, L.: In vitro packaging of a λ *Dam* vector containing EcoRI DNA fragments of *Escherichia coli* and phage P1. Gene 1, 255-280 (1977)

Stevens, W.F., Adhya, S., Szybalski, W.: Origin and bidirectional orientation of DNA replication in coliphage lambda. In: The Bacteriophage Lambda (ed. A.D. Hershey), pp. 515-533. New York: Cold Spring Harbor Laboratory 1970

Tait, R.C., Rodriguez, R.L., Boyer, H.W.: Altered tetracycline resistance in pSC101 recombinant plasmids. Submitted for publication, 1977

Taketo, A.: Ba^{2+}-induced competence for transfecting DNA. Naturforschung 30O, 520-522 (1975)

Telford, J., Boseley, P., Schaffner, W., Birnstiel, M.: Novel screening procedure for recombinant plasmids. Science 195, 391-393 (1977)

Thomas, M., Cameron, J.R., Davis, R.W.: Viable molecular hybrids of bacteriophage lambda and eukaryotic DNA. Proc. Nat. Acad. Sci. (Wash.) 71, 4579-4585 (1974)

Thomas, M., White, R.L., Davis, R.W.: Hybridization of RNA to double-stranded DNA: formation of R-loops. Proc. Nat. Acad. Sci. (Wash.) 73, 2294-2298 (1976)

Tiemeier, D., Enquist, L., Leder, P.: Improved derivative of a phage λ EK2 vector for cloning recombinant DNA. Nature (London) 263, 526-527 (1976)

Timmis, K.N., Andres, I., Cabello, F., Synenki, R., Cohen, S.N., Burkhart, H.: Replication functions of antibiotic resistance plasmid R6-5. In: Microbiology - 1978. Washington, D.C.: ASM Publications. In press, 1978

Timmis, K.N., Andres, I., Slocombe, P.M.: Plasmid incompatibility: cloning analysis of an FII incompatibility determinant. Submitted for publication (1978)

Timmis, K.N., Cabello, F., Cohen, S.N.: Utilization of two distinct modes of replication by a hybrid plasmid constructed in vitro from separate replicons. Proc. Nat. Acad. Sci. (Wash.) 71, 4556-4560 (1974a)

Timmis, K., Cabello, F., Cohen, S.N.: Cloning, isolation, and characterization of replication regions of complex plasmid genomes. Proc. Nat. Acad. Sci. (Wash.) 72, 2242-2246 (1975)

Timmis, K., Cabello, F., Cohen, S.N.: Covalently closed circular DNA molecules of low superhelix density as intermediate forms in plasmid replication. Nature (London) 261, 512-516 (1976)

Timmis, K.N., Cabello, F., Cohen, S.N.: Molecular cloning of plasmid DNA: properties of recombinant plasmids in transformed bacteria. In: Modern Trends in Bacterial Transformation and Transfection (eds. A. Portoles, R. Lopez and M. Espinosa), pp. 263-276. Amsterdam: Elsevier/ North Holland 1977a

Timmis, K.N., Cabello, F., Cohen, S.N.: Properties and regulation of DNA replication origins and associated functions in multi-origin replicons. In: Theoretical and Medical Problems of Antibiotic Resistance (eds. L. Rosival and V. Krcmery), pp. 417-424. Prague: Avenicum. Berlin-Heidelberg-New York: Springer 1977b

Timmis, K.N., Cabello, F., Cohen, S.N.: Cloning by *insertional inactivation* and characterization of EcoRI and HindIII restriction endonuclease-generated fragments of antibiotic resistance plasmids R6-5 and R6. Submitted for publication, 1977c

Timmis, K., Early, P.W., Marvin, D.A.: Filamentous bacterial viruses. IX Protein synthesis inhibitors and DNA replication. Biochem. Biophys. Res. Commun. 57, 263-270 (1974b)

Timmis, K., Marvin, D.A.: Filamentous bacterial viruses. XV. DNA replication and abortive infection. Virology 59, 281-292 (1974)

Timmis, K., Winkler, U.: Gene dosage studies with pleiotropic mutants of *Serratia marcescens* superactive in the syntheis of marescin A and certain other exocellular proteins. Mol. Gen. Genet. 124, 207-217 (1973)

Tomizawa, J.-I., Sakakibara, Y., Kakefuda, T.: Replication of colcin E1 plasmid DNA in cell extracts. Origin and direction of replication. Proc. Nat. Acad. Sci. (Wash.) 71, 2260-2264 (1974)

Tomizawa, J.-I., Sakakibara, Y., Kakefuda, T.: Replication of colicin E1 plasmid DNA added to cell extracts. Proc. Nat. Acad. Sci. (Wash.) 72, 1050-1054 (1975)

Uhlin, B.E., Nordström, K.: Plasmid incompatibility and control of replication: copy mutants of the R-factor R1 in *Escherichia coli* K-12. J. Bacteriol. 124, 641-649 (1975)

Wackernagel, W.: Genetic transformation in *Escherichia coli*: the inhibitory role of the recBC DNAse. Biochem. Biophys. Res. Commun. 51, 306-311 (1973)

Warren, R.W., Womble, D.D., Barton, C.R., Easton, A., Rownd, R.H.: Replication of R plasmid DNA, RoR12, in *Proteus mirabilis*. Microbiology 78 (in press, 1978)

Wensink, P.C., Finnegan, D.J., Donelson, J.E., Hogness, D.S.: A system for mapping DNA sequences in the chromosomes of *Drosophila melanogaster*. Cell 3, 315-325 (1974)

Willetts, N.: The genetics of transmissible plasmids. Ann. Rev. Genet. 6, 257-268 (1972)

Williams Report. Report of the working party on the practice of genetic manipulation. London: Her Majesty's Stationery Office 1976

Zubay, G., Chambers, D.A., Cheong, L.C.: Cell-free studies on the regulation of the lac operon. In: The Lactose Operon (eds. J.R. Beckwith and D. Zipser), pp. 375-391. Cold Spring Harbor: Cold Spring Harbor Laboratory 1970