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Characterization of argF Specialized Transducing Derivatives of Bacteriophage P1

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CHARACTERIZATION OF *argF* SPECIALIZED TRANSDUCING DERIVATIVES OF BACTERIOPHAGE P1

by

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VITA

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Mrs. York is the co-author of two abstracts presented on this research: "Specialized transducing bacteriophage P1*argF*," Annual Meeting of the American Society of Microbiology, 1979; and "Formation of P1*argF* specialized transducing bacteriophage: Involvement of IS*1*," Twelfth Annual Winter Symposium of the University of Miami, 1980.

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LIST OF ABBREVIATIONS

Arg	Arginine biosynthesis
Cm^r	Chloramphenicol resistant
Cm^s	Chloramphenicol sensitive
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
Δ	Deletion
EDTA	Ethylene diamine tetraacetic acid
HFT	High frequency transducing
kb	Kilobases or kilobase pairs
Lac	Lactose utilization
LFT	Low frequency transducing
m.o.i.	Multiplicities of infection, multiplicity of infection
mRNA	Messenger ribonucleic acid
OTC	Ornithine carbamoyltransferase
PEG	polyethylene glycol
pfu	plaque forming unit(s)
Pro	Proline biosynthesis
RNase	Ribonuclease
SDS	Sodium dodecyl sulfate
SSC	Standard saline citrate
Tris	Tris (hydroxymethyl) aminomethane

LIST OF GENE SYMBOLS

Escherichia coli K12[a]

argF	isoenzyme of ornithine carbamoyl-transferase (EC2.1.3.3)
argI	isoenzyme of ornithine carbamoyl-transferase (EC2.1.3.3)
argR	regulatory gene of arginine biosynthesis
gpt	guanine-xanthine phosphoribosyl transferase (EC2.4.2.8)
lac	lactose utilization
proAB	block prior to L-glutamate semialdehyde in proline biosynthesis
recA	competence for genetic recombination and repair of radiation damage

Phage P1

c	clear plaque (number following *c* indicates genetic locus)
Cm	chloramphenicol resistance determinant
g	less of a requirement for Ca^{+2} for adsorption
k	improved efficiency of plating on *E. coli* K12 strains
lox	locus of exchange
mod	modification
res	restriction
ts	temperature sensitive
vir	virulent

[a] From Bachmann and Low, 1980.

CHAPTER I

INTRODUCTION

Segments of chromosomes are physically exchanged through genetic recombination. This process conserves the order of genes in normal recombinations between homologous chromosomes. There are also aberrant or "illegimate" recombinations between homologous or heterologous chromosomes in which the order of genes is not conserved. Such recombinations result in the reassortment of genetic information in nature and the evolution of chromosomal structures in both higher and lower organisms.

Both eukaryotic and prokaryotic viruses have functions that promote aberrant recombination. The consequences of these recombinations include integration of viral genes into the host chromosome, integration of host genes into the viral genome, and the rearrangement of genetic information in the chromosome. Mechanisms involved in these viral mediated aberrant recombinations have been the subject of active research. Much work has been done to elucidate the mechanisms of integration of bacteriophage λ and P22 into specific sites on the Escherichia coli chromosome, (Morse et al., 1956a, b; Campbell, 1971; Smith-Keary, 1966; Hoppe and Roth, 1974) and the integration of bacteriophage Mu into random sites within the E. coli genome (Taylor, 1963; Bukhari, 1976). The tumor virus SV40 is integrated into the animal host genome during neoplastic transformation, while DNA transcripts of

RNA tumor viruses have been shown to integrate during viral replication (Bishop and Varmus, 1975; Croce and Koprowski, 1975).

One example of viral mediated aberrant recombination is the formation of specialized transducing bacteriophage. In these bacteriophage derivatives, genes acquired from a foreign chromosome are an integral part of the phage chromosome (Morse et al., 1956b). Preliminary investigations on the formation of the specialized transducing derivative, P1*argF*, indicate that these hybrids of phage P1 and the *argF* bacterial gene occur with an unusually high frequency (Stodolsky, unpublished). This observation prompted the study presented in this dissertation.

The purpose of this work has been to examine the events involved in the formation of P1*argF* genomes mediated by various P1 strains and to characterize the resulting transductants. Standard genetic techniques, restriction endonuclease cleavage analysis, and electron microscopy of heteroduplex DNA are the predominant methodologies used. The results presented here provide some insight into the factors involved in the formation of aberrant recombinational derivatives of bacteriophage P1. One of these factors, the involvement of the transposable element, IS*1*, is presented in great detail.

CHAPTER II

REVIEW OF THE RELATED LITERATURE

Genetic and physical structure of bacteriophage P1 DNA.

Bacteriophage P1 was originally isolated by Bertani (1951) from a lysogenic strain of *Escherichia coli*. While P1 has been adapted to grow lytically with varying efficiency on many *E. coli* strains, *Shigella dysenteriae* is considered a better host for plaque formation by the phage (Lennox, 1955). Lysogens with P1 prophage are easily isolated from both *E. coli* and *Shigella* strains. Because of its capacity for generalized transduction, phage P1 has been an invaluable tool for the genetic mapping of the *E. coli* K12 chromosome (Lennox, 1955; Ikeda and Tomizawa, 1965a).

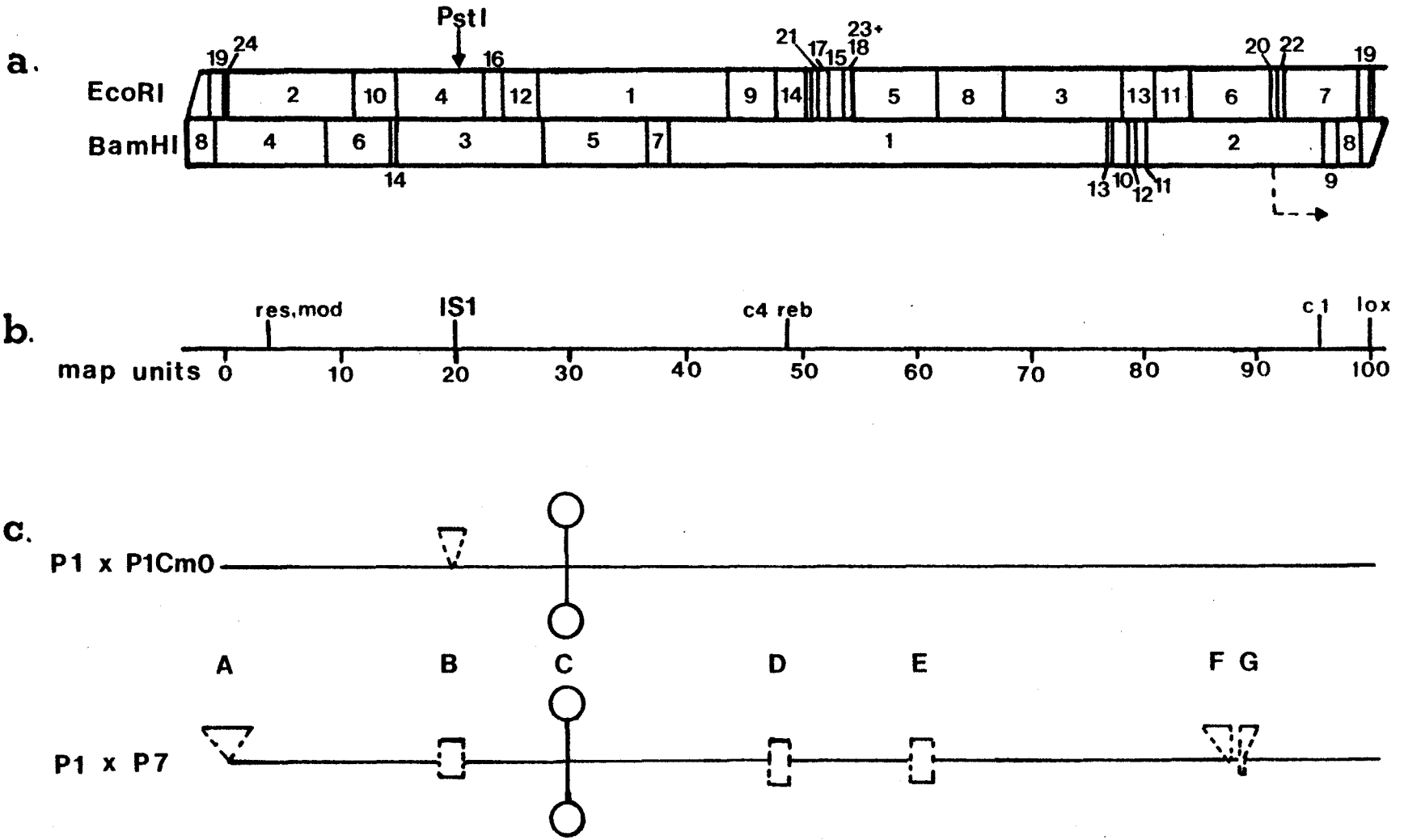
The genome of P1 exists in two forms: as a 90 kb double-stranded DNA plasmid in lysogenic bacteria and as a linear duplex DNA chromosome of approximately 100 kb in P1 virion particles (Ikeda and Tomizawa, 1968; Yun and Vapnek, 1977). The enlargement of the virion chromosome over that of the prophage is due to a terminal repetition of 7 to 12% of the genome (Ikeda and Tomizawa, 1968; Yun and Vapnek, 1977). Virion chromosomes have also been shown to be permuted; that is, different bacteriophage chromosomes begin with different P1 genes. Because of these structural features, virion chromosomes are thought to be incorporated into the phage particles by a "headful" packaging system acting on concatemeric DNA (Streisinger *et al.*, 1967).

Concatemer formation has been demonstrated (Bornhoeft, Ph.D. thesis, 1979). The "headful" packaging system explains the presence of both plaque-forming (PIB) and small-head (PIS) particles (Ikeda and Tomizawa, 1965b; Walker and Anderson, 1970), as well as generalized transducing particles in P1 lysates (Ikeda and Tomizawa, 1965a). After infection, the virion DNA circularizes by a recombinational event, loses its terminal redundancy, and forms a plasmid which replicates autonomously under stringent control (Ikeda and Tomizawa, 1968; Prentki et al., 1977).

Although the P1 chromosome is large enough to encode approximately 100 genes, only about 20 have identified as mutable loci or sites of chromosomal action (Yarmolinsky, 1977; Mural et al., 1979; Sternberg, 1979). A linear genetic map (Fig. 1) has been constructed from the results of three point bacteriophage crosses (Scott, 1968; Walker and Walker, 1976). This linearity is due to the functioning of a site-specific recombination system (Lox) acting at a site corresponding to the genetic ends of the P1 map. The lox recognition site and genes for the protein(s) involved in the recombination reaction are closely linked (Sternberg, 1978).

There are two genetic loci in P1, c1 and c4, which code for functions responsible for repression of the phage lytic cycle. Mutations in either of these genes result in loss of the ability to establish lysogeny (Scott, 1970). The c1 gene, located near the right end of the genetic map, produces a protein that directly represses lytic functions. The c4 gene, located near the middle of the genetic map, encodes a protein which represses an adjacent activator gene, termed reb (for repressor bypass), the reb protein, if expressed, is able to

Fig. 1. Genetic and physical maps of P1 and its derivatives. Heteroduplex map (c) of P1, P7 and P1CmO (from Yun and Vapnek, 1977) is aligned to the restriction endonuclease cleavage map (a) constructed by Bachi and Arber (1977) and Iida and Arber (1979) for enzymes EcoRI and BamHI. Genetic markers (b) have been aligned to the cleavage map. Broken arrow indicates origin and direction of DNA packaging. The cleavage fragments depicted in (a) are numbered from largest to smallest according to Bachi and Arber (1977).


a.
PstI
EcoRI
BamHI
b.
res,mod
IS1
c4 reb
c1
lox
map units
c.
P1 x P1Cm0
P1 x P7
A
B
C
D
E
F G

overcome the repression by c1. Together the c4 and reb genes are termed the Immunity I region of the P1 chromosome (Scott, 1975; Scott et al., 1978).

Upon infection of non-lysogenic strains, some P1 phage will establish lysogeny and others will enter the lytic cycle depending on how quickly the c1 and c4 encoded repressor proteins are produced. Upon infection of P1 lysogenic strains, phage particles adsorb and inject their DNA (Bertani and Nice, 1954), but they do not lytically propagate. This immunity to superinfection by P1 is due to the presence of the c1 repressor protein in the lysogenic strain. There are P1 deletion mutants that lack c1 and other genes lethal to the host. These mutants are unable to prevent vegetative growth of incoming phage, especially if the incoming phage also lack a functional c1 gene. Strains harboring these deletion mutants are therefore not P1 immune (Scott, 1975).

Another class of P1 deletion mutants have normal c1 function but do not confer immunity to superinfection by P1. These mutants lack both c4 and reb. As prophage, they are unable to prevent vegetative growth of incoming phage, because the incoming reb gene can be expressed and overcome the resident c1 repressor protein (Scott et al., 1978).

There are also P1 mutants which are temperature sensitive (ts) for c1 function (Scott, 1970; Rosner, 1972). Inactivation of the c1 repressor protein at the non-permissive temperature results in the induction of the phage lytic cycle in strains lysogenic for P1c1ts prophage.

Bacterial strains lysogenic for P1 resist infection by lytic phages such as T1, T3, and T7, and virulent (vir) mutants of temperate

phages λ and P2 (Bertani and Nice, 1954). This is due to the expression of a restriction (res) and modification (mod) system encoded in the P1 chromosome (Glover et al., 1963; Rosner, 1973). Both functions are expressed by the virus in the prophage state and are located near the left end of the P1 genetic map (Fig. 1).

The P1 physical map is divided into 100 units; each unit corresponds to approximately 0.9 kb (Bachi and Arber, 1977). Restriction endonuclease PstI cleaves the P1 chromosome once (Iida et al., 1978; DeBruijn and Bukhari, 1978). The cleavage site is within the naturally occurring IS1 sequence of P1 at map unit 20, and is used for alignment of the genetic and physical maps. Encapsidation of concatemers during the lytic cycle starts at a position corresponding to map unit 92 and proceeds to the right (Bachi and Arber, 1977).

Bacteriophage P7, isolated by Smith (1972), is a close relative of phage P1. It possesses six regions of non-homology with P1, as manifested in P7-P1 heteroduplexes (Yun and Vapnek, 1977). These structural differences make P7-P1 heteroduplexes extremely useful for localizing gross alterations in P1 chromosomes.

The P7 prophage is a 100.4 kb plasmid (Yun and Vapnek, 1977). The enlargement of P7 over P1 is due largely to the presence in the P7 chromosome of a transposon conferring ampicillin resistance. This transposon is identified as non-homology region A in P1-P7 heteroduplexes (Fig. 1). There is also non-homology between the two chromosomes at map unit 20. Phage P7 lacks the IS1 sequence that is present in P1 chromosomes at this locus (Iida and Arber, 1979).

Both P1 and P7 possess a 3.1 kb segment of DNA flanked by

inverted repeats (map unit 32). The region anneals as a C-loop structure in single-stranded molecules (Lee et al., 1974; Bachi and Arber, 1977). It is also represented as a region of non-homology in annealed P1 homoduplexes, when complementary strands have their C regions in opposite orientations. The intramolecular inversions that cause this orientation switch are due to reciprocal recombination between the homologous inverted repeat sequences (Lee et al., 1974).

Phages P1 and P7 also differ at the Immunity I locus, encoding c4 and reb (Chesney and Scott, 1975; Scott et al., 1977; Wandersman and Yarmolinsky, 1977). This is represented as non-homology region D and is responsible for the heteroimmunity between the two phages (Yun and Vapnek, 1977). That is, P1 will propagate lytically in P7 lysogens, and conversely, P7 phage will grow in P1 lysogens. The c1 repressor protein of P1 is interchangeable with that of P7. It is expression of the incoming reb gene, not repressed by the resident c4 gene product, that can overcome c1 repression and result in lytic cycle expression (Wandersman and Yarmolinsky, 1977; Scott, et al., 1978).

Bacteriophage P1 is well known for its involvement in generalized transduction, in which segments of the bacterial chromosome are encapsidated in P1 virions (Lennox, 1955; Ikeda and Tomizawa, 1965a). Specialized transduction mediated by P1 occurs less frequently. In this process, the transduced genes are aberrantly integrated into the P1 genome (Luria et al., 1960) and replicate thereafter as part of the prophage. During the lytic cycle of such specialized transducing derivatives (P1std), the integrated host genes are packaged, producing high frequency transducing (HFT) lysates (Luria et al., 1960).

Because the P1 genome is smaller than the virion DNA content, a single P1 phage particle can contain up to 12 kb of acquired host genes, in addition to the entire P1 chromosome. Lysates produced by such P1 derivatives are normal in P1 functional characteristics, if the acquired host segment does not interrupt essential P1 genes. A P1std genome can be greater in size than the virion chromosome. Virion populations derived from such prophage still contain the entire P1std genome, but not within single phage particles. Consequently, single virions are not proficient as plaque forming bacteriophage (Rae and Stodolsky, 1974; Iida and Arber, 1977). Formation of such "overlarge" P1std prophage initially requires multiple infection of recipient strains, so that the full complement of P1 and host genes is provided. Alternatively, recipient strains can be singly infected if they are lysogenic for P1, since the recipient prophage provides missing P1 genes (Rae and Stodolsky, 1974).

Several P1 derivatives that have acquired E. coli chromosomal genes have been characterized: P1dlac (Luria et al., 1960; Rae and Stodolsky, 1974), P1dpro (Rosner, 1975; Stodolsky, 1973), and P1argF (Schultz and Stodolsky, 1976). Other P1 derivatives have acquired their foreign genes from R plasmids with drug resistance determinants. These include P1CmO (Kondo and Mitsuhashi, 1964), P1Tc (Mise and Arber, 1976) and P1CmSmSu (Iida and Arber, 1977).

Integration of the P1 prophage into the bacterial chromosome is a rare event. Most of the unions of an integrated P1 chromosome and the host genome occur at sites which map at the ends of the P1 genetic map (Chesney et al., 1978). In contrast to prophage integration, the

majority of P1std that have been genetically or physically characterized have at least one of the unions of P1 and the acquired genes near or within the naturally occurring IS1 sequence of P1 (Schultz and Stodolsky, 1976; Yun and Vapnek, 1977; Iida et al., 1978; Iida and Arber, 1980; for review see Yarmolinsky, 1977). However, precise localization of acquired genes within IS1 has only been done for P1Cm0 (Yun and Vapnek, 1977; Iida et al., 1978; DeBruijn and Bukhari, 1978), P1Cm246 (Iida et al., 1978), and P1CmSmSu (Walker et al., 1979). These P1std were formed by transposition from R factors which also encode IS1. Consequently, insertion of these drug resistance determinants at the P1 IS1 locus involves a site-specific recombination (Iida and Arber, 1980).

The only physical characterizations of P1std derived from unions with the host chromosome are those reported by Schultz and Stodolsky (1976) and Schultz (Masters thesis, 1977). These P1argF derivatives were isolated from a single transduction experiment, using E. coli K12(P1) as the donor strain. The argF gene in this strain is encoded within the host chromosome. Cleavage analyses indicated that P1argF genomes constitute a diverse population having two common features. All argF insertions were within the same EcoRI segment of the P1 chromosome and all produced one common EcoRI cleavage fragment. The results reported in this thesis confirm and extend the observations of Schultz and Stodolsky (1976).

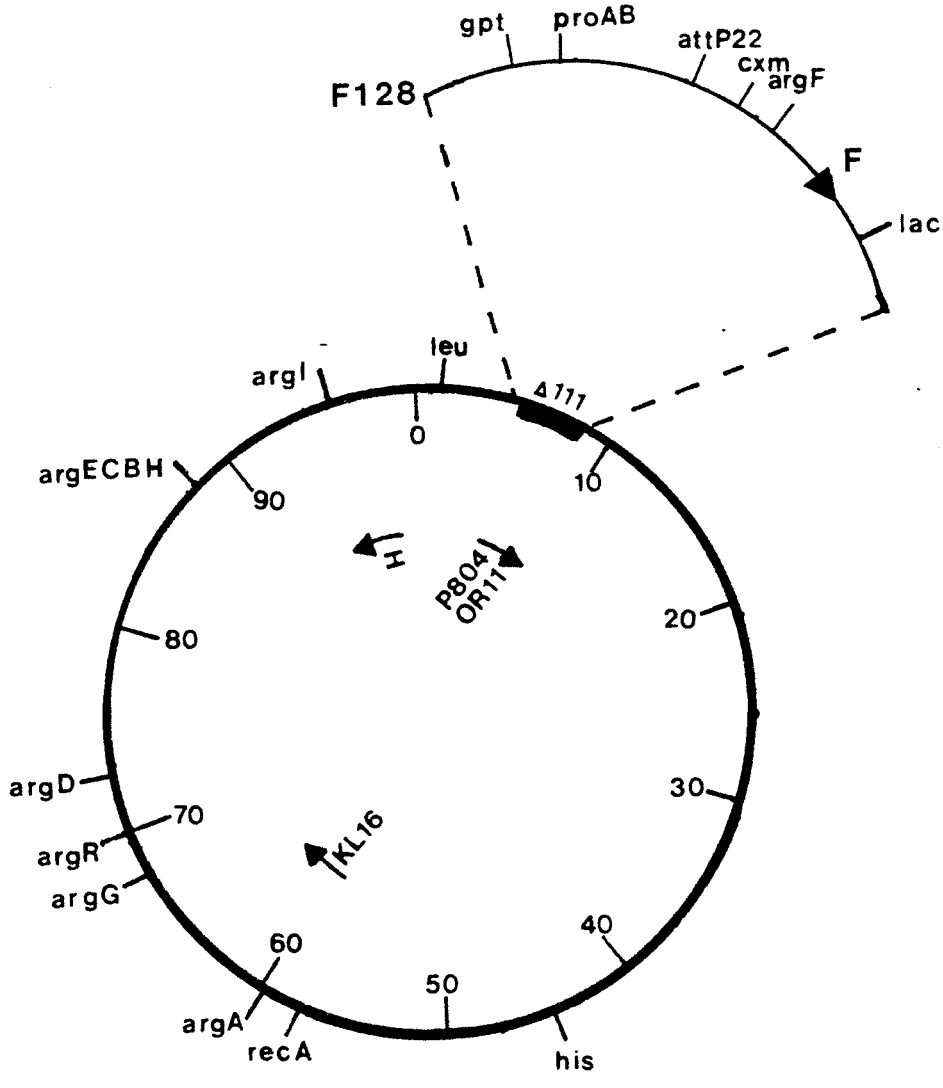
Properties of the argF gene of E. coli K12.

There are nine genes involved in arginine biosynthesis. Even

though they are scattered aroung the E. coli chromosome (as illustrated in Fig. 2) they all respond to the common regulatory protein located at argR (Maas, 1961; Gorini et al., 1961; Jacoby and Gorini, 1969). The argF gene, encoding ornithine carbamoyltransferase (OCT), catalyzes the sixth step of arginine biosynthesis in which ornithine is converted to citrulline (Gorini et al., 1961). Some years ago it was found that E. coli K12 strains contain two distinct genes, argF and argI, that are able to produce a functional ornithine carbamoyltransferase (Glansdorff et al., 1967). Gene argI is located at 96 minutes on the E. coli K12 linkage map and is present in E. coli strains W, B, and K12 (Legrain et al., 1972), as well as in strains of Salmonella and Proteus (Kikuchi and Gorini, 1976). The argF gene, located at 6 minutes on the E. coli K12 map, is unique to this strain (Glansdorff et al., 1967; Legrain et al., 1972). Why strain K12 should possess two OCT genes is not clear, although it is postulated that argF arose by transposition from the argI locus (Kikuchi and Gorini, 1976).

There are several lines of evidence to indicate that both genes evolved from a common ancestral argI gene. Both genes are under control of the regulatory argR gene (Glansdorff et al., 1967; Cleary et al., 1977) and both produce an inactive gene product which associates with two other identical subunits to form an active trimeric enzyme. The protomers from both genes can interact within the cell to form any of four possible trimeric molecules (Legrain et al., 1972). Products from both genes cross react immunologically but differ slightly in size and isoeletric point (Legrain et al., 1972; Cleary et al., 1977). A trimeric molecule composed only of argI subunits is more heat resistant

Fig. 2. Linkage map of Escherichia coli K12 (reproduced in part from Bachmann and Low, 1980). Numbers indicate map intervals in minutes. One minute corresponds to approximately 39 kb. Inside the circle, the transfer origins of pertinent Hfr strains are indicated. Genes of the arginine biosynthetic pathway are noted. The heavy boxed area indicates the extent of the proAB-argF-lac deletion X-ray 111 of strain SC1800. Plasmid F128 is derived from Hfr P804. In Hfr P804 the F plasmid is integrated between argF and lac, transferring argF first. The aberrant excision generating F128 joins gpt and lac (Low, 1972; Hoppe and Roth, 1974).


gpt
proAB
attP22
cxm
argF
F128
F
lac
leu
argI
4777
0
10
argECBH
90
H
P804
OR11
20
80
argD
30
70
argR
KL16
argG
40
60
50
argA
recA
his

than that composed of argF subunits (Kikuchi and Gorini, 1975). Subunits coded by argI also have a faster rate of association than those coded by argF (Cleary et al., 1977). Other kinetic and affinity constants are identical (Kikuchi and Gorini, 1975).

The similarity between the two genes was demonstrated by analysis of their DNA by heteroduplex formation. Specialized transducing λ bacteriophage carrying either the argI region or the argF region have been selected. Complementary single-stranded DNA from these phage annealed to form a 1.2 kb region of homology attributable to the OCT gene (Kikuchi and Gorini, 1975). Heteroduplex analysis also demonstrated that the genes surrounding argF and argI are completely different. This suggests that only a small segment of DNA could have been transposed from the argI locus to that of argF. Hybridization of argF mRNA to a labeled DNA probe carrying argI demonstrated that between 25 and 40% of the base pairs in the two genes have changed since they diverged (Sens et al., 1977).

Genes argF and argI also differ slightly in the segments produced by restriction endonuclease cleavage analysis of their DNA. An EcoRI cleavage site in the DNA from the λ vector carrying argF is lacking in the DNA of the vector carrying argI (Kikuchi and Gorini, 1976). Recently, a segment of DNA encoding the argF gene has been cloned into pBR322. Preliminary studies with this hybrid plasmid indicate that the argF promoter is located approximately 30 base pairs in from the EcoRI restriction site (Moore et al., 1978). The argF gene with its promoter occupies a region of approximately 1160 base pairs (Moore and James, 1979).

Transposable elements and the insertion sequence IS1.

Transposable elements comprise a group of distinct DNA segments found in prokaryote organisms (for reviews see Kleckner, 1977; Bukhari et al., 1977). These elements are capable of repeatedly inserting into a few or many sites within bacterial, plasmid, or phage genomes (Campbell et al., 1979). Translocation events mediated by these elements are independent of generalized recombination functions, since they occur in bacteria in which homologous recombination is eliminated by the recA mutation (Rubens et al., 1976).

Transposable elements are divided into three categories based on their complexity (Campbell et al., 1979). Insertion sequences (IS elements) are generally shorter than 2 kb and contain no known genes unrelated to insertion function. They are so named because their insertion within a gene causes a mutation of the gene (Jordan et al., 1967). Transposons (Tn elements) contain genes unrelated to insertion function and are larger than 2 kb. They often contain the same IS element at each end. Lastly, episomes are self-replicating transposable elements such as the F plasmid and phages Mu and λ.

While studying mutations in the Tn3 transposon encoding resistance to ampicillin, Arthur and Sherratt (1979) isolated intermediates in the translocation process that have led to the development of a general model for transposition (Grindley and Sherrat, 1978; Arthur and Sherratt, 1979; Shapiro, 1979). Their work has shown that when transposition of an element occurs from one replicon to another, a cointegrate molecule containing both replicons, separated by directly repeated copies of the element, is first produced. Such a process is

called replicon fusion. The intermediate is then resolved into separate replicons, each with a copy of the transposon. This resolution can be mediated either by recA dependent recombination or by a site-specific recombination specified by the transposon itself (Arthur and Sherratt, 1979). Thus the transposition process results in the covalent linear insertion of the element into one replicon and the reconstruction of the other replicon with its original element.

If the transposition process is intramolecular, that is, within the replicon encoding the transposable element, there are two possible outcomes. First, the replicon can remain intact, but there is an inversion of the genes between the duplicated transposable elements. Second, two circular molecules can be generated, each with a copy of the transposon and each carrying the genetic complement of the other. Commonly, one of these will be replication deficient and the other will effectively be a replicon carrying a deletion of the parental chromosome (Arthur and Sherratt, 1979). Either circular molecule can insert into another functional replicon through the action of its transposon. Such insertion creates a "sandwich" structure at the new location with a copy of the transposed element on either side of the inserted DNA. This type of transposition has been proposed as one pathway for the translocation of drug resistance genes from R factors to other plasmids (Iida and Arber, 1980). In addition, "sandwich" structures can be amplified by the site-specific recombination function of the transposon, generating tandem duplications of the integrated genes (see Meyer and Iida, 1979).

At least for Tn3, the transposon encodes both a transposase and

a repressor that regulates transposase synthesis (Gill et al., 1979; Arthur and Sherratt, 1979). The transposase controls both the insertion of the transposable element at the new site and the site-specific recombination function. The nucleotide sequences at the ends of the element are essential for transposition (Heffron et al., 1977). In addition, there is a distinct internal site required for the site-specific recombination. Mutants lacking this site are unable to resolve fused replicons in a recA host even if the transposase is present in the cell (Arthur and Sherratt, 1979).

Insertion sequence IS1 is the smallest known transposable element. It is estimated that there are 8 to 14 IS1 sequences within the genome of E. coli (Saedler and Heiss, 1973; Chow, 1977). The entire nucleotide sequence (768 base pairs) is known (Ohtsubo and Ohtsubo, 1978; Johnsrud, 1979). It is unlikely from the size of IS1 that it encodes more than one protein, but no gene product has yet been isolated or even demonstrated.

The transposon Tn9 encodes the chloramphenicol resistance determinant, chloramphenicol acetyl transferase (cat), flanked by direct repeats of IS1 (MacHattie and Jackowski, 1977; DeBruijn and Bukhari, 1978). The initial P1 specialized transducing phage carrying Tn9 was isolated by Kondo and Mitsuhashi (1964) and is now designated P1CmO (Iida et al., 1978). Recently, Alton and Vapnek (1979) have sequenced the 1102 base pair region between the directly repeated IS1 sequences of Tn9. Comparison of the DNA sequencing data to the amino acid sequence of the cat protein (Shaw et al., 1979), indicates that it is unlikely that any other genes besides cat and IS1 are encoded by Tn9.

Principles of DNA spreading techniques.

Use of spreading techniques to visualize DNA molecules for electron microscopy was first developed by Kleinschmidt (1968). The technique is based on two principles. First, many globular proteins in solution are capable of producing an insoluble monomolecular film of unfolded polypeptides, through denaturation on the surface of an aqueous solution. Second, negatively charged DNA or RNA molecules are adsorbed irreversibly to the basic side chains of these proteins. When the protein film is formed, the adsorbed nucleic acid molecules are spread on it, converting them from a three-dimensional to a two-dimensional position. This surface film is elastic and can be compressed or decompressed without breaking. The adherence of the film to a solid support is greater than the adherence of the film to the aqueous solution. Consequently, the protein-DNA complex can easily be removed to an electron microscope grid for visualization (Kleinschmidt, 1968).

In practice, the DNA is adsorbed to a basic protein, usually cytochrome C, in a solution of high salt concentration, referred to as the hyperphase. This solution is spread dropwise down a ramp onto an aqueous solution of lower ionic strength called the hypophase. The protein-DNA film is then picked up on a coated grid, stained and rotary shadowed to give sufficient contrast for observation with the electron microscope (Davis et al., 1971).

The aqueous DNA spreading technique has been very useful for determining the structure of DNA genomes and their replicative forms (for review see Younghusband and Inman, 1974). Size determinations of DNA molecules can also be made from such spreads if a molecule of known

size is included for comparison (Davis et al., 1971). The ratio of the lengths of the two DNA molecules as seen by electron microscopy is equal to the ratio of the number of kilobases present in each of the two molecules. Determinations of the molecular weight of DNA molecules by this technique correlates well with size determinations calculated from the sedimentation coefficients of the DNA (Sharp et al., 1972).

Westmoreland et al. (1969) adapted the procedure of Kleinschmidt to observe single-stranded DNA and RNA and differentiate it from double-stranded molecules. In this procedure, formamide is added to both the hyperphase and hypophase solutions to reduce random annealing of single-strands. Formation of hydrogen bonds between the formamide and the unpaired single-stranded bases blocks intrastrand hydrogen bonding and resultant aggregation.

Formamide is a denaturing agent that lowers the mean thermal denaturation temperature (T_m) or melting temperature of the DNA. The T_m of a particular DNA is the temperature at which half the base pairs are dissociated. Every 1% increase in formamide concentration corresponds to a lowering of the T_m of double-stranded DNA by 0.7°C (McConaughy et al., 1969). Conversely, an increase in the ionic strength of the solution raises the T_m (Massie and Zimm, 1969). Since the salt concentration of the hyperphase is necessarily high, the formamide concentration must also be high to maintain the same denaturing conditions as the concentrations of salt and formamide in the hypophase. These conditions, known as isodenaturing conditions, assure that the DNA is mounted for electron microscopy under true equilibrium conditions and not trapped in some metastable state (Davis and Hyman, 1971).

The formamide technique of spreading DNA is useful for heteroduplex analysis. A heteroduplex is defined as a double-stranded molecule formed by annealing the denatured opposite strands of two partially complementary DNA molecules (Younghusband and Inman, 1974). Under renaturation conditions, regions of near homology will form duplexes, while regions of non-homology will remain single-stranded. Denaturation of the DNA is accomplished by heat, alkali, or organic compounds such as formamide. The rate of renaturation follows second order kinetics and is maximal at temperatures between 15 and 30°C below the T_m of the DNA (Wetmur and Davidson, 1968). This is best accomplished by addition of formamide to the reaction without raising the temperature of the solution, since depurination of the DNA and subsequent chain scission is increased at elevated temperatures (Davis and Hyman, 1971).

Heteroduplex techniques have been used for mapping differences between genomes; identifying inversions in segments of DNA within a genome; and detecting inverted repeat sequences, such as the C-loop structure of P1 (Lee et al., 1974). The permutedness and terminal redundancy characteristics of phage genomes are also demonstrated by heteroduplexing techniques. Denatured linear DNA molecules from virion populations will form duplex circular structures if the virion DNA is terminally repetitious. If the genome is also permuted, two linear single-stranded regions will radiate from separate locations on these circular molecules. Such structures have been reported for bacteriophage P1 (Ikeda and Tomizawa, 1968).

CHAPTER III

MATERIALS AND METHODS

Bacteria and bacteriophage.

Shigella dysenteriae 16, the indicator strain for plaque assays, is described in Luria *et al.* (1960). All other bacterial strains are derivatives of *E. coli* K12, and are listed in Table 1. Bacteriophage P1 strains used in this study are derived from P1*kc*, a clearer plaque mutant (*c* character) of the original turbid plaque forming P1 isolate, selected for improved efficiency of plating on *E. coli* K12 strains (*k* character) (Lennox, 1955). Phage strains are further described in Table 2. Mutants with the temperature sensitive *c1* alleles: *c1*.100, *c1*.225, and *c1*.9*ts* are thermally inducible as prophage at temperatures above 40°C.

For brevity, allelic designations will be omitted when they are not significant to the discussion.

Media and culturing conditions.

For routine use, bacteria were grown in L broth (Lennox, 1955), containing 1% Bacto-tryptone, 0.5% yeast extract, and 0.5% NaCl adjusted to pH 7.2 with 1 M NaOH. When appropriate, L broth was supplemented after sterilization with $MgCl_2$ to a final concentration of 0.01 M, to stabilize phage particles, or with $CaCl_2$ to a final concentration of 0.005 M, to enhance phage adsorption. For colony growth, L agar (L broth plus 1.5% agar) was used. When chloramphenicol resistant

TABLE 1

Bacterial Strains

Strain	Relevant markers	Source	Comments
SC1800	argI90 proAB-argF-lac deletion X-ray 111 (Δ111)	This laboratory	The argI allele is non-reverting.
TJC77	argI90 proAB-argF-lac Δ111/ F'128 proAB argF lac	W. Epstein	SC1800 x F'128
SC7100	SC1800 $proAB^+$ $argF^+$ lac^+	This laboratory	The Δ111 locus is replaced by conjugation with HfrH.
TJC26	argI90 proA argF	W. Epstein	This argF allelle is revertable.
D5	SC1800(P1argF5c1.100)	This laboratory	Transduction of SC7100(P1c1.100) into SC1800(P1c1.100)

TABLE 2

Bacteriophage Strains

Phage strain	Reference or source	Comments
P1CmO	Kondo and Mitsuhashi, 1964	Recombinant of P1kc and R factor pSM14 (Novick, 1974); contains the Tn9 transposon
P1CmOc1.100	Rosner, 1972	Mutant of P1CmO with a temperature sensitive c1 gene product [This strain differs from those described by DeBruijn and Bukhari (1978) and Iida and Arber (1979) which possess a duplication of Tn9.]
P1c1.100	Rosner, 1972	Cm^S revertant of P1CmOc1.100
P1c1.225	Scott, 1968	Mutant of P1kc isolated following hydroxylamine mutagenesis having a temperature sensitive c1 gene product
P1Cm13c1.225	Iida and Arber, 1977	Recombinant of P1c1.225 and R plasmid NR1 [The Cm^r gene segment flanked by IS1 is at map unit 4 (Arber et al., 1978).]
P7c1.9ts	Yarmolinsky	Mutant of P7 with temperature sensitive c1 gene product
P1vir	Scott, 1968	Mutant of P1kc that grows on P1 lysogens
P1clg	N. Franklin	Mutant of P1kc lacking the c1 repressor protein and requiring less Ca^{2+} for adsorption than wild type P1 (g character).
P2vir	Our stocks	Virulent mutant of phage P2

(Cm^r) colonies were selected, chloramphenicol was added to a final concentration of 25 μg/ml. Ampicillin was added at a concentration of 50 μg/ml to select P7 lysogens. For plaque assays, L agar was supplemented with 0.01 M $MgCl_2$ (final concentration) after sterilization (L-Mg agar).

Minimal selective media contained Ozeki buffer (Ozeki, 1956), described in Table 3 and appropriate supplements which were prepared separately and mixed after sterilization. The final concentrations of supplements were 12 g/l glucose or lactose, 2 μg/ml thiamine, 15 g/l agar, and 40 μg/ml D, L-amino acids as required for various selections (Stodolsky et al., 1972). For genetic crosses, minimal selective media lacking the appropriate amino acid and/or containing the appropriate antibiotic were used. Ozeki buffer with 0.65% agar (Ozeki soft agar) was the embedding medium for selecting and scoring transductants. MacConkey agar was used to score Lac phenotypes.

Before use, all cultures of E. coli K12 were exponentially grown in L broth at 30°C to a titer of 2×10^8 cells/ml. A turbidity corresponding to 2×10^8 cells/ml was measured as 40 Klett units at 540 nanometers using the Klett-Summerson photoelectric colorimeter.

A soft agar embedding medium (0.8% Bacto nutrient broth, 0.5% NaCl, and 0.65% agar) was used as an overlay for plaque forming assays. Soft agar overlays were prepared from E. coli cultures by the following standard procedure. Aliquots containing 2×10^7 bacteria from actively growing cultures were added to 2.5 ml of soft agar at 45°C. This suspension was vortexed and layered onto L-Mg agar. For soft agar overlays of Shigella 16, an aliquot of 0.1 ml from an overnight culture grown at

TABLE 3

Buffer Solutions

Buffer name	pH	Salt concentrations
Ozeki	7.3	10.5 g/l K_2HPO_4, 4.5 g/l $MgSO_4$, 1 g/l NH_4SO_4, 0.47 g/l NaCl
Phage	7.4	0.1 M Tris, 0.01 M $MgCl_2$, 0.01 M NaCl
DNA	8.0	0.01 M Tris, 0.001 M EDTA
Phage lysis	8.5	0.04 M Tris, 0.04 M EDTA
Electrophoresis	8.4	0.09 M Tris, 2.8 mM Na_2EDTA, 0.09 M boric acid
20x SSC	7.0	3.0 M NaCl, 0.3 M Na_3citrate

25°C was used.

Buffers and solutions.

Buffers used in this study are listed in Table 3. Sodium azide was added to a final concentration of 0.02 M to all purified phage and DNA samples to prevent bacterial contamination. Samples were then stored at 4°C.

Preparations of phage lysates.

Thermally inducible phage stocks were prepared by either induction of lysogens or infection of sensitive strains. For the former, 0.01 M $MgCl_2$ (final concentration) was added to bacterial cultures at the time of induction. Cultures were incubated at 43°C for 30 minutes. The temperature was then lowered to 37°C for 90 minutes or until clearing was apparent (Iida and Arber, 1977). For the latter, bacterial cultures containing 2 x 10^8 cells/ml were concentrated by centrifugation at 2000 rpms for 5 minutes (25°C) to one-tenth the original volume and incubated with phage at an m.o.i. of 0.3. After a 20 minute adsorption, infected bacteria were diluted into L broth to their original concentration and the cultures were heat induced.

To produce lysates from lysogens with defective prophage, P1 helper phage were adsorbed before induction (Luria et al., 1960). Bacterial cultures were concentrated 10-fold by centrifugation. P1 helper from SC1800(P1c1.100) was added at an m.o.i. of 2. After adsorption for 20 minutes, the infected bacteria were diluted and heat induced as above.

Bacteriophage without heat inducible alleles, (P1Cm0, P1c1g,

P1vir, and P2vir) were prepared by the confluent lysis method (Swanstrom and Adams, 1951). Aliquots of 2×10^7 sensitive bacteria were added to 2.5 ml of soft agar along with various dilutions of phage stocks. This suspension was layered onto L-Mg agar and incubated 18 hours at 37°C. Five ml of L broth was poured onto plates showing confluent lysis of the bacterial lawn. After 10 minutes, the broth containing phage particles was collected.

Lysates were routinely sterilized with a few drops of $CHCl_3$ and stored at 4°C. Stocks were freed of $CHCl_3$ by dilution or by 30 minutes of aeration at 30°C before use (Lennox, 1955).

Phage titers.

Phage titers were determined as described by Rosner (1972). Soft agar overlays of Shigella 16 were prepared with various dilutions of phage lysates. After 18 hours incubation at 40°C, visible plaques were counted. Phage titers were expressed as plaque forming units (pfu)/ml.

Isolation of strains lysogenic for P1 or P7.

Cultures of the bacterial strain to be lysogenized were prepared in soft agar overlays. Samples of several dilutions of phage lysates were spotted on the bacterial lawn. Plates were incubated for 18 hours at 30°C. An aliquot from the lysed area produced from the lowest dilution giving a turbid plaque was suspended in L broth and incubated for two hours at 30°C. The resulting culture was diluted and plated onto L agar with a hockey stick to obtain isolated colonies. Plates were incubated at 30°C for 18 hours and then replica plated to

L agar. Duplicate plates were incubated at both 30 and 40°C.

For recognition of P1c1.100 strains, colonies that grew at 30°C but not at 40°C were tested further for ability to produce phage. For recognition of P1c1.225 strains, colonies that grew at 30°C but formed smaller colonies at 40°C were tested further. Colonies from P1c1.225 lysogens that did not form visible colonies at 40°C proved to be lysogens with defective phage.

Alternatively, bacterial cultures were incubated with phage particles at a multiplicity of infection (m.o.i.) of 1 at 30°C for one hour, diluted and plated for isolation. Replica plates were then prepared and selection made as described above. Lysogens of P1Cm or P7 were selected on L agar containing chloramphenicol or ampicillin, respectively. Drug resistant transductants were tested further for phage production.

Transduction frequencies.

Recipients for transduction, either SC1800 or SC1800(P1), were infected as described by Rosner (1972). The amount of phage lysate used to infect recipients depended on the m.o.i. desired; usually this was 0.3. For increased accuracy, phage titers were determined no more than two days before transduction experiments.

Phage were incubated with recipient bacteria (2×10^8/ml) at 25°C for 30 minutes in L broth with 0.005 M $CaCl_2$. Infected bacteria were concentrated by low speed centrifugation (8000 rpms at 4°C for 10 minutes). The supernatants were removed and replaced with an equal volume of saline (0.85% NaCl). After resuspension, a second low speed

centrifugation was performed. It was necessary to include a saline wash step to remove traces of L broth. Washed bacteria were suspended in saline to a concentration of 1 x 10^9 cells/ml, diluted into 2.5 ml of Ozeki soft agar at 45°C, and quickly plated on Ozeki minimal selective medium.

If the recipients per plate exceeded 8 x 10^8, the size of transductant colonies diminished. Hence, to maintain optimal detectability, no more than 5 x 10^8 recipients were plated. Frequencies were calculated as the number of transductants per input pfu after 48 hours incubation at 30°C.

Rapid assay for HFT lysates.

The ability to produce a high frequency transducing (HFT) lysate was determined by a rapid assay. Lysates of Arg^+ transductants were prepared as described previously with and without helper phage. Phage particles at an m.o.i. of approximately one were adsorbed to 2 x 10^8 bacteria/ml from the indicator strains. After 30 minutes at 25°C, samples of approximately 0.02 ml were spotted onto Arg selective medium. Prolific transductant colony formation after 48 hours incubation at 30°C was evidence of an HFT lysate. Indicator strains were either SC1800 or TJC26, as indicated. Known low frequency transducing (LFT) lysate controls did not produce transductant colonies on the indicator strains with this protocol.

Rapid assay for normal bacteriophage production.

To determine if Arg^+ transductants yielded normal P1 plaque forming titers, suspected lysogenic cultures were thermally induced as

described previously. Sterile aliquots containing approximately 0.02 ml of the heat induced cultures were diluted into wells of 25 well plates containing 0.2 ml L broth. Several successive 10-fold dilutions were made in this manner. Dilutions were spotted onto a soft agar overlay of Shigella 16 and examined for lysis after 18 hours incubation at 40°C. Cultures that produced plaques from the undiluted heat induced sample were plaque formers. Cultures yielding plaques from 0.02 ml aliquots after eight 10-fold dilutions were considered to have normal plaque forming titers. Laboratory strains of P1c1.100 and P1c1.225 usually produced titers between 10^9 and 10^{10} pfu/ml from induced lysogens. To see if non-plaque forming cultures produced virions, a phage purification protocol was performed, as described below. If no phage bands were visible in the final CsCl gradient, the cultures were considered to be defective in virion production.

Tests of P1 immunity, restriction, and modification.

Bacterial cultures were tested for P1 immunity as described by Iida and Arber (1977). Soft agar overlays were prepared from the test bacteria and spotted with P1c1g and P1vir phages. After a 24 hour incubation at 30°C, test bacteria which resisted P1c1g but were lysed by P1vir were classified as P1 immune.

Tests for P1 restriction and modification were as described by Arber and Wauters-Willems (1970). Modified P2vir was prepared by growth of P2vir on a P1 lysogen defective in P1 phage production. Unmodified P2vir was prepared on a non-lysogen. For restriction assays, cultures of the test bacteria were suspended in soft agar overlays and

spotted with P1 modified and unmodified stocks of bacteriophage P2vir. Lysis of the test bacteria by modified P2vir but not by unmodified P2vir indicated that the test strain was Res^+. Test bacteria that were Res^- were lysed by both P2 phage stocks.

For modification assays, P2vir propagated on the test bacteria were spotted onto soft agar overlays of strains SC1800 and SC1800 (P1c1.100). If both strains were lysed by the P2 phage, the test bacteria were Mod^+. If strain SC1800 was lysed but not SC1800(P1), the bacteria were Mod^-.

Plaque center test.

The procedure for the plaque center test followed the general method of Kondo and Mitsuhashi (1964). In this test, plaques were initiated by infection with a single phage particle from a P1argF lysate. These plaques were tested for the presence of Arg^+ lysogens by the following technique. Appropriate dilutions of P1argF phage were prepared in soft agar overlays with SC1800 bacteria. Plaques were developed at 30^oC to allow for P1c1ts lysogenization. After 18 hours the center of the plaque was stabbed with a sterile toothpick and transferred to Arg selective medium. Growth of Arg^+ colonies in 48 hours at 30^oC indicated presence of Arg^+ transductants and a positive plaque center test.

Bacteriophage purification.

Crude lysates of bacteriophage were prepared from 250 ml cultures by heat induction as described. Phage were concentrated with polyethylene glycol (PEG-6000) according to the method of Yamamoto et al. (1970). Lysates were adjusted to 0.5 M NaCl and PEG was added with

continuous stirring to a final concentration of 10%. The resulting solution was refrigerated overnight at 4°C, followed by a low speed centrifugation to pellet the high molecular weight complexes of PEG, host DNA, and phage. The pellet was suspended in 4 ml of phage buffer (Table 3), using a rubber policeman to remove the residue from the sides of the centrifuge bottle. A 1:50 dilution of a stock enzyme solution containing 500 μg/ml DNase I and 500 μg/ml pancreatic RNase in phage buffer was added to the suspension. After incubation at 37°C for 45 minutes, a low speed centrifugation was used to remove cellular debris. The supernatent was then layered onto a stepwise CsCl gradient according to the method of Ting (1962). The gradient consisted of 4 ml of 1.4 gm/cm^3 CsCl and 2 ml of 1.6 gm/cm^3 CsCl in phage buffer in a 12 ml centrifuge tube.

Centrifugation was for 3 hours at 4°C and 35,000 rpms in a SB-269 swinging bucket rotor of an IEC B-60 centrifuge. Phage bands of mature virion particles formed near a density of 1.47 gm/cm^3 (Ikeda and Tomizawa, 1965b; Walker and Anderson, 1970). These were removed with a 19 gauge needle and syringe and were dialyzed against three changes of phage buffer.

Purification of virion DNA.

The DNA was extracted from purified virions for restriction endonuclease cleavage analysis. Pronase type VI was self-digested at 37°C for 2 hours in phage lysis buffer (Table 3) at a concentration of 10 mg/ml to destroy contaminating nucleases. Treated Pronase was added to the virions at a final concentration of 1 mg/ml. Virions were incubated

for 2 hours at 37°C and then dialyzed for 3 to 16 hours at 25°C against phage lysis buffer containing 1% SDS. The dialysis was continued against phage lysis buffer without SDS for two additional hours. An equal volume of phenol saturated with lysis buffer was added to the virion DNA. Samples were gently mixed for 10 minutes and centrifuged at 2000 rpms for 5 minutes. The phenol layer was removed and the extraction was repeated. Samples were dialyzed exhaustively against DNA buffer to remove traces of phenol until the dialysis buffer had no absorbance at 270 nm using the unused dialysis buffer as the reference blank. Concentrations of DNA were calculated from absorbancy at 260 nm; an absorbance of 20 is equivalent to 1 mg/ml of DNA (Warburg and Christian, 1941).

Isolation of DNA from thermally induced lysogens.

This method was applicable to prophage whose vegetative replication can be induced. The induction increased the molar ratio of P1 to host genomes. In subsequent cleavage analysis using total purified DNA from the induced lysogens, the restriction fragments derived from the P1 chromosomes, but not the host chromosome, were detected on agarose gels.

Bacterial cultures (50 ml) were thermally induced as described previously. Twenty minutes after induction, 100 µg/ml of chloramphenicol was added. This treatment deferred lysis of the bacteria while it permitted continued replication of P1 chromosomes. After 2 hours at 37°C, bacteria were collected by low speed centrifugation. An adaptation of the procedure of Katz et al. (1973) was used for lysate preparation. Details of the procedure are in Table 4. The DNA from the

TABLE 4

Bacterial Lysis Procedures For DNA Isolation

Step No.	Isolation of DNA from induced lysogens		Isolation of plasmid DNA		Procedure
	Solution added[a]	Amount (ml)	Solution added[a]	Amount (ml)	
1.	0.15 M Tris	0.6	0.25 M Tris 0.25% sucrose	5.6	Suspend pellet
2.	5 mg/ml lysozyme in 0.25 M Tris	0.08	8 mg/ml lysozyme in 0.25 M Tris	0.75	Reaction time 5 minutes
3.	0.25 M Tris 0.25 M EDTA	0.16	0.25 M Tris 0.25 M EDTA	1.5	Reaction time 5 minutes
4.	10% sarcosyl 0.06 M Tris 0.06 M EDTA	0.84	10% Triton X100 0.0625 M Tris 0.0625 M EDTA 200 µg/ml EtBr[b]	7.5	Mix gently until the suspension clears.

[a] All solutions were at pH 8. Reactions were at 4°C.

[b] This step required visualization using a red light source only.

cell lysate was purified from cellular protein by Pronase digestion and phenol extraction as described for virion DNA purification.

Plasmid DNA isolation.

Plasmids were isolated by a modification of the CsCl-ethidium bromide (EtBr) density gradient centrifugation technique of Bauer and Vinograd (1968). Bacterial cultures (200 ml) were grown to 2 x 10^8 cells/ml and centrifuged at low speed. Pellets were suspended in buffer (see Table 4) and transferred to a 30 ml centrifuge tube. A cell lysate was prepared as described in Table 4. The resulting lysate was underlaid with 15 ml of a solution of CsCl (1.625 g/cm^3) and ethidium bromide (100 µg/ml) using a syringe with a large bore needle. Fractionation proceeded for 18 hours at 32,000 rpms and 4°C in an A-211 angle head rotor of an IEC B-60 centrifuge.

In this fractionation system, an aggregate of cell debris, host DNA and ribosomes pellet , while plasmid chromosomes band in the supernatant of the CsCl-EtBr gradient (Bornhoeft, personal communication). The pellet was aspirated from the gradient, and the plasmid fraction was collected with a 12 gauge needle and transferred to a 15 ml centrifuge tube. The tube was filled with CsCl-EtBr in the same concentrations as above, and centrifuged to isopycnic equilibrium (60 hours). Supercoiled plasmid DNA was removed from the gradient with a 12 gauge needle. Ethidium bromide was extracted with equal volumes of isoamyl alcohol or isopropanol saturated with 20x SSC. The purified plasmid DNA was then dialysed against DNA buffer.

Cleavage analysis and gel electrophoresis.

Purified DNA was digested with restriction enzymes, PstI or BamHI, in 0.05 M NaCl, 6mM Tris (pH 7.4), 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol and 100 µg/ml bovine serum albumin (Brown and Smith, 1976; Wilson and Young, 1975). For EcoRI restriction the same reaction mixture was used except the Tris concentration was increased to 100 mM (Hedgpeth et al., 1972). If DNA had been isolated from induced lysogens, RNase at a final concentration of 2 µg/ml was also added to the reaction mixture. Mixtures of enzymes and DNA samples were incubated at 37°C for 1 to 2 hours. Restriction was terminated by addition of one part 0.1 M Tris, 0.1 M EDTA (pH 8.5), 0.07% bromphenol blue to 10 parts of restriction mixture, followed by a 5 minute incubation at 60°C. For DNA isolated from induced lysogens, 5 µg of DNA was used per gel pocket; for purified virion DNA, 1 µg was used. The digested DNA samples were mixed with 0.15% agarose and 5% sucrose (final concentrations) in electrophoresis buffer and layered into pockets of 0.7% agarose gels.

Electrophoresis was carried out at 1.3 or 2.6 V/cm for 18 to 36 hours as indicated. Gels were stained with 0.5 µg/ml ethidium bromide. The fluorescing bands were illuminated with long wave ultraviolet light, and photographed with Polaroid type 55 film. Restriction fragments of P1 DNA, often from the same gel pocket, were used as standards for size determinations of new fragments. Sizes of P1 restriction fragments in map units have been reported by Bachi and Arber (1977). One map unit equals 1/100 of the P1 genome length. The size of the P1 genome is 91.48 kilobase pairs (kb), as determined by electron microscopy (see Chapter IV, Table 7).

For fragments less than 1.9 kb, PstI and EcoRI:PstI cleavage fragments of the Tn9 transposon were used as standards for size determinations. Sizes in kilobases of these fragments were from the nucleotide sequence determinations of Alton and Vapnek (1979).

Heteroduplex techniques.

Heteroduplex formation was performed by the method of Davis et al. (1971). For optimal results, equal concentrations of the participant chromosomes were desirable. Since purified virions were the source of the chromosomes, equal concentrations of the virions were used in amounts equal to 1 to 3 μg of DNA. For this determination, an absorbance of 40 at 260 nm was equivalent to about 1 mg/ml of encapsidated DNA. All reactions were performed in dialysis tubing to minimize manipulation of denatured DNA. Virion samples were added to 0.25 ml of 0.1 M NaOH, 0.02 M EDTA is dialysis tubing, to disrupt the virus particles and denature their DNA. After 10 minutes, the pH was lowered to 8.3 by adding 20 μl of 2 M Tris-HCl. Formamide was added to a final concentration of 50% and the denatured DNA was dialyzed against 1 M NaCl, 0.1 M Tris, 0.1 M EDTA (pH 8.5), and 60% formamide for 1 to 2.5 hours at 25°C depending on the concentration of the DNA. These conditions of renaturation were equivalent to 25°C below the T_m of phage DNA (Davis and Hyman, 1971). Renaturation was terminated by dialysis against DNA buffer at 4°C for at least 2 hours. The heteroduplexes were then spread by the formamide technique for DNA visualization.

DNA spreading techniques.

The aqueous DNA spreading technique of Davis et al. (1971) was

used for mounting plasmid DNA for size determinations and the formamide technique of Davis et al. (1971), as adapted by Yun and Vapnek (1977), was used for heteroduplex spreading. The 30%-60% formamide concentrations used for heteroduplex spreading were equivalent to 17°C below the T_m of phage DNA at the salt concentrations used in the hypophase and spreading solutions (Davis and Hyman, 1971). It has been estimated that about 1.5% randomly distributed mismatched bases result in a lowering of the T_m by 1°C (Laird et al., 1969). Therefore, under the conditions for the formamide spreading, a possible 25% (1.5% x 17) level of mismatched bases could exist in what appears to be homologous regions in the heteroduplexes (Laird et al., 1969).

Solutions for DNA spreading are listed in Table 5. All solutions were made with triple distilled water and filtered through 0.2 micron HAWP Millipore filters before use. Filters were freed of residual detergent by discarding the first few milliliters of filtrate. Cytochrome C solutions were not filtered, but were made shortly before use in either filtered water or buffer, as indicated in Table 5. Care was taken in making the Tris and EDTA buffer to avoid excess Na^+ ions. The EDTA was brought to pH 8.5 with NaOH before adding the Tris (Sharp et al., 1972). Final adjustments of the pH were made by adding Tris-HCl or Tris base to the buffer.

Formamide (99%) was preferably recrystalized by refrigeration at 4° C. The crystals formed at this temperature were collected and liquified at 25°C for use. Formamide was added to aqueous solutions just before spreading, to prevent pH changes due to the decomposition of

TABLE 5

DNA Spreading Solutions

Method	Hypophase	Spreading solution	Stocks for spreading solution	Amount of stock used for spread
Aqueous	0.25 M NH_4Ac (pH 8.5)	0.1 mg/ml cyto C	1 mg/ml ctyo C in H_2O	5 µl
		0.5 M NH_4Ac	1 M NH_4Ac (pH 8.5)	25 µl
		0.5 µg DNA (approximate)	DNA sample in DNA buffer DNA standard in DNA buffer	20 µl
Formamide	0.01 M Tris (pH 8.5) 0.001 M EDTA (pH 8.5)	0.1 mg/ml cyto C	1 mg/ml cyto C	
		0.1 M Tris	1 M Tris (pH 8.5)	5 µl
		0.01 M EDTA	0.1 M EDTA (pH 8.5)	
	30% formamide	60% formamide	Recrystalized formamide	30 µl
		0.5 µg DNA (approximate)	DNA sample in DNA buffer DNA standard in DNA buffer	15 µl

Abbreviations: NH_4Ac, ammonium acetate; cyto C, cytochrome C; Tris, hydroxymethyl aminomethane; EDTA, ethylene diamine tetraacetic acid; DNA buffer, 0.01 M Tris, 0.001 M EDTA (pH 8.5).

formamide into formic acid.

A stock of 5×10^{-3} M uranyl acetate in 50 mM HCl was made fresh weekly by adding 50 µl of concentrated HCl and 25.5 mg of uranyl acetate to 12 ml of distilled water. This solution was stored in the dark for 24 hours before use to completely dissolve the uranyl acetate. Before each use the stain was filtered through a 0.2 micron Millipore filter. The filtered stain was diluted 1:100 in 90% ethanol within one hour of DNA spreading.

Solid parlodian was baked for 24 hours at 90°C and then dissolved in isoamyl acetate to a final concentration of 3½%. Several plastics were found to be soluble in isoamyl acetate. Therefore care was taken not to pipet this chemical with plastic disposable pipets. Dissolved plastic caused black circular spots on the grids even before staining. Parlodian solutions were stored away from light in bottles with Poly-seal or Teflon liners.

Grids (300 mesh) were washed before use by 3 minute sonications in 3 changes each of acetone, 95% ethanol and filtered distilled water, followed by drying at 50°C. Parlodian coated grids were freshly prepared on the day of spreading. Grids were placed on a clean wire screen below the surface of filtered water. The surface of the water was swept with lens paper and a drop of Parlodian solution was dispensed through a wide bore pipet on the surface. After the film stabilized, the water level was lowered to deposit the Parlodian film on the grids. Grids were dried at 60°C for approximately 30 minutes.

In the spreading process, an acid cleaned, thoroughly rinsed glass microscope slide was dipped in 0.25 M NH_4 acetate and allowed to

air dry. A Petri dish was filled with 20 ml of hypophase and the glass slide was positioned in the hypophase to form a ramp. The spreading solution was prepared in a small weighing boat. Capillary pipets (50 µl size calibrated in 5 µl amounts) were useful for measuring the solutions used in the hyperphase.

The spreading solution was delivered on the glass slide through a 50 µl Eppendorf pipet with 2 mm of the tip cut off, using a back and forth motion across the slide about 2 mm above the meniscus. The film was allowed to spread for at least one minute. A grid was touched, Parlodian side down, to the surface of the water about 3 mm from the meniscus of the ramp and the solution. The grid was immediately placed in uranyl acetate stain for 25 seconds without agitation. Movement of the grid in the stain caused uranyl oxide precipitates to form. The grid was then quickly dipped in isopentane for 10 seconds and allowed to air dry. Several grids were prepared in this way from a single spread.

Grids were rotary shadowed with 1 to 2 cm of platinum-palladium (80:20) 23 gauge wire rapped around a tungsten filament. A glass slide with double stick tape was used to secure the grids by touching them to the edges of the tape. Grids were placed 7 cm away and 0.7 cm above the wire and shadowed just until a slight change in color was visible on a white cardboard placed in the vacuum jar. A light carbon coat was evaporated on the grids to give them strength under the electron beam.

Electron microscopy.

Specimens were observed in an RCA EMU 3 electron microscope operating at an accelerating voltage of 50 kv. Micrographs were projected using an Omega enlarger to a final magnification of at least 50,000x. Molecules were traced and tracings were measured with a Deitzgen 1718 Plan Measure.

Single-stranded øX174 phage DNA and purified øX174 RF II (replicative form II) DNA, supplied by R. Westro, were used as single and double-stranded standards, respectively. The øX174 chromosome is 5386 bases in length, as determined by the nucleotide sequence of øX174 DNA (Sanger et al., 1977). Internal C-loops and regions of non-homology between P1 derivatives and P7 were used as internal single-stranded standards. The single-stranded region of the C-loop is 3.1 kb (Bachi and Arber, 1977), while the sizes of the P1-P7 regions of non-homology have been determined by Yun and Vapnek (1977).

Materials.

Egg white lysozyme, cytochrome C VI from horse heart, Pronase type VI, and pancreatic RNase and DNase I from beef pancreas were purchased from Sigma. Isopentane, isoamyl acetate, parlodian, and formamide (99% A.C.S.) were from Mallinckrodt Chemical Company. Polyethylene glycol (Carbowax PEG-6000) was obtained from Union Carbide and restriction enzymes were from New England Biolabs.

CHAPTER IV

STRUCTURAL ANALYSIS OF BACTERIOPHAGE P1*argF* CHROMOSOMES

Introduction.

Preliminary results in this laboratory have provided the following information with which this investigation was started. Lysogens with P1*argF* prophage are selected by transduction from the donor strain SC7100(P1*c1*.100) to the recipient strain SC1800Δ111(P1*c1*.100). The genetic region spanned by deletion *proBA-argF-lac* (Δ111) is larger than the size of the P1 virion chromosome. Thus, fragments transduced by P1 from the donor cannot be integrated by generalized recombination, since they do not genetically overlap both ends of the deletion. With this selective system, the frequency of *arg*$^+$ transduction is approximately 10^{-6} (Stodolsky, personal communication).

Among 40 transductants from such a genetic cross, about half of them produce HFT lysates for *argF*. This is phenotypic evidence for lysogeny by P1*argF* prophage in the transductants. Virions from 10 of the transductants yielding HFT lysates have been characterized by restriction endonuclease cleavage analysis (Schultz and Stodolsky, 1976; Schultz, Masters Thesis, 1977). Seven phage chromosomes produced indistinguishable digestion fragments when cleaved with *Eco*RI endonuclease. The *Eco*RI-4 fragment of wild type P1 (see Fig. 1) was replaced by a larger 13.8 kb fragment and a smaller 3.1 kb fragment. Digestions of the other three P1*argF* chromosomes also lacked *Eco*RI-4 and contained

the 3.1 kb fragment. However, they had other fragments totaling more than 13.8 kb.

Because it represents the predominant class isolated by M. Stodolsky, the prophage designated P1argF5 of transductant D5 has been characterized further. Plasmid DNA from strain D5 transforms SC1800 to a P1argF lysogen (Stodolsky, personal communication). Lysates of D5 have normal plaque forming titers, indicating that the prophage genome is not overlarge and can be packaged genetically intact in single virion particles.

Electron microscopic analyses presented in this chapter demonstrate that the P1argF5 prophage contains an insertion of approximately 11 kb attributable to the argF gene segment. The insertion is precisely at the IS1 locus of the P1 chromosome and is flanked by direct repeats of IS1. Restriction endonuclease cleavage mapping of the argF gene segment is also presented and permits localization of the argF gene within the insertion.

Transducing activity of P1argF5 lysates.

Since P1argF5 lysates were normal in plaque forming ability, it was of interest to determine if the virions were capable of transmitting the P1argF genome by single infection. Lysates containing 3×10^9 pfu/ml were used to transduce strain SC1800 to the Arg^+ phenotype. For the most part, arg^+ transduction into the SC1800 recipient is accomplished by lysogenization with P1argF prophage (see Chapter V). Approximately 35 Arg^+ transductants/100 pfu were produced whether the SC1800 recipient was lysogenic or non-lysogenic for P1 (Table 6). Virion chromosomes from eight Arg^+ transductants were

TABLE 6

Transducing Activity of P1*argF5* Virions[a]

Recipient strain	M.o.i. of helper	Transduction frequency[b]
SC1800	0	.35
SC1800	3	.17 (.57)[c]
SC1800(P1)	0	.31

[a] Recipient strains were infected at an m.o.i. of 0.1.

[b] Frequency is expressed as transductants per input pfu with 93% of the input phage adsorbing to recipient bacteria.

[c] Value in parenthesis is corrected for the killing effect due to helper infection.

digested with EcoRI endonuclease. Cleavage patterns produced by these digests were identical to that produced by the P1argF5 chromosome depicted in Fig. 6. At the m.o.i. used for the infection only 5 transductants per 100 pfu could result from multiple infection, as calculated from the Poisson distribution. Therefore, the rest (30/100) resulted from single infection of recipients. If P1 virions were added as helper along with the transducing particle, the transduction frequency was only slightly increased (less than a factor of 2). These results showed that multiple infection of recipients was not necessary for the establishment of lysogeny with a P1argF prophage. Consequently, this experiment confirmed that in general the prophage genome was not overlarge.

P1argF5 prophage size determinations.

To determine the actual size of the P1argF5 genome, plasmid chromosomes isolated from strain D5 were characterized using the aqueous DNA spreading technique of electron microscopy. In order to compare the size of the P1argF5 plasmid with that of its parental genome, the size of the parental P1c1.100 plasmid was determined by the same technique. Results in Table 7 showed that the P1argF5 genome was approximately 10% larger than the P1c1.100 genome.

Phage P1 chromosomes are 7 to 11% larger than prophage chromosomes (Ikeda and Tomizawa, 1968; Yun and Vapnek, 1977). Consequently, the P1argF5 plasmid genome was shown to be nearly the same size as the P1 virion chromosome.

TABLE 7

Electron Microscopic Measurements

A. Size Determinations of Plasmid DNA from P1 Derivatives

Genome	Kilobase pairs ± standard deviation	No. measured
P1*argF5c1*.100	100.7 ± 3.3	20
P1*c1*.100	91.5 ± 2.1	21

B. Heteroduplex analysis of P1*argF* Virion DNA

Heteroduplex	Long arm	Short arm	No. measured
P1*argF5* x P1	10.8 ± 0.8[a]	0	8
P1*argF5* x P7	13.3 ± 1.0[b]	2.5 ± 0.5[b]	6
P1*argF5* x P1Cm0	10.8 ± 0.4	1.3 ± 0.3	6

[a] Kilobases ± standard deviation.

[b] Measurements are for the B substitution loop only.

Heteroduplex analysis of P1argF5 chromosomes.

Virion chromosomes were used for heteroduplex analysis to determine the size and location of the argF gene segment. Heteroduplexes were first prepared from the chromosomes of P1argF5 and its P1c1.100 parent. A micrograph of a heteroduplex is presented in Fig. 3. Results of several measurements are summarized in Table 7. A single-stranded insertion of 10.8 kilobases (kb) was observed approximately 9.3 kb from the C-loop present in P1 DNA (Lee et al., 1974). Because there was no other insertion in the P1/P1argF5 heteroduplexes, the 10.8 kb insertion was interpreted to represent the argF gene segment. The insertion was not accompanied by any detectable deletion of P1 genes, since no unpaired single-stranded region was observed on the other strand at the insertion site. Thus the heteroduplex analysis showed that the increase in size of the P1argF plasmid genome was due to the insertion of a single large segment of DNA.

Virion DNA from P1argF was annealed with itself to determine if the argF insertion is capable of inverting in the same manner as the C-loop of P1. No substitution loops except those the size of the C-loop were found in 40 homoduplex molecules examined, indicating that the argF gene segment did not invert. In addition, insertion loops were not observed. These structures would indicate that tandem duplications of DNA sequences were present within the virion chromosome population.

To identify the site of the argF insertion, heteroduplexes of P1argF and P7 chromosomes were examined. An electron micrograph of a P1argF/P7 heteroduplex is in Fig. 4 and the results of the analysis are

Fig. 3. Heteroduplex of P1argF5 and P1c1.100. An insertion, presumed to be the argF gene segment, is approximately 9.3 kb from the P1 C-loop region (C), represented as a substitution loop in this heteroduplex. The small plasmid, ϕX174 RFII is a size reference. Bar in this and all succeeding micrographs is equivalent to one kilobase of double-stranded DNA.

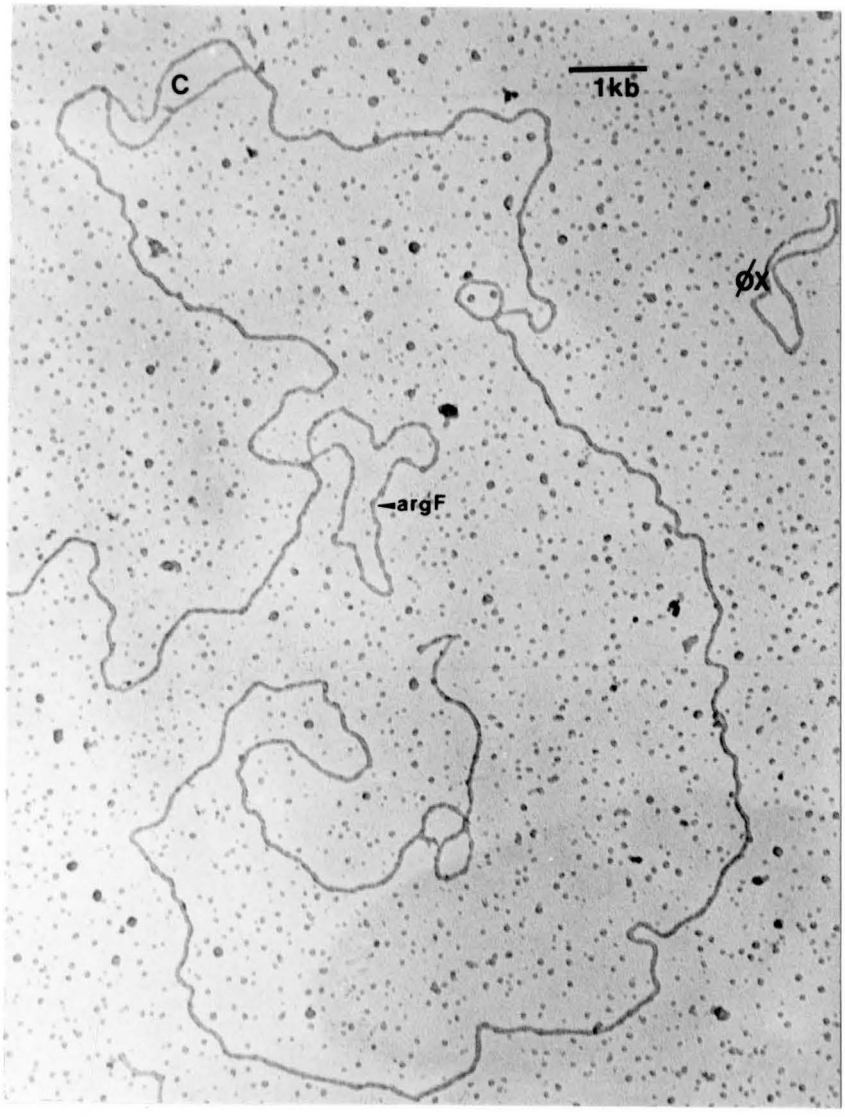
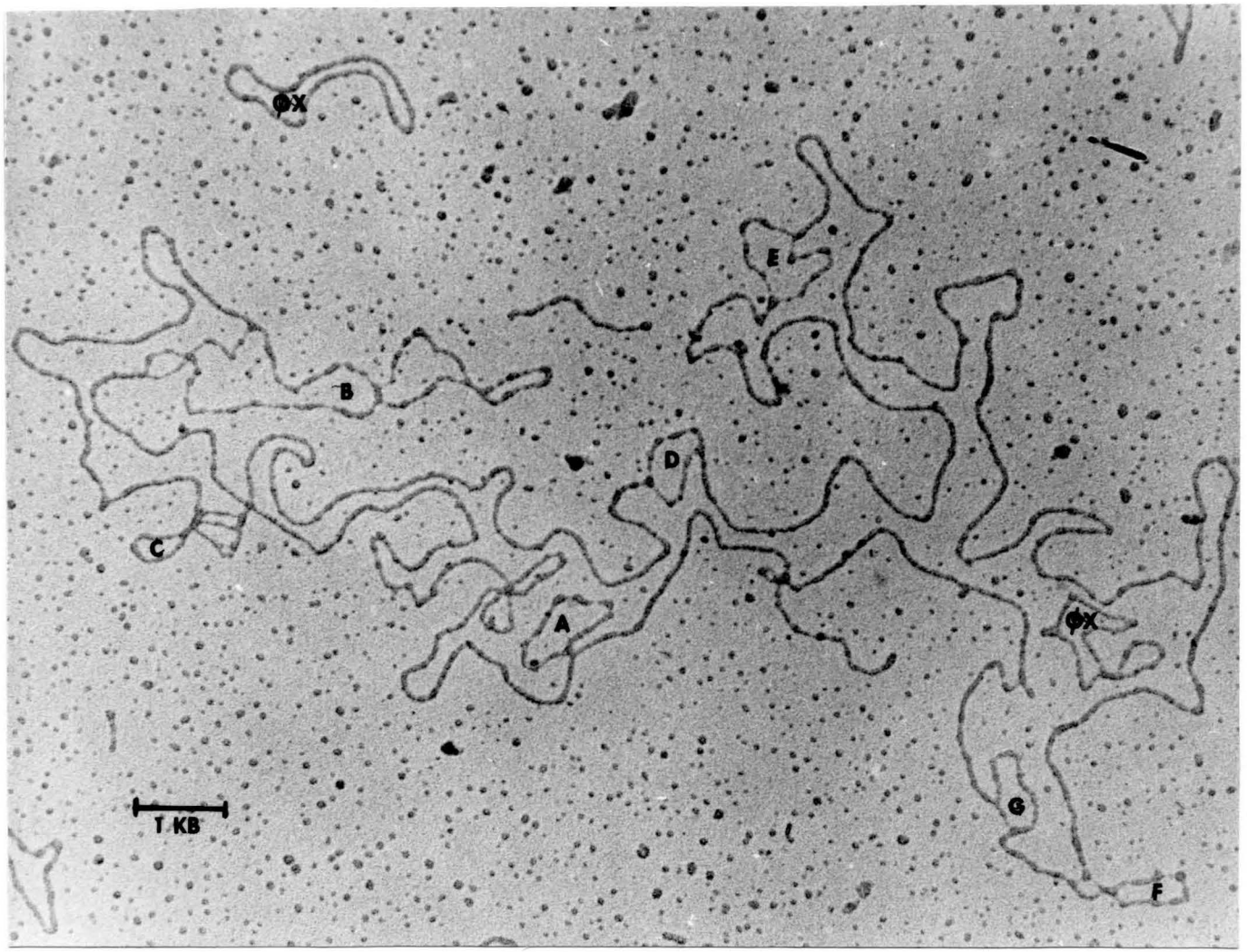

C
1kb
øX
argF

Fig. 4. Heteroduplex of P1argF and P7 virion chromosomes. Regions of non-homology are labeled according to Fig. 1. The enlargement of one arm of the B substitution loop is attributed to the argF insertion. Phage ϕX174 RFII DNA is present in the field.


ΦX
E
B
D
C
A
ΦX
1 KB
G
F

presented in Table 7. In P1/P7 heteroduplexes, each arm of the B substitution loop is 2.4 kb (Yun and Vapnek, 1977). In P1argF/P7 heteroduplexes one arm was 2.5 kb while the other was enlarged to 13.3 kb. All other regions of non-homology between P1argF and P7 were indistinguishable from those reported for P1/P7 heteroduplexes (see Fig. 1). Therefore the enlargement of the B substitution loop was attributed to the argF insertion.

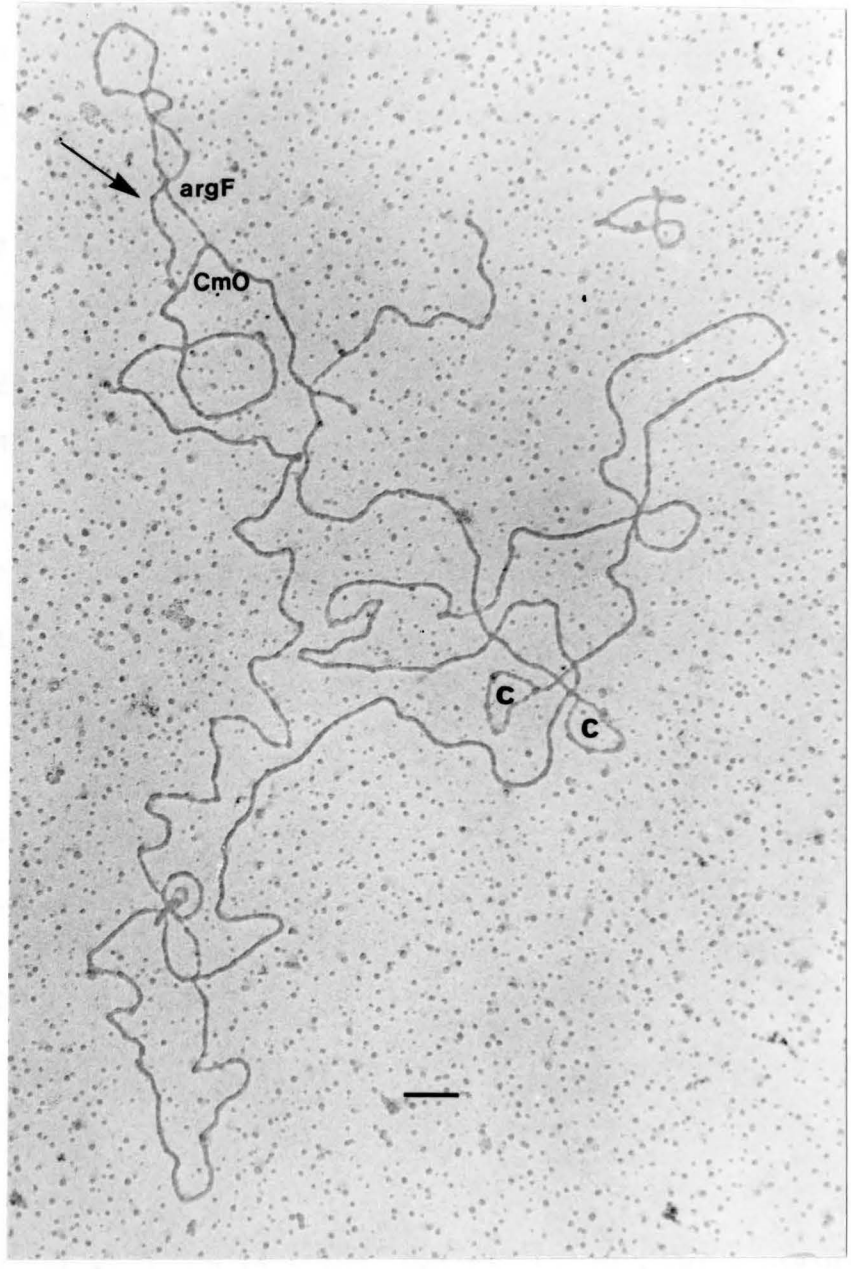
In order to localize the insertion site even more precisely, heteroduplexes of P1argF with P1CmO virion DNA were analyzed. Only one region of non-homology was seen in the heteroduplex depicted in the micrograph in Fig. 5. This was an asymmetric substitution loop approximately 10 kb from the C-loop structure of P1. The large arm was attributed to the argF insertion and the small arm to the Cm^r determinant of P1CmO. Sizes of the two arms are presented in Table 7.

These results established that both insertions were at the same site on the P1 chromosome or that there were too few base pairs between the argF and CmO insertions to permit stable base pairing. If the two insertions were not at the exact same site, two distinct insertion loops would be seen in the micrograph. Since it has been found that wild type P1 has an IS1 sequence at the CmO insertion site, (DeBruijn and Bukhari, 1978; Iida and Arber, 1980), these results located the argF insertion at the site of the IS1 sequence of P1.

Restriction endonuclease cleavage analysis with EcoRI endonuclease.

Chromosomes of P1argF were further characterized by restriction endonuclease cleavage analysis to verify the electron microscopic

Fig. 5 Heteroduplex of P1argF and P1CmO. The asymmetric substitution loop (arrow) is attributed to the insertions of the argF and CmO determinants into P1. The C-loop (C) has annealed showing its inverted repeat structure in this heteroduplex. The bar represents 1 kb.


argF
CmO
C
C

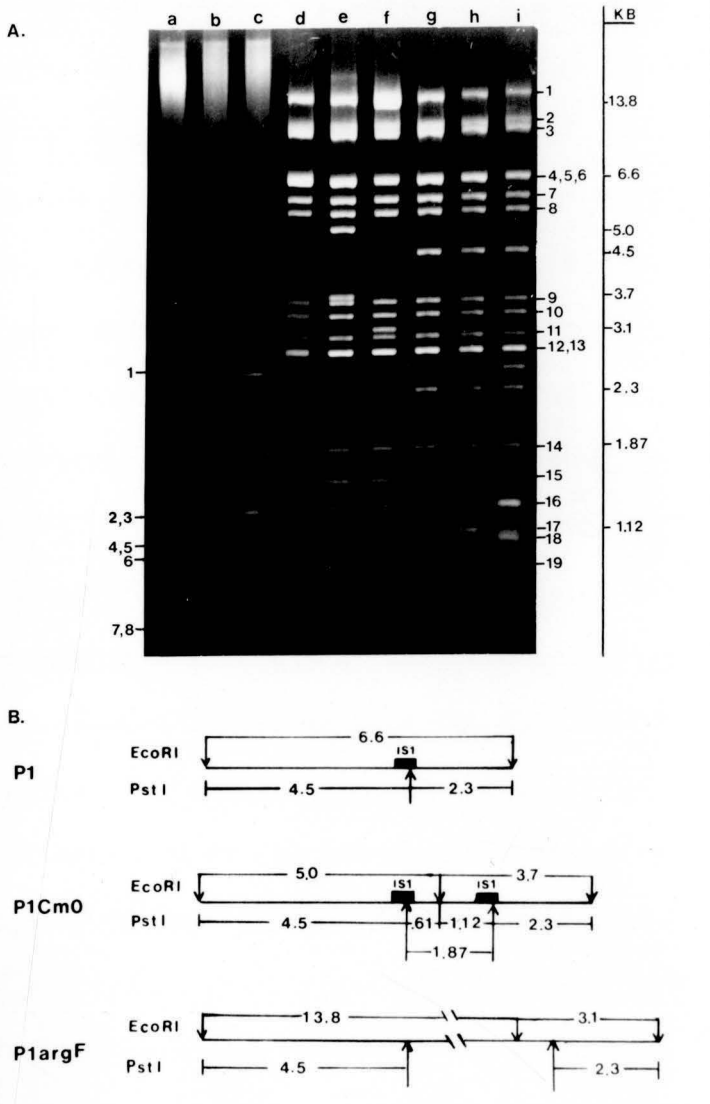
interpretations by another technique. Digests produced with EcoRI contained two novel fragments of 13.8 and 3.1 kb, as seen in Fig. 6 (slot f). Thus the argF gene segment encoded a single EcoRI cleavage site. The digest lacked the EcoRI-4 fragments, which band as a triplet with EcoRI-5 and 6; EcoRI-4 contains the IS1 sequence of P1 (DeBruijn and Bukhari, 1978; Iida et at., 1978). All other features of the P1c1.100 and P1argF5 EcoRI digests were indistinguishable, indicating that the added DNA in P1argF5 chromosomes was due to a single insertion within EcoRI-4.

The EcoRI-4 fragment was also missing in a digest of P1CmOc1.100 chromosomes run for comparison (Fig. 6, slot e). Two novel fragments of 5.0 and 3.7 kb were present, since the CmO determinant contains one EcoRI cleavage site (Schultz and Stodolsky, 1976). Some P1CmO strains carry two tandem copies of Tn9; fractionated EcoRI digests of these strains have a doublet at EcoRI-14 (DeBruijn and Bukhari, 1978). Since such a doublet was not observed in the digests of P1CmOc1.100 used in this study, the CmO determinant was not duplicated in this strain.

Analysis of EcoRI:PstI double digests.

Digestion with PstI was advantageous because wild type P1 DNA is cleaved once by this enzyme. The cleavage site is within its IS1 sequence at map unit 20 (Iida et al., 1978). Consequently EcoRI:PstI double digests of P1 DNA lack the EcoRI-4 fragment but contain two new fragments of 4.5 and 2.3 kb, representing the EcoRI-4 chromosomal segments to the left and right of the PstI cleavage site within IS1 (Iida et al., 1978). The 4.5 and 2.3 kb fragments are also present in digests

Fig. 6. Digestions of P1c1.100, P1CmO, and P1argF virion chromosomes with EcoRI and PstI endonucleases. (A) Electrophoretic separations of virion chromosomes: digested with PstI, slots (a) P1c1.100, (b) P1CmO, and (c) P1argF5; digested with EcoRI, slots (d) P1c1.100, (e) P1CmO, and (f) P1argF5; and digested with EcoRI:PstI, slots (g) P1c1.100, (h) P1CmO, and (i) P1argF5. Electrophoresis was at 1.3 V/cm for 18 hours. Numbers on left of gel indicate the PstI cleavage fragments of P1argF5 DNA (see Table 8). Numbers on the right identify the EcoRI cleavage fragments of P1 DNA according to Bachi and Arber (1977). The kilobase sizes of pertinent restriction fragments are also indicated on the right. (B) Physical map of the EcoRI-4 segments of the P1 chromosome and the alterations in this segment in P1CmO and P1argF chromosomes. PstI and EcoRI cleavage sites are indicated. Orientations are deduced from the gel data or from previous studies (Iida et al., 1978; Alton and Vapnek, 1979). Numbers represent kilobase sizes.


A.
a b c d e f g h i
KB
1
2
3
4,5,6
7
8
9
10
11
12,13
14
15
16
17
18
19
1
2,3
4,5
6
7,8
13.8
6.6
5.0
4.5
3.7
3.1
2.3
1.87
1.12
B.
P1
EcoRI
PstI
6.6
IS1
4.5
2.3
P1Cm0
EcoRI
PstI
5.0
IS1
IS1
3.7
4.5
.61
1.12
2.3
1.87
P1argF
EcoRI
PstI
13.8
3.1
4.5
2.3

of P1CmO DNA, because an IS1 sequence encoding a PstI cleavage site is present at each end of the CmO insertion (Iida and Arber, 1980; Fig. 6, slot h). A physical map of this chromosomal region is included in Fig. 6.

Phage P1c1.100, the parent chromosome of P1argF5, is a chloramphenicol sensitive deletion mutant of P1CmOc1.100 (Rosner, 1972). It was of interest to determine if the chromosome of this revertant was the same as that of the wild type P1. Digests of P1c1.100 virion DNA with EcoRI contained the EcoRI-4 fragment (Fig. 6, slot d). Double digests with EcoRI and PstI lacked EcoRI-4, but contained the 4.5 and 2.3 kb digestion products of the PstI cleavage present in wild type P1 (Fig. 6, slot g). This demonstrated that P1c1.100 DNA encoded a single PstI cleavage site and therefore most likely retained the IS1 sequence of wild type P1. Digests produced with BamHI were also indistinguishable from those of the wild type P1 chromosome (Fig. 7). These results suggested that the P1c1.100 chromosome was formed by reciprocal recombination between the IS1 sequences of P1CmO, resulting in the excision of the CmO determinant and one IS1 sequence.

The EcoRI:PstI double digestion of P1argF virion chromosomes was particularly informative in precisely localizing the argF insertion independent of electron microscopy. Two fragments of 4.5 and 2.3 kb, not seen in the EcoRI digest, were present (Fig. 6, slot i). These were attributed to the left and right portions of the P1 EcoRI-4 segment containing IS1 base sequences in each fragment. Verification that one terminus of each of these fragments was generated by EcoRI digestion

was shown by the PstI digest alone (Fig. 6, slot c). Neither the 4.5 or the 2.3 kb fragment was present in this digest. Similar results with BamHI:PstI digests showed the left and right segments of BamHI-3 (4.7 and 7.2 kb respectively) in both P1 and P1argF5 DNA (Fig. 7). The BamHI-3 segment of P1 encodes IS1 (see Fig. 1). This confirmed that the internal EcoRI cleavage site of the argF insertion did not produce the 4.5 or the 2.3 kb fragments of the EcoRI:PstI digest. Consequently neither the EcoRI-4 nor the BamHI-3 segment of P1 DNA was altered by the argF insertion except at the IS1 locus. These results demonstrated that the argF gene segment was inserted precisely at this locus.

Moreover, within the resolution of the gel fractionation, the argF insertion encoded a PstI cleavage site on both sides of the insertion. Since one IS1 sequence was presumed to be present in the P1c1.100 parent chromosome, one PstI site was assigned to IS1. The PstI site on the other side suggested that an IS1 sequence was on the other side of the argF insertion, as observed with the CmO insertion.

This interpretation was not definitive since the argF insertion encoded 11 PstI cleavage sites. Ten novel bands, ranging in size from 2.5 to 0.23 kb and totally 11.2 kb, were identified by PstI cleavage analysis (Table 8). All but the 0.53 and 0.23 kb fragments were retained by the gel in Fig. 6 (slot c). Bands of 1.45, 1.20, and 0.75 kb were doublets. One of the 0.75 kb fragments contained the EcoRI:PstI cleavage site, since it was reduced to 0.73 kb in the EcoRI:PstI double digest. Since there is a PstI cleavage site on either side of the argF insertion, these 10 fragments represent the entire argF insertion.

TABLE 8

Characterization of PstI Cleavage Fragments of the argF Insertion

Fragment No.	Size in kilobases		Comments
	P1argF5	P1argF51	
1	2.5	-	Cleaved by BamHI
2	1.45	1.45	Only PstI fragment in P1argF51 large enough to encode argF gene
3	1.45	-	
4	1.20	1.20	Cleaved by BamHI
5	1.20	-	
6	1.15	1.15	Cleaved by BamHI
7	0.75	0.75	Cleaved by EcoRI
8	0.75	-	
8'	-	0.63	Novel fragment in P1argF51
9	0.53	0.53	
10	0.23	ND	

ND, not demonstrated.

The P1 chromosomal region of P1argF5 virion DNA produced a smeared fluorescent background at the top of the gel (see Fig. 6, slot c). This background represented digestion fragments with one end produced by PstI cleavage and the other by headful packaging cleavage of the permuted viron chromosome population. Such fragments are, for the most part, heterogeneous in size and do not form discrete bands in fractionated digests. Since P1c1.100 chromosomes encoded only one PstI site, virion DNA from P1c1.100 contained no discrete fragment band within the fluorescent background. In contrast, P1CmO virion DNA, having two PstI cleavage sites, had a discrete DNA fragment the size of EcoRI-14 (Fig. 6, slot b), encoding the entire CmO insertion (Alton and Vapnek, 1979).

Results of these cleavage analyses permitted orientation of the two novel EcoRI fragments in P1argF5 chromosomes. In order to account for the 4.5 and 2.3 kb segments of EcoRI-4, the 3.1 kb segment cannot be positioned to the left of the P1 IS1 sequence. Therefore, the 13.8 kb fragment is on the left of the 3.1 kb fragment in the P1argF5 physical map (see illustration, Fig. 6).

Cleavage analysis with BamHI endonuclease.

A cleavage map of P1argF5 with BamHI was constructed to further characterize the argF insertion, since the 13.8 kb fragment of the EcoRI digests could not be resolved from EcoRI-1. The BamHI-3 fragment spans the EcoRI-4 segment of the P1 chromosome and was therefore missing in the BamHI digest of P1argF5 DNA (Fig. 7 and Fig. 8). There were 4 new BamHI fragments (3b, 4a, 6a, and 6b) in the digest, which were

Fig. 7. Fractionated digests of P1, P1argF5, P1argF51, and P1Cm13argF5 virion chromosomes with BamHI and PstI endonucleases. Virion chromosomes: digested with BamHI, slots (a) P1c1.100, (b) P1argF5, and (c) P1argF51; digested with BamHI:PstI, slots (d) P1c1.100, (e) P1argF5, and (f) P1argF51; digested with EcoRI, slots (g) P1c1.100 and (h) P1argF51; digested with PstI, slots (i) P1Cm13argF5 and (j) P1argF51. Numbers on left indicate BamHI cleavage fragments of P1 and P1argF5 DNA according to the cleavage map in Fig. 9. Numbers on right represent PstI cleavage fragments of P1argF5 DNA as listed in Table 8. The fragment encoding the Cm13 determinant in the PstI digest is identified. Sizes of the BamHI:PstI cleavage fragments of P1 DNA are identified in kilobases on the left. Arrows indicate the novel fragments of P1argF51 in the BamHI (c), BamHI:PstI (f), EcoRI (h), and PstI (j) digests. The (<) identifies the 1.0 and 1.05 kb BamHI cleavage products of PstI fragments 5 and 6. Electrophoresis was at 1.3 V/cm for 18 hours.

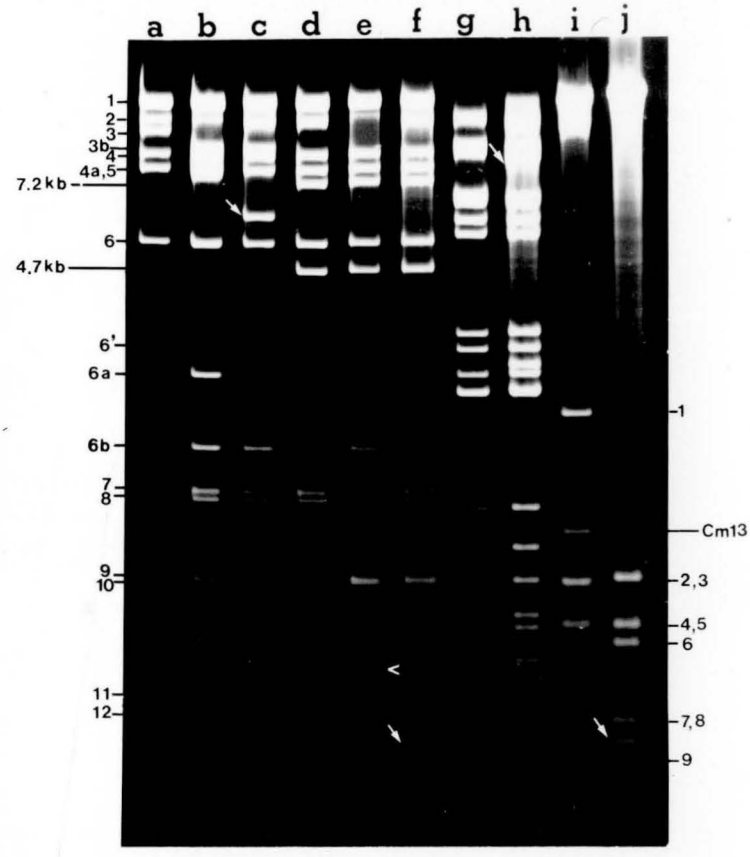
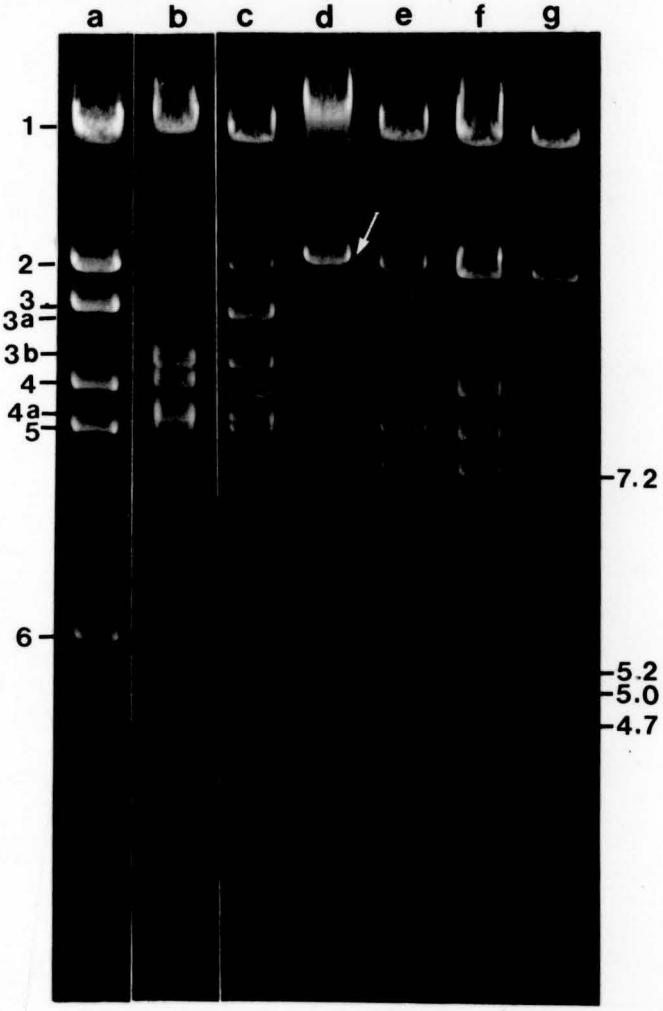

a b c d e f g h i j
1
2
3
3b
4
4a,5
7.2 kb
6
4.7 kb
6'
6a
6b
7
8
9
10
11
12
1
Cm13
2,3
4,5
6
7,8
9

Fig. 8. Fractionated digests of P1, P1argF5, P1Cm13, and P1Cm13argF5 virion chromosomes with BamHI and PstI endonucleases. Virion chromosomes: digested with BamHI, slots (a) P1c1.100, (b) P1argF5, and (c) P1Cm13argF5; digested with PstI, slot (d) P1Cm13argF5; digested with BamHI:PstI, slots (e) P1Cm13argF5, (f) P1c1.100 and (g) P1Cm13. Numbers on the left indicate the BamHI cleavage fragments of P1, P1Cm13, and P1argF5 DNA according to the cleavage map in Fig. 9. Arrow indicates the 14.5 kb fragment annealed in Fig. 10. Sizes of the BamHI:PstI cleavage fragments of P1 and P1Cm13 are indicated on the right in kilobases (kb). Electrophoresis was at 2.6 V/cm for 18 hours.


a
b
c
d
e
f
g
1
2
3
3a
3b
4
4a
5
6
7.2
5.2
5.0
4.7

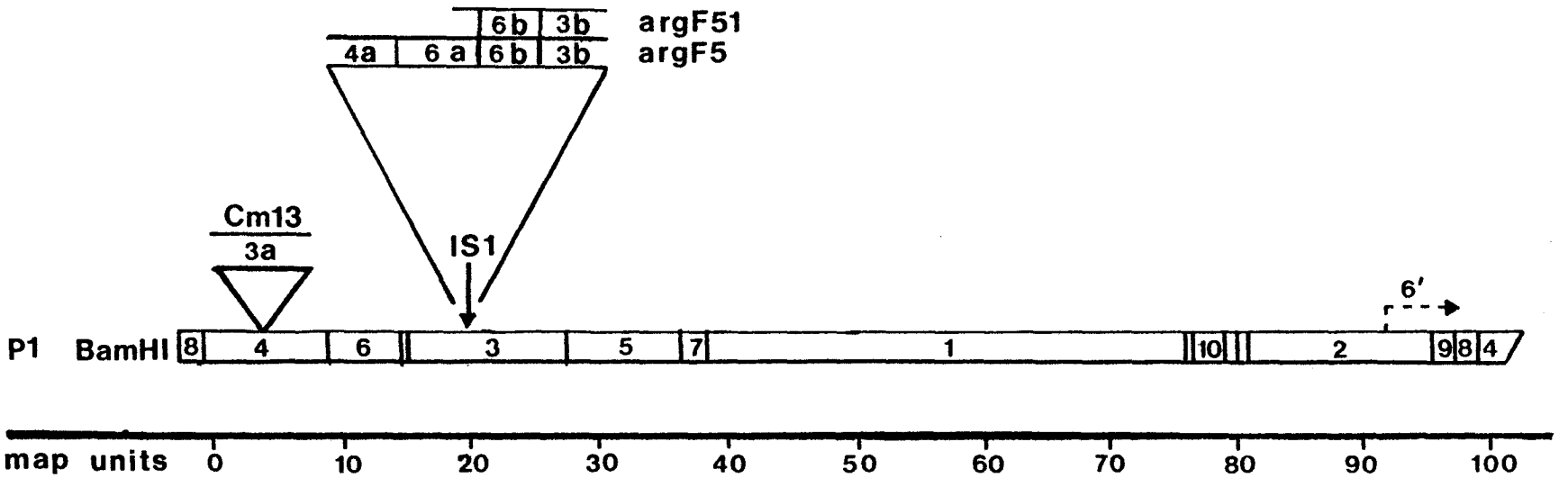
attributed to the argF gene segment and the BamHI-3 segment of P1.

These fragments were ordered by deletion mapping. A lysogen containing a deletion mutant of P1argF5 was isolated as an Arg^+ transductant from the center of a turbid plaque produced on strain SC1800. Chromosomes of this P1argF5 derivative, designated P1argF51, were digested with EcoRI and found to be deleted in the 13.8 kb fragment (Fig. 7, slot h). A new smaller fragment was present as well as the 3.1 kb fragment. This result indicated that there was a deletion of the left side of the argF insertion.

Digests of P1argF51 produced with BamHI contained the 3b and 6b fragments, but not the 4a or 6a fragments (Fig. 7, slot c). The interpretation of this result was that the left side of the argF insertion was contained in the 4a and 6a segments. The 6a and 6b segments must be the internal segments of the argF insertion since they were too small to encode portions of the argF insertion and the P1 BamHI-3 chromosomal segment on either side of the insertion. Thus all four cleavage fragments were ordered as illustrated in the map in Fig. 9.

An interesting phenomenon was observed in the BamHI digests of P1argF virion chromosomes. The content of intact BamHI-2 fragments was low, relative to that of the other fragments in the digest. This was not observed in P1 digests. (Compare slots a and b in Fig. 8). Headful packaging processes generated a minor BamHI cleavage fragment (6') in digests of P1 and P1argF5 virion chromosomes. This fragment encodes the right portion of BamHI-2 from map unit 92 (see Fig. 9 for illustration). Its presence indicates that packaging of concatemers

Fig. 9. Cleavage map of P1 and its derivatives with BamHI endonuclease. The P1 map was reproduced from Bachi and Arber (1977). The order of the P1argF5 and P1argF51 segments was determined as described in the text. Arrow indicates the first cut site and direction of headful packaging.


6b
3b
argF51
4a
6a
6b
3b
argF5
Cm13
3a
IS1
6'
P1
BamHI
8
4
6
3
5
7
1
10
2
9
8
4
map units
0
10
20
30
40
50
60
70
80
90
100

is initiated at map unit 92 and proceeds to the right (Bachi and Arber, 1977). The P1argF5 genome was approximately the size of the virion chromosome. Therefore packaging of full size chromosomes that began at map unit 92 probably terminated within BamHI-2, resulting in a depletion of intact BamHI-2 segments. The smaller genome of P1 is packaged with a 7 to 12% terminal repetition. Thus, packaging of P1 virion chromosomes that began at map unit 92, terminated at a site that extended beyond the right boundary of the BamHI-2 segment. Consequently, a normal yield of this fragment resulted after BamHI digestion.

Mapping of the PstI digestion fragments of P1argF5 chromosomes.

The PstI fragments of P1argF5 could be partially characterized, using the deletion mutant P1argF51. Digests of P1argF51 chromosomes with PstI and BamHI:PstI are depicted in Fig. 7. There was no evidence that P1 genes were deleted in the formation of the P1argF51 mutant, since the 4.7 and 7.2 segments of BamHI-3 were present in the BamHI:PstI digests of the mutant.

Comparison of the BamHI:PstI digest with that of PstI alone permitted identification of all three BamHI cleavage sites within the PstI digested fragments. This analysis is summarized in Table 8. The 1.2 and 1.15 kb fragments in the PstI digest were reduced in the BamHI:PstI digests to 1.0 and 1.05 kb fragments (plus fragments not seen in Fig. 7).

The PstI digest of P1argF5 chromosomes contained a 2.5 kb fragment that was not present in the PstI digest of P1argF51

chromosomes. This localized the 2.5 kb fragment in the left hand portion of the argF insertion. A fragment, the size of BamHI-6b (2.2 kb), was present in the BamHI:PstI double digest of P1argF5 DNA. This fragment was not observed in the double digest of P1argF51 chromosomes. Thus the 2.2 kb BamHI:PstI fragment was a cleavage product of the 2.5 kb PstI fragment. All the doublet bands of PstI digested P1argF5 DNA were singlets in the PstI digests of P1argF51 DNA. In addition, PstI digests of P1argF51 chromosomes contained novel 0.63 kb fragments, which contained the boundaries of the deletion of P1argF51.

Location of IS1 sequences in P1argF5 chromosomes.

One question still to be definitively resolved was whether the argF insertion was flanked by IS1 sequences. An electron microscopic approach was designed to answer this question. Detection of IS1 sequences could be demonstrated by forming heteroduplexes between P1argF5 chromosomes and a DNA probe encoding IS1. Because of the small size of IS1 (758 base pairs) relative to the 100.7 kb P1argF5 chromosome, such annealings were kinetically less favorable than homoduplex annealings. If the IS1 sequences of the probe were in the P1argF5 chromosome, intramolecular IS1 annealing would be kinetically favorable. A P1argF5 derivative was constructed such that intrastrand annealing would occur between inserted IS1 sequences and any IS1 sequences of the argF insertion.

This principle was used by Iida et al. (1978) to demonstrate the presence of IS1 elements in a P1 derivative, P1Cm13. In this derivative the Cm13 determinant is within the BamHI-4 segment of the

P1 chromosome, and its flanking IS1 sequences are inverted with respect to the natural IS1 sequence of P1 which is within BamHI-3 (Iida et al., 1978; Meyer and Iida, 1979).

A recombinant of P1Cm13 and P1argF5 was produced by the following procedure. Strain D5(P1argF5) was infected with bacteriophage P1Cm13, and subsequently the lytic cycle was induced. Virions from the resulting lysate were used to transduce SC1800(P1c1.100) to the Arg^+ phenotype. A lysogenic recipient for transduction was used to supply a complement of P1 genes. The desired P1Cm13argF5 recombinant genome was expected to be larger than the infecting virion chromosome. Thus recombination with the recipient's plasmid would be necessary to reconstitute a non-defective P1Cm13argF genome. An m.o.i. of 0.01 was used for the transduction to minimize double infection of recipients with bacteriophage P1Cm13 and P1argF5.

With this protocol, potential lysogens harboring the recombinant P1Cm13argF5 were selected as Arg^+ transductant colonies and were replica plated to score for Cm^r. One Arg^+ Cm^r transductant was subcloned and scored for revertants to the Arg^- or Cm^s phenotype. Neither revertant type was found in over 100 colonies scored. The reversion rate would have been high if the transductant prophage had a co-integrate P1argF5:P1Cm13 genome. Such co-integrates are resolved by recombination into two genomes at a high frequency.

To determine if the recombinant chromosome had the desired genotype, P1Cm13argF5 and control P1 virion chromosomes were purified and compared by cleavage analysis. Digests of P1Cm13argF5 produced with BamHI lacked the BamHI-3 and BamHI-4 fragments of P1 chromosomes

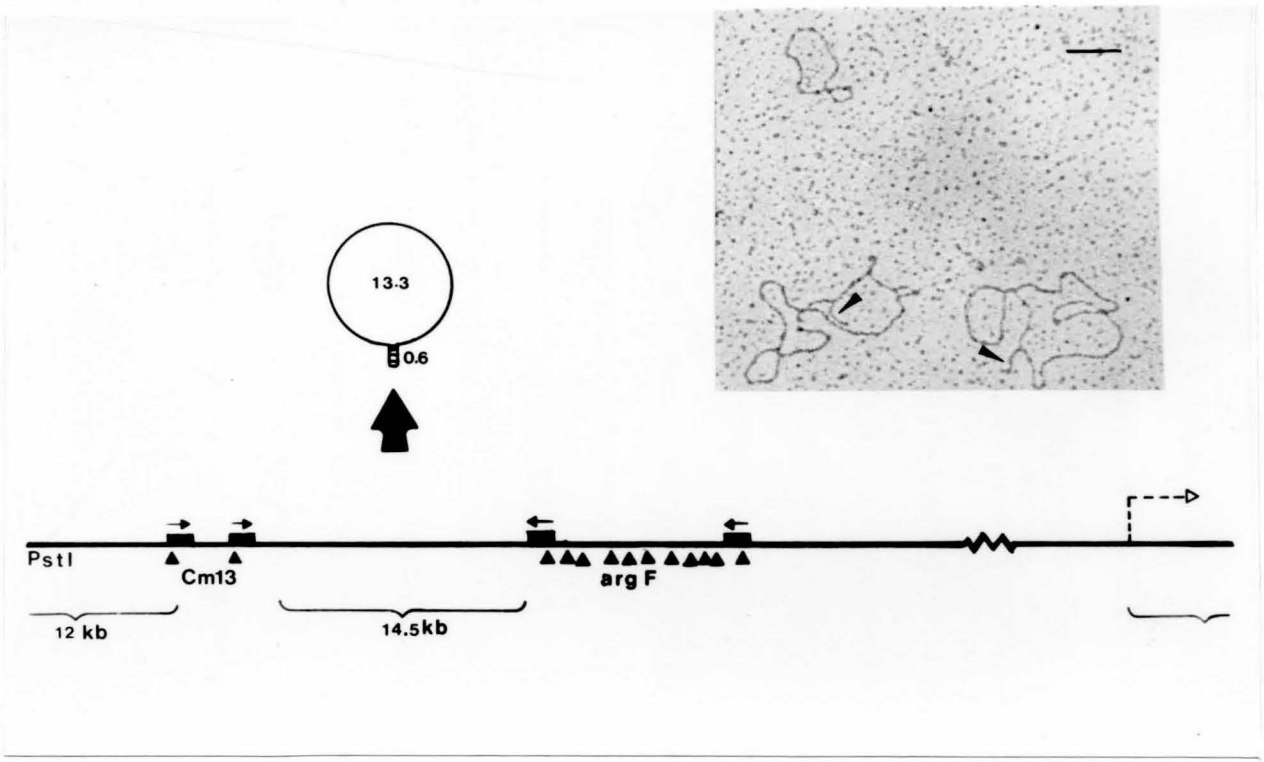
(Fig. 8, slot c). Thus the prophage was not a co-integrate, since P1Cm13 had a normal BamHI-3 segment and P1argF5 had a normal BamHI-4 segment (see map, Fig. 9). One or both of these segments would be present in a co-integrate chromosome.

The digest did contain the 3a fragment encoding the Cm13 determinant of P1Cm13 chromosomes and the 3b and 4a fragments of P1argF5 DNA. As expected, these three fragment bands were absent in PstI:BamHI double digests, since both the Cm13 and argF insertions contained PstI cleavage sites (Fig. 8, slot e). Consequently, the BamHI cleavage analysis confirmed that P1Cm13argF5 had the desired genotype and was formed by generalized recombination between P1Cm13 and P1argF5 genomes. This careful cleavage analysis was necessary to verify that the Cm13 transposon did not transpose to a novel locus in the P1argF5 chromosome.

Fractionated PstI digested P1Cm13argF5 chromosomes produced three bands seen in Fig. 8 (slot d) and 11 bands smaller than 2.5 kb visible in Fig. 7 (slot i). Fragments in the largest band presumably represented the longer distance between the argF and Cm13 insertions, while the 14.5 kb fragment band spanned the smaller distance. A minor fragment band the size of BamHI-3a (12 kb) was interpreted to encode the region from the first cut of headful packaging at map unit 92 to the PstI cleavage site on the left side of the Cm13 insertion. These fragments are schematically illustrated in Fig. 10.

Of particular interest was the 14.5 kb fragment whose left end was generated by PstI cleavage within one IS1 sequence of the Cm13

Fig. 10. Virion chromosomes from P1Cm13argF5 cleaved with PstI and self-annealed. Arrows in the micrograph indicate double-stranded stems. Small circular molecule is single-stranded øX174 virion chromosome. Interpretation of the PstI cleavage fragments of the P1Cm13argF5 chromosome is below the micrograph. Origin of the large circular molecules seen in the micrograph is illustrated.

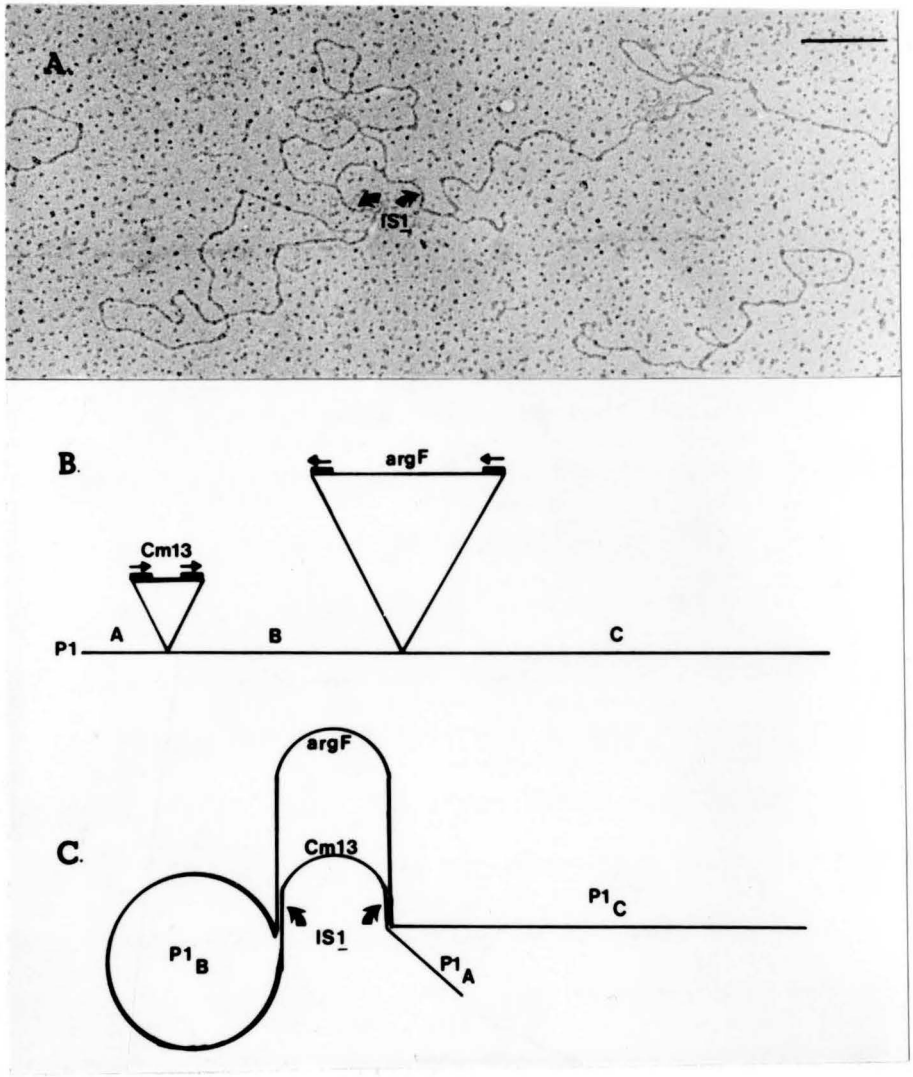

13.3
0.6
PstI
Cm13
arg F
12 kb
14.5kb

determinant. Based on sequence analysis of IS1, 588 base pairs of IS1 remained on the left side of the 14.5 kb fragment after PstI digestion (Ohtsubo and Ohtsubo, 1978; Johnsrud, 1979; Iida et al., 1978). The PstI cleavage site on the other side represented the left end of the argF insertion. This fragment was used to determine if IS1 was present on the left end of the argF insertion.

Virion chromosomes of P1Cm13argF5, digested with PstI, were denatured and annealed as described for heteroduplex analysis. Annealed mixtures were then spread by the formamide technique and observed in the electron microscope. As seen in the micrograph in Fig. 10, circular single-stranded molecules measuring 13.4 $\pm$ 1.3 kb (21 molecules) were present. Each had a double-stranded stem of 0.62 $\pm$ .07 kb. These molecules were interpreted to be the 14.5 kb fragments of the PstI digestion. The annealed stem structures demonstrated that there were complementary DNA sequences at the ends of the fragments. Since 588 base pairs of IS1 were present on the left end of the fragment, complementary IS1 bases were present on the right end. These latter bases belonged to the left end of the argF insertion. Hence, an IS1 sequence was present on the left side of the argF gene segment.

In order to ascertain if IS1 was present on the right side of the argF insertion, the entire P1Cm13argF5 chromosome was denatured and the single-strands allowed to reanneal with themselves. Two 0.83 kb double-stranded regions were observed in the molecule shown in Fig. 11. These duplex regions were attributed to the presence of 4 IS1 sequences in the recombinant genome. Two were from the Cm13

Fig. 11. Demonstration of IS1 sequences in P1Cm13argF5.
(A) Self-annealed single-stranded DNA from P1Cm13argF5. Double-stranded regions interpreted as IS1 elements are identified. Each measures 0.83 kb. The Cm loop in this molecule measures 1.03 kb; the argF loop is 10.3 kb; and the P1 loop is 13.2 kb.
(B) Physical map of the P1Cm13argF5 recombinant chromosome. Retangular boxes indicate IS1 sequences. Arrows indicate orientations of the four IS1 elements. (C) Diagramatic representation of the chromosome in (A).


A.
IS1
B.
argF
Cm13
P1
A
B
C
C.
argF
Cm13
P1 C
IS1
P1 B
P1 A

determinant and the other two were assigned to the argF insertion. Measurements of the single-stranded regions representing the Cm13 and argF insertions and the P1 genome between these two insertions agreed with expected sizes determined from previous heteroduplex and cleavage analysis. The origins of the single-stranded loops are schematically represented in Fig. 11. These results confirmed that the P1argF5 chromosome contained two IS1 sequences as direct repeats in the same orientation as the naturally occurring IS1 sequence of wild type P1.

Discussion and summary.

The P1argF plasmid chromosome, as measured by electron microscopy is approximately the same size as the P1 virion chromosome. However, two genetic observations presented in this chapter indicate that many P1argF virions contain chromosomes with a full complement of P1argF genes and some terminal repetition at the ends of the chromosome. (1) Plaque forming titers are normal. Titers of overlarge P1 derivatives are at least 100 times lower than normal (Iida and Arber, 1977). (2) Lysogens with non-defective P1argF prophage are frequently formed from single virion infections. In singly infected cells, productive infection and lysogeny require that the virion chromosome be terminally redundant, so that it can efficiently circularize and/or replicate (Ikeda and Tomizawa, 1968).

The presence of only two minor fragment bands (BamHI-2 and BamHI-6') in fractionated BamHI digests of P1argF virion DNA demonstrates that mature virion chromosomes vary in size. Full size chromosomes that begin at map unit 92 in the BamHI-2 segment of the

chromosome, also end within this BamHI segment. This interpretation accounts for the two minor BamHI bands as explained previously. Therefore, plaque forming virion chromosomes digested with BamHI must contain fragments with one cut from cleavage of the left-hand cleavage site of BamHI-2 and the other from the final packaging cut somewhere between map unit 92 and the right-hand BamHI-2 cleavage site (see Fig. 9 for illustration). Such fragments band between BamHI-2 and BamHI-3b. However, no discrete minor band is visible in fractionated BamHI digests in this region. This result is interpreted to mean that these fragments must be too heterogeneous in size to form a discrete band. Consequently, the virion chromosomes that produced these fragments vary in size. Such an interpretation is in agreement with an observation made by Ting (1962) who demonstrated that plaque forming P1 virions are heterogeneous in density.

Both the heteroduplex and cleavage analyses indicate that P1argF5 genomes possess a single insertion in the P1 chromosome of approximately 11 kb. The additive sizes of the bands observed in fractionated PstI digests of P1argF5 virion DNA total 11.2 kb. These bands represent only the inserted DNA. Since gels employed in this study did not retain fragments less than 0.23 kb, there could be additional fragments produced from the argF insertion that are not included in the 11.2 kb total. By tabulating all the electron microscopic heteroduplex measurements (20 molecules), a value of 11.0 ± 0.8 kb is assigned to the argF insertion. Thus both techniques are in close agreement on the size of the insertion.

The cleavage analysis and the heteroduplex analysis demonstrate

that the insertion is localized at the IS1 locus of P1. In addition, three lines of evidence demonstrate that IS1 is present at both ends of the argF insertion. (1) The two 800 base pair duplex regions present in self-annealed P1Cm13argF5 single-stranded DNA provide the strongest evidence for duplicated IS1 sequences. (2) The PstI cleavage site on either side of the argF insertion is compatible with the duplication since IS1 sequences encode a PstI cleavage site. (3) The P1CmO:P1argF heteroduplexes indicate a region of non-homology on the P1CmO strand of 1.3 kb ± 0.3 kb. Alton and Vapnek (1979) have sequenced the CmO insertion and found it to be 1.87 kb. This discrepancy suggests that the two chromosomes have homology at one or both ends. Since the CmO insertion encodes an additional IS1, this is compatible with the argF gene segment having IS1 sequences at each end.

Other explanations for the size of the small arm in the region of non-homology between P1CmO and P1argF have been ruled out. Cleavage analyses presented in this chapter demonstrate that there is no deletion within the CmO insertion in the P1CmO strain used for heteroduplex analysis. The cleavage patterns of this P1CmO strain agree with those reported by other laboratories (Meyer and Iida, 1979; DeBruijn and Bukhari, 1978). However, such IS1 mediated deletions in CmO are common and can exist without altering the Cm^r phenotype of P1CmO lysogens. MacHattie and Jackowski (1977) have demonstrated that the internal CmO determinant can be as small as 890 base pairs and still retain the Cm^r phenotype.

The size of the small arm in the P1CmO/P1argF heteroduplexes

could also be explained if the P1c1.100 parent chromosome of P1argF5 retained some of the CmO determinant upon excision. However, P1c1.100 cleavage patterns with three different restriction enzymes are indistinguishable from those of wild type P1 chromosomes. Therefore, the homology in the heteroduplexes is attributed to direct repeats of IS1 at the ends of both the CmO and argF determinants.

The involvement of IS1 in P1argF formation is unique in that the transferred genes are of host chromosomal origin, rather than of plasmid origin. Transposable genes of plasmid origin that are flanked by IS1 include several antibiotic resistance determinants (Iida and Arber, 1977) and the heat stable toxin of E. coli (So et al., 1979). It is quite likely that many other host genes are frequently transferred to P1 via an IS1 mediated recombination. Because there is no strong selection for retention of these transferred genes by P1, these P1 derivatives are not detected. In addition, unless recipient chromosomes themselves are deleted in these genes, they will be lost by reciprocal recombination between the two copies of the gene.

The argF insertion has the structure of a transposon, since it is flanked by IS1 and encodes genes not involved in the transposition process (Campbell et al., 1979). A Tn number, Tn2901, has been assigned to the insertion in P1argF5 by the central registry of Tn elements. Further studies are necessary to determine the extent of the similarity between Tn2901 and other transposable elements. However, several researchers have concluded that any DNA sequence between two IS1 sequences is transposable (Iida and Arber, 1980; Rosner and Guyer, unpublished).

The P1argF51 derivative provides evidence that deletions in Tn2901 occur. This mutant was selected from the lysogens within a plaque. No mutagenesis was involved. Unless the P1argF51 was formed by an aberrant event near but not involving IS1, there are two possible mechanisms by which this chromosome was generated. Either there was an IS1 mediated intramolecular transposition event or, alternatively there was an intramolecular site-specific recombination between two IS1 sequences within the P1argF chromosome. Because IS1 encodes a PstI site, this latter interpretation would not generate a new PstI cleavage fragment in digests of the deletion mutant chromosomes. Since there is a novel 0.63 kb fragment in the PstI digest of P1argF51, the event generating the deletion mutant was probably a transposition event involving IS1.

The argF gene, which is unique to E. coli K12 strains, encodes one of the ornithine carbamoyltransferase isoenzymes. The other is encoded by the argI gene (Glansdorff et al., 1967). That both genes are from the same ancestor gene is indicated by their extensive DNA homology (Kikuchi and Gorini, 1975). It has been postulated that argF arose in K12 strains by transposition from argI, since many other bacterial species contain argI but only E. coli K12 strains contain both argF and argI (Kikuchi and Gorini, 1976). Interest in this gene has prompted Moore et al. (1978) to clone a segment encoding argF into pBR322. Moore and James (1979) have reported a detailed cleavage analysis of the gene, which is reproduced in Fig. 12.

The argF gene with its promoter occupies a region of

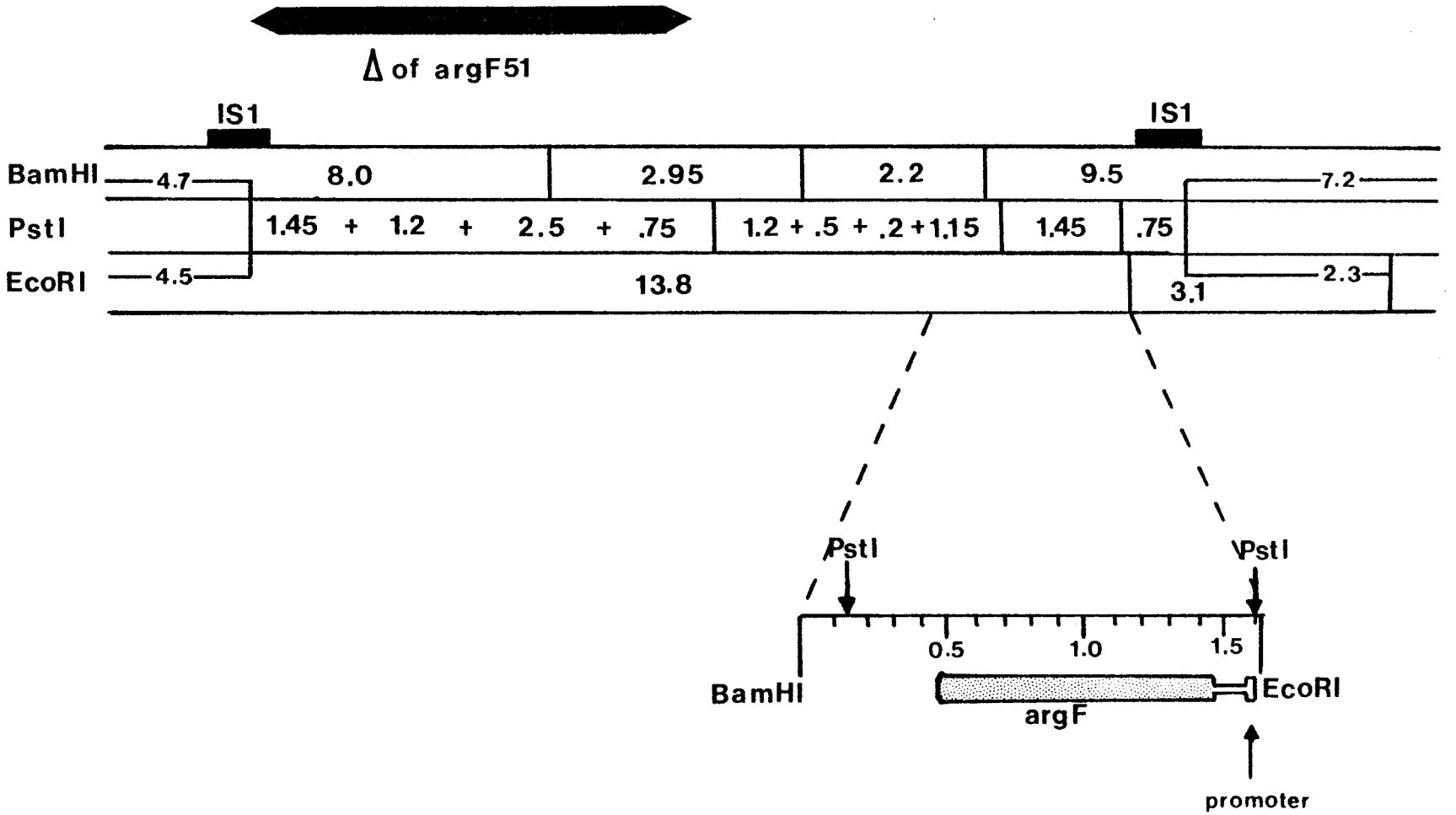
approximately 1160 base pairs and encodes no PstI, BamHI, or EcoRI cleavage sites within the gene (Moore and James, 1979). The only fragment of the argF51 insertion that was larger than 1.16 kb in the BamHI:PstI double digest was the 1.45 kb fragment. The cloned fragment encoding argF that has been characterized by Moore and James (1979) contains the argF gene between two PstI cleavage sites 1.45 kb apart. Therefore, the 1.45 kb fragment of PstI digested P1argF51 chromosomes was interpreted to encode the argF gene.

A comparison of the cleavage analysis of the cloned argF gene with the cleavage analysis of P1argF5 chromosomes permitted localization of the argF gene within the insertion in P1argF5. A summary of the interpretation of the cleavage analysis of P1argF5 DNA is presented in Fig. 12. The 1.45 kb fragment from P1argF51 has no BamHI cleavage sites. Therefore it can only be located within BamHI-6b (2.2kb) or 3b (9.5 kb), which encodes 3.3 kb of the argF insertion. If it is positioned within 3b, the cleavage data with BamHI, EcoRI and PstI are compatible with the data for the cloned argF gene segment.

Placing the 1.45 kb fragment within BamHI-3b localizes the 0.75 kb PstI fragment of Tn2901 to the right of the 1.45 kb fragment, since the 0.75 kb fragment encodes the only EcoRI site in Tn2901. The 0.75 kb PstI fragment is cleaved by EcoRI generating a 0.72 fragment and a 20 to 30 base pair fragment. In the cloned gene segment, there is a PstI site 20 base pairs from the EcoRI site (see Fig. 12).

Either the 1.15 or the 1.2 kb PstI fragments of Tn2901 must be on the left of the 1.45 kb fragment since they contain BamHI cleavage

Fig. 12. Cleavage map of the argF insertion with BamHI, EcoRI and PstI restriction enzymes. The deletion in P1argF51 is represented by the double ended arrow. The probable location of the argF gene is aligned to the cleavage map of the cloned argF gene segment as reported by Moore and James (1979). Numbers are kilobase sizes.


Δ of argF51
IS1
IS1
BamHI
PstI
EcoRI
4.7
4.5
8.0
2.95
2.2
9.5
7.2
1.45 + 1.2 + 2.5 + .75
1.2 + .5 + .2 + 1.15
1.45
.75
13.8
3.1
2.3
PstI
PstI
0.5
1.0
1.5
BamHI
EcoRI
argF
promoter

sites. Data presented in this chapter demonstrate that cleavage of either of these PstI fragments with BamHI generates small fragments between 100 and 200 base pairs long. This result is in agreement with the 165 base pair fragment produced from BamHI:PstI cleavage of the cloned argF gene. Thus the cleavage analysis of Tn2901 with three different restriction enzymes is compatible with that of the cloned argF gene. In addition, the EcoRI cleavage site in the cloned argF gene is 30 base pairs from the argF promoter (Moore and James, 1979). Because the only EcoRI site of Tn2901 is 140 base pairs from the start of the right IS1 sequence, the argF gene in Tn2901 is presumably located 170 base pairs from IS1.

Such close proximity between argF and IS1 has prompted speculation that IS1 was involved in the transposition of argF from argI. This hypothesis does, however, depend on the demonstration of an IS1 sequence near argF on the E. coli K12 chromosome. The 14.9 and 3.6 kb EcoRI fragments of F128 chromosomes (see Hadley and Deonier, 1979) encode sequences homologous to IS1 (Deonier, personal communication). Both fragments also anneal to P1argF DNA. In addition, Deonier has also demonstrated by heteroduplex analysis that there is a 12.3 kb region of homology between an F'argF derived from Hfr OR11 (see Fig. 2) and a P1argF derivative, P1CmOargF10 (Deonier, personal communication). The 12.3 kb homologous region is large enough to encode the argF insertion with 2 flanking IS1 sequences. These results suggest not only that IS1 is present near argF on the E. coli K12 chromosome but also that the entire Tn2901 transposon is present on this chromosome.

CHAPTER V

ANALYSIS OF THE EVENTS INVOLVED IN P1*argF* FORMATION

Introduction.

The formation of P1*argF* chromosomes involves recombination mediated by IS*1*. This conclusion is based solely on the structure and location of the *argF* insertion. Experiments described in this chapter are designed to provide genetic evidence of the involvement of IS*1*. In addition, the role of the P1 vector in P1*argF* formation is characterized. For these experiments the F128 episome has been chosen as the donor chromosome of the *argF* gene segment, since this chromosome has been characterized and could easily be isolated for further study. Approximately 130 kb of acquired *E. coli* K12 host genes, including all of the *proAB-argF-lac* region is encoded in F128 (Hoppe and Roth, 1974). Genetic studies on P1*argF* chromosomes are also pursued to determine if any qualities of transposons are exemplified by the *argF* insertion.

Results of this investigation show that the union of P1 and the *argF* gene segment occurs most efficiently in the lytic cycle. Phage P1 derivatives with varying numbers of IS*1* sequences vary in their frequency of P1*argF* formation. Lastly, chromosomes of P1*argF* display changes characteristic of transposons such as deletions and excisions. These changes are again more frequent during the P1 lytic cycle.

Review of genetic terminology.

At the onset of this chapter, it is well to recall several genetic terms. Transduction is a genetic event involving a donor, a vector, and a recipient. The donor bacterial strain supplies the transduced genes. The vector is the bacteriophage used to transfer the donor genes, and the recipient is the bacterial strain that is infected with the vector. A transductant therefore has the genotype of the recipient, plus the acquired genes from the donor chromosome. If alleles of the acquired genes are present in the recipient chromosome, a substitution of the acquired alleles for the resident alleles occurs (Lederberg et al., 1951).

If the transducing vector is a temperate bacteriophage, the recipient may also have acquired the genome of the vector. In such cases, the transductant is considered lysogenic for the phage vector (Lederberg et al., 1951; Lennox, 1955). If the vector itself contains the transduced genes as part of its genome, it is called a specialized transducing bacteriophage. In this case, the transductant produces a high frequency transducing (HFT) lysate for the transduced genes, when a lytic cycle is induced (Morse et al., 1956a, b.) If the specialized transducing phage is deficient in virion maturation functions, production of an HFT lysate requires superinfection with non-defective "helper" bacteriophage (Luria et al., 1960).

Preliminary control tests.

Several experiments were designed to determine the validity of the arg^+ transduction assay. Transductant colonies of minute size were

observed on plates containing either large numbers of recipients or large numbers of transductants. This minute colony problem was overcome by washing the recipient bacteria and by plating less recipients per plate (see Materials and Methods). However, it was important to verify whether all the transductants were being detected in the recipient background with this procedure. An experiment summarized in Table 9 was designed to determine this. Recipient bacteria were grown and washed as described in Materials and Methods. Immediately before plating on Arg selective medium, three consecutive 1:10 dilutions of strain D5(P1argF5) bacteria were added to 1×10^9 recipients. Control plates of D5 without recipient bacteria were also plated on Arg selective medium. After incubation, the number of Arg^+ colonies was scored on each plate. There was essentially no difference in the number of Arg^+ colonies detected with or without recipient bacteria present (Table 9, Experiment 1). Hence in this assay system essentially all the transductants present in the recipient background were being detected. Conversely, these results indicated that recipient bacteria were not supplying any nutrients promoting colony formation by the transductants, since each consecutive ten-fold dilution of D5 without recipients contained ten-fold less transductants (Table 9, column 1).

In another experiment the effect of washing the recipient bacteria was evaluated (Table 9, Experiment 2). Two dilutions of strain D5 (one 5-fold less than the other) were prepared. The diluted bacteria were added to separate recipient cultures either before or

TABLE 9

Effect of Recipient Bacteria on Transductant Detection

Experiment No.	No. of input Arg^+ bacteria[a]	No. of Arg^+ colonies detected[b]	No. detected after washing[c]
1.	865[d]	820[d]	-
	70	77	-
	9	2	-
2.	253	245	164[d]
	57	56	31

[a] Input Arg^+ bacteria were supplied from strain D5(P1argF5).

[b] Detection was in a recipient background of 1×10^9 SC1800 per plate.

[c] Detection was in a recipient background of 1×10^9 SC1800 per plate. After the Arg^+ bacteria were added to the recipient culture, cells were washed in saline, centrifuged and plated as described in Materials and Methods.

[d] Colony detection was on Arg selective media after 48 hours incubation. Ozeki soft agar was used as an overlay to disperse bacteria on the Arg selection plates.

after the washing step. The washed bacteria were then plated on Arg selective medium and scored for Arg^+ colonies. Tests were done in triplicate. The results in Table 9, Experiment 2 indicated that there was approximately a 40% loss of transductants in the wash step. However, fluctuating results which would have indicated aggregation of the transductants were not observed in the triplicate platings.

It was desirable to determine if the lysates used for transduction experiments contained substantial numbers of killer particles. Recipient bacteria were incubated for 30 minutes with P1CmOc1.100 virions at an m.o.i. of 0.36, the highest m.o.i. used for transduction experiments. The number of viable cells was then determined by embedding appropriate dilutions of recipients in soft agar, and scoring the resulting colonies after 24 hours incubation. No significant difference was seen in the number of survivors of the infection compared to the uninfected control (2.6×10^8 vs. 2.5×10^8 ml, respectively). Therefore, no substantial killing of recipients occurred from non-plaque forming virions.

Previous arg^+ transduction frequencies had been determined with SC7100 as the donor. In this strain the argF gene is encoded within the host chromosome. To determine whether argF transduction with the TJC77 donors occurred with frequencies similar to those observed with SC7100 donors, lysates were prepared from SC7100 (P1CmOc1.100) and TJC77(P1CmOc1.100). The resulting virions were used to infect the non-lysogen, SC1800, at various m.o.i. Observed frequencies of arg^+ transduction were the same with either strain within a factor of 2

(Table 10). The decrease in transduction frequency with decreasing m.o.i. reflected a requirement for multiple infection of recipients (see also Chapter VI, Fig. 17). Results of this experiment indicated that the use of F128 as the donor chromosome did not introduce a variable affecting *arg*$^+$ transduction frequency.

Production of an HFT lysate for a gene is phenotypic evidence of integration of the gene in the prophage chromosome (Morse *et al.*, 1956a, b). Using TJC77(P1Cm0*c1*.100) as the donor, 22 Arg$^+$ transductants were selected from experiments where different m.o.i. were used. These transductants were scored for the ability to produce HFT lysates. The assay for HFT lysates was as described in Materials and Methods (Chapter III), with TJC26*argF*$^-$(P1) as the indicator strain. All 22 transductants produced HFT lysates for *argF*. In addition, the 15 transductants that were Cm^r also produced HFT lysates for the Cm0 determinant. Since all the HFT lysates were prepared without superinfection with P1 virions as helper genomes, the prophage of the transductants were proficient in virion production.

These results indicated that all 22 transductants contained P1*argF* or P1Cm0*argF* prophage. Such a finding had great utility for subsequent analysis. It established that the TJC77 donor -SC1800 recipient system selected almost exclusively for P1*argF* lysogens.

In control experiments, lysates produced by TJC77(P1) were not HFT for *argF* when assayed on the TJC26(P1) indicator strain. Since some of the TJC77(P1) lysates tested had transduction frequencies as high as 10^{-5} (see Table 12), the HFT lysate test conditions required

TABLE 10

Comparison of *argF* Transduction Frequencies with Different Donors

Multiplicity of infection	*argF* Transduction frequencies[a]	
	SC7100(P1Cm0*c*1.100) Donor	TJC77(P1Cm0*c*1.100) Donor
.18	1.3×10^{-6}	1.8×10^{-6}
.072	1.0×10^{-6}	2.6×10^{-6}
.032	1.4×10^{-6}	7.2×10^{-7}
.018	4.5×10^{-7}	2.8×10^{-7}
.018	not done	4.1×10^{-6}[b]

[a] The recipient for transduction was SC1800. Frequencies are expressed as the number of transductants per input pfu.

[b] The recipient for transduction was SC1800(P1) in this case only.

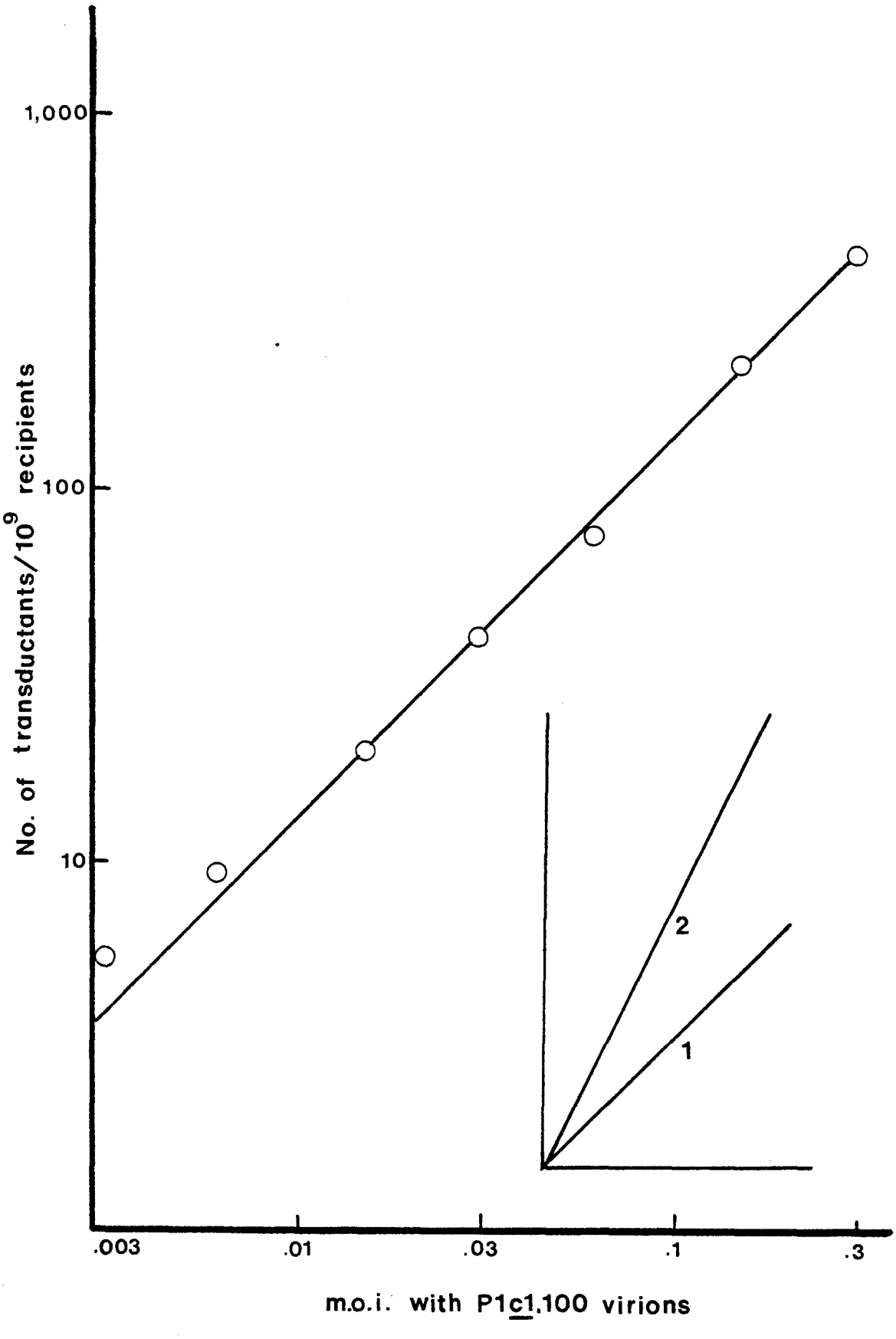
frequencies of transduction greater than this value for a positive result. Consequently, lysates of TJC77 will be referred to as LFT lysates in this study.

Virion requirements for arg^+ transduction.

The validity of the TJC77-SC1800 transduction assay was further confirmed in the following experiment. The dependence of $\underline{arg}^+$ transduction on m.o.i. was determined using TJC77(P1c1.100) as the donor and SC1800(P1) as the recipient. The P1 prophage in this recipient provided any P1 genes which the transducing genomes may require for lysogeny. On a double logarithmic plot, a slope of one persisted over a 100-fold m.o.i. range (Fig. 13). Since the slope was one, the curve of transductants against m.o.i. was linear. This result confirmed the validity of the transduction assay and established that one virion was sufficient to generate an Arg^+ transductant in a lysogenic recipient. A similar curve was generated if the donor was TJC77(P1CmOc1.100) (See Chapter VI, Fig. 17). Since most Arg^+ transductants in this system contained P1argF prophage, these results indicated that only one virion need be adsorbed to generate a P1argF prophage in a lysogenic recipient.

An experiment was performed to determine if the union of the argF gene and the P1 genome occurred in the donor for transduction. Virions from TJC77(P1CmOc1.100) were used to infect the SC1800(P1) recipient at an m.o.i. of 0.03. Fifty Arg^+ transductants from the infection were scored for coinheritance of the CmO allele. Approximately 66% had the phenotype Arg^+ Cm^r. The probability that any

Fig. 13. Dependence of *arg*$^+$ transduction on m.o.i. with P1*c1*.100 transducing vectors. Transductants were isolated from infection of SC1800(P1) recipients with virions produced in TJC77(P1*c1*.100) donors. Insert indicates the ideal slopes of one and two.


1,000
100
10
No. of transductants/10^9 recipients
.003
.01
.03
.1
.3
m.o.i. with P1c1.100 virions
2
1

infected recipient will be doubly infected at an m.o.i. of 0.03 is less than 1 in 100, according to the Poisson distribution. Since *arg*$^+$ transduction was not dependent on double infection, 99 out of 100 Arg$^+$ transductants came from single infection. Because 66% were also Cm^r, the CmO gene and the *argF* gene were in many cases transported by a single virion. This indicated that the union of *argF* and P1CmO occurred primarily in the donor for transduction before virion packaging.

Transduction frequencies with various P1 derivatives.

Several derivatives of phage P1 and its close relative P7 differ in the number and location of IS*1* sequences. Since the IS*1* locus of P1 was the site of insertion of the *argF* gene segment, these differences might affect the *arg*$^+$ transduction frequency mediated by these phage vectors. Virions of each of these vectors were prepared by induction of appropriate TJC77 donor lysogens. The resulting lysates were used to infect SC1800(P1) or SC1800(P7), as appropriate. Frequencies of *arg*$^+$ transduction were tabulated (Table 11). A lysogenic recipient was used for these experiments to assure that transductant yield was proportional to m.o.i.

The fraction of input virions adsorbed to recipients under these experimental conditions was determined in separate experiments. This value was used to correct transduction frequencies (transductants/pfu) for unadsorbed virions. Non-lysogenic SC1800 was used as the recipient in determining adsorption efficiency, since cultures of lysogens contained a low titer of virions in their media. The

TABLE 11

Transduction Frequencies for *argF*[a]

Transducing vector	No. of IS*1* sequences[b]	Transductants/10^9 pfu[c] Observed	Adjusted	Fraction of phage adsorbed
P1Cm0*c1*.100	2	4100	5200	.79
P1*c1*.100	1	2900	3100	.93
P1*c1*.225	1	620	2800	.22
P1Cm13*c1*.225	3	37	92	.40
P7*c1*.9*ts*	none known	3	5	.57

[a] Donors for transduction were TJC77/F128 lysogenic for the transducing vector. Transductions were performed at an m.o.i. of 0.3 into SC1800 lysogenic for either P1 or P7, as appropriate. Titers of LFT lysates were 10^9 or higher.

[b] The number of IS*1* sequences was determined by Iida *et al*. (1978), DeBruijn and Bukhari (1978), or this research.

[c] Values represent the average of three determinations using different LFT lysates. All values greater or less than other values by a factor of 2 differ significantly ($P < .01$). Separate experiments were normalized by comparison with a standard P1*c1*.100 donor lysate run concurrently. Adjusted values are observed values corrected for the fraction of virions adsorbed.

fraction of virions adsorbed was determined by comparing the input pfu with the pfu in the supernatent fluid after a 30 minute infection of SC1800. As observed in Table 11, the adsorption efficiency varied considerably among the bacteriophage strains. Adsorption was good with P1*c1*.100, while P1*c1*.225 adsorbed to recipients poorly.

The frequencies of *arg*$^+$ transduction varied among the P1 derivatives (Table 11). The highest frequency was observed with P1Cm0*c1*.100, which contains two IS*1* sequences at the natural IS*1* locus of P1 (DeBruijn and Bukhari, 1978; Iida *et al*., 1978). Phage P1*c1*.225 and P1*c1*.100, the Cm^S revertant of P1Cm0*c1*.100, had similar frequencies (when the values were adjusted for the poor adsorption of the P1*c1*.225 virions). Both of these strains contain one IS*1* sequence at map unit 20 (Iida *et al*., 1978). Phage P1*c1*.225 is considered wild type at this locus because, unlike P1*c1*.100, it is not known to have ever had an insertion at map unit 20 (Iida *et al*., 1978).

Bacteriophage P1Cm13*c1*.225 encodes the Cm13 resistance determinant flanked by direct repeats of IS*1* at map unit 4, in addition to the natural IS*1* at map unit 20 (Iida *et al*., 1978). This strain had an *arg*$^+$ transduction frequency 30 times lower than its P1*c1*.225 parental strain. There is no IS*1* sequence at map unit 20 in the genome of P7 (Iida *et al*., 1978; Iida and Arber, 1979), which rarely mediated complete *arg*$^+$ transduction to SC1800(P7). As a control, P1 virions produced in strain SC1800(P1) were unable to transduce this strain to the Arg$^+$ phenotype. Thus P1 infection by itself cannot generate an *arg*$^+$ gene.

Phage P1c1.100, P1c1.225, and P1Cm13 produced in TJC77 were also assayed for pro$^+$ transduction on SC1800(P1) recipients. In each case, no pro$^+$ transductants were isolated from 3 x 10^9 lysogenic recipients infected at an m.o.i. of 0.3. Therefore the pro$^+$ transduction frequency was less than 10^{-9} in this donor-recipient system.

Comparison of LFT lysates from induction and from infection.

Since P1argF were primarily formed in the donor for transduction, it was of interest whether they were formed during passage and growth of the P1 prophage or during the lytic cycle of the phage. Lysates were prepared by infection of P1 sensitive donors with various P1 vectors. The arg$^+$ transduction frequencies of these LFT lysates were compared with the frequencies obtained with lysates prepared by thermal induction of lysogenic donors. As seen in Table 12, LFT lysates produced by infection of the donor had arg$^+$ transduction frequencies 3 to 36 times greater than those produced by induction of lysogenic donors. Establishment of lysogeny was therefore not necessary for P1argF formation in the donor. In fact, the cytoplasm of a P1 immune cell appeared to be less favorable to P1argF formation than that of a P1 sensitive cell.

Genetic analysis of Arg$^+$ transductants.

Transductants produced with different vectors were characterized. Since the union of argF and P1 vector occurred in the donor, each Arg$^+$ transductant chosen for analysis was produced from a separate LFT lysate of the donor. This guaranteed that the argF elements of

TABLE 12

Comparison of *argF* Transduction Frequencies of LFT Lysates from Infection of Donors and Induction of Lysogenic Donors.[a]

Transducing vector	Fraction of phage adsorbed	Arg^+ transductants/10^6 pfu[b]		Ratio of infection to induction
		From induction	From infection	
P1CmO*c1*$^+$	.45	ND[c]	6	-
P1CmO*c1*.100	.79	5.2	101	19
P1*c1*.100	.93	3.1	10	3
P1*c1*.225	.22	2.8	22	8
P1*rev9c1*.225	.29	6.5	235	36

[a] Donors were TJC77/F128. Transductions were performed into SC1800(P1) at an m.o.i. of 0.3.

[b] Values represent the average of three determinations using different LFT lysates. All values greater or less than other values by a factor of 2 differ significantly ($P < .01$). Separate experiments were normalized by comparison with a standard P1*c1*.100 donor lysate run concurrently. Values have been corrected for the fraction of virions adsorbed.

[c] Not determined.

each transductant were not genetic sisters. All transductants analyzed were produced in the non-lysogenic SC1800 recipient, so that the recipient's prophage would not introduce a genetic variable. The m.o.i. of infection was 0.3 or below, to prevent extensive killing of recipients which was observed at m.o.i. above one.

Ten Arg$^+$ transductants produced with TJC77(P1c1.100) as donor were initially scored for ability to grow at 40^{o}C. Inability to grow at this temperature was presumptive evidence that the transductant acquired the P1c1.100 prophage with the thermal-sensitive c1.100 allele. No transductant grew at this temperature even though the m.o.i. used for infection of recipients was 0.03. In addition none of the transductants coinherited pro$^+$ or lac$^+$.

Each transductant produced lysates which had normal plaque forming titers, and were HFT for argF (even when assayed on SC1800). With SC1800 as the indicator strain for recognizing HFT lysates, the conditions of the assay were stringent; conversion of SC1800 to the Arg$^+$ phenotype, for the most part, required P1argF lysogeny. Conversion of TJC26argF$^-$ to the Arg$^+$ phenotype does not require lysogeny, since the incoming argF gene can be integrated into the recipient chromosome. Thus, these results indicated that all 10 Arg$^+$ transductants contained P1argF prophage.

Seven Arg$^+$ transductants were selected from separate experiments with P1c1.225 as the transducing vector. All seven produced lysates containing plaque forming particles. These lysates were also HFT for argF, as assayed on SC1800. Consequently, the transductants

probably contained P1argFc1.225 prophage. Transductants with P1Cm0 and P1Cm13 as vectors had more diverse characteristics and will be discussed in Chapter VI.

Successful arg$^+$ transduction was rare with P7 as the vector. Only seven transductants from a single LFT donor lysate were characterized. Properties of these transductants are listed in Table 13. Two transductants coinherited pro$^+$ but not lac$^+$ with arg$^+$. All contained P7 prophage but some of the prophage were defective in virion functions. Only one transductant produced an HFT lysate for argF. Apparently P7argF formation was an extremely rare event. The results confirmed that in the absence of argF integration into a prophage, arg$^+$ transduction in the TJC77 donor-SC1800 recipient system is rare.

Physical characterization of P1argF chromosomes.

Genetic evidence presented above indicated that all the Arg$^+$ transductants analyzed with P1c1.100 and P1c1.225 as the transducing vectors contained P1argF prophage. Virion chromosomes from several of these transductants (six from each vector) were analyzed by restriction endonuclease cleavage analysis, to determine their physical structure. Half the transductants analyzed were chosen from experiments in which the donor lysate was prepared by induction of lysogenic TJC77 strains, and half were from experiments with donor lysates prepared by infection of the P1 sensitive TJC77 donor. Virion chromosomes from all 12 transductants had BamHI cleavage patterns identical to P1argF5c1.100, characterized in Chapter IV. Digests of chromosomes from several of these P1argF derivatives are presented in Fig. 14.

TABLE 13

Characteristics of Arg$^+$ Transductants Produced by P7 as the Transducing Vector[a]

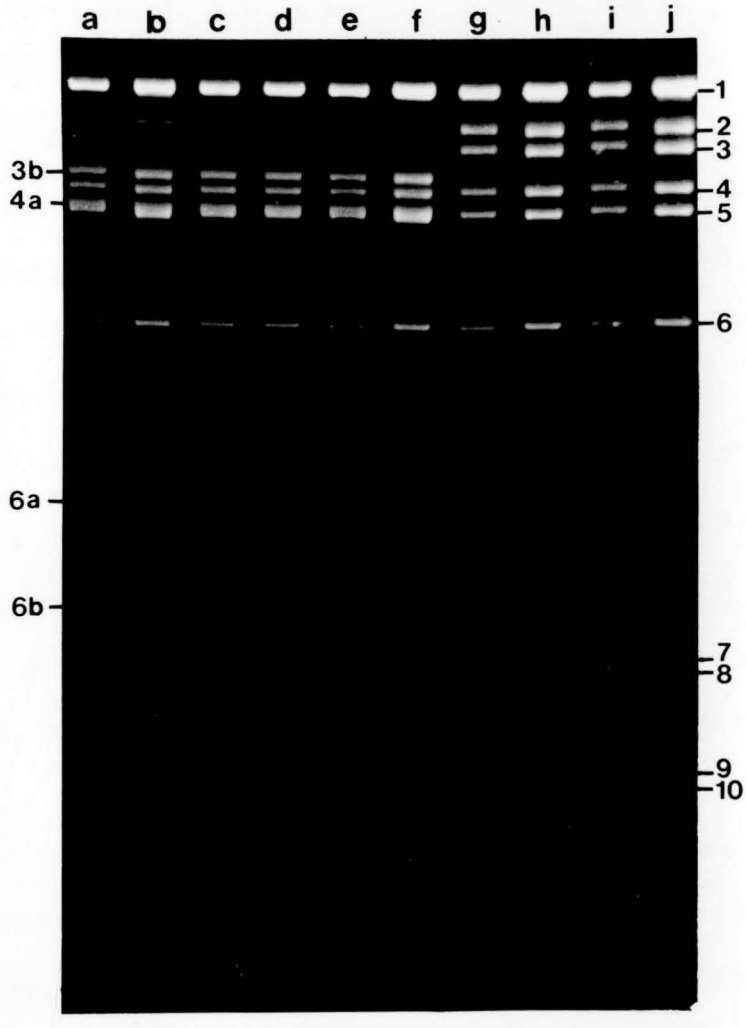
Transductant No.	Parental recipient for transduction	Pro$^+$ Phenotype	Lysate characteristics[b]	
			Plaque forming titer	HFT for c *argF*
1	SC1800(P7)	-	normal	-
2	SC1800(P7)	-	normal	-
3	SC1800(P7)	+	normal	-
4	SC1800	-	normal	-
5	SC1800	-	low	-
6	SC1800	-	low	+
7	SC1800	+	low	-

[a] The donor for transduction was TJC77(P7). All seven transductants were ampicillin resistant and had a Lac$^-$ phenotype.

[b] Assays were performed by the rapid assay technique described in Materials and Methods.

[c] Results of tests for HFT lysate production were the same with either TJC26(P7) or SC1800 as the indicator strain.

Fig. 14. Fractionated BamHI digests of virion chromosomes from several Arg^+ transductants and their Arg^- revertants. (a) P1argF2c1.100, (b) P1argF5c1.100, (c) P1argF4c1.100, (d) P1argF10c1.100, (e) P1argF15c1.100, (f) P1argF9c1.225, (g) P1c1.100, (h) P1rev9c1.225, (i) P1rev5c1.100, and (j) P1rev4c1.100. Numbers on the right indicate BamHI cleavage fragments of P1 DNA according to the cleavage map in Fig. 9. Numbers on the left indicate unique fragments produced by P1argF chromosomes as designated in Fig. 9. Electrophoresis was at 1.3 V/cm for 18 hours.

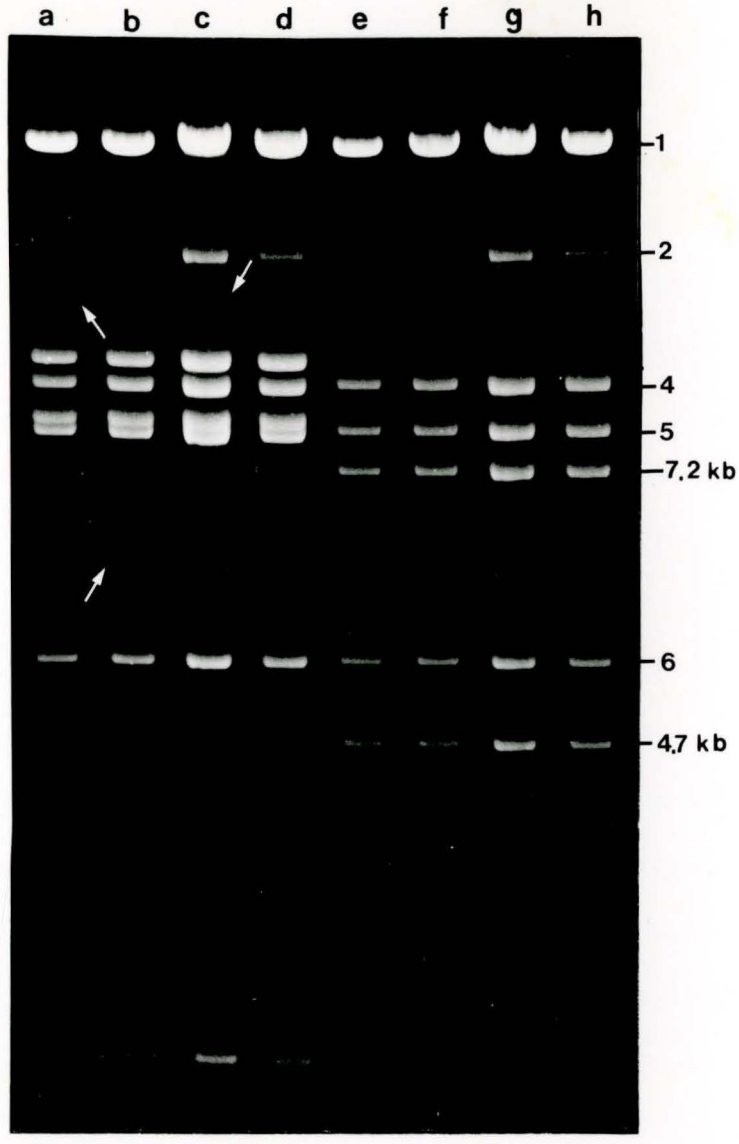

a
b
c
d
e
f
g
h
i
j
1
2
3
3b
4a
4
5
6
6a
6b
7
8
9
10

In all cases, the BamHI-3 fragment encoding IS1 was replaced by 4 new fragments (3b, 4a, 6a, and 6b). No matter how the original LFT lysate was produced or which P1 phage vector was used, the P1argF genomes that were formed were indistinguishable by cleavage analysis.

The presence of the minor cleavage fragments, BamHI-2 and 6', were explained previously. Other minor cleavage fragments were present in digested DNA from P1argF5, P1argF10, and P1argF15 (Fig. 14, slots b, d, and e), even though 3 subcultures preceded isolation of the virion chromosomes. These minor fragments were not always present in digests of virion chromosomes from the same strain isolated in different experiments. As seen in Fig. 15, the minor cleavage fragments in the BamHI digests were reduced to lower molecular weight fragments in BamHI:PstI double digests. The only Pst cleavage sites in P1argF chromosomes are within the argF insertion or within its flanking IS1 sequences (see Chapter IV). These fragments were therefore interpreted to encode either portions of the argF gene segment or an IS1 element.

Such an interpretation suggested that these fragments resulted from IS1 mediated deletions in the argF gene segment. The minor fragments in P1argF5 and P1argF15 digested DNA were the same size as BamHI-3. The BamHI-3 fragment of P1 chromosomes encodes the IS1 sequence at the insertion site of the argF gene segment (see Fig. 9). The data were therefore interpreted to mean that the entire argF gene segment was excised from some of the virion chromosomes. The minor fragments in P1argF10 were the size of the novel BamHI fragment seen

Fig. 15. Fractionated digests of virion chromosomes from several P1*argF* derivatives, demonstrating minor cleavage fragments. Virion chromosomes: digested with *Bam*HI, slots (a) P1*argF*15, (b) P1*argF*10, (c) P1*argF*5, and (d) P1*argF*9; digested with *Bam*HI:*Pst*I, slots (e) P1*argF*15, (f) P1*argF*10, (g) P1*argF*5, and (h) P1*argF*9. Arrows indicate the minor cleavage fragments in slots a, b, and c. Numbers on right represent *Bam*HI cleavage fragments of P1 DNA according to the cleavage map in Fig. 9. The 7.2 kb and the 4.7 kb fragments produced by *Pst*I cleavage of *Bam*HI digested P1 DNA are noted. Electrophoresis was at 2.6 V/cm for 18 hours.


a
b
c
d
e
f
g
h
1
2
4
5
7.2 kb
6
4.7 kb

in digested P1argF51 DNA. The P1argF51 derivative was characterized in Chapter IV and found to be deleted in the left portion of the argF insertion. The deletion was probably an IS1 mediated transposition event. Therefore the presence of this minor band suggested that a subclone of IS1 mediated deletion mutants was present in the P1argF10 virion population.

Plaque center tests.

If some of the minor bands in the digests were derived from excision of the argF gene segment, the virions in a P1argF lysate should contain a mixture of P1 and P1argF chromosomes. To test this hypothesis, the plaque center test was performed. In this test, a plaque was derived from a single virion proficient in lytic cycle functions. The plaque contained both progeny virions from this single phage particle and lysogens derived from infection of bacteria in the plaque with these progeny virions. Plaques were produced by infection of SC1800 with P1argF virions of several different phage strains. These plaques were scored for the presence of Arg^+ lysogens by subculture of the plaque center to Arg selective medium. Only 13 of 72 (18%) plaques from different P1argF lysates contained Arg^+ lysogens. Therefore argF was frequently lost during plaque development.

Reversion rate of strains harboring P1argF prophage.

To determine if the excision of the argF segment was a property of passage and growth of lysogens, the reversion rate during non-selective passage was examined. Clonal populations of strains

lysogenic for P1*argF* were scored for Arg$^-$ revertants. Results are in Table 14. Taken collectively, the reversion rate of the five strains examined was 0.4%.

Four independently selected revertants were examined for P1 functions. Three produced lysates upon induction that had normal plaque forming titers. The prophage of these revertants are designated P1*rev*. The Arg$^-$ revertant from the other strain had lost P1 genes. This revertant did not produce virions visible in CsCl gradients after induction of the culture. It was further tested and found to be *res*$^-$, *mod*$^-$, and P1 sensitive, suggesting that the strain was cured of the entire P1*argF* prophage.

Virion chromosomes from the P1*rev* strains were digested with *Bam*HI and fractionated on 0.7% agarose gels (Fig. 14). Restriction patterns were indistinguishable from those of the ancestral P1 chromosome. Endonuclease *Eco*RI and *Eco*RI:*Pst*I double digests also gave electrophoretic patterns identical to that of wild type P1 chromosomes on gels with resolution of fragments greater than 350 base pairs (Fig. 16). These results suggested that the entire *argF* gene segment had been excised. The single *Pst*I cleavage site of P1 DNA was present in the *Eco*RI:*Pst*I double digests. Since this site is within IS*1*, a single IS*1* element probably remained in the revertant chromosomes after the excision of the *argF* gene segment.

Several minor cleavage bands were visible in gel pockets 2 through 8, in the digests of Fig. 16. These bands resulted from a technical error in the digestion procedure. The *Eco*RI and *Eco*RI:*Pst*I

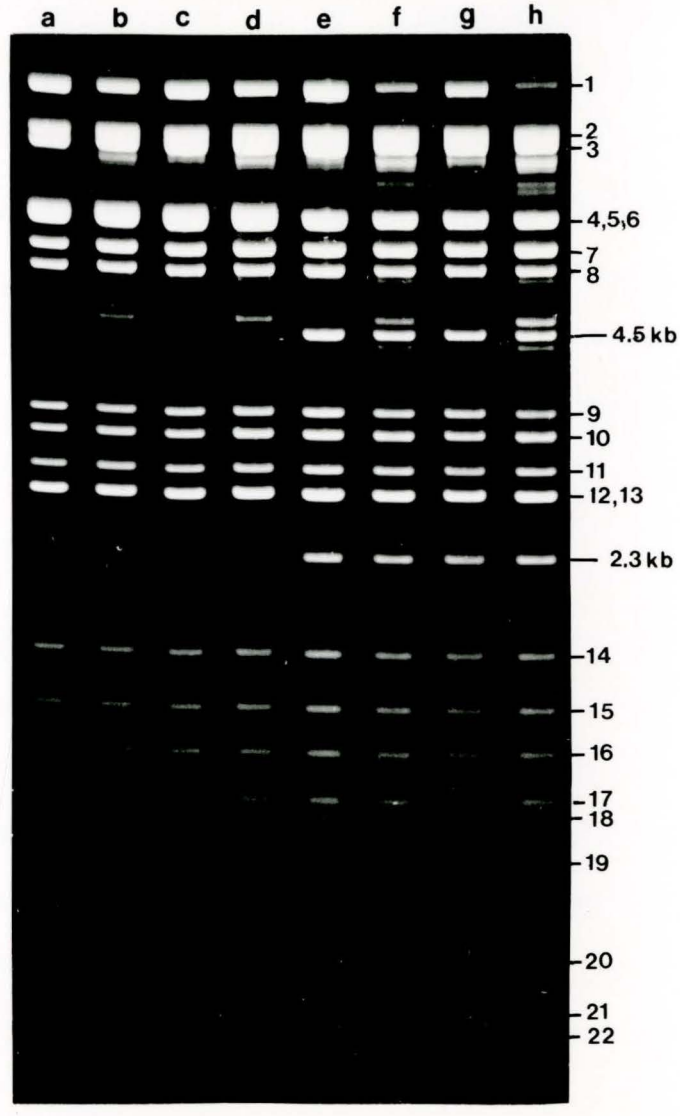
TABLE 14

Loss of *argF* by P1*argF* Lysogens

Prophage	No. Arg$^-$ colonies/ No. colonies scored
P1*argF2c1*.100	2/441
P1*argF4c1*.100	1/227
P1*argF5c1*.100	1/476
P1*argF9c1*.225	5/762
P1*argF25c1*.225	0/516

Isolated Arg$^+$ colonies from strains lysogenic for P1*argF* were grown for 3 generations in L broth and plated for isolation on L agar. After overnight incubation, colonies were replica plated to Arg selective medium. Colonies which failed to grow on the Arg selective medium but grew on L agar were checked to verify their Arg$^-$ phenotype.

Fig. 16. Fractionated digests of virion chromosomes from P1 and P1rev mutants. Virion chromosomes: digested with EcoRI, slots (a) P1, (b) P1rev4, (c) P1rev5, (d) P1rev9; digested with EcoRI:PstI, slots (e) P1, (f) P1rev4, (g) P1rev5, (h) P1rev9. Numbers on the right represent EcoRI cleavage fragments of P1 DNA according to Bachi and Arber (1977). The 4.5 kb and 2.3 kb fragments produced by PstI cleavage of EcoRI digested P1 DNA are noted. Electrophoresis was at 1.3 V/cm for 18 hours.


a
b
c
d
e
f
g
h
1
2
3
4,5,6
7
8
4.5 kb
9
10
11
12,13
2.3 kb
14
15
16
17
18
19
20
21
22

digestions were performed in the buffer recommended for PstI digestion of DNA. In 100 mM Tris a specific hexanucleotide sequence is recognized and cleaved by EcoRI endonuclease. However, in Tris buffers less than 25 mM, EcoRI also recognizes a specific tetranucleotide sequence (Polisky et al., 1975). Because the molar concentration of Tris was low (6 mM) in the PstI digestion buffer, these tetranucleotide sequences were cleaved by the EcoRI enzyme. The tetranucleotide digestion was apparently incomplete, since the additional digestion only produced minor fragment bands (see Fig. 16). These minor bands did not, however, interfere with interpretation of the cleavage analysis, because they were consistent and present in the P1 control as well as the P1rev mutants.

Isolation of a mutant in P1argF formation.

Selection of P1argF genomes in arg$^+$ transduction experiments may also be a selection for mutants more reactive in P1argF formation. To test this hypothesis, arg$^+$ transduction frequencies were determined using P1rev strains as the transducing vectors. One P1rev, designated P1rev9c1.225 from P1argF9c1.225, was 10 times more reactive in P1argF formation than its P1c1.225 ancestral phage. As observed in Table 12, the frequency of P1argF formation by P1rev9 was higher than that of any other P1 derivative. The increase in arg$^+$ transduction frequency was most pronounced if the LFT lysate was prepared by infection of the TJC77 donor. If the LFT lysate was from induction of TJC77(P1rev9) the frequency was only slightly higher than that of the other P1 derivatives.

Discussion.

Successful transduction of the argF gene by bacteriophage P1 is accomplished in one of three ways: (1) The gene is integrated into the recipient host chromosome by generalized recombination. This type of transduction cannot occur for the argF gene in SC1800 recipients, since the chromosomal locus corresponding to the transduced gene is within the recipient's deletion. (2) The transduced gene is integrated into the P1 chromosome. Such integration is manifested by production of HFT lysates by the transductant, if the P1 prophage is non-defective in lytic cycle funtions. (3) The gene is aberrantly integrated into the host chromosome. The most common type of aberrant integration is integration by addition, as described by Stodolsky (1974) and reviewed by Anderson and Roth (1977). In such integrations the transducing chromosomal fragment finds homology at one end of the deletion and an unstable tandem duplication is formed at this locus.

This latter type of integration is apparently rare in the donor-recipient system described in this chapter. This conclusion is based on the fact that in this system pro$^+$ transduction frequencies are less than 10^{-9}. In addition, practically all of the Arg$^+$ transductants analyzed produced HFT lysates for argF, unless they were defective in phage production. Different results are obtained in the system in which the E. coli host chromosome is the donor for transduction and SC1800 is the recipient. Transduction frequencies for pro$^+$ are 10^{-8} and only 50% of the Arg$^+$ transductants produce HFT

lysates for argF (Stodolsky, personal communication). The Pro^+ transductants and the Arg^+ transductants yielding LFT lysates probably acquire the genes through integration by addition. Since such transductants were rarely formed in the TJC77 donor-SC1800 recipient system, the acquired host gene segment of the F128 plasmid probably does not genetically overlap the deletion of the recipient. This system is therefore quite useful for studying specialized transduction for genes within the deletion of the SC1800 recipient.

All of the Arg^+ transductants analyzed from P1c1.100 and P1c1.225 vectors produced HFT lysates for argF, indicating that they contain P1argF prophage. The normal plaque forming titers of these HFT lysates demonstrate that the P1argF chromosomes are not overlarge. Those chromosomes that were analyzed in detail are indistinguishable from P1argF5, which contains its argF gene segment between flanking IS1 elements at the natural IS1 locus of P1 (see Chapter IV). These results indicate that the P1 IS1 locus is the preferred integration site for the argF gene segment.

Of the various phage strains used as vectors, all those that encode at least one IS1 sequence produce P1argF genomes with varying but high frequencies (as compared to the frequency of P1pro formation). Bacteriophage P7 rarely mediates successful P7argF formation. This phage lacks the IS1 sequence of P1 at map unit 20 (Iida et al., 1978; Iida and Arber, 1979). The P7 phage encodes an ampicillin resistance transposon (Smith, 1972: Yun and Vapnek, 1977), which apparently is unable to react with the argF region of the donor chromosome with high

frequency. Therefore, IS1 appears to be specifically responsible for the frequent integration of the argF gene into the P1 phage genome.

Phage P1 and P7 encode a site-specific recombination function (Lox), which has been implicated in rare recombinations between these phage and their host chromosomes (Chesney et al., 1978). The site on the P1 and P7 chromosome for integration mediated by lox is thought to be at the end of the phage genetic map (Sternberg, 1978). Since no P1argF have been isolated in which this locus is the site of integration of the argF gene segment, recombination events mediated at lox are not involved in most P1argF formations.

The presence of more than one IS1 element appears to have an effect on successful P1argF formation. This conclusion is based on comparison of the arg^+ transduction frequencies observed with P1Cm0c1.100, which contains two IS1 elements, and its Cm^S revertant, P1c1.100 having only one IS1 (Table 12). The advantage in P1Cm0c1.100 may be merely in the presence of a second IS1 element or it may be that the second IS1 element is more reactive in P1argF formation than the IS1 remaining in P1c1.100.

The IS1 elements at map unit 4 in P1Cm13 are disadvantageous to successful P1argF formation. There are two alternative explanations for this observation. Either the presence of the additional IS1 elements inhibits stable P1argF formation or it promotes formation of lethal deletion mutants accompanying or following argF insertion. There is little direct evidence to support either hypothesis. However, two of the three P1Cm13argF derivatives characterized by cleavage

analysis in the next chapter have deletions of large segments of the P1 chromosome. Plaque forming titers of P1Cm13 virion populations are normal. Therefore deletions of P1 genes are not common in P1Cm13 virions although they are seen in the P1Cm13argF virions.

The low frequency of arg$^+$ transduction from P1CmOc1$^+$ compared to that of P1CmOc1.100 was surprising (Table 12). However, there is a variable present in the transduction assays with these two strains. Donor lysates from P1CmO were prepared by confluent plate lysis, while lysates of P1CmOc1.100, even if they were from infection of sensitive strains, were prepared by thermal induction. The major difference in these two preparations is that virions from thermal induction are produced from one phage lytic cycle followed by host cell lysis. Virions prepared from confluent plate lysis are from several rounds of infection and host cell lysis. In the process argF insertions could be formed and excised repeatedly.

The union of the P1 chromosome and the argF gene segment occurs primarily in the donor for transduction. Evidence for this is that the CmO and argF determinants are often transported by single virions of the donor lysate. The mode of production of the LFT lysate has a significant effect on arg$^+$ transduction frequency. Lysates produced by infection have significantly higher frequencies than those produced by induction of lysogenic donors. This observation indicates that the initial events of P1argF formation occur primarily during the lytic cycle in the donor and that the prophage state is not essential for the event. Consequently, the formation of P1argF specialized

transducing phage is different from that of λ specialized transducing phage. A λ phage integrates into a specific site in the donor chromosome as a prophage in order to transduce the surrounding genes with high frequency (Morse et al., 1956a, b).

That lysogeny by P1 is not needed for P1argF formation would be a valid conclusion even if arg$^+$ transduction frequencies of lysates produced by infection were the same as those of lysates produced by induction. However, lysates from infection have higher frequencies than those from induction. Consequently, it appears that P1 function(s) expressed in the prophage state have a repressing effect on P1argF formation or transmission, even after induction of the lytic cycle. This repressor function is apparently expressed to a lesser extent or not at all upon infection of P1 sensitive bacteria. Possibly the repressor function is a repressor of the recombination between P1 and argF.

An unusually high frequency of arg$^+$ transduction is mediated by one revertant, P1rev9. As observed with the P1rev$^+$ strains, this increased activity is repressed in the prophage state. Thus, the rev9 mutation does not affect the repressor function. The presence of both the rev function and the distinct repressor function demonstrates the existence of at least two loci within the P1 chromosome that are involved in the formation or transmission of P1argF recombinants. One function must account for the increased activity and another must account for repression of that activity. Genetic mapping of the rev9 mutation will establish whether it is distinct from IS1. If rev9 maps at the IS1 locus, the mutation will be useful for studying IS1 function.

Since IS1 is involved in P1argF formation, these observations

could be interpreted to suggest that the P1 chromosome encodes a function that promotes an IS1 mediated recombination during the P1 lytic cycle and a function that represses an IS1 mediated recombination during the prophage state. This interpretation is in agreement with the observation of Chow and Bukhari (1978) in which they show involvement of bacteriophage Mu in Tn9 transposition during the lytic cycle of Mu, but not during the Mu lysogenic state.

Minor fragments were found in several digests of virion chromosomes of P1argF lysogens. Their presence is explicable in terms of IS1 promoted deletions and excisions. Clonal populations of 10^9 P1argF lysogens contained about 0.4% Arg^- bacteria. Loss of argF usually results from excision of the entire argF insertion. The rate of argF excision agrees with that reported by Rosner (1972) for loss of CmO in strains lysogenic for P1CmOc1.100. However, these reversion rates are too low to explain the presence of minor bands in the fractionated digests. A fragment produced from only 0.4% of the digested chromosomes would not be visible by ethidium bromide staining of the fractionated digestion products. Consequently, the chromosomes yielding minor fragments are probably generated during the lytic cycle. Thus, it appears that IS1 mediated excision of the argF gene segment is more common during the lytic cycle, than during passage and growth of lysogens.

This interpretation is also suggested from the results of the plaque center tests. Only about 20% of the plaques initiated with P1argF virions contained lysogens with P1argF prophage. The lack of

P1argF lysogens in 80% of the plaques is not due to the lack of P1argF genomes in the virions that initiate the plaques. Fragments representing the argF gene segment were seen in normal or near normal molar ratios in digests of these virion chromosomes.

Therefore, the argF genes are deleted during plaque formation, probably through IS1 mediated excisions and deletions. One Arg^+ lysogen (P1argF51) isolated from a plaque has been examined. It has a deletion of a segment of the argF insertion which can be attributed to IS1 action (see Chapter IV). Plaques result from multiple lytic cycles and IS1 mediated deletions of argF can occur within each of the constituent lytic cycles. Deletion mutants would have a growth advantage within the plaque since they replicate faster. Another possible explanation for the plaque center test results is that loss of the argF gene segment may be promoted by multiple infection of bacteria, which occurs during plaque formation.

Kondo and Mitsuhashi (1964) report that 79 to 92% of the plaque centers from P1CmO virions contain Cm^r lysogens. This result is even more striking since their virions were obtained through multiple lytic cycles, whereas the P1argF virions were produced in a single lytic cycle. In addition, neither this laboratory nor others (DeBruijn and Bukhari, 1978; Meyer and Iida, 1979; Iida and Arber, 1980) have reported the presence of minor fragment bands in digests of P1CmO virion chromosomes. These observations suggest that excision of the CmO determinant during the phage lytic cycle is much less common than excision of the argF gene segment. Possibly

excision of CmO is less steriochemically favored because of the smaller size of the CmO transposon. Alternatively, the regulation of IS*1* action with the Tn*9* transposon and the Tn*2901* transposon may be quantitatively distinct. That is, the nucleotide sequences of their IS*1* elements may differ.

CHAPTER VI

CHARACTERIZATION OF P1CmO*argF* AND P1Cm13*argF* DERIVATIVES

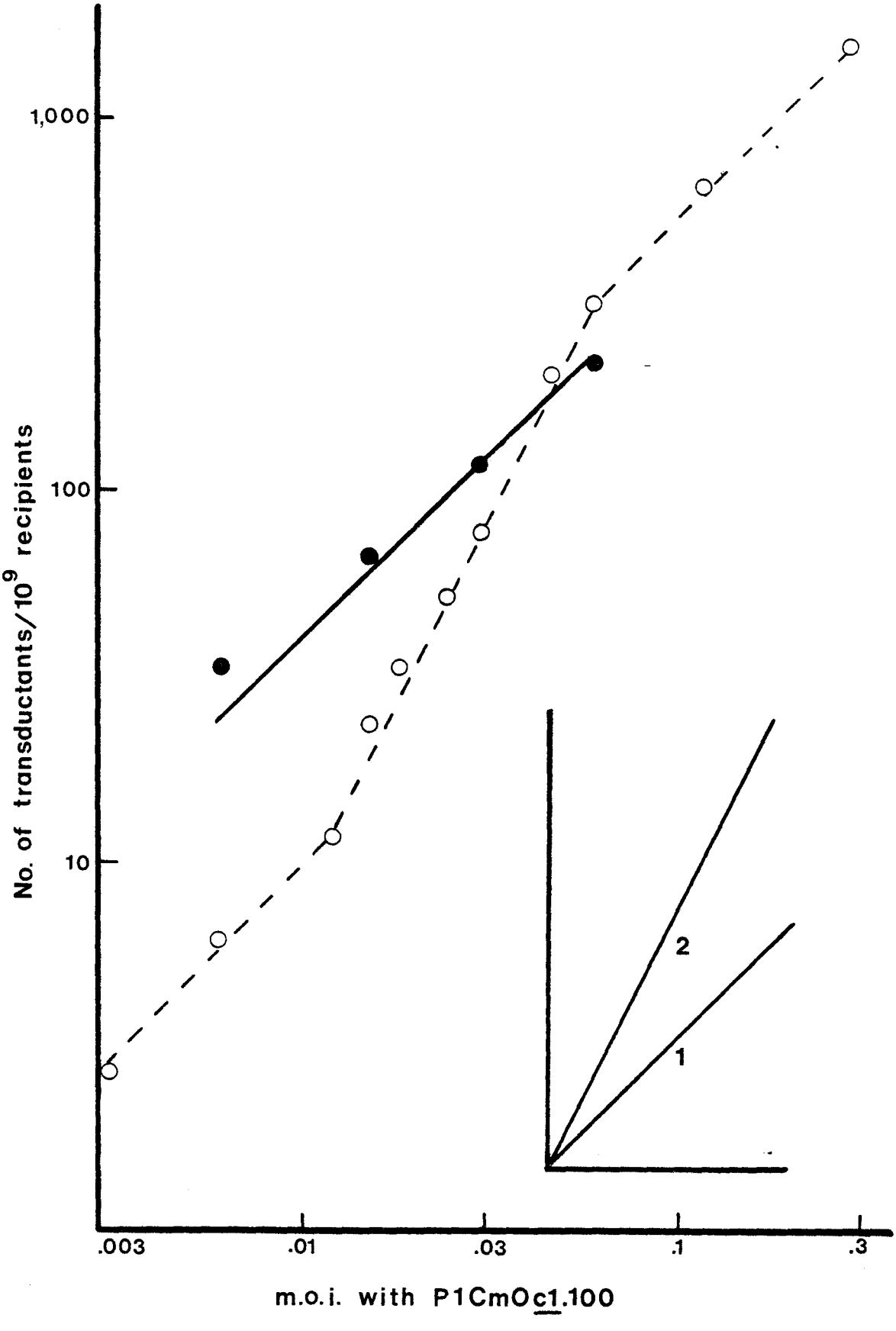
Introduction.

Analysis of several transductants derived from P1CmO*c1*.100 and P1Cm13*c1*.225 vectors was undertaken to evaluate the effects of the Cm transposon on P1*argF* formation. Of the P1 derivatives analyzed in Chapter V, with the exception of P1*rev*9, virion populations of P1CmO*c1*.100 had the highest frequencies of *arg*$^+$ transduction, while those of P1Cm13 had the lowest frequencies. The analyses presented in this chapter demonstrate that defective *argF* specialized transducing phages are more commonly isolated from infections with P1CmO and P1Cm13 transducing vectors than from infections with other P1 vectors. Characterization of these derivatives provides some insight into both P1 functions and IS*1* mediated recombination events.

Characterization of the *arg*$^+$ transduction event mediated by P1CmO.

To determine how many virion particles were required for *arg*$^+$ transduction from P1CmO, the number of transductants of SC1800 recipients was determined at various m.o.i. When the data were analyzed on a double logarithmic plot, an initial slope of one was observed (Fig. 17). This slope indicated that virions capable of transducing *argF* by single infection were present in the lysate. From this low m.o.i. region, six transductants were analyzed. All lysed upon thermal induction without helper. One lysate was LFT for *argF* and had only

Fig. 17. Dependence of *arg*$^+$ transduction on m.o.i. with P1CmOc1.100 transducing vectors. Virions were produced in TJC77/F128(P1CmOc1.100). Recipients for transduction were SC1800 [o—o] and SC1800(P1) [•—•]. Insert indicates the ideal slopes of one and two.


1,000
100
10
No. of transductants/10^9 recipients
.003
.01
.03
.1
.3
m.o.i. with P1CmOc1.100
2
1

about 500 pfu/ml. The other five lysates were HFT for argF indicating that they had argF prophage with proficiency in lytic cycle functions.

The slope of two, observed at higher m.o.i., indicated that simultaneous infection with P1CmO greatly facilitated Arg^+ transductant formation (Fig. 17). All five transductants analyzed from these m.o.i. produced HFT lysates for argF without helper. At higher m.o.i., the slope returned to one, indicating that the recipients were saturated with virions acting as helper particles. The transduction frequency at these high m.o.i. was 5×10^{-6} transductants/pfu, while at the lowest m.o.i. tested, the frequency was five times lower. Thus, P1 helper increased transduction efficiency 5-fold.

If the recipient was a P1 lysogen, a slope of one was observed, representing an arg^+ transduction frequency of 4×10^{-6} (Fig. 17). Thus, only one virion was needed to generate a P1argF prophage, demonstrating that a P1 prophage can substitute for an infecting P1 helper particle.

From infection of the non-lysogenic SC1800 recipient, three transductants produced at each m.o.i. were analyzed for Cm^r. Of 50 transductants scored, 22 had lost the CmO resistance determinant.

Analysis of Arg^+ transductants derived from P1CmO vectors.

Low frequency transducing lysates were prepared from 7 clonal populations of TJC77(P1CmOc1.100) and TJC77(P1c1.100) for comparison. These lysates were used to infect the non-lysogen SC1800 at an m.o.i. of 0.3 and 0.03. Three Arg^+ clones were analyzed from each transductant population. All the transductants were Pro^- and Lac^-. Other

characteristics are listed in Table 15 and discussed below.

There was a substantial correlation between m.o.i. of infection and defectiveness of the prophage derived from the P1CmOc1.100 vectors. From the infections at an m.o.i. of 0.3, 19 out of 21 transductants failed to form colonies at 40°C, indicating that they harbored a prophage with the c1.100ts allele. In contrast, only 12 of the 21 transductants produced at an m.o.i. of 0.03 demonstrated this phenotype. The rest presumably contained prophage too defective to kill the host efficiently. Since defective prophage were less frequent at the higher m.o.i., infections of recipients with helper probably supplied complements of P1 genes lacking in the transducing phage.

The analysis suggested that there was clonal variation in the donor population. Usually if one transductant was defective in P1 functions, other transductants produced with the same virion population were also defective (see Table 15). If the defective genotype was generated during the lytic cycle, a homogeneous distribution of the defects among the lysates would be expected. Since this distribution was not observed, it appeared that the defective genotype pre-existed in the donor population. All the Arg^+ transductants derived from P1c1.100 vectors failed to grow at 40°C, even if they were produced at an m.o.i. of 0.03.

Several Arg^+ transductants derived from P1CmOc1.100 vectors were analyzed further. One Cm^r and one Cm^s transductant produced from each transductant population was chosen. All produced lysates that contained some plaque forming particles. Approximately half of the

TABLE 15

Growth at 40°C by Arg$^+$ Transductants Derived from Vectors Having the c1.100ts Allele[a]

Donor lysate No.	No. of Arg$^+$ transductants that grew at 40°C			
	P1CmOc1.100 vectors		P1c1.100 vectors	
	M.o.i. of 0.3	M.o.i. of 0.03	M.o.i. of 0.3	M.o.i. of 0.03
1	0	0	0	0
2	0	1(+)[b]	0	0
3	0	3(-)	0	0
4	0	0	0	0
5	0	0	0	0
6	0	3(-)	0	0
7	2(+)	2(+)	0	0

[a] Donors were either TJC77(P1CmOc1.100) or TJC77(P1c1.100). Recipients were SC1800. Growth at 40°C was determined by colony formation on L agar at that temperature in 24 hours. Three transductants were scored for each experimental condition.

[b] One transductant that grew at 40°C from each experimental condition was analyzed further. Symbol in parenthesis indicates whether that transductant produced any plaque forming virions after induction, even though none of the cultures visibly lysed.

plaque forming titers were lower than normal but there was no correlation between a low titer and the Cm^r phenotype. All the transductants produced HFT lysates for argF and for CmO (if they were Cm^r), without superinfection with helper. Strain SC1800 was the indicator strain for HFT lysate production. Thus the transductants contained P1argF or P1CmOargF prophage.

Physical characterization of a P1CmOargF derivative.

Virion chromosomes from one Arg^+ and one Arg^+ Cm^r transductant were analyzed by cleavage analysis. The BamHI digest of the virion chromosomes from the Arg^+ transductant was identical to that of P1argF5, characterized in Chapter IV. Virion chromosomes of the Arg^+ Cm^r transductant lacked only the 4a fragment of P1argF5 (Fig. 18). A novel band of 9.9 kb was present, which presumably represented the 4a segment plus the CmO determinant. The digest contained the 3b fragments which correspond to the right-hand segment of the argF insertion (see map Fig. 9). Thus the CmO determinant was located to the left of the argF determinant on the P1 physical map. This phage derivative is designated P1CmOargF10.

Digestions with EcoRI were in agreement with the interpretation of the BamHI digest. This analysis is illustrated in Fig. 19. In addition, the EcoRI:PstI double digest showed that the left (4.5 kb) and right (2.3 kb) portions of the EcoRI-4 segment of P1 were intact (Fig. 20). This indicated that both the CmO and argF insertions were at the IS1 locus of P1 and that a deletion of P1 genes probably did

Fig. 18. Virion chromosomes from (a) P1argF5 and (b) P1CmOargF10 digested with BamHI. Cleavage fragments of P1argF5 DNA are designated on the left according to the BamHI cleavage map in Fig. 9. The novel 9.9 kb fragment of P1CmOargF10 is noted. Electrophoresis was at 1.3 V/cm for 18 hours in this and all subsequent fractionated digests.

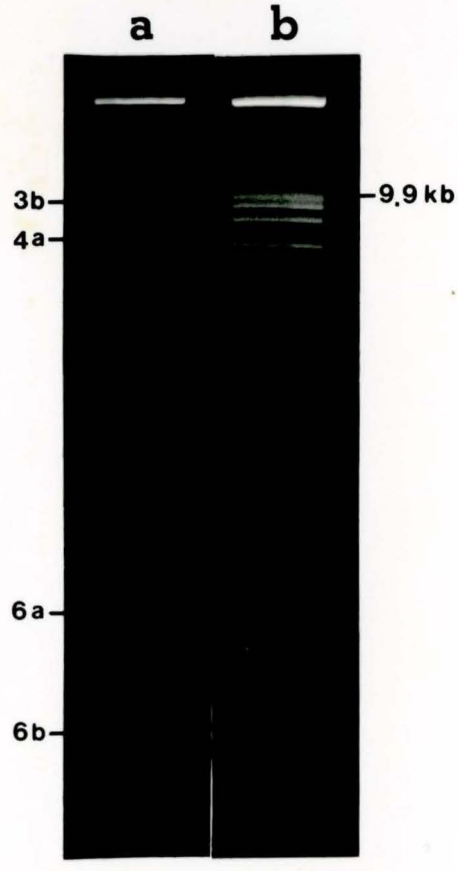

a
b
3b
4a
6a
6b
9.9 kb

Fig. 19. Virion chromosomes from P1, P1*argF*5, P1CmO and P1CmO*argF*10 digested with *Eco*RI. Relevant fragments are noted. Physical map of the *Eco*RI-4 segment of the P1 chromsome and the alterations in this segment in the P1 derivatives is below the fractionated digests. The order of the CmO and *argF* insertions in P1CmO*argF*10 is determined from the common fragments that were shared with P1CmO and P1*argF*.

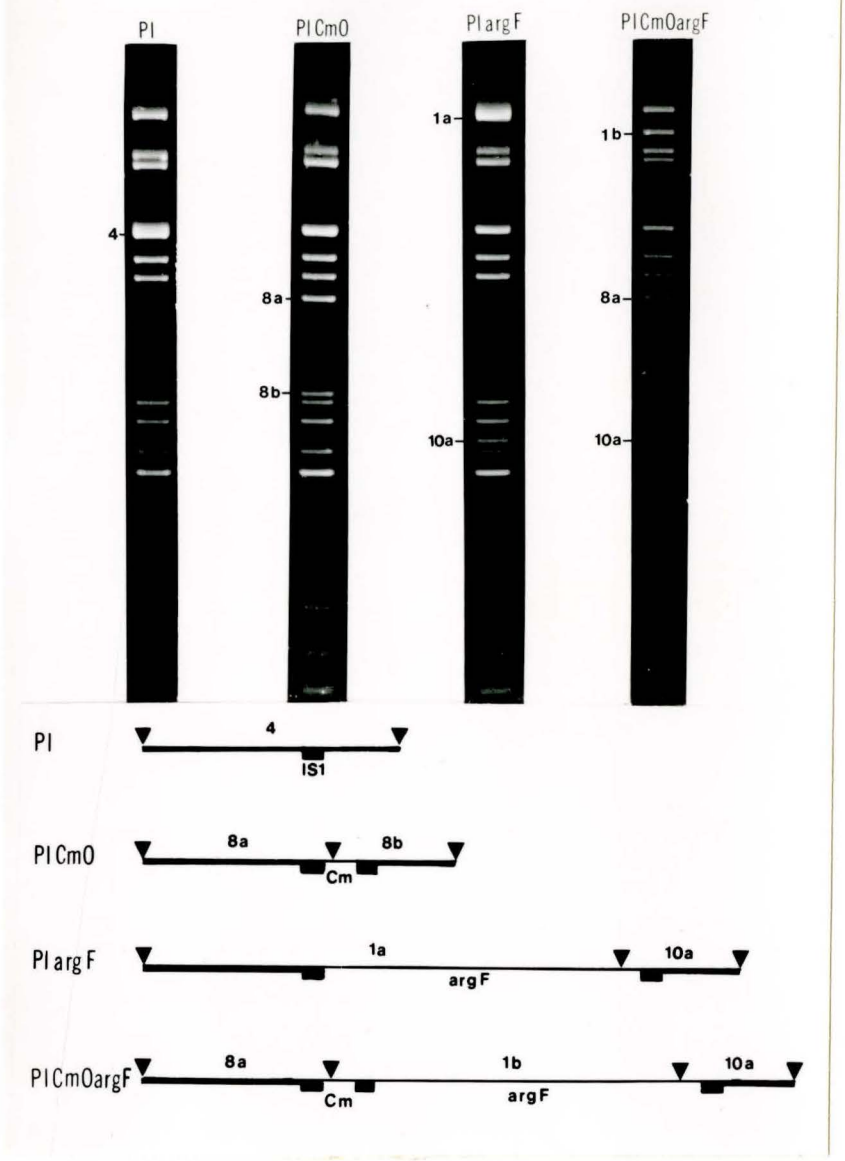
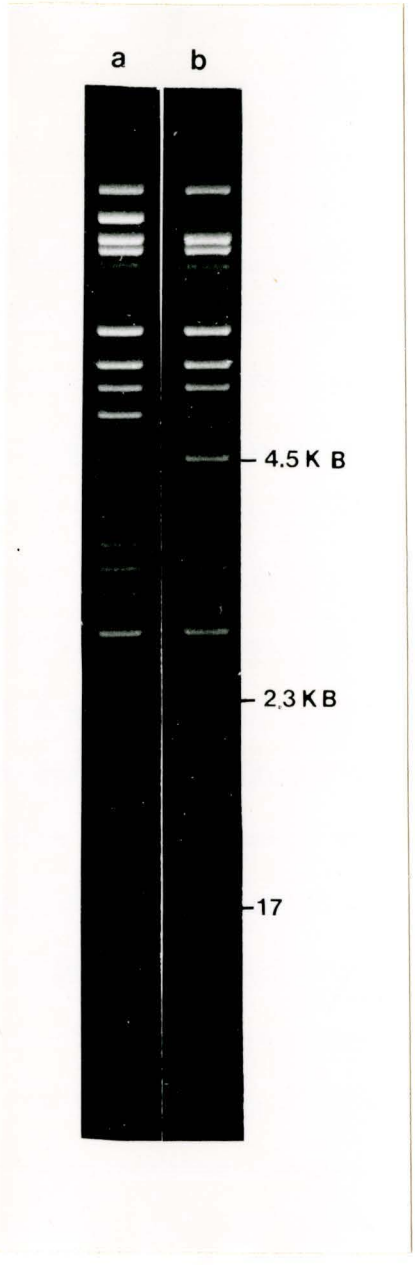

P1
P1CmO
P1argF
P1CmOargF
4
8a
8b
1a
10a
1b
8a
10a
P1
4
IS1
P1CmO
8a
8b
Cm
P1argF
1a
10a
argF
P1CmOargF
8a
1b
10a
Cm
argF

Fig. 20. Virion chromosomes of P1CmOargF10 digested with (a) EcoRI and (b) EcoRI:PstI. The 4.5 and the 2.3 kb PstI cleavage products of the EcoRI-4 segment of P1 DNA and the doublet at EcoRI-17 are noted.


a
b
4.5 K B
2.3 K B
17

not accompany the insertion. Fragments in the doublet at EcoRI-17 in the EcoRI:PstI digest represented the right-hand segment of the CmO determinant cleaved by PstI (see Chapter IV, Fig. 6). This suggested that an IS1 element was present between the argF and CmO insertions in P1CmOargF10.

Genetic characterization of P1Cm13argF prophage.

Seven Arg^+ transductants derived from P1Cm13c1.225 were characterized. These transductants were produced from two separate LFT lysates of TJC77(P1Cm13c1.225). Recipient SC1800 bacteria were infected at an m.o.i. of 0.3. All seven transductants had different phenotypic characteristics, as listed in Table 16. Unlike the transductants derived from P1CmO vectors, most of the transductants from P1Cm13 infected recipients were Cm^r.

Only the transductant N541 produced an HFT lysate for argF without superinfection with P1 as helper. An unusual result was obtained with this transductant. This strain expressed the res gene of P1. However, the Cm13 determinant of P1Cm13 is inserted such that it prevents expression of the res and mod genes of P1 (Iida and Arber, 1977). Consequently, strains harboring P1Cm13argF prophage should not be able to express the P1 restriction and modification system.

While most of the transductants were unable to produce HFT lysates without helper, several lysates prepared with P1 superinfection were HFT for argF, as assayed on SC1800(P1). This result was evidence that the Arg^+ transductants contained defective prophage that encoded the argF gene segment.

TABLE 16

Characterization of Arg^+ Transductants from P1Cm13 Transducing Vectors[a]

Transductant Strain	Cm^r	Res^+	Mod^+	P1 immune	Lysis[b]	Plaque formation	HFT lysate production for argF	
							Without helper[c]	With helper[d]
N541	+	+	ND[e]	+	+	+	+	+
N542	+	-	-	+	+	-	-	+
N543	+	-	-	+	±	+ (rare)	-	-
N544	+	-	-	+	+	+	-	+(rare)
N517	+	-	-	+	+	+	-	+
N518	+	-	-	-	-	-	-	+
N519	-	-	-	-	-	+ (rare)	-	-

[a] All tests were as described in Materials and Methods.

[b] Lysis indicates whether there was visible clearing of broth cultures two hours after induction.

[c] Indicator strain for detection of HFT lysates was SC1800.

[d] Helper (P1c1.100) was added before induction at an m.o.i. of 3.0. The indicator strain for detection of HFT lysates was SC1800(P1).

[e] Not determined.

Two of the Arg^+ transductants lacked immunity to superinfection with P1*clg*. One of these, N518, produced an HFT lysate for *argF* if helper was present during the phage lytic cycle. The other (N519) produced an occasional plaque forming particle.

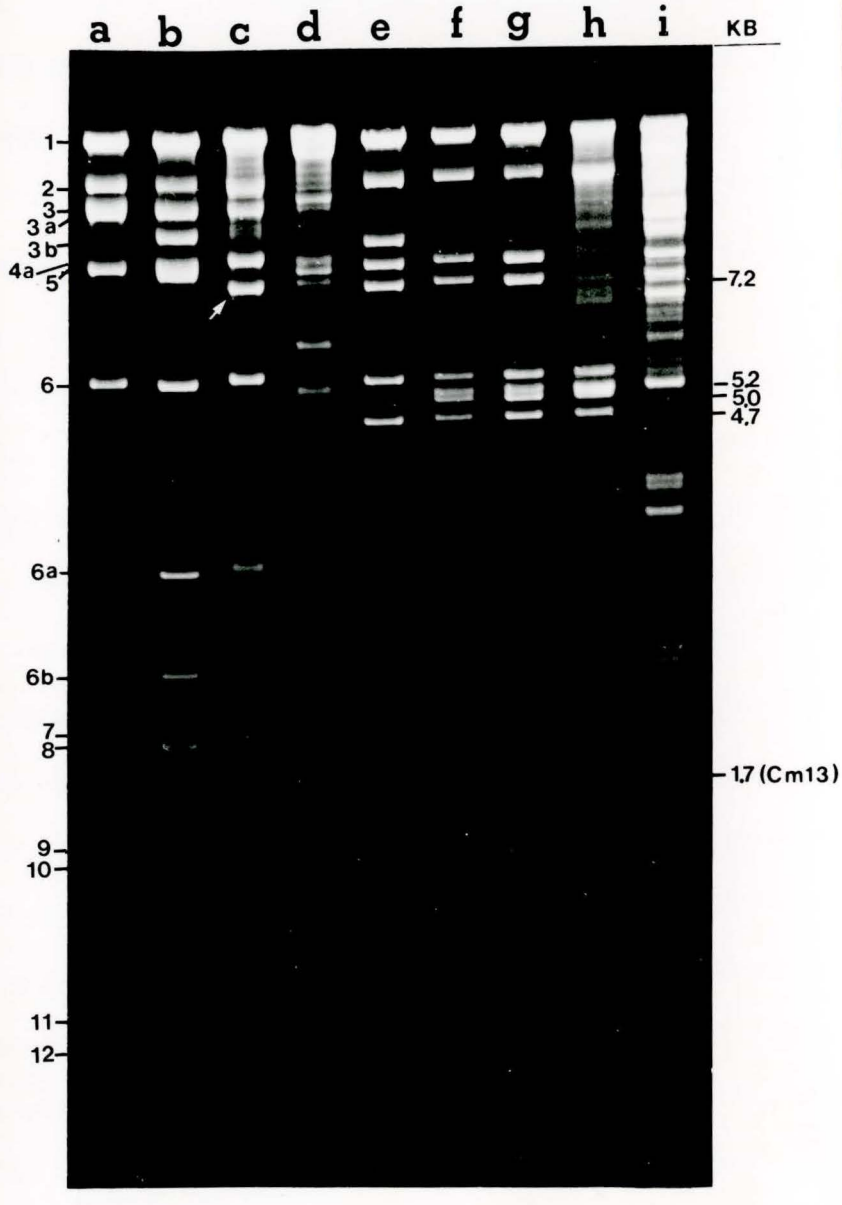
Transductant N543 showed a slight decrease in turbidity 2 hours after induction of a broth culture. However, cultures of this strain did not visibly lyse if helper was added. This protocol was repeated several times with the same result. Cultures of all other transductant strains listed in Table 16 lysed when superinfected with P1 helper.

Physical analysis of P1Cm13argF chromosomes.

The P1Cm13 genomes were analyzed from strains N541, N542, and N543. The P1Cm13 derivatives harbored by these strains were designated P1Cm13*argF*1, P1Cm13*dargF*2, and P1Cm13*dargF*3, respectively. Because the latter two derivatives were defective in virion production (*d* character) their chromosomes were purified by isolation of total cellular DNA following thermal induction (see Materials and Methods). Digests of DNA from these P1Cm13 derivatives are depicted in Fig. 21.

Fragments from P1Cm13*argF*1 chromosomes produced with *Bam*HI and *Bam*HI:*Pst*I were indistinguishable from those of P1Cm13*argF*5, the recombinant phage constructed for electron microscopic analysis of IS*1* sequences in P1*argF* chromosomes (see Chapter IV). The 3a fragment of P1Cm13 encoding the Cm13 determinant and *Bam*HI-4 of P1 was present, as well as all 4 fragments of the *argF* insertion (3b, 4a, 6a, and 6b). Thus this phage encoded the Cm13 transposon at map unit 4 and the *argF*

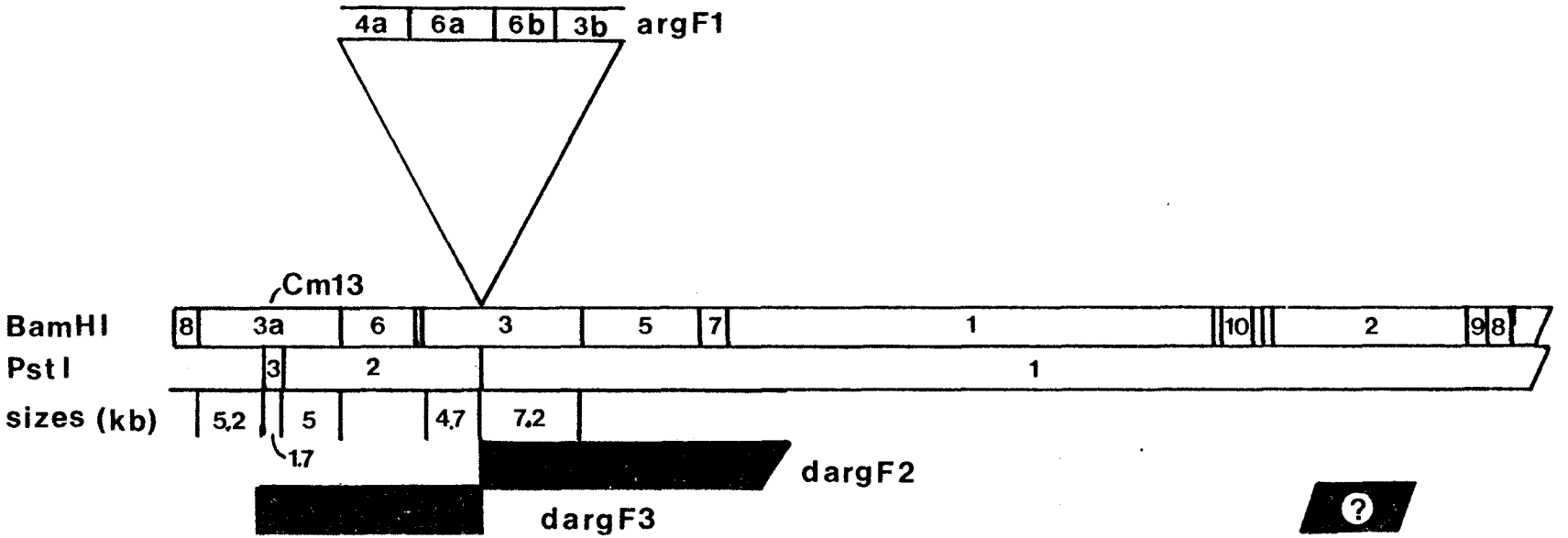
Fig. 21. Digests of P1, P1Cm13, and P1Cm13argF derivatives with BamHI and PstI endonucleases. Chromosomes: digested with BamHI, slots (a) P1Cm13, (b) P1Cm13argF1, (c) P1Cm13dargF2 and (d) P1Cm13dargF3; digested with BamHI:PstI, slots (e) P1, (f) P1Cm13, (g) P1Cm13argF1 (h) P1Cm13dargF2 and (i) P1Cm13dargF3. Cleavage fragments of P1Cm13 and P1argF DNA are listed on the left, according to the map in Fig. 22. The 5 kb, 1.7 kb and 5.2 kb PstI products of BamHI-3a are noted, as well as the 7.2 and 4.7 kb PstI products of BamHI-3. The 1.7 kb fragment encodes the Cm13 determinant. The novel fragment (5a) in the BamHI digest of P1Cm13dargF2 is indicated by the arrow.


a
b
c
d
e
f
g
h
i
KB
1
2
3
3a
3b
4a
5
6
6a
6b
7
8
9
10
11
12
7.2
5.2
5.0
4.7
1.7 (Cm13)

insertion at map unit 20 with no detectable deletion of P1 genes (see map Fig. 22). However, these results could not explain why the res and mod genes of this phage were expressed while the P1Cm13 parent phage was unable to restrict other DNA or modify its own DNA.

Phage P1Cm13dargF2 was defective in production of plaque forming particles. This observation was confirmed by the BamHI cleavage analysis. The digests lacked the BamHI-3b fragments of P1Cm13argF1 and the BamHI-5 and 7 fragments of P1 DNA. All other cleavage bands in the fractionated digest were indistinguishable from P1Cm13argF1. These results were interpreted to mean that the P1Cm13dargF2 derivative had a deletion of P1 genes corresponding to the region from the argF insertion at map unit 20 through BamHI-5 and 7 and probably into BamHI-1 (see Fig. 22 for illustration). A small deletion in the 36 kb BamHI-1 fragment would not alter the mobility of this fragment significantly. However, the novel fragment (5a) in the digest of P1Cm13dargF2 is too large to encode only part of BamHI-7 and the right-hand segment of the argF insertion up to the first BamHI cleavage site to the left (see Fig. 22 for illustration). This suggested that the deletion extended into BamHI-1. Digestion with BamHI:PstI confirmed the extent of the deletion of P1 genes. The 7.2 kb product of the right portion of BamHI-3 was not present though a fragment of 4.7 kb, the size of the left half of BamHI-3 was visible in the fractionated digest (Fig. 21). A band was also present that was presumed to represent the Cm13 determinant and one IS1 element. This fragment is present in PstI digests of P1Cm13

Fig. 22. Cleavage map of P1Cm13 and its derivative chromosomes with BamHI and PstI endonucleases. Order of fragments was determined by Bachi and Arber (1977) and results in Chapter IV. The PstI cleavage products of BamHI-3 of P1 DNA and BamHI-3a of P1Cm13 DNA are below the PstI cleavage map in kilobases. The argF insertion of P1Cm13argF1 is illustrated. Proposed deletions of P1Cm13dargF2 and P1Cm13dargF3 are indicated by solid bars.


4a
6a
6b
3b
argF1
Cm13
BamHI
8
3a
6
3
5
7
1
10
2
9
8
PstI
3
2
1
sizes (kb)
5.2
5
4.7
7.2
1.7
dargF2
dargF3
?

chromosomes (Meyer and Iida, 1979) and is 1.7 kb in size.

Chromosomes of P1Cm13dargF3 produced an unusual cleavage pattern. Because this digest contained so many large molecular weight fragments which may have indicated partial digestion, the digestion was repeated with 5 times as much BamHI and PstI endonucleases as normally used. However, the results were the same. Bands BamHI-2,3,4, and 6 of P1 DNA were missing and at least 8 novel fragment bands were produced. Fragments the size of one PstI cleavage fragment of BamHI-4 (5.2 kb) and the right-hand PstI cleavage fragment of BamHI-3 (7.2 kb) were visible in the BamHI:PstI double digests (see fractionated digest in Fig. 21). Since the BamHI-4,6,3 segment of P1 DNA is contiguous, this result was compatible with a deletion of P1 genes from the Cm13 insertion at map unit 4 to the IS1 element of P1 at map unit 20 (see Fig. 22 for illustration).

The internal BamHI-6a and 6b fragments of the argF insertion and a PstI fragment the size of the fragment that encodes the Cm13 insertion were present in the BamHI and BamHI:PstI digests of P1Cm13dargF3 DNA. This suggested that the chromosome did encode both insertions although it was not clear where either was located. The data were not compatible with placing either the Cm13 insertion or the usual argF insertion within the proposed deletion of BamHI-4,6,3.

Possibly one or both insertions were located within the BamHI-2 segment of P1, which was the only other P1 chromosomal segment not accounted for in the digests of P1Cm13dargF3 DNA. Even though BamHI-2 was missing in the digest, the N543 transductant strain was immune to

P1 superinfection and its vegetative cycle was inducible at high temperature. Genes responsible for these functions are encoded within BamHI-2 (Scott, 1970; Iida and Arber, 1979). The additional BamHI cleavage fragments also suggested that a larger argF gene segment had been transferred from the TJC77 donor than had been previously observed in other P1argF derivatives.

Discussion.

The analysis of P1CmOargF derivatives provides some interesting information on P1CmO-argF recombination. Some P1CmOargF derivatives are defective in phage maturation functions. These are mostly isolated under conditions of recipient infection at low m.o.i. where single infection is possible. This observation agrees with the conclusion that establishment of P1argF lysogeny is facilitated by multiple infection with P1CmO vectors. A non-defective P1argF prophage can be produced by recombinations between a defective P1dargF and a non-defective P1 helper chromosome if both are present in the recipient. Multiple infection may not be advantageous for P1argF formation from P1c1.100 vectors, since defective P1argF chromosomes are rarely isolated from infections with this vector.

The presence of defective P1CmOargF derivatives was also correlated with certain donor lysates. This result suggests that the defects occurred in the donor which is also where union of argF and the P1 chromosome usually occurs. The formation of a defective phage therefore may accompany the insertion of the argF gene segment.

Defective phage may not initially result from deletions in P1 genes in the donor upon argF insertion. Loss of P1 genes may occur during packaging of chromosomes, if the recombinant of P1CmO and the argF gene segment is too large to be packaged intact.

Of the specialized Arg^+ transducing phage formed from P1CmO vectors, almost half are Cm^S. The cleavage analysis of virion chromosomes from two Arg^+ transductants from P1CmO vectors demonstrate that the argF gene segments are usually inserted at the CmO locus. Thus recombination resulting in the deletion of CmO should be an extremely rare event. That it was frequent suggests that an IS1 mediated deletion event commonly accompanies or follows insertion of the argF gene segment. The deletion of the CmO determinant without argF insertion is an uncommon event during the lytic cycle of the phage, since most plaque forming virions in P1CmO lysates contain the CmO determinant (Kondo and Mitsuhashi, 1964).

Most of the P1Cm13argF derivatives that were analyzed were defective in P1 functions. This is probably due to the deletion of substantial segments of the P1 chromosome, as confirmed for two derivatives characterized by cleavage analysis. The mechanism of formation of these deletions in the case of P1Cm13dargF2 is compatible with an IS1 mediated transposition followed by the deletion. In the case of P1Cm13dargF3, the deletion is more complex since it appears to have occurred between two IS1 elements in opposing orientations. If this latter deletion is common, it would account for the low frequency of P1argF formation with P1Cm13 vectors since an argF insertion at map

unit 20 would be deleted by the same mechanism. Possibly the only reason the P1Cm13dargF3 derivative was detected is because argF probably is inserted at a different P1 locus. Further analyses are necessary to confirm these hypotheses. However, the P1Cm13dargF derivatives provide a diverse group of chromosomes for the study of IS1 mediated events.

In addition, selection of Arg^+ transductants from P1Cm13 vectors is evidently a selection for P1 deletion mutants. Such mutants are useful for genetic mapping of the P1 chromosome and studying the effects of mutations on P1 functions. For example, it is apparent that the genes necessary for plasmid maintenance and vegetative replication are not encoded in the segments of the P1 chromosome that are missing in either P1Cm13dargF2 or P1Cm13dargF3.

A novel type of genetic defect is manifested by P1Cm13dargF3. This prophage is proficient in vegetative chromosomal replication because its chromosome is amplified upon thermal induction. However, it is deficient in some step leading to host cell lysis. The addition of P1 helper does not promote lysis. Since the P1 helper is proficient in lytic functions, the P1Cm13dargF3 defect is preventing normal P1 expression in trans. Further characterization of this mutant would be a worthwhile endeavor.

CHAPTER VII

SUMMARY AND MODELS

The translocation of a specific host chromosomal gene, *argF*, to the chromosome of the plasmid-bacteriophage P1 has been characterized as a model of virus mediated aberrant recombination. To select P1*argF* recombinants, transductions were performed into recipients with a large *proAB-argF-lac* deletion. This reduced the possibility of successful generalized recombination. In addition, with the F128 episome as the donor of the *argF* gene, virtually all the *arg*$^+$ transductants were P1*argF* or P1d*argF* lysogens. The frequencies of *argF* transduction in this system ranged from $1/10^8$ pfu to $1/10^4$ pfu, depending upon the P1 vector strain and how the lysates were prepared. In the same system, *proAB* transduction frequencies were less than $1/10^9$ pfu.

Cleavage analyses of 14 independently isolated P1*argF* indicated that each contains the same *argF* gene segment inserted at the naturally occurring IS*1* locus of P1. The genome of P1*argF5* was characterized further by electron microscopic heteroduplex analysis. It has an 11.2 kb insertion precisely at the P1 IS*1* locus. Although the P1*argF5* genome is approximately the same size as the P1 virion chromosome, genetic evidence indicates that it can be packaged intact in one virion. A P1Cm13*argF5* recombinant of P1*argF5* and P1Cm13 has intrastrand annealing properties which establish that the *argF* gene segment is flanked by directly repeated IS*1* elements. The *argF5* insertion was assigned the

designation Tn2901.

Of the various phage strains used as vectors, all those that encode at least one IS1 sequence were able to produce P1argF genomes with high frequency. Phage P7, a close relative of P1 having no known IS1 element, rarely formed P7argF. Thus IS1 appears to be specifically necessary for high frequency argF specialized transduction.

The presence of more than one IS1 element on the P1 chromosome affected the frequency of P1argF formation. For P1CmO vectors with two IS1 elements, the frequency was increased; however, P1dargF were occasionally formed. For P1Cm13 vectors with three IS1 sequences, the frequency was decreased considerably and most transductants contained P1dargF with large deletions of P1 genes.

Genetic studies indicated that P1argF formation does not require establishment of lysogeny in the donor. However, unions of P1 and argF did occur in the donor for transduction, as manifested by co-transduction of CmO and argF. Lysates produced by induction of established lysogens had lower argF transduction frequencies than those produced by infection of P1 sensitive donors. Thus there is a P1 function which affects P1argF formation or isolation. Such a function might repress IS1 action in induced lysogens. A P1 mutant was isolated from which P1argF are much more frequently obtained. This mutation may be in a gene affecting IS1 mediated recombination.

Clonal populations of 10^9 P1argF lysogens contained about 0.4% Arg^- bacteria. However, lysates from these bacteria contained higher proportions of virions which had lost the argF gene. Therefore, loss of

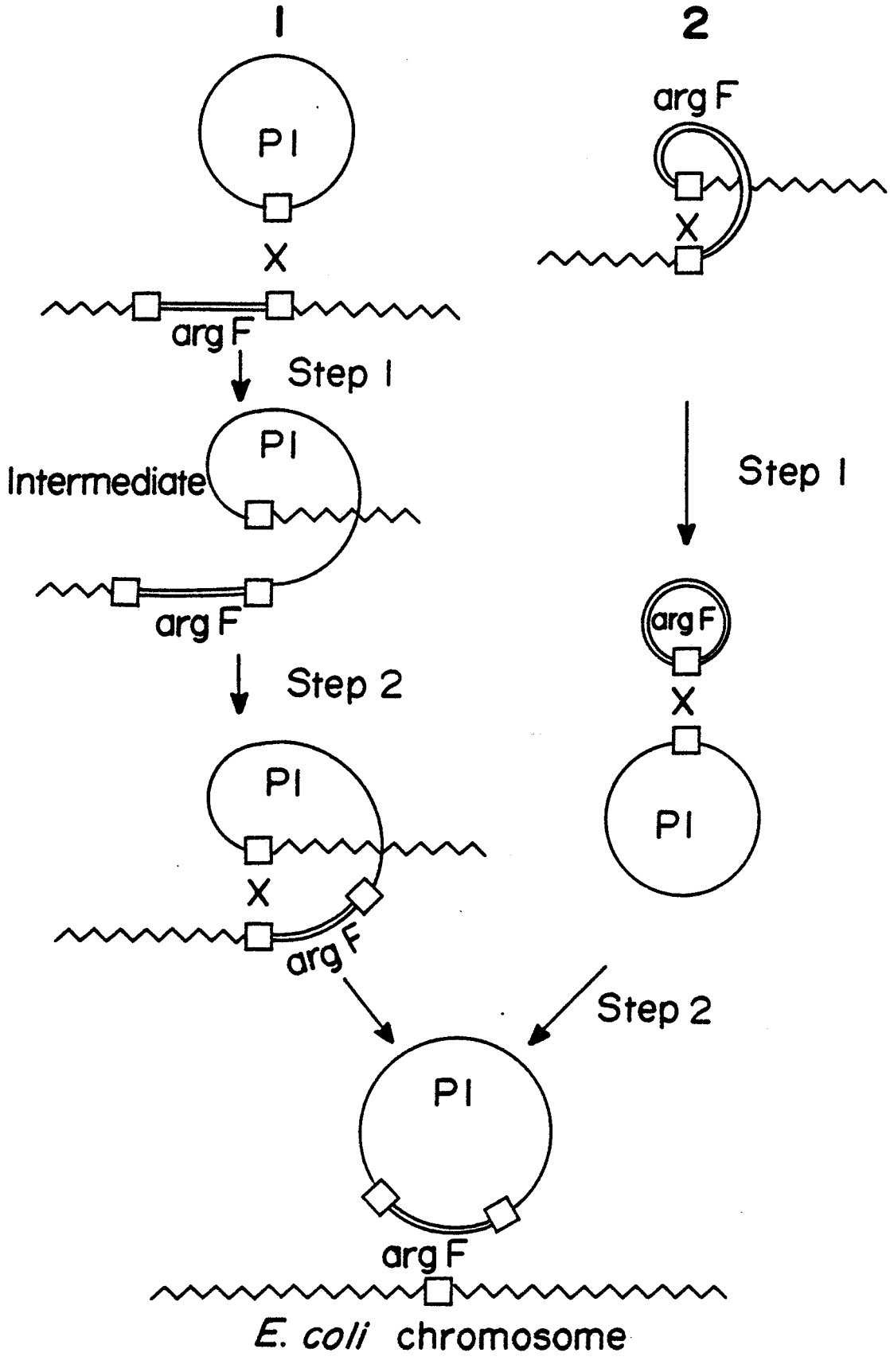
argF occurs most rapidly during the lytic cycle of P1. In most cases, an excision of the entire argF insertion including one IS1 element occurred. Such excisions are common to genes present between two IS1 elements. Deletions of part of the argF insertion were also detected among lytically propagated P1argF. Consequently, the argF segment appears to be both inserted into P1 and excised from P1argF most often during the phage lytic cycle, rather than during the lysogenic state.

Two mutually compatible models for the transfer of the argF gene segment to the P1 chromosome are presented in Fig. 23. In both models site-specific recombination events between IS1 sequences occur to generate the P1argF chromosome. In the first model, the E. coli chromosome and the P1 chromosome recombine at their IS1 loci. Another recombination between the P1 IS1 and a second E. coli IS1 element resolves the two chromosomes into a P1argF and an E. coli chromosome deleted in argF.

Alternatively, an argF excision occurs first within the E. coli chromosome, resulting in loss from the E. coli genome of a small circular segment of DNA containing argF and one IS1 sequence. Evidence indicates that such deletions of argF are common in P1argF chromosomes during the phage lytic cycle and thus they may also be common in the E. coli chromosome during the P1 lytic cycle. The circular argF molecule can then be inserted into the P1 chromosome at its IS1 locus. The recombination events of each model are the same; it is only the order of the events that varies.

These models depict P1 as a plasmid. Since unions of P1 and argF frequently occur in the phage lytic cycle, the participating P1 genome

Fig. 23. Models for P1*argF* formation. IS1 elements are depicted as squares; *E. coli* DNA as jagged lines; and P1 DNA as solid lines.


1
2
P1
X
arg F
Step 1
Intermediate
Step 2
arg F
P1
X
arg F
Step 1
arg F
X
P1
Step 2
P1
arg F
E. coli chromosome

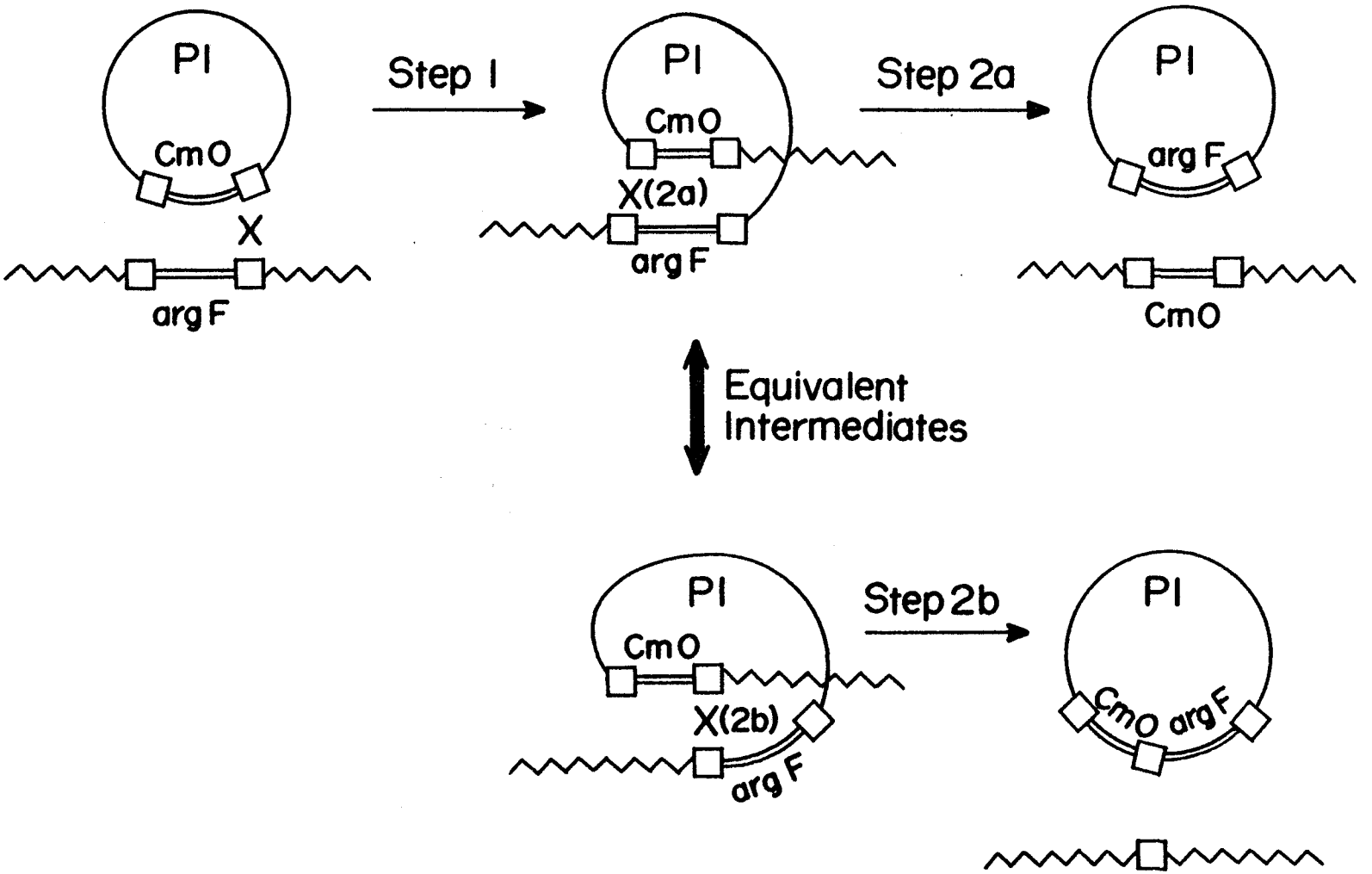
could be in its concatemeric form. The recombinations are the same whether P1 is in monomeric or concatemeric form.

It is possible that the second recombination producing the P1*argF* genome in model 1 (Fig. 23) occurs in either the donor or the recipient. Multiple infection of recipients facilitates P1*argF* formation, at least for P1CmO vectors. This supports the theory that the second recombination may occur in the recipient. If a portion of the P1-*argF* intermediate molecule is packaged in the donor, a recombination between this chromosome and a second P1 chromosome would generate a non-defective P1*argF* genome in the recipient.

A model is presented in Fig. 24 which explains the observation that approximately half of the CmO determinants are lost from P1CmO vectors during recombination with the *argF* segment. In step 1, recombination occurs between the right IS*1* sequences. In step 2a, the recombination occurs between the left IS*1* elements to complete the exchange of *argF* and CmO. This results in a P1*argF* genome and the insertion of CmO into the *E. coli* chromosome. Alternatively, (step 2b) the final recombination could occur between the terminal IS*1* sequences of the intermediate, yielding a P1CmO*argF*. This hypothesis argues in favor of the first model of Fig. 23 being the predominant model, since CmO would not be deleted in the second model of Fig. 23.

Data presented in Chapter IV (Discussion) from R. Deonier are compatible with these models. His observations strongly suggest that Tn*2901* exists intact on the *E. coli* K12 chromosome with flanking IS*1* sequences.

Fig. 24. Model for P1CmΔargF formation. IS1 elements are depicted as squares; E. coli DNA as jagged lines, and; P1 DNA as solid lines.


PI
CmO
X
argF
Step 1
PI
CmO
X(2a)
argF
Step 2a
PI
argF
CmO
Equivalent
Intermediates
PI
CmO
X(2b)
argF
Step 2b
PI
CmO argF

The models proposed above are similar to those proposed by Iida and Arber (1980) for specialized transduction involving the formation of P1r-det (resistance determinant), in which the r-det is flanked by IS1. However, these authors report integration of the r-det at several loci on the P1 chromosome, in addition to the IS1 locus of P1. With P1argF, the preponderance of integration at the P1 IS1 locus obscures any other events.

Formation of P1argF chromosomes has evolutionary significance for the exchange of genetic information. It represents a unique case in vivo of the translocation of a specific host chromosomal gene encoding an enzyme of amino acid biosynthesis to a bacteriophage genome. Both the translocation event and the excision of the translocated gene occur with high frequency during the phage lytic cycle. Other genes may be transferred from the host chromosome by a similar mechanism, but so far none have been detected.

Why this translocation system has evolved in such a fashion is not clear. However, it seems that it is desirable for a bacteriophage in its dying host to take some host genes with it to its next host. If these genes are part of the phage genome, they can replicate, independently of integration into the next recipient chromosome. It is conceivable that the genes could be integrated into the new host chromosome by a transposition event during passage and growth of the prophage. This could have been the mechanism of transfer of argF to the E. coli K12 chromosome from an ancestral argI location.

If a strong selection does not exist for the translocated gene of the P1 chromosome, the gene will be progressively lost during

successive lytic cycles of the phage. Such excision eliminates wasteful transfer of useless genetic information. It also allows the bacteriophage to then acquire genes from its present host at its IS1 sequence without becoming overlarge.

The above hypothesis does not apply to the transfer of drug resistance determinants. They are retained during the phage lytic cycle with high frequency. Such retention is seen for the ampicillin resistance determinant of P7 (Smith, 1972) and the chloramphenicol resistance determinant of P1CmO (Kondo and Mitsuhashi, 1964). Apparently these recombinations are controlled by mechanisms different from those controlling argF retention. Possibly their persistence through multiple lytic cycles is more beneficial to the bacterial population as a whole and thus also to their bacteriophage.

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The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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