THE *CIS*-REQUIRED DNA SEQUENCES FOR BACTERIOPHAGE MU DNA TRANSPOSITION AND MATURATION.

by

© JOSEE HAREL

A thesis submitted to the Faculty of Graduate Studies and Research, McGill University, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Department of Microbiology and Immunology
McGill University
Montréal, Québec
Canada

March, 1986

"Aller vers l'obscur et l'inconnu par ce qui est plus obscur et inconnu encore."
Devise alchimique.

("Go toward the darkness and the unknown by darker and more unknown ways").
Alchemist motto.

(Marguerite Yourcenar, 1968, 'L'oeuvre au noir', 4th edition, Gallimard, collection Folio, Paris).

A Luc.

ABSTRACT.

Transposable elements are stretches of discrete DNA segments capable of rec-A independent translocation while maintaining their physical and functional integrityThe DNA of temperate bacteriophage Mu embodies the properties of both a transposable element and a phage. Its 37 kilobase linear double-stranded DNA is capable of catalysing and undergoing the full spectrum of DNA rearrangements characteristic of prokaryotic mobile elements.

Mu DNA transposition requires the presence of the Mu encoded A and B gene products act upon sequences at the extremities of the Mu genome and catalyse the concerted nicking-ligation reactions inherent to most current models of DNA transposition.

We developed two strategies in order to define the cis-acting DNA sequences which affect Mu DNA transposition and maturation. The first involved in vitro deletion mutagenesis of the cloned Mu ends and the second involved the subcloning of the sequences at the ends of Mu DNA.

Using a Mini-Mu Amp cloned into pSC101 (pMD861), we used a combination of exonuclase III and SI nuclease to generate a series of deletions extending toward the Mu DNA sequences. These mutants were tested for their ability to transpose in vivo. We found a class of Mini-Mu transposition mutants in which alterations in the neighboring plasmid sequences of pMD861 near the Mini-Mu extremities exert a modulatory influence in the extent of transposition of Mini-Mu when the helper

prophage contains a polar insertion in its semi-essential early region. This region of the Mu genome is expressed early during the Mu lytic cycle along with the A and B genes required for transposition and encodes for functions that may affect DNA transposition. It is possible that one or several of these functions may act on sequences adjacent to Mu to allow maximal Mu DNA transposition from virtually any location.

We constructed various size Micro-Mu plasmids in order to define the minimal DNA sequences required for transposition and packaging. We monitored the transposition and maturation properties of the Micro-Mu's carried *in vivo* by plasmids with the transposition and maturation functions provided *in trans*.

We found that the transposition domains at the left extremity of the Mu genome are composed of a necessary sequences within the region from 1 bp to 55 bp and auxiliary sequences within the region from 126 bp to 203 bp and the transposition domain at the right extremity of the Mu genome to reside within the rightmost 62 bp. The packaging recognition site was found to be located within the leftmost 55 nucleotides of Mu genome. Thus, during its evolution, the giant transposable Mu bacteriophage has integrated various functional domains to behave as an efficient transposable phage.

RESUME

Les éléments de transposition sont des segments de DNA capables de translocation indépendante du gène recA et gardent leur integrité physique et fonctionelle durant ces mouvements. Le bactériophage tempéré Mu accomplit les fonctions de phage ainsi que d'élément de transposition. Son DNA est linéaire, double-brin, et est long d'environ 37 kilobase. Le DNA de Mu peut accomplir toute la gamme de réarrangements génétiques caractéristiques des éléments mobiles de transposition.

Les produits des gènes A et B codés par Mu, des enzymes codés par des gènes de la région semi-essentielles du génome de Mu ainsi que des enzymes de la cellule hôte sont nécessaires pour que Mu transpose. Les enzymes de transposition interagissent avec des séquences aux extrémités du génome de Mu et ainsi catalysent des réactions de 'bris et ligations' proposées dans plusieurs modèles de transposition.

Nous avons développé deux stratégies afin de localiser les séquences qui agissent en cis sur la transposition du DNA de Mu ainsi que la maturation de Mu. La première consiste à mutagéniser in vitro les extrémités clonées de Mu en utilisant un système de favorisant les délétions. La seconde stratégie implique le sous-clonage des séquences minimales nécessaires pour la transposition et les autres activités biologiques de Mu.

Les extrémités du génome de Mini-Mu furent soumises à des délétions. Nous avons utilisé le DNA de Mini-Mu Amp cloné dans un vecteur

pSC101 (pMD861). Les extrémités du génome de Mini-Mu Amp ont été traitées à l'exonucléase III et à la nucléase SI afin de produire une série de délétions allant vers l'intérieur du génome de Mini-Mu Amp. Les mutants furent testés pour leur capacité à transposer in vivo.

Nous avons caractérisé une classe de mutants de Mini-Mu. Les altérations du DNA chez ces Mini-Mu se retrouvent dans les séquences adjacentes au génome de Mini-Mu. Lorsqu'un plasmide Mini-Mu possède ces altérations adjacentes à proximité de l'extrémité droite du génome de Mini-Mu, la transposition du Mini-Mu n'est diminuée que lorsque le prophage auxilliaire possède une insertion polaire qui affecte l'expression des gènes de la région précoce semi-essentielle. Cette région est exprimée durant le stade précoce du cycle lytique de Mu ainsi que les gènes A et B qui sont nécessaires pour la transposition de Mu. Cette région semi-essentielle pour la transposiiton de Mu code pour des fonctions reconnues comme influençant l'efficacité de transposition de Mu. Nous suggérons que certaines des fonctions de cette région interagissent avec les séquences adjacentes au génome de Mu afin de maximiser l'efficacité de transposition de Mu.

Nous avons créé des plasmides portant différentes longueurs des extremités du génome de Mu; ceci, afin de délimiter les séquences de DNA minimales requises pour la transposition de Mu, de disséquer les différents domaines des extrémités de Mu et déterminer leurs rôles biologiques durant le cycle du bacteriophage Mu. Nous avons testé in vivo les propriétés de transposition et de maturation des plasmides portant des Micro-Mu, les fonctions de transposition et de maturation étant fournies

en trans. Nous avons pu déterminer l'extrémité gauche du génome de Mu est composée de séquences essentielles dans la région comprenant les nucléotides 1 à 55 et de séquences auxilliaires dans la région comprenant les nucléotides 126 à 203. Le domaine de transposition à l'extrémité droite de Mu est situé dans 62 dernières bases. Le site d'encapsidation de Mu a été localisé à l'intérieur des derniers 55 nucléotides à l'extrémité gauche de Mu. Ainsi, durant son évolution le bactériophage Mu a su intégrer différentes fonctions afin de se déguiser efficacement en transposon viral.

Acknowledgements.

I thank Dr M.S. DuBow who accepted me in his laboratory and whose contagious enthusiasm for Mu, transposition, and molecular genetics contributed to the bloom of interest in this aspect of biology. I did appreciate his long-sighted and effervescent guidance.

And of course deeply in my heart I acknowlegde the whole laboratory population, the faithful members as well as the more fugitive students, for their support, advice, unestimable help and their long-lived friendship. In laboratory disorder: Heather and David Bonthwick-Hoff, Linda Duplessis, Daphne Goring, Jeff Kahn, Annie Kupelian, Allan Jarjour, Manon Lalumiere, Martine Lapointe, David Levin, Alex Lussow, Mike MacLeod, Gayle Shinder, Rowen Shinder, Katia Sol, George Szatmari, Peter Tolias, Paul Zanga and Mike's cats and others.

I gratefully acknowledge Jeffrey S. Kahn for his aid in sequencing the E series plasmids, Alexander R. Lussow for the computer analysis of base composition of the TaqI region comprising the PvuII site of pMD861 and punky and eye-opening ideas on transposition in relation to DNA biophysical properties and composition, Annie Kupelian who spent a whole summer running to take time points of desperately non-lytic LF1037 derivatives. I thank A. Bernardi and F. Bernardi for their welcoming and fruitful discussions on the role of IS102 and adjacent sequences in Mini-Mu transposition.

I greatfully ackowledge Jeffrey S. Kahn for the creation of pJK43 as well as the initial cloning of the Micro-Mu Sau3A, Heather

Bonthwick-Hoff for preliminary data obtained on Micro-Mu transduction and Manon Lalumière and Linda Duplessis for pulling off the last data on Micro-Mu characterizations.

I appreciated the plasmid pUC71K supplied by Stanley Brown, Mucts62pf7701 phage supplied by Martha M.Howe and pSAS1206 supplied by A. Sasarman and F. Shareck. I am very greatful to Luc DesGroseillers for his large contribution to make the schemas and tables and his patience.

I am grateful to the staff of the Microbiology and Immunology department whose friendly help eased this studying term, as well as the members of the department whose cheerfulness and discussions were appreciated.

Finally I thank La Société de Recherches sur le Cancer de Montréal to have sponsored me financially during most of the years of the ph.D. term.

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LIST OF ABBREVIATIONS.

^{r}, resistant.

^{s}, sensitive.

Amp, ampicillin.

Kan, kanamycin.

Spc, spectinomycin.

Tet, tetracycline.

bp, base pair.

Kb, kilobase pair.

nt, nucleotide.

gp, gene product.

DNA, deoxyribonucleic acid.

RNA, ribonucleic acid.

attL, attachment site left.

attR, attachment site right.

pac, packaging recognition site.

SEER, semi-essential early region.

ccc,covalently closed circle.

oc,open circle.

CHAPTER I. GENERAL INTRODUCTION.

A- Plan of the thesis.

The thesis presents two different aspects of the cis-requirements for Mu transposition and is therefore constructed in the following manner. A long introduction covers a general overview of temperate bacteriophage Mu a) as a phage and b) as transposon and in relation to other transposons. In chapters III and IV, my experiments on the effects of adjacent sequences on Mu transposition and cis-required sequences for Mu maturation and transposition, respectively, are presented with their own introduction, results and discussions.

B. Overview.

B-1.Transposable elements.

Transposable elements are discrete genetic entities that can move to many different sites within and between replicons. They may also mediate other DNA rearrangements inherent to the mechanism by which transposition events occur such as inversions, deletions, duplications and translocations of DNA sequences (Kleckner, 1981) (Table 1). The transposable element responsible for these rearrangements is present at the point of DNA rearranged. And each time a DNA rearrangement occurs, the element may duplicate itself or insert itself without duplication. All of these events occur by pathways independent of general homologous recombination. The transposable elements when inserted into a gene, can either block gene expression and thus act as a polar mutation or activate the gene. Thus, the transposable elements have the potential for influencing gene expression during the life time of an organism either as a direct consequence of their transposition mechanisms or indirectly by acting as mobile sites of genetic homology for recombination catalyzed by the cellular homologous recombination system (Krowleski et al., 1982).

The basic molecular mechanisms of DNA transposition may be similar among all life forms (Figure1). It is likely that all transposons move by a combination of "break and join" processes combined with DNA replication. These events are promoted by element-encoded "transposases" and many host functions. Thus, there are two main requirements for the transposition of a mobile element: 1) The end sequences of the transposon

Table 1.

Properties of mobile genetic elements. Modified from Shinder et al., 1983.

1. They are discrete nucleotide segments with a similar physical organization and often bounded by the dinucleotides 5' TG...CA 3'.

2. The integration site on the element is specific (somewhere near or at the extreme ends).

3. The ultimate chromosomal orientation of the integrated DNA is random.

4. The DNA sequences at the ends of the element are often identical and inverted.

5. The integration event generates a duplication of a short host DNA sequence at the target integration site whose length is characteristic for each element.

6. The integration target site is usually random.

Figure 1.

Some properties of the duplicative insertion pathway of mobile genetic elements (modified from Shinder et al., 1984).

A. Movement occurs via in situ direct duplication or reverse transcription from RNA intermediates.

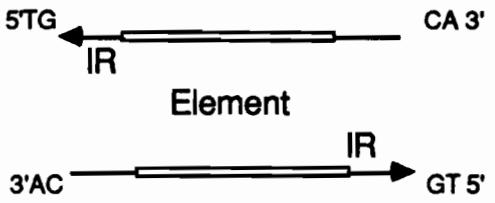
B. The insertion reactions (nicking, ligation, duplication) utilize only the element termini and are catalyzed by both element encoded and host encoded enzymes.

C. Integration results in direct duplication of a short host sequences at the random target site; the length of the duplicated sequence is a characteristic of each element.

Consequences: Altered gene structure and function due to :

- genetic rearrangements (insertion, deletion, inversion).
- Generalized recombination among dipersed elements.

Movable element showing Inverted Repeats and dinucleotides 5'TG...CA 3' boundaries



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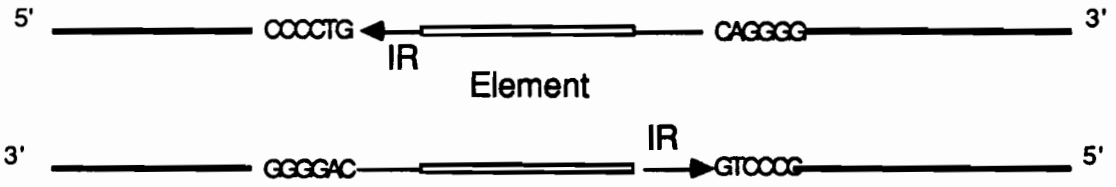

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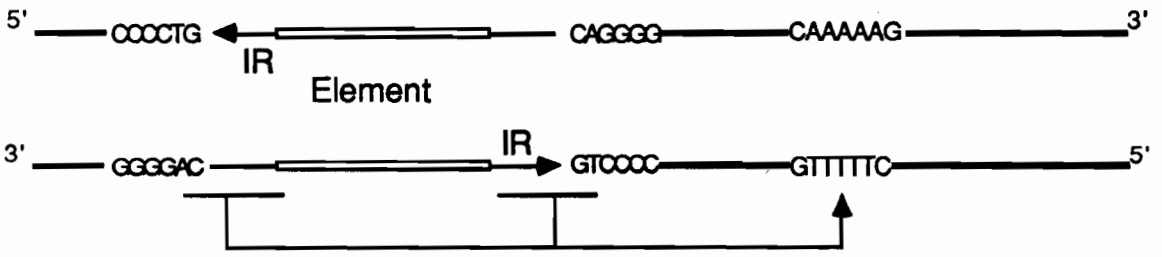

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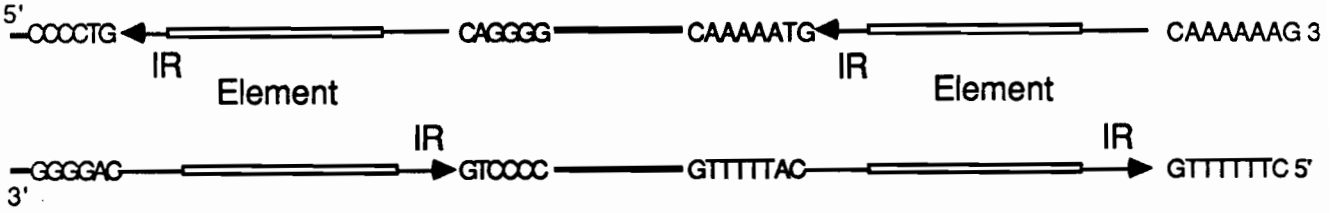


(Transposable elements require the DNA sequences of their extremities in cis to transpose. These sequences fall within the terminal inverted repeats sequences found at the ends of many mobile elements). 2) A protein (the transposase) that recognizes the transposon extremities to promote transposition.

The interest in transposable elements can be attributed to : 1) their ubiquity; 2) the novel DNA sequence recognition, cleavage, and joining reactions that their movement entails; 3) the insertion mutation, genome rearrangements and changes in gene expression they cause; 4) their role in genome evolution; and finally 5) their usefulness in the genetic analysis and manipulation of many organisms.

B-2. Brief review of movable elements.

Transposable elements were first identified in maize plants during the 1940's as a result of genetic studies done by Barbara McClintock of the Carnegie Institute of Washington, at Cold Spring Harbor, New York. McClintock (1957) showed that unstable mutations can be caused by the insertion into and excision from a gene by transposable elements. Some of these mutants displayed different levels of gene expression depending on the presence or absence of trans-acting transposable genetic elements elsewhere in the genome. From these observations, McClintock developed

not only the idea of transposable genetic elements but also the concept that the expression of genes can be regulated by these mobile genetic elements (McClintock, 1967).

Until well into the 1970's, an organism's genome was considered to be quite stable. The genomes of all organisms, far from being stable computer tapes of hereditary information, were capable of undergoing a wide variety of rapid and extensive rearrangements during an organism's or cell's lifetime (McClintock, 1984; Shinder et al., 1984). Much of this instability can be attributed to the presence within the genome of endogeneous tranposable elements. These elements are capable of duplicating their genetic information and moving a product of their duplication reaction to random new sites within the genome by an integration mechanism which recognizes specific DNA sequences at the extremities of the element (Calos, 1980).

The awareness that movable genetic elements are both abundant and ubiquitous is recent. Although transposition was first deduced from McClintock's genetic studies of maize, her results remained in an intellectual void until the discovery of transposition in bacteria. In the past 20 years, movable elements have been discovered in every kind of organisms that has been examined, from bacteria to fruit flies.

The first indication of transposable elements in bacteria came in the mid-1960's, when certain peculiar mutations were found to be the result not of small changes in the sequence nucleotides but of the insertion in a gene of a sizable piece of foreign DNA. The first example of insertion mutations was provided by bacteriophage Mu when Taylor (1963) reported the discovery of a mutator phage which caused mutations in <u>Escherichia</u>

coli by inserting its DNA into various genes. Later, other mutations in Escherichia coli were found to be caused by insertion of specific DNA sequences. It was subsequently found that multiple transferable drug resistance genes in bacteria were evolving by the action of mobile genetic elements. The widespread use of antibiotics in animals and humans has resulted in the emergence of bacterial strains simultaneously resistant to several antibiotics. It was discovered that the genes conferring antibiotic resistance were carried from one strain to another through bacterial plasmids. It became clear that the antibiotic resistance genes are passengers on small mobile elements called transposons, picked up by the plasmids as they travel from cell to cell.

Indeed, transposable elements have played an active role in the evolution of many bacterial plasmids. On occasion, a cellular or phage genome may have imprisoned a transposable function for its own purpose. The bacteriophage Mu is considered as a giant viral transposon.

It now appears that many transposons do not move from their initial location but rather give rise to a new copy of their information that becomes inserted elsewhere. A 'classical' example of a bacterial transposable element is Tn3. Tn3 confers resistance to the antibiotic ampicillin as it carries a gene which specifies the ampicillin degrading enzyme beta-lactamase. Tn3 carries two other genes, the transposase and the resolvase genes, which function to promote genetic movement or transposition. After transposition, a new copy of Tn3 is found at a new integration site but the old copy is still found at its original site. During the transposition process, a new copy of the transposon is inserted into another replicon via a fused structure, called a cointegrate, in which the

fusion of the donor DNA molecule and the recipient molecule (flanked by direct copies of the transposon) occurs. Creation of a duplication of DNA sequences at the site of insertion is a signature of transposition reaction. For example, one of the consequences of Tn3 transposition is the duplication of 5 bp at the insertion target site. To promote transposition, the transposase, in conjunction with host functions, acts on the extremities of the element. Transposable elements require certain DNA sequences in *cis* for transposition. These sequences fall within the terminal repeated sequences which are found at the ends of most mobile elements. The 38 bp inverted repeats at the extremities of Tn*3* are the reactive sequences in transposition and the symmetry that they exhibit is perhaps important for the transposition process. The ends must be in the correct orientation for transposition to occur. In the case of Tn*3*, the cointegrate is resolved by the Tn*3* resolvase into the donor molecule and the recipient molecule each containing a copy of the transposon.

It is suspected now that movable genetic elements are a major feature of all DNA genomes. In eukaryotes, the mobile nature of some elements is well documented in yeast, in *Drosophila*, and mammalian retroviruses.

The existence of transposable elements in *Drosophila* was first inferred from observations analogous to those that identified the first insertion sequences in *Escherichia coli*. There arose some evidence that several highly mutable genetic loci might be associated with movable control elements. Several types of transposable elements are found in *Drosophila* such as the copia elements, the Fold Back elements and the P elements. A phenomenon known as hybrid dysgenesis results from the

mobilization of DNA sequences called P elements in Drosophila embryos. When a sperm from P-carrying strain fertilizes an egg from a non-P strain, the P elements are zygotically induced to transpose throughout the genome, usually disrupting vital genes. The P element is approximately 3 kb in length with 31 bp inverted repeats at its ends. In its basic structure, the P element closely resembles bacterial transposons such as Tn3.

The mating type of yeast (a or alpha) is determined by the expression of either the a or alpha gene at the mating-type locus. However copies ("cassettes") of the mating-type genes exist at two other loci (HML and HMR) on the same yeast chromosome. Copies of these silent genes can be inserted into the mating locus so that the sex of the yeast changes. The mechanism for moving copies of silent genes and inserting them into the mating-type locus has clear parallels with the movement of transposons (Hicks et al., 1979). In both processes, a copy of the parental genetic element is inserted at new site in the chromosome while the parental element remains intact and in place. An enzyme that makes a very specific cut at the beginning of the active mating-type locus has already been found. Though, it appears that homology plays a key role in this site-specific gene conversion (Roeder and Fink, 1983).

Retroviruses have single-stranded RNA genomes that are replicated by reverse transcription into a double-stranded DNA intermediate. DNA copies are inserted into the cellular genome by a transposition-like event (Flavell,1981; Temin, 1981). Sequence analysis of the ends of integrated proviral genomes indicated that they are often flanked by 4-6 base pair direct repeats of the host target site. In addition, there are identical blocks at the two ends of the proviral genome in direct orientation. The

retrovirus family uses a transposition-like mechanism to insert DNA copies of the viral RNA genome onto the chromosome of its host cell (Varmus, 1983).

The Ty1 element in yeast contains directly repeated DNA sequences, called delta's and, at its site of integration, is flanked by direct repeats. Structural similarities between the proviral forms of retrovirus, *copia* elements in *Drosophila* and Ty1 in yeast (Boeke et al.,1985) suggested a common mechanism of movement between these elements. The movements of *copia*- and Ty1-like elements, retroposition, involves RNA intermediates that in turn are converted by reverse transcriptase back into cytoplasmically located DNA proviral forms prior to their eventual insertions into new chromosomal sites (Baltimore, 1985). In contrast, the movement of bacterial transposons like Mu or Tn*3*, the P element of Drosophila, and the *Ac* element of corn is thought to occur through formation of fused chromosomal DNA segments, and whose potential subsequent replication can generate daughter transposons.

In maize, considerable information exists about the relationships between individual members of a transposon family as well as on the effects of insertion at particular loci and various outcomes of the transposition event (Fedoroff, 1983). Families of controlling elements have been defined by the interactions between autonomous and non-autonomous elements. A non-autonomous element can be activated in *trans* only by certain autonomous elements that are to be members of the same family. One of the best charaterized family of controlling elements is the *Ac*-*Ds* family originally discovered by B. McClintock; the *Ds* element remains stationary within its chromosomal site unless the *Ac* element is

present. In the process of movement, Ds elements can induce DNA rearrangements such as insertion, breakage, and deletion. Ds represents a deleted form of the larger Ac element, which is believed to be a complete transposon coding for its own transposase and to have the same basic genomic structure as the P element of Drosophila and Tn3 in Escherichia coli.

However, analogous transposable elements to that of the maize system have not been detected as components of animal cell genomes, but footprints of transposition events are found in the form of direct target repeats flanking dispersed repetitive sequences. Dispersed repetitive DNA sequences are present at many location in the chromosomes of species. It is hypothesized that repetitive elements are or were mobile elements at some time in the evolutionary history of the species (Jagadeeswara et al., 1981). The induction of some mutations and cancers has been suggested to be caused by mobile genetic instabilities (Georgiev et al., 1980; Cairns, 1981; Klein, 1981; Rechavi et al., 1982). The structures of some pseudogenes (Sharp, 1982) and other sequences such as members of the Alu family, imply that a pathway analogous to retroposition may be followed by cellular sequences.

The gamut of genomic rearrangements in the eukaryotic genome runs from random transposition to tissue-specific reconstruction (recombination in immunoglobulins is used to construct active genes in the appropriate somatic cells). The molecular mechanisms of eukaryotic transposition and other DNA rearrangements, however, are not defined in the same details as those of bacteria but the presence of features such as

direct repeats at the target site in many classes of repetitive DNA sequences suggests some generalized step(s) of the transposition process. Whether all movable elements have a common DNA ancestor cannot yet be answered. A better understanding of how transposons move at the molecular level may give us a more comprehensive picture of their evolutionary history.

B-3. Mu as a transposition model.

Mu is an important transposition model system because its genome is both of a virus as well as a transposon. The bacteriophage Mu displays many characteristics of transposable elements. It uses transposition to replicate its DNA and it can cause the same DNA rearrangements (inversions, deletions, duplications, and translocations) associated with other movable genetic elements. Mu is a giant transposable element (37 Kb) capable of packaging itself into phage particles.

Mu has many useful advantages over other insertion sequences (IS) and transposons (Tn): It is not a normal constituent of the bacterial genome, so that isogenic bacteria with and without a Mu can be compared. Mutants of Mu(cts) are inducible. This allows one to provoke at will transposition events at high frequency and create advantageous experimental conditions. Bacteriophage Mu transposes 100 times per cell during its lytic cycle. This potential for highly efficient transposition, in contrast to the very low transposition frequencies of other prokaryotic transposable elements (10^{-3}-10^{-5} events /cell/generation), makes Mu an

attractive system for analysis of the transposition mechanism. However the complexity due to the presence of a whole set of functions related to its phage part can interfere (Toussaint and Resibois, 1983).

C- Bacteriophage Mu.

C-1. Generalities.

Bacteriophage Mu was discovered by A.L. Taylor in 1963 (Taylor, 1963). The unusual mutagenic capability of this bacteriophage prompted the name of Mu for mutator. It became clear from a number of studies that bacteriophage Mu is a temperate phage which behaves as a transposable element (see reviews by Bukhari, 1976; Kleckner, 1981; Toussaint and Resibois, 1983). The transposition process is an obligatory feature in the Mu life cycle, hence its mutagenic potential is inherent to its transposition properties. A related phage to Mu, D108, also uses transposition as a normal mechanism for vegetative replication of the phage genome during lytic growth and for prophage insertion during lysogeny. The two phages are heteroimmune but they share 90% DNA sequence homology, similar functional organization and partial functional cross-reaction. Mutator phages that may be analogous to Mu and D108 have been identified in Vibrio cholerae and Pseudomonas aeroginosa. Mu can infect many enterobacteriaceae including strains of Escherichia coli, Citrobacter freundii, Erwinia, Shigella and Salmonella typhimurium. The phage adsorbs to a lipopolysaccharide component of the bacterial outer membrane (Sandulache et al., 1983).

Figure 2.

A- The Mu phage particle.

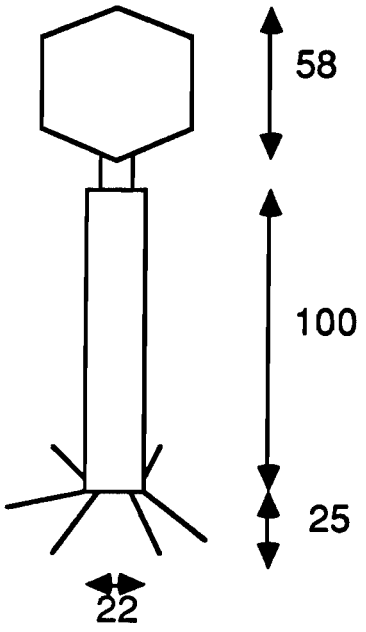
Diagrammatic representation of Mu phage particles with extended (A) and contracted (B) tails. The measurements are in nanometers. (Grundy and Howe, 1985).

B-Physical map of the Mu genome.

The major structures of Mu DNA seen under electron microscopy after denaturation and renaturation of the Mu phage DNA. The alpha (33 kb), G (3 kb) and beta (1.7kb) regions refer to distinguishable regions of Mu DNA heteroduplexes. The non-complementary sequences at the termini are bacterial DNA segments which differ from one DNA molecule to the next.

A)

1)



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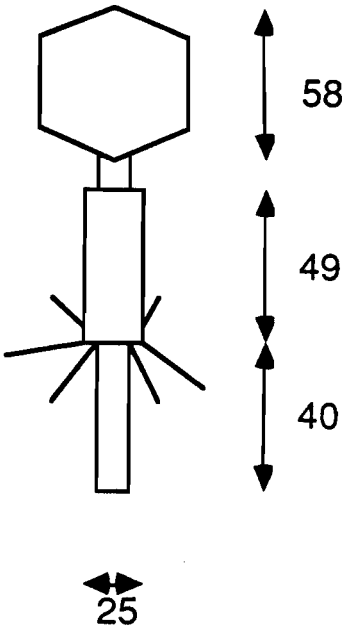

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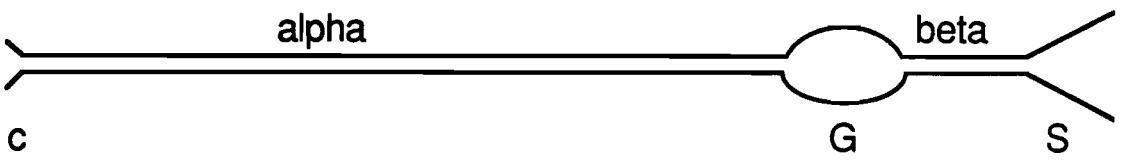


C-2. Description of the viral properties.

C-2.a. The phage structure.

The Mu phage particle consists of an icosahedral head of 540 Å in diameter, a contractile tail with six tail fibers and a knob like structure at the junction of the head and the tail. The tail sheath has a length of 1000 Å long by 180 Å wide in the extended state and 560 Å long by 180 Å wide in the contracted state (To et al., 1968; Grundy et al., 1984) (Figure 2). The intact phage particle has a buoyant density in CsCl of 1.468 (To et al, 1966), corresponding to a DNA/protein weight ratio of 40/60. The DNA can be extracted by phenol treatment, is linear, double-stranded, has a lenght of 39 kb (Bukhari, 1976) and a GC content of 51% (Martuscelli et al., 1971). Electron microscopy of phage DNA and of disrupted particles has revealed that DNA is encapsulated within the head by a headful mechanism (Bukhari and Taylor, 1975) with the right end of the genome adjacent to the attached tail in the finished virion (Inman et al., 1976).

C-2.b. The phage DNA.

DNA extracted from mature Mu particles is a linear duplex of about 39 kb. After denaturation and renaturation, Mu DNA displays unusual characteristcs. From these, the primary physical map of the virus genome was drawn. It consists of four regions, the alpha region or the left end (also called the c end), the G loop, the beta region or the right end (also called the S end), and the splitted ends (Daniell et al., 1973a) (Figure 2).

The alpha region (31 kb) contains the immunity gene (c) and most of the essential Mu genes. The G region (3 kb) is an invertible phage sequence containing genes S and U and S' and U' involved in host range specificity. The beta region (1.7 kb) contains the non-essential genes gin (G inversion) and mom (modification of Mu). At the right end of the mature Mu chromosome there is a region of non-homology known as the split end . The right end contains bacterial host sequences which vary in length between 500 and 3200 bp (Bukhari and Taylor, 1975). Bacterial sequences of 50 to 150 bp are also detected at the left end of the Mu chromosome (George and Bukhari, 1981). The G loop is an internal region of non-complementarity. Approximately 50% of all renatured Mu DNA molecules from phage propagated by induction of a lysogen contain the characteristic single stranded G bubble . The single stranded bubble observed in heteroduplexes is caused by an inversion of the G segment (Hsu and Davidson, 1974). The orientation of the G loop is responsible for the phage host range. The G segment is 3 kb long and its boundaries are located 30.7 kb from the left end and 1.7 kb from the Mu split end junction on the right. Heteroduplexstudies also revealed a double loop structure in the G loop (Hsu and Davidson, 1974; Daniell et al., 1973b). This double loop structure was formed by the presence of two sets of inverted repeats. The inverted repeats at the end of the G region were estimated to be 50 nucleotides long. The internal inverted repeats sequences are about 300 nucleotides long. It is suggested that inversion of the G segment is produced by intramolecular recombination between the inverted repeats at the ends of the G region (Figure4). gin, a phage encoded function, is involved in G inversion, and is able to act in trans.

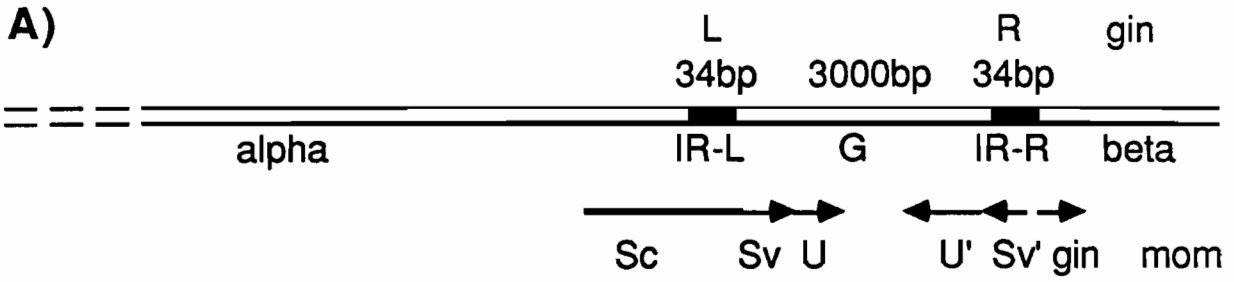
Figure 4.

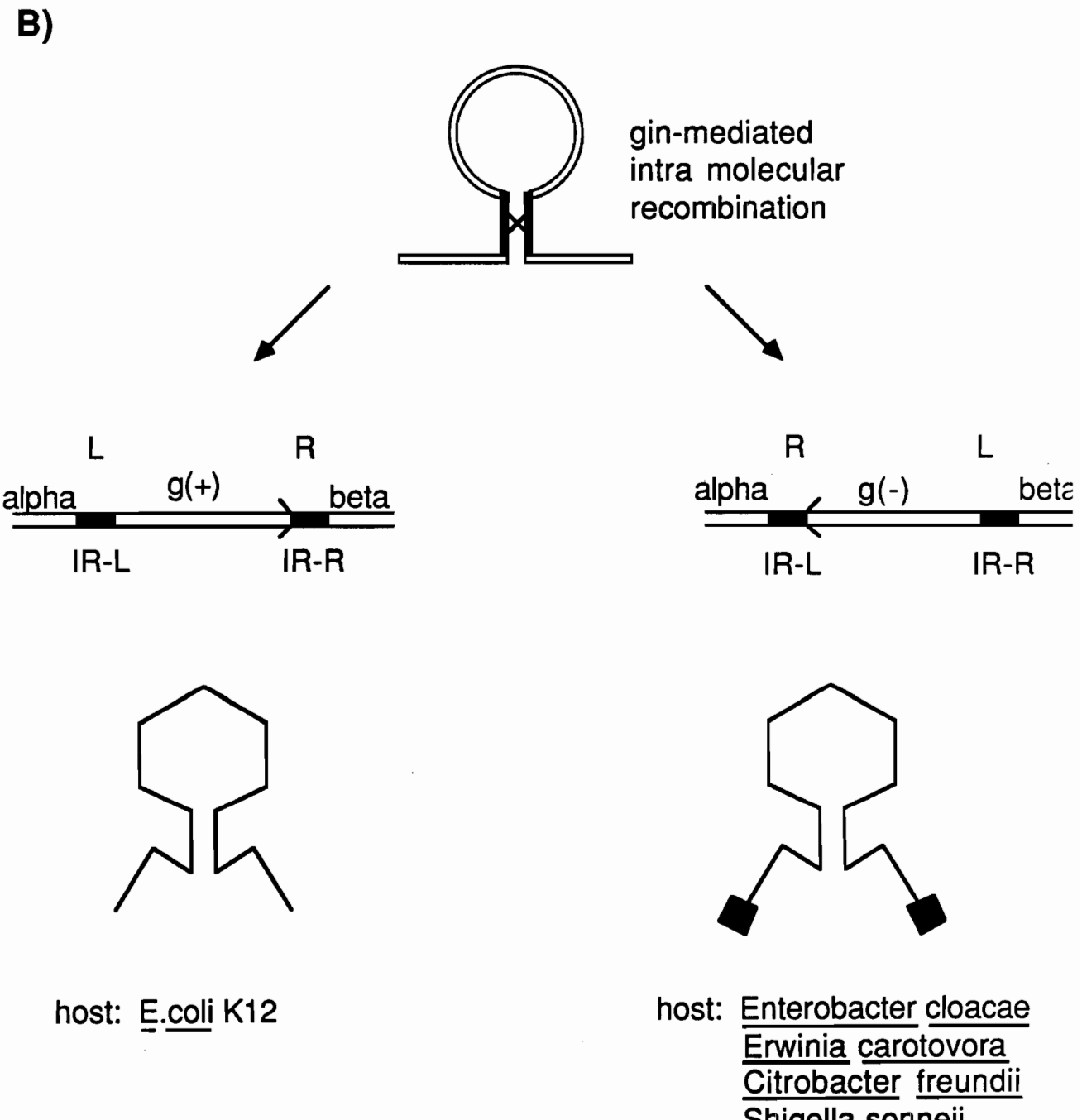
Inversion of the G loop.

Diagram showing inversion of the G segment in bacteriophage Mu DNA.

In A) the genetic organization of the invertible G segment is shown in the (+) orientation; genes S and U are expressed. The two sets of genes S and U, and S' and U' in the inverted orientation code for genes involved in tail-fiber synthesis. The promoter and a small coding region lie outside upstream from the inversion region. IR's (inverted repeats) flank the invertible segment and are the cis-acting sites for inversion.

B) Mu host range is determined by a genetic rearrangement. The inversion of the G segment via site-specific recombination mediated by the gin gene product (the gin gene is located outside the the inversion) results in a switch in host range. Phages with a G segment in the G(+) orientation are infectious for E.coli K-12, that is if gene products S and U are present; phages with G in the G(-) orientation are infectious for other gram-negative bacteria (Kahmann et al., 1984) that is if gene products S'and U' are present.


A)
L
34bp
3000bp
R
34bp
gin
alpha
IR-L
G
IR-R
beta
Sc
Sv
U
U'
Sv'
gin
mom


B)
gin-mediated
intra molecular
recombination
L
R
alpha
g(+)
beta
IR-L
IR-R
R
L
alpha
g(-)
beta
IR-L
IR-R
host: E.coli K12
host: Enterobacter cloacae
Erwinia carotovora
Citrobacter freundii
Shigella sonneii

C-2.c. The genetic map of Mu and gene functions.

Unlike lambda, the Mu gene order is identical for both mature phage and the prophage DNA; prophage and phage DNA are colinear (Abelson et al., 1973). The genetic map of Mu can be divided into two general areas based on the function and temporal expression of the genes (Figure 3). The early region comprises the leftmost 8-9 kb of the Mu genome. The early region includes genes that are expressed early in Mu development and are involved in Mu regulation of transcription, integration and replication of the Mu genome. The late region comprises the genes expressed in the late phase of the phage lytic cycle, downstream from the leftmost gene of this region, gene C. The late genes are involved in completion of Mu development and phage morphogenesis.

The early region extends from the immunity gene c at the left end of Mu DNA to just before the C gene. It is 8 to 9 kb long and is presumed to be transcribed as one long polycistronic messenger RNA (Giphart-Gassler et al., 1981a). Immediately adjacent to the left end of the Mu genome is the repressor gene c (c gp is 22-26 Kd) and ner (ner gp is 8.7 Kd) which regulate early Mu transcription. Then follow two essential early genes, A (the transposase) (A gp is approximatively 70 Kd) and B (lytic replication) (B gp is approximatively 33 Kd) which are both intimately involved in transposition and replication. The products of the two early genes A and B are essential for the replication of phage DNA. In the case of the A gene, this inhibition in Mu DNA synthesis is coupled with a complete block at the genetic level, in that neither after infection nor induction do A^- mutants show detectable signs of any of the characteristic Mu-mediated DNA rearrangement and Mu transposition. Because of this inertness the product

of A gene is thought to be a transposase which recognizes the ends of the Mu genome (O'Day et al., 1978; Faelen et al., 1978; Craigie et al., 1985). The evidence in connection with B^- mutants is less clear cut (Coelho et al.,1982; Faelen et al., 1978). There is a consistent, but low, level of Mu-mediated rearrangements and transpositions in B^- mutants. The role of the B gene in Mu development is part of the normal transposition complex (Harshey, 1983). In addition, Mu DNA replication does not occur in A^- or B^- mutants (Wiffelman and Lotterman, 1977; Waggoner et al., 1981). Recently, Chaconas et al., 1985, demonstrated that a truncated form of gpB lacking 18 amino-acids from the carboxy terminus blocks replicative transposition but not conservative integration.

Between 5 kb and 10 kb from the left end is a further early region (read on the same transcript as the A and B genes), which codes for a collection of gene products (approximatively 12 to 15) (Giphart-Gassler et al., 1981)whose roles are unknown but modify the Mu life cycle without being essential for phage production. Because of the semi-essential nature of these genes , it has been difficult to obtain amber mutations in them, rendering it arduous to perform complementation tests between mutants. The genes in the semi-essential early region are proposed on the basis of their phenotypic properties, and since delineation of the various phenotypes into separate genes has not been possible. In the semi-essential early region are the genes that affect immunity (cim) (Zipser et al., 1977), cause death of lysogens (kil) (Westmaas et al., 1976; Van de Putte et al., 1977), amplify Mu replication (arm) (Waggoner et al., 1981; Goosen et al., 1981), are able to complement host and T4 mutants deficient in ligase (lig) and gyrase activities (topo) (Ghelardini et al., 1979; Ghelardini et al., 1980), protects Mu DNA from exonuclease attack

Figure 3.

Genetic and physical maps of Mu DNA.

A- Genetic map of Mu DNA.

The solid line represents Mu-specific DNA; the broken lines at the left and right ends represent attached host DNA .

The position of the genes were obtained from the correlation of the genetic map and the physical map of Mu.

The exact order of the cim, kil, arm, gam, sot and lig functions has sitll to be confirmed.

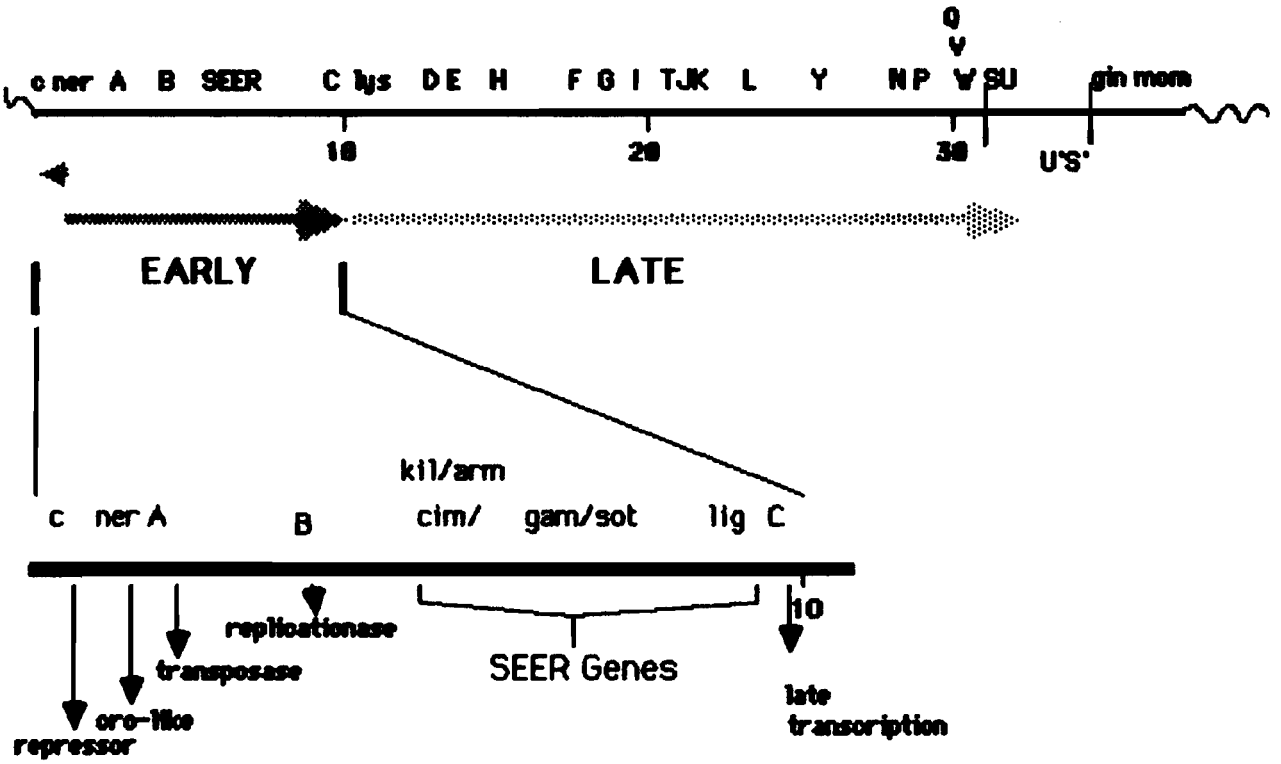
Wavy lines represent the variable host DNA covalently attached to the Mu ends.

The arrows represent the repressor, early region, and late transcription units.

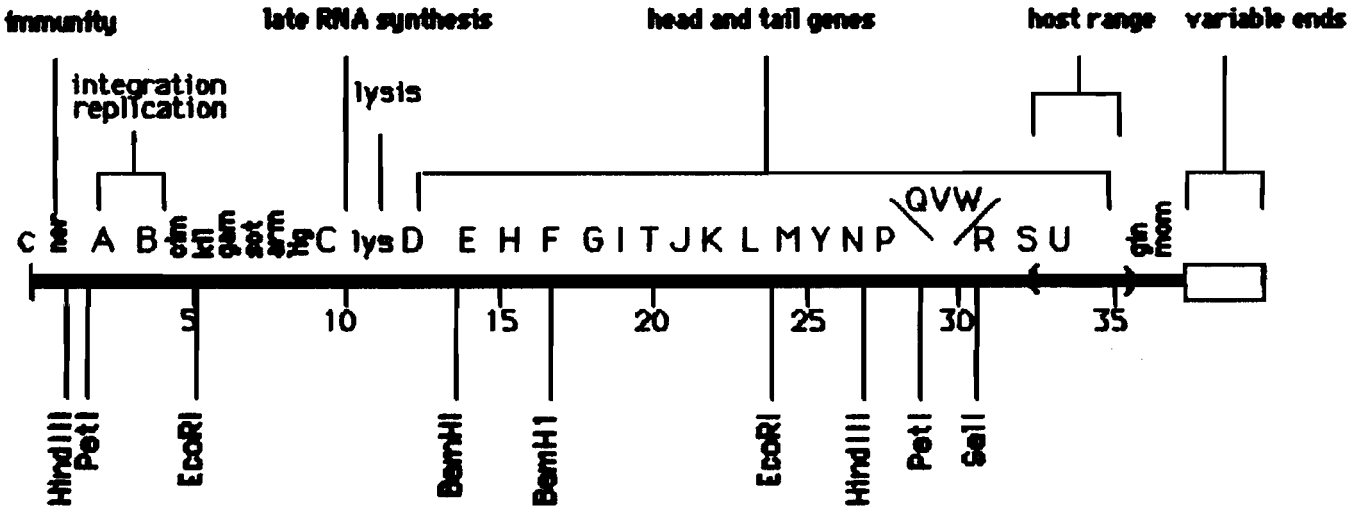
(Grundy and Howe, 1985; Schumm et al., 1980).

B- Restriction map of the Mu genome.

A)


Q
V
c ner A B SEER C lys D E H F G I TJK L Y N P W SU gin mom
10
20
30
U'S'
EARLY
LATE
kil/arm
c ner A B cim/ gam/sot lig C
10
replicationase
transposase
ore-like
repressor
SEER Genes
late
transcription

B)


immunity
late RNA synthesis
head and tail genes
host range
variable ends
integration
replication
lysis
QVW
c ner A B C lys D E H F G I T J K L M Y N P R S U gin mom
5
10
15
20
25
30
35
HindIII
PstI
EcoRI
BamHI
BamH1
EcoRI
HindIII
PstI
SalI

(sot, gam) (Akroyd et al., 1985). Sot is defined in terms of transfection experiments (van de Putte et al., 1977) and gam by analogy to the gam gene of lambda whose product is an exonuclease inhibitor (van Vliet et al., 1978).

Transcription of the genes of the late region requires gene C expression at the rightmost of the early region.The product of the lys gene effects cell lysis, allowing the release of complete phage particles (Faelen and Toussaint, 1973). Gene I encodes the major structural protein of the phage head (Shore and Howe, 1982) and the gene L and Y encode the major tail protein (Giphart-Gassler et al., 1981b). The K gene product is needed for the production of normal length tails (Admiraal and Mellema, 1976). Genes S, U, S' and U' are involved in the determination of Mu host range and are required for the production of phage tail fibers (Grundy et al., 1984). In vitro complementation assays have allowed the assignment of most of the Mu late genes into two functional groups. Genes D, E, I, J are in one group as head defective mutants while G, K, L, M, P, Q, R and S in a second group as tail defective mutants (Giphart-Gassler et al., 1982; Grundy and Howe, 1985).

In the beta region are the non-essential genes, gin and mom. gin protein is the only known phage function necessary for the inversion of the G region (Schumann and Bade, 1979; Schumann et al., 1980) (Figure 4). Thus it is involved in the determination of the phage host range. mom (modification of Mu DNA) (Toussaint, 1976) is not restricted to Mu DNA only, it affects all DNA molecules in the cell. mom acts in conjunction with the host function dam (deoxyadenosine methylase), which is involved

in the methylation of deoxyadenosine residues. Modification of DNA occurs only when the phage is mom$^+$ and the host dam$^+$ (Bukhari and Ambrosio, 1978; Plasterk et al., 1983). The modification generated on the DNA by mom is not methylation; it is a new type of adenine residue in a specific sequence that is C/G A G/C N Py (Hattman, 1979). mom expression is under tight regulatory control which requires the presence of the active host Dam methylase and the expression of a transacting phage gene designated dad now identified as C (Chaconas et al., 1981; Hattman and Ives, 1984). A putative cim II function lies as well in the beta region and is thought to be involved in immunity (van de Putte et al., 1979; Chaconas et al., 1981).

C-2.d. Phage cycle.

Mu conforms to the classic mode of temperate bacteriophages. After infecting its host Escherichia coli, it can either go through lytic cycle or follow the lysogenic cycle. In the lytic cycle, Mu functions for DNA replication and morphogenesis are expressed and the bacterial cells are killed.The process generates 50 to 100 phage particles per cell (Figure 5). In the lysogenic cycle, the functions needed for productive Mu growth are repressed. Instead, the phage DNA is inserted into the host genome and remains dormant as a prophage in the lysogenic cells. About 3% of the lysogens have acquired mutations since Mu integrates at random locations in the host chromosome, thus inactivating the target gene (Taylor, 1963; Jordan et al., 1968).

Figure 5.

The life cycle of the temperate bacteriophage Mu.

Mu temperate bacteriophage can either enter lytic cycle upon infection or lysogenize its host.

During infection of a bacterium with phage Mu, the phage attaches to the bacterium by its tail. The linear Mu DNA is injected into the bacterium, S end first. Forms of circularized Mu DNA non-covalently held by proteins are found prior to integration. An infecting Mu DNA molecule first integrates into host DNA regardless of whether it will undergo lytic transposition or establish its lysogenic state.

A-Lysogeny.

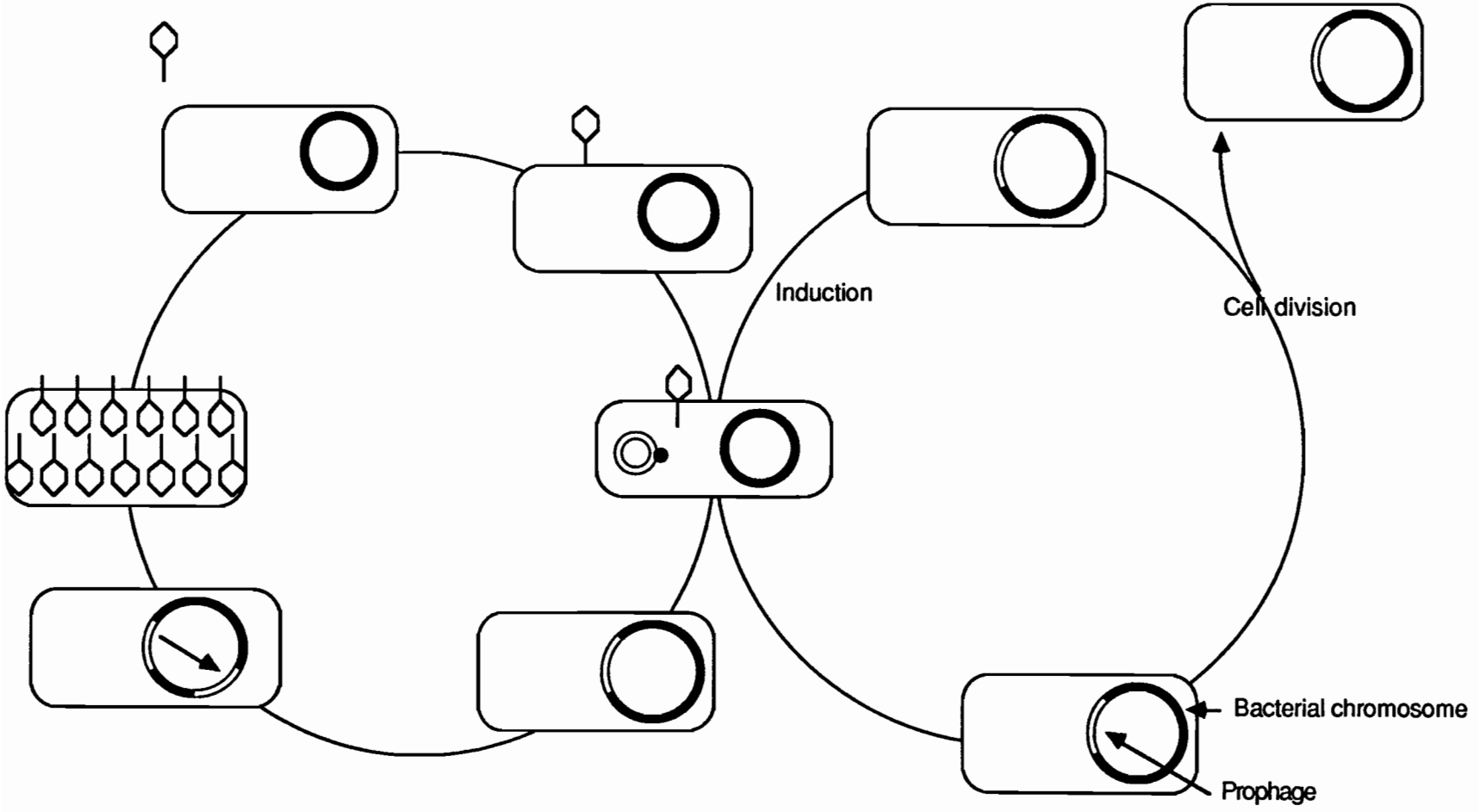
In the prophage state Mu DNA replicates in conjunction with the bacterial chromosome and remains quiescent. Almost all viral transcriptions are repressed in the lysogenic state. Only the c gene is expressed. The c gene product represses transcription of the early genes. Muc<u>ts</u>62 bacteriophage carrying a temperature sensitive mutation in the c gene is able to establish and maintain the lysogenic state as long as the cells are propagated at a temperature that allows the c gene product to repress transcription of the early genes. If the repressor activity is inactivated, the transcription of the early genes is resumed.

Figure 5 (continuation).

B- Lytic cycle.

Mu prophage undergoes lytic cycle when the c repressor is inactivated. Then the Mu lytic cascade begins. The lytic pathway requires early and late gene functions. The expression of the A and B early genes involved in Mu transposition are expressed their expression is controlled by the ner gene (negative regulator of the early genes). The C gene regulatory protein allows the expression of the entire late region which contains many genes involved in head and tail assembly and cell lysis.

Mu DNA replicates by duplicative transposition, and is integrated randomly throughout the bacterial genome. The lytic process generates 50 to 100 particles of Mu phage per cell.



Lytic response

Lysogenic response

A-2.e. Integration of Mu DNA.

In Mu both lytic and lysogenic pathways require integration of Mu DNA into host DNA. Following infection with phage Mu, transposition of the parental DNA occurs to yield simple insertion in what is a conservative event (Liebart et al., 1982; Chaconas et al., 1983; Harshey, 1984). Using an infecting phage with completely methylated DNA, a dam$^-$ (DNA adenine methylase) host and a combination of restriction enzymes that can either cut fully methylated or unmethylated DNA but not hemimethylated DNA, Harshey (1984) demonstrated that transposition of the phage DNA into the host does not involve a duplication of its phage DNA. In contrast, transposition of Mu DNA which is already resident in the host cell generates cointegrates, which are replicative by nature (Chaconas et al., 1981). Although the first transposition event from an infecting phage is not replicative, the very next event is replicative (Harshey, 1984). It is becoming clear that some transposable elements can alternate their modes of transposition, forming either cointegrates and/or simple inserts . The switching of modes could be governed by any of these possibilities or their combinations:

1) It may depend upon differential expression of proteins involved in transpositon following infection versus prophage induction. For example, the availability of the proteins that act at the ends of the element. In Mu, the A gene product, the transposase, is essential for both types of transposition (O'Day et al., 1974; Toussaint and Faelen, 1974). The B protein seem to be responsible for the high frequency of transposition which distinguishes Mu from other transposons. However in the absence of B gene product, integration of Mu from an infecting phage is efficient and

replicative transposition still occurs but at a reduced frequency of about100 fold. It was suggested that the B protein may play a role in determining whether the end products are simple inserts or cointegrates (Harshey, 1983), but data on this are as yet contradictory and no clear conclusions can be drawn.

2) The differences in the physical state of infecting Mu DNA (linear) as compared with the resident prophage (superhelical). Mu DNA isolated from infected cells and mini-cells was shown to be circularized into twisted and open circular form DNA's and held non-covalently by proteins (Harshey and Bukhari, 1981; Puspurs et al., 1983).

3) The injection of packaged proteins from the Mu virion into the host cell along the Mu phage DNA. It is noteworthy that a 64 000 dalton protein which enters the host cell following infection with Mu has been reported (Harshey and Bukhari, 1981; Chase and Benzinger, 1981; Puspurs et al., 1983) to place the Mu DNA extremities in close proximity.

Though the factors necessary to achieve simple insertions seem to be a subset of the Mu factors required to achieve replicative transposition, the mechanisms of these two processes are likely to involve common intermediates (Please see chapter on models of transposition).

C-2.f. The lytic cycle of Mu.

Mu, like many viruses, replicates its DNA efficiently within a short span of time during its lytic cycle. The replication of phage Mu DNA has been found to be coupled to the transposition of phage Mu DNA. Multiplication of Mu viral DNA involves repeated cycles of insertion into the host DNA (Harshey and Bukhari, 1981). Over 100 transposition events

occur per cell during the lytic cycle of Mu. Upon infection of a sensitive host, or heat induction of a Mu prophage containing a temperature sensitive repressor mutation, all phage functions are expressed, the virus replicates and synthesizes its coat proteins, the DNA is packaged, and the host cell is lysed, releasing 50-100 phage particles.

For temperate bacteriophage Mu, the Escherichia coli genes dnaB, dnaC, dnaE, dnaK, dnaG and dnaA are needed for phage replication (Toussaint and Faelen, 1979; Teifel and Schmieger, 1981; Toussaint and Resibois, 1983), and host RNA polymerase is required for phage transcription (Table 2). Mu requires host functions for protein synthesis and energy metabolism.

C-2.g. Replication of Mu DNA.

Mu DNA replication requires the Mu early transcript encoding proteins A (the transposase) and B (replicationase), and the ends of the phage genome. The level of replication is also dependent on the Mu encoded function Arm which resides in the semi-essential early region. The requirement for continuous synthesis of A protein during the lytic cycle suggests that the transposase is unstable and raises the possibility that it is used stoichiometrically rather than catalytically (Pato and Reich, 1984). Mu B protein greatly enhances replicative transposition. A shortened form of gpB was shown to uncouple conservative integration from replicative transposition (Chaconas et al., 1985) and block replicative transposition. Mu replication is largely dependent on host enzymes (Table 2).

Table 2.

Host mutants affecting functions of bacteriophage Mu.

Gene	location on E.coli K-12 (min)	Effects	References
himA	38	inhibits Mu growth .	1
hip (himD)	19	inhibits Mu growth	2
himB	62	partially inhibits Mu growth	3
polA	86	DNA polymerase.	4
tnm	90-100	inhibits Mu growth	5
yup		inhibits Mu growth	6
hek		inhibits Mu kil expression	7
recA		interfers with Mu early expression	8
dnaC		Replication functions	9,10
dnaB		Replication functions	9,10
dnaE		Replication functions	9,10
dnaZ		Replication functions	9,10
dnaG		Replication functions	9,10
dnaA		Replication functions	11

1. Miller and Friedman, 1980.
2. Goosen et al., 1984.
3. Yoshida et al., 1982.
4. Clements and Syvanen, 1981
5. Ilyina et al., 1981
6. Hopkins et al., 1982
7. Waggoner et al., 1984.
8. Patterson et al., 1984.
9. Toussaint and Faelen, 1974.
10. Toussaint and Resisbois, 1983.
11. McBeth and Taylor, 1982.

In vitro studies show that Mu replication is semi-conservative and semi-discontinuous (Higgins and Olivera, 1984). Mu controlled replication forks have the basic characteristics of host replication forks except that Mu has overlaid a special initiation and termination event at the Mu sequence boundaries; the special initiation event takes place at the left end of Mu. It is assumed that Mu DNA is replicated by components found in the replication fork such as DNA *Pol*III holoenzyme, the primosome complex, etc. (Kornberg, 1982). An *in vitro* transposition reaction for bacteriophage Mu DNA was developed by Mizuuchi(1983); the reaction requires a donor DNA carrying the two ends of Mu in the proper relative orientation, extracts containing the *A* and the *B* gene products of Mu, and host factor(S). Two methods were used to detect transposition products: The first one consisted of lambda DNA as the target of transposition and a plasmid DNA containing the ends of Mu DNA and an ampicillin resistance gene as a donor. After the reaction, *in vitro* lambda packaging allows the scoring of *Amp*^{R} transducing phages generated by transposition. The second method involved the identification, by gel electrophoresis, of the DNA products made in the presence of radioactive precursors. The host factors used in the transposition reaction are the enzyme fractions that are required for the replication of the *Escherichia coli* chromosome. RNA synthesis was not required. This suggests that the generation of the 3' termini of the polynucleotide necessary for the initiation of transposition replication is carried out by strand transfer and that RNA polymerase is probably not required. DNA gyrase is needed ; it is not clear whether DNA gyrase is required to maintain the superhelicity of DNA or whether it is more directly involved in the reaction., DNA ligase is also shown to be required as the use of inhibitor NMN (nicotinamide mononucleotide)

inhibits the transposition reaction. Addition of the four dideoxynucleoside triphosphates in place of the deoxynucleoside triphosphates inhibits the reaction. The dideoxynucleosides triphosphates are known inhibitors of DNA synthesis; indicating that DNA synthesis is occurring in this reaction. Mg^{++} is necessary as is ATP and an ATP generating system.

Mu directed DNA synthesis begins by 6-8 minutes in vivo after induction at 42^0C. Most of the Okazaki fragments purified at different times after induction of Mu hybridize with the r strand of Mu. This led to the conclusion that Mu replication proceeds from the left end of the prophage, unidirectionally toward the right terminus. However, experiments by Pato and Reich (1984) on the direction of the first round of Mu DNA replication indicate that initiation can occur at either end of the Mu genome, but proceeds predominantly from the left towards the right end. One explanation for the preferential use of the left end is the proximity of the transposase gene (gene A) to the left of the Mu genome. Since the transposase of Mu appears to work predominantly in cis i.e. on the DNA strand encoding for it the transposase may more frequently encounter the left end and initiates replication there. Although the replication process is asymmetric, the S end is required in the correct orientation for replication. It is not clear whether it is necessary for the initiation of replication.

Upon induction, a Mu prophage is not excised, yet copies of Mu DNA are found inserted at new places (Ljungquist and Bukhari,1977). Mu is always found associated with the host DNA, no evidence of free replicating copies of Mu have been obtained (Ljungquist et al., 1978). A continuous association of Mu DNA with host DNA follows also from the observation that large, covalently closed, DNA molecules containing both Mu and host

DNA appear 15-20 minutes after the start of Mu development (Waggoner et al., 1974; Schroeder et al., 1974). Circles that are heterogeneous in length and contain one or a number of copies of Mu covalently linked to many different host segments have been found to constitue 3% of the Mu DNA molecules in vivo (Parker and Bukhari, 1978; Harshey and Bukhari, 1982). Key structures (circles of variable sizes attached to tail of variable length) are seen after induction of Mu replication and are identified as results of intrachromosomal transposition events which link replicating Mu DNA to different host sequences (Harshey and Bukhari, 1982; Symonds, 1983). This process of integrative replication does not lead to simultaneous amplification or alterations of the host DNA adjacent to either end of the prophage (DuBow and Bukhari, 1980). By 10-15 minutes after induction, host cells become irreversibly committed to the lytic process, and 90% of the induced cells are ultimately killed (Waggoner et al., 1984).

C-2.h. Regulation of Mu gene expression.

Similar to lambda in many respects, Mu is a temperate bacteriophage which can make one of two choices following infection of its host. It can adopt the lysogenic state or the lytic cycle. However, unlike other temperate bacteriophages, an infecting Mu DNA molecule first integrates into host DNA to give simple Mu DNA insertions regardless of whether it will undergo lytic transposition or establish the lysogenic state. It is not known whether the decision of lysogeny or lytic growth is made prior to or after the integration event.

Figure 6.

Regulatory mechanism of Mu expression.

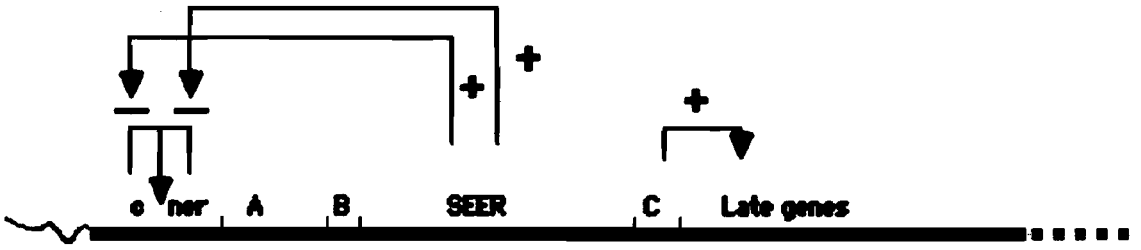
A- Schematic representation of the regulatory mechanism of the Mu early and late functions (Toussaint and Resibois, 1983)

+, positive regulation.

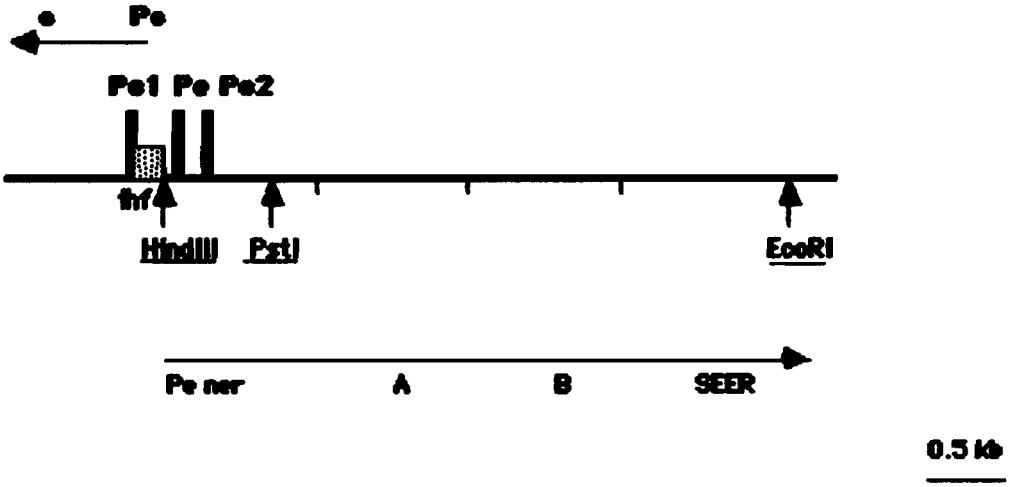
-, negative regulation.

B- Map of the early region of Mu and regulation of its transcriptions. Directions of the early transcript (rightward) and of the repressor (leftward) are indicated. The consensus sequence for the binding of IHF is represented by a box (Goosen et al., 1984).

A)


+
+
+
e
ner
A
B
SEER
C
Late genes

B)


1.0 kb
e
Pe
Pe1
Pe
Pe2
tnf
HindIII
PstI
EcoRI
Pe ner
A
B
SEER
0.5 kb

The genetic switch and the genes which determine whether lysis or lysogeny will be the selected mode of infection are located at the left end or immunity region of the Mu map (Abelson et al., 1973) (Figure 6). Lysogeny, and immunity to further viral infection of the host, are maintained by the product of the repressor gene, c (Schumann et al., 1979). Synthesis of the Mu repressor product of the c gene is essential for the establishement of lysogeny. The regulation of replication of Mu DNA shows similarities with that of lambda (van de Putte et al., 1981). The replication functions are transcribed from a promoter situated on the r strand (Pr), whereas the repressor c is transcribed on the l strand. The c gene is the only known Mu gene to be transcribed in the leftward direction. The c gene encodes a 24 000 dalton repressor protein. This molecule functions in negatively regulating early gene transcription, presumably by binding to one or more sites in the regulatory region that lies between its structural gene and the beginning of the ner gene (Kwoh and Zipser, 1979; Schumann et al., 1979; Priess et al., 1982). Within 1100 bp from the left end, there are two repressor promoters (Pc-1 and Pc-2) located close to each other. The RNA initiated from Pc-2 overlaps with the rightward transcript from the early promoter, Pe (Pe straddles the left HindIII restriction site in the Mu genome.) (Priess et al, 1981; Krause et al, 1983; Goseen et al., 1984; Tolias and DuBow, 1986) (Figure 6). The organization of the repressor and transposition functions is comparable to that of Tn3. In Tn3, transcription of the transposase gene (InpA) and the repressor gene (InpR) starts within a region located between the InpA and InpR coding frame and proceeds in opposite directions (Wisbard et al., 1983). Repressor synthesis is thought to be controlled in at least three ways. First, synthesis is autoregulated (van de Putte et al., 1981). Secondly, it is

negatively regulated by the product of the ner gene (van Leerdam et al., 1982) comparable to the role of cro in lambda. Finally, repressor synthesis is apparently stimulated by cim I and a putative cim II function (van de Putte et al., 1981).

The Escherichia coli functions himA and himD are required for Mu lytic growth (Miller and Friedman, 1980).Fusion protein experiments have identified two host proteins affecting the expression of the Mu early operon : the IHF (Integration Host Factor) complex which is composed of two subunits himA (an 11 Kd polypeptide) and himD (9.5 Kd polypeptide), known to regulate Pc-1, Pc-2 and Pe, and recA (Goosen et al., 1984). IHF is known to play a role in the regulation of phage lambda integration.

Upon inactivation of a thermosensitive repressor by thermoinduction of a Mu prophage, transcription of the early region begins and occurs from the r strand. Expression of the A and B genes from Pe is positively regulated by a host function himD (Giphart-Gassler et al., 1979; Goosen and Van de Putte, 1984). The transcript is under the control of different operators which compete with one another. Early transcription continues in, or just before, the C gene. In the next 4 to 5 minutes, the amount decrease presumably due to the production of the ner protein. The ner gene product regulates negatively the transcript of the early transposition functions of the Mu phage (van Leerdam et al., 1982). Therefore, the ner gene seems similar to that of the cro gene of phage lambda. Both cro and ner turn off CI and c transcription respectively and regulate the expression of the early genes.

C-2.i. Mu packaging.

Mu DNA is assembled into phage particles, by headful packaging of DNA from maturation precursors that contain DNA covalently linked to both ends of Mu DNA. In mature Mu particles both ends of Mu are heterogeneous in length and sequences owing to the presence of host DNA (Daniell et al., 1973a; Bukhari and Taylor 1975) (Figure 7). The right end contains host sequences which vary between 500 bp and 3 200 bp, while at the left end host sequences correspond to about 50 to 150 bp. George and Bukhari (1981) found that the minimal size of host sequences attached to the left of mature Mu DNA is 56 bp, host sequences longer than 144 bp are rare. From the left end of Mu host sequences are packaged into discrete blocks. Host DNA from the left end of Mu seems to be measured from the left end in units of helical turns (10 bp) for packaging with a minimum of 56 bp. It has been observed that the phage tail is attached to the right end of Mu DNA (Inman and Howe, 1976), supporting the theory that the right end of Mu is the last to be packaged and the first to be ejected.

The headful packaging mechanism of Mu implies that packaging starts specifically at the left end of Mu DNA in a maturation precursor, that it proceeds unidirectionally toward the right end, and that the Mu genome alone cannot fill the heads adequately and therefore some host DNA covalently linked to Mu DNA is packaged during morphogenesis. The model leads to the prediction that the length of the host DNA attached to the right end depends on the size of Mu DNA. Bukhari and Taylor (1975) found that the length of foreign DNA at the right end of Mu<u>cts</u> <u>X</u> mutants, which harbor an IS (insertion sequence) in the <u>B</u> gene, is decreased proportionally (Bukhari et al, 1976). This is in agreement with the headful model starting

Figure 7.

Headful packaging of Mu DNA.

A- Diagram of inserted Mu DNA showing how the cutting process can generate host sequences at the termini. Packaging of Mu DNA involves the recognition of a pac site at the left end of the Mu genome and packaging begins from the left end (c end) of Mu and a headful is packaged. This amount of DNA is greater than the length of the Mu genome; thus the rightmost terminus of the DNA within the phage contains bacterial host DNA.

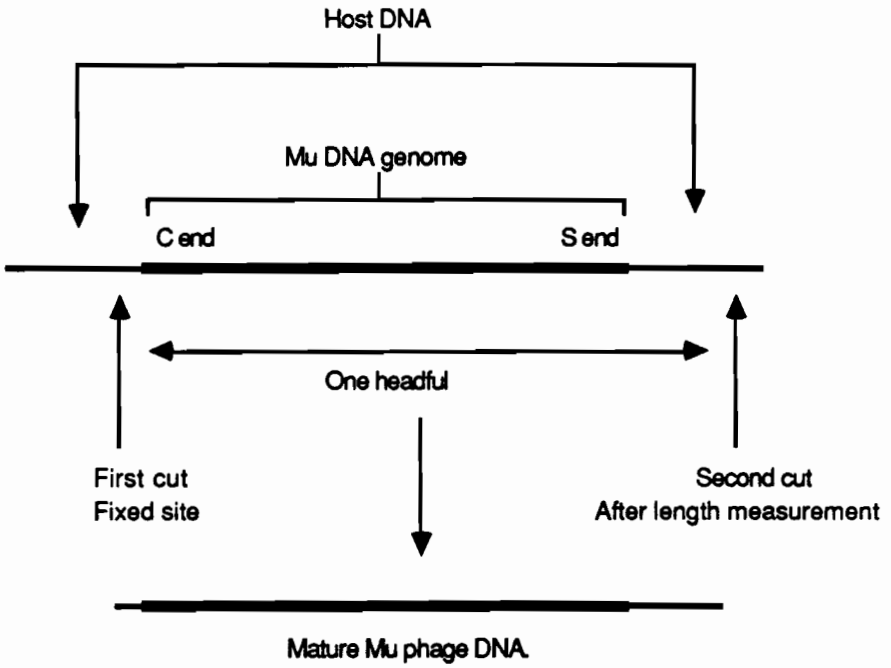
B- Models for packaging of Mu DNA.

Two possible models are based on the assumption that the maturation precursor contains E. coli DNA linked at both ends of Mu DNA.

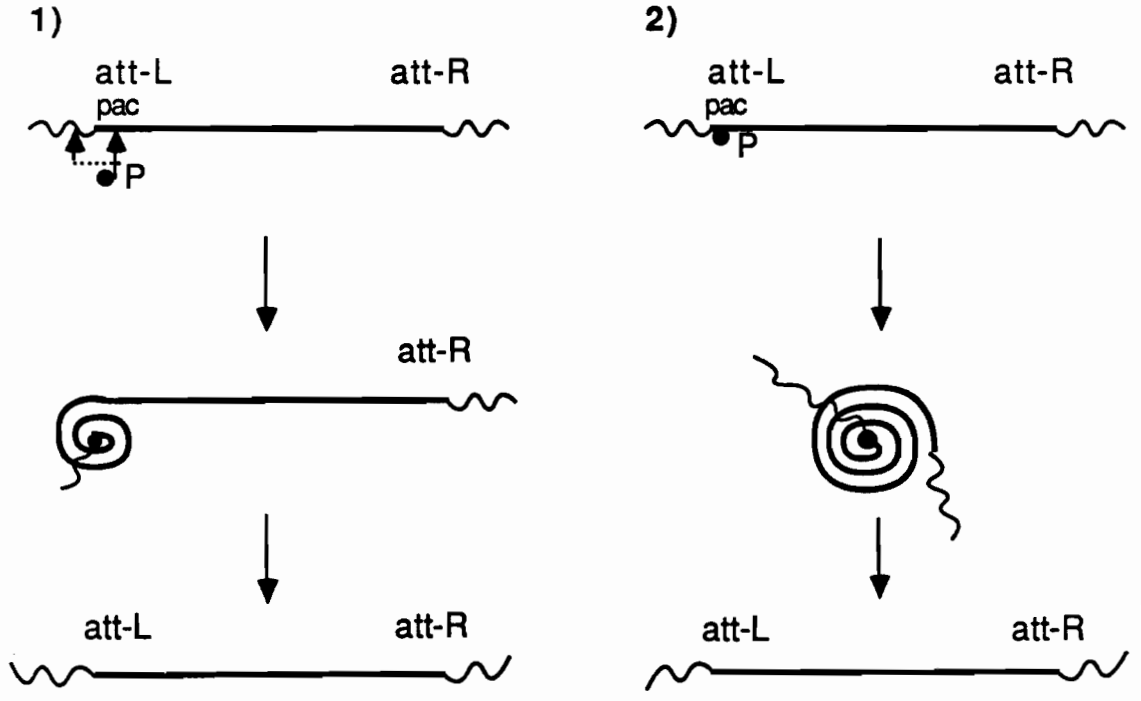
A) A protein (P) recognizes a specific pac site at the c end but cuts to the left of the prophage DNA within the host. The DNA is then folded until a headful is reached past the S end then a cut in the DNA sequences is made.

B) (P) recognizes the pac site at the c end and serves as a nucleation center for condensing the DNA. When a headful is reached, the cuts in DNA are made. The cut into E.coli DNA near the c end is made because the nucleation center is large, covering a part of the host DNA at the c end. (Bukhari et al., 1976).

A)


Host DNA
Mu DNA genome
C end
S end
One headful
First cut
Fixed site
Second cut
After length measurement
Mature Mu phage DNA

B)


1)
att-L
pac
att-R
P
att-R
att-L
att-R
2)
att-L
pac
att-R
P
att-L
att-R

from the left end of the Mu genome. Apparently the packaging proteins recognize a specific sequence on the c end, the pac site, but during packaging the DNA is cut to the left of the primary recognition site, always leaving some host sequences attached to the phage. Two mechanisms have been proposed to explain this step in packaging (George and Bukhari, 1981; Bukhari et al., 1976) (Figure 7). Either the left end is condensed first and cut, after which the rest of the DNA is packaged, or the whole genome is packaged and then the left end and the right end cuts are made. The first mechanism suggests that the first event is cutting of the left by an enzyme behaving like a type I restriction enzyme. This enzyme would recognize a site at the left end and start cutting only after 56 bp of host DNA. In the second mechanism, the DNA is rolled or packaged first in such a way that the first 56 host bp are not available for cutting.

DNA packaging is highly specific for Mu phage DNA. The pac signal of Mu is assumed to be located within the first 101 bp of the left end of Mu (Goodchild et al., 1985; Teifel-Greding, 1983; Groenen and van de Putte, 1985). The capacity of Mu for generalized transduction is rather low (Howe, 1973). Yet a small number of particles are filled with bacterial DNA of mature Mu DNA length (Teifel and Schmieger, 1979; 1981). Studies on Mu mediated transduction of multicopy plasmid pBR322 indicate that the plasmid is transduced as a head to tail oligomer, in which the rec system participates. The packaging of this plasmid into Mu heads is presumed to be initiated at specific sites resembling the packaging signal of Mu (Teifel-Greding, 1983).

D- Prokaryotic transposable elements and the viral transposon Mu.

D-1. Prokaryotic transposable elements.

D-1.a. Definition.

Prokaryotic transposable elements are stretches of non-permuted DNA segments capable of inserting at different sites in the genome. They have many properties that distinguish them (Bukhari, 1976; Calos and Miller, 1980; Kleckner, 1981).

1. They insert their DNA via a recA independent pathway (illegitimate recombination).

2. When they insert, they can interrupt a gene order causing a polar effect by turning off a gene or turning on a gene.

3. They promote DNA rearrangements such as inversions, deletions, duplications (figure 8).

4. They can serve to fuse two replicons giving a cointegrate structure (Figure 9).

5. At their site of insertion, there is duplication of oligonucleotide sequences. For example, Mu generates a 5 bp duplication of the host target site (Figure 13).

6. Their ends consist of perfect or nearly perfect inverted repeats. They are considered to be the reactive DNA sequence in the transposition process, since the transposable elements exhibit a very strict cis dependence on their ends.

7. Although there is a strict specificity of the transposable elements' termini, the specificity of insertion site selection seems to be an

Figure 8.

Type of transposon promoted events.

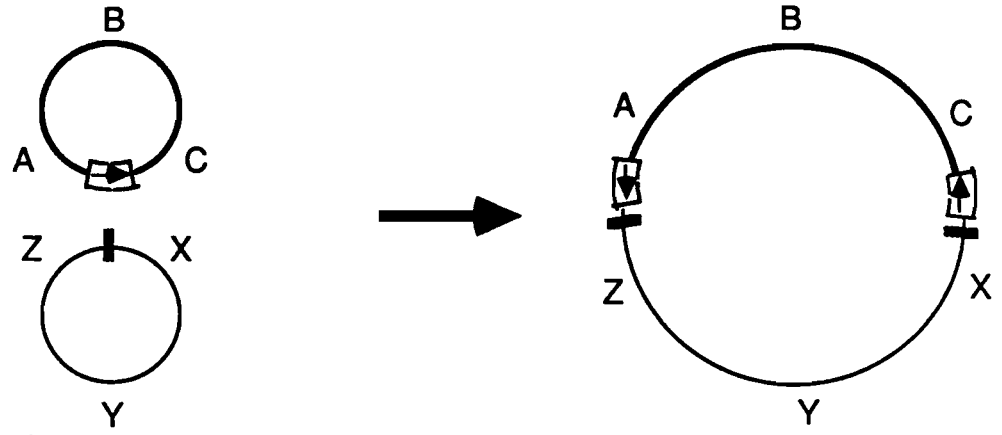
This figure shows three of the most common events promoted during transposition. Fusions, deletions and inversions can be mediated by a transposon such as Mu. Mu is depicted as a box containing an arrow. The location of the 5 bp target sequence and the duplicated copies are shown as narrow boxes.

A) Fusions. During cointegration, a 5 bp target sequence is duplicated at either end of the two transposons and the two replicons are fused together flanked by direct repeats of the transposon.

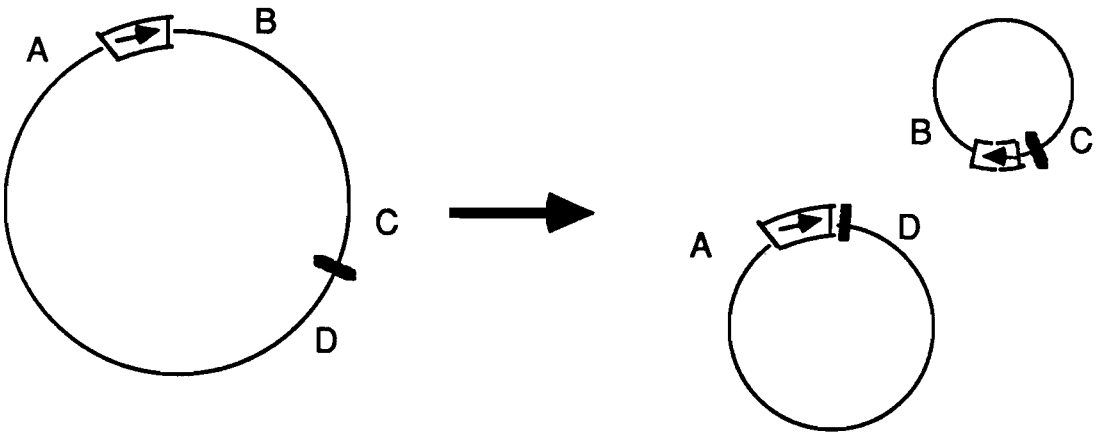
B) and C) The predicted locations of the 5 bp duplication is shown for the other two events, inversion and adjacent deletion. Resolution by a site-specific strand exchange between two transposons or exchange in homologous sequences yield a deletion B) or an inversion C).

C) During replicative inversion, the DNA intervening between the two copies of the transposon is also inverted.

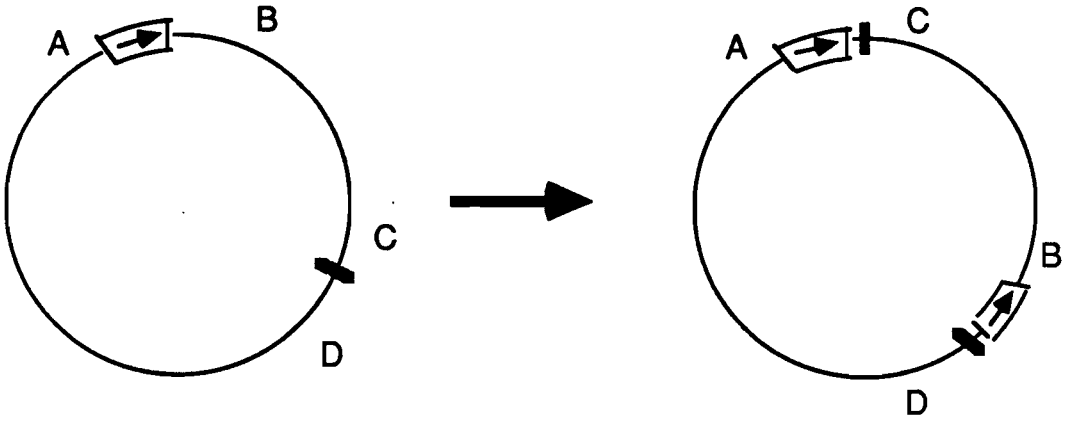
A) Fusions



B) Deletions



C) Inversions



intrinsic property of a transposable element. Transposable elements show various degree of specificity on their choice of DNA target site. Some are found at one or very few sites only , others insert at a larger but still limited number of sites and a third category seems to transpose more randomly.

8. There is at least one open reading frame between the two ends. This open reading frame codes for a protein that must be expressed before the sequence can transpose. The gene product necessary for movement is called the transposase. The transposase is essential for transposition and specific to the transposable element's own ends.

D-1.b. Classes of transposable elements.

Transposons are normal constituents of most bacterial genomes and of many extrachromosomal plasmids and bacteriophages. They are widespread, diverse and highly evolved in both mechanistic and regulatory properties. Prokaryotic transposable elements can be divided in three classes on the basis of mechanistic differences, genetic organization, and DNA sequence homology (Kleckner, 1981).

Class 1. The IS like modules and their composite elements.

Class 2. The Tn3 family.

Class 3. The transposable bacteriophages, Mu and D108.

The IS (for Insertion Sequences) elements vary in length between about 700 and 1800 bp. Their common structural characteristics are that they have perfect or nearly perfect inverted repeats at both ends and they have the internal capacity for coding one or a few polypeptides (Iida et al.,

1984). The IS elements are normal constituents of bacterial chromosomes and plasmids. It is estimated that 1 to 2% of the Escherichia coli K12 chromosome represents IS sequences. The composite transposons are mobile DNA elements carrying physiologically detectable genes which renders their detection easier than the detection of IS elements, but these genes are unrelated to insertion functions. Composite transposons are flanked by IS elements in direct repeat or inverted repeat orientation, on whom their transposition relies (Calos and Miller, 1980).

The Tn*3* class (Grindley, 1983) and the bacteriophages class (Toussaint and Resibois, 1983) represent more complex transposable elements. All elements in the Tn*3* class are more than 5 kb long and encode for accessory determinants in addition to transposition functions. They have 38-40 bp inverted repeats and generate a 5 bp duplication at the target site. Tn*3* is well studied, it codes for a beta-lactamase responsible for ampicillin resistance. It encodes for the transposase *tnp*A and a regulatory protein *tnp*R. This latter protein, also called the resolvase, has a dual function. It represses the synthesis of the transposase and catalyzes recombination between two Tn*3* sequences. Tn*3* has an internal resolution system that resolves transposition intermediates such as cointegrate structures into final separation of donor and target DNA molecules, each harboring the transposon. The separation is accomplished by a site-specific recombination event which implicates a separate function of *tnp*R operating on the internal resolution sites after completion of DNA replication (Reed, 1981).

The third class, phages Mu and D108, are large transposable elements of 38 kb and also temperate bacteriophages of Gram negative bacteria. As part of their life cycle they catalyze the full spectrum of processes

attributed to transposons (Bukhari, 1976; Toussaint et al., 1977). The phages propagate their DNA via an *in situ* integrative replication mechanism, i.e. by transposition. The phages integration and vegetative DNA replication appear to be linked. Although the first transposition event at the integration step is conservative, during lytic growth Mu propagates its DNA by replicative transposition, and cointegrates are the main transposition products. A major difference between these bacteriophages and the other transposons lies in the efficiency of their respective transposition systems. The transposition process occurs at a frequency of 100 events per cell, which is at least four orders of magnitude higher than for most prokaryotic moveable elements (their transposition frequency ranges between 10^{-4} to 10^{-7}).

In addition to host factors, only the Mu A gene product and the two Mu ends are required for integration of the phage genome into the host DNA. Efficient replicative transposition during the lytic cycle also requires these proteins plus the B gene product; a number of non-essential early functions are also implicated in enhancing the efficiency of transposition (O'Day et al., 1978; Faelen et al., 1978; Wijffelman and Lotterman, 1977; Waggoner et al, 1981; Goosen et al., 1982). Unlike the Tn3 family, Mu and D108 do not have a site specific recombination system to resolve their cointegrate structure. Transposition is an obligatory feature in the life cycles of Mu and D108 phages and thus Mu and D108 are considered as viral transposons.

D-1.c. Evolution of the prokaryotic transposeable elements.

Several variants and relatives of a number of transposable elements were found either in the same or different organisms. The members of such families usually differ from each other by small DNA sequence alterations. In analogy to the situation found with related phage genomes such as the lambdoid family, recombination within the homologous segments may result in viable hybrid transposable elements. For example IS102 and IS903 are related (Bernardi and Bernardi, 1981). Nucleotide differences between these two elements are found only in their two left halves. In the Tn3 family, the similarity in structure and function suggest a common evolutionary origin. The temperate bacteriophages Mu and D108 also share functional and structural characteristics and are 90% homologous in their DNA sequences.

Transposable elements are basic agents for genetic variability at the population level. Through DNA rearrangement, they act in vertical as well as in horizontal gene exchange, and they may represent one of the principal devices for evolution. Transposable elements are discrete DNA segments that move to new sites on a genome without requiring extensive DNA sequence homology. It has been speculated that transposable genetic elements may represent degenerate replicons that have preserved just the minimal unit for self-replication (Doolittle and Sapienza, 1980). Transposable elements may confer neither advantage nor disadvantage on the phenotype but could constitute 'selfish DNA' concerned only with their own propagation. According to this concept, the relationship of the transposon to the genome resembles that of a parasite with its host. Presumably the spreading of the elements by duplication and transposition

is balanced by the harm done if a transposition event inactivates a gene or if the number of transposons becomes a burden on cellular systems. In the other hand, nature may have taken advantage of these elements to use them as drivers in evolution by using their capacities to rearrange molecules and exchange genome segments between different organisms. Any transposition event conferring selective advantage will lead to preferential survival of the genome carrying the active transposon. They may act in parallel with mechanisms of evolution (e.g. nucleotide substitutions, formation of small deletions, and sequence duplications).

D-2. Transposition Mechanism.

D-2.a. Common features of DNA transposition.

Movable elements are responsible for a variety of biological phenomenom in both procaryotes and eucaryotes. Movable genetic elements in procaryotic organisms can be grouped in two classes. One class carries out conservative site-specific recombination reaction such as the integration-excision reaction of phage lambda. these reactions require neither DNA replication or synthesis of DNA (Nash,1981) . The transposons form a widespread class of elements. Transposable genetic elements are pieces of DNA which have the ability to change their location in a genome without requiring homologous sequences for recombination. However, certain features of transposition of DNA are common in the systems studied so far:

First, the transposon is a self contained unit which usually encodes

the protein responsible for its own movement, the transposase. Host fuuctions have also been shown to be involved in the transposition process.

Second, the nucleotide sequences at the ends of the transposon are the only DNA sequences required *in cis* for the transposition event to occur.

Third, at the site of insertion a small number of base pairs of target DNA are duplicated during the transposition event.

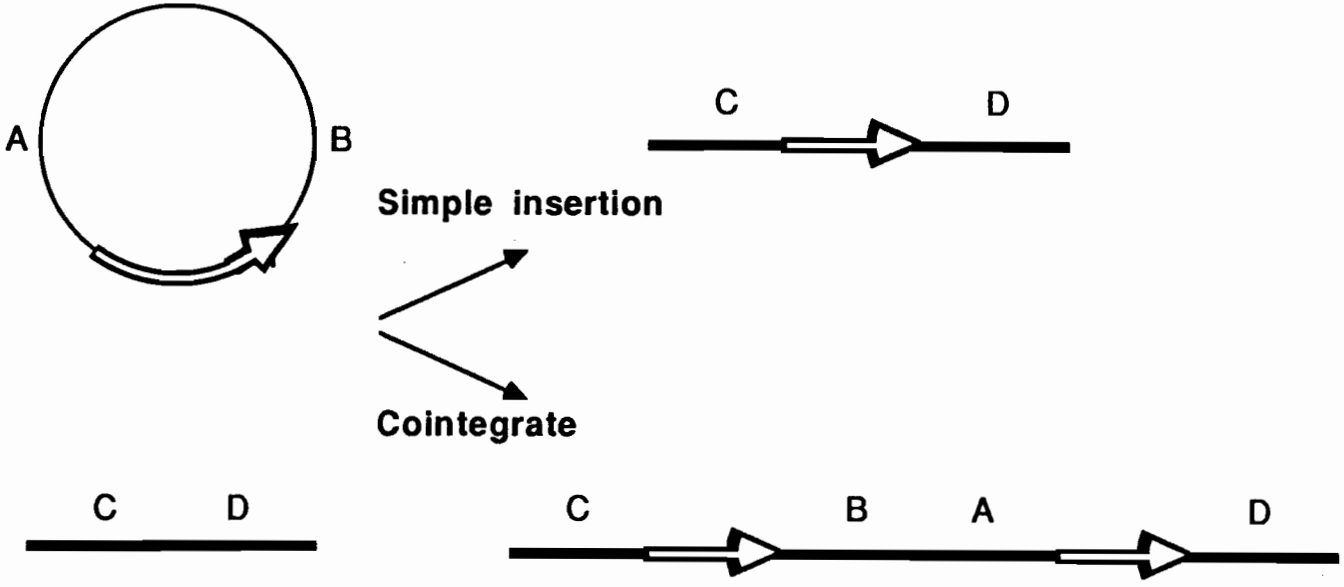
Fourth, a specific nucleotide sequence of the target DNA at the insertion site is not usually required although some transposons insert at "hot-spot"sites and others exhibit "regional specificity".

It has become clear that transposition can occur via a replicative process, as first proposed by Ljungquist and Bukhari (1977), or by a conservative mechanism. The end products of transposition are of two kinds. Cointegrates consist of a fused donor and target molecules with duplicated copies of the transposon at the junctions, while simple inserts consist of one copy of the transposon inserted into the target molecule (Figure 9). One group of transposons carries out a replication transposition reaction, in which replication of the transposable element is an integral part of the transposition reaction. Typical examples of such elements are the tranposons Tn*3* and Gamma Delta, which produce a so called cointegrate structure almost exclusively as the primary product of transposition (Grindley, 1983). During the Mu lytic cycle, cointegrates are the majority of its transposition end products. The replicative nature of the reaction is apparent since a cointegrate structure contains two copies of the transposable element within a single molecule generated after one cycle of transposition starting from a replicon containing only one copy of

Figure 9.

Simple inserts versus cointegrates.

Transposition of transposable elements to an intermolecular site lying between C and D can result in the generation of either a simple insertion (top) or a cointegrate structure (replicon fusion) (bottom). (Weinert et al., 1984).


A
B
C
D
Simple insertion
Cointegrate
C
D
C
B
A
D

the element. However, the cointegrate structure is resolved via a resolvase system to simple inserts, as seen with Tn3 and related transposons.

Another group of transposons is believed to carry out transposition exclusively through an essentially non-replicative process. Tn5 is an example of such an element, as tested genetically by its inability to produce cointegrates (Berg,1983). Kleckner et al.(1984), have shown that the majority of Tn10 transposition products are made through a non-replicative process. A typical product of non-replicative transposition is a simple insert, consisting of one copy of the transposable element transposed to a target DNA without the accompanying vector part of the donor DNA. Simple inserts are produced in this case directly without extensive DNA replication by a set of cutting and strand transfer reactions followed by gap repair.

Between the two extreme groups, there are a number of transposons , including Mu, that produce as their primary products both cointegrates and simple inserts ,with different ratios. The initial integration of Mu after infection is of the simple insertion type and does not involve replication of the element (Liebart et al., 1982; Harshey, 1983; Akroyd and Symonds, 1983). During the phage lytic cycle Mu propagates its DNA via replicative transposition and the transposition end-products, in this case, are essentially cointegrates.

D-2. b. Models of transposition.

Current models of replicative transposition (Grindley and Sherratt,

1978; Shapiro, 1979; Galas and Chandler, 1981) envisage a staggered cut at the target site and a nick at each end of the transposable element, with a pair of single stranded DNA transfers between the donor and the target molecules, generating a replication fork at each end of the element.

One feature of the reaction carried out by the transposable elements is the generation of a short duplication at the target site sequences at the ends of the transposed element (Kleckner,1981). This suggested that a staggered cut is made in the target DNA, and that its protruding ends are joined to the ends of the transposing element during the process of transposition (Grindley and Sherratt,1978). The first models for transposition were proposed by Grindley and Sherratt (1978) and Shapiro (1979). Shapiro's model was based on the observation made by Ljungquist and Bukhari (1977) who found that the original Mu linkage in a chromosome persisted late into the replicative cycle, indicating that the provirus was never efficiently excised although it was duplicated many times.

Biochemical studies on in vitro transposition of bacteriophage Mu support Shapiro's view (Mizuuchi, 1984). The in vitro transposition reaction developped by Mizuuchi (1983) requires a donor DNA carrying the two ends of Mu in the proper relative orientation, extracts containing the A and the B gene products of Mu, and host factor(s). The enzyme fractions used are the ones isolated for the replication of the Escherichia coli origin region of the chromosome. The analysis of the distribution of newly synthesized DNA strands in the in vitro transposition products of Mu indicated that the transpositon events in Mu are initiated by a pair of strand transfer reactions that attach the 3' ends of the Mu DNA to the 5' protruding strand of the target DNA.

The current model of replicative transposition is envisioned the following way (Figure 10):

The two strands of the target DNA are cut with a short stagger. The donor molecule is also cut on one strand at each end of the transposable element. The free single-strand termini at each end of the transposable element are joined to the protruding strand termini of the target DNA. The transposition intermediate consists of a synaptic junction having the 3' strands at the left and the right ends of the element broken and rejoined to the target site, whereas the 5' strands at each end remained linked at the original position. This generates a set of branched structures with the properties of replication forks from which replication can be initiated. Replication of this structure results in duplication of the element and cointegrate formation if the reaction proceeds between two circular chromosomes. The cointegrate structure is composed of two semi-conservatively replicated daughter copies of the transposable element separated by the old DNA flanked sequences and the new DNA flanked sequences. If the reaction takes place between two sites on a single circular chromosome, the products are either deletions or inversions bounded by replication of the transposable element copies.

In theory, simple inserts can be made by a totally separate reaction pathway or via a pathway branched from the cointegration pathway either before or after the round of replication. In the case of Mu transposition, it is known that simple inserts are not made from an intermediate that has completed a full round of DNA replication, because at most one third of Mu in a simple insert is newly synthesized (Mizuuchi,1983). Genetic evidence with IS1 and IS10 indicate that simple insertions arise when the element

Figure 10.

A model for transposition to generate cointegrate structure and simple inserts.

Replicative transposition.

A- single stranded cuts are made at the ends of the transposon on the donor molecule and staggered cuts 5 bp apart in the target DNA (the recipient molecule) producing a 5 bp protruding 5'ends.

The nicks are indicated by the arrows.

B- Each transposon is attached to a protruding target DNA end. Each of the 5' ends of the target site are transferred to the 3'end of Mu DNA on each strand. From the strand separation between the nicks and joining of non-homologous strands, two replication forks result.

C1 and D1- If successful initiation of replication, by using the 3' end originating from the target DNA as a primer for leading strand (C1), takes place at either one or both ends of Mu DNA, a cointegrate structure is produced in D1.

Figure 10.

A conservative model of transposition.

A- Double-stranded breaks are formed at each end of the DNA segement to be transposed, and single strands at each end of the element are joined to nicked target sequences.

B- If the structure fails to initiate replication, cleavage of the junction between flanking DNA of the donor molecule and Mu DNA will produce a simple insert with a pair of gaps on each strand at the 5' ends of the Mu DNA .

C2 and D2- Repair DNA synthesis fills in the single-strand gaps and generates the target sequence duplication, but the element itself is not copied during transposition.

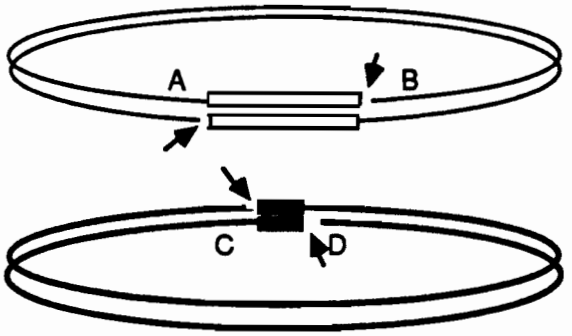
The vector from which the element is clipped is not recircularized and is ultimately lost through exonucleolytic degradation.

Implicit in this diagram is the idea that the element-encoded transposase acting in a complex of generalized host factors is likely to be responsible for the specific binding and cutting at the ends of the element and the target DNA. (Shapiro, 1979; Mizuuchi et al., 1984; Berg et al., 1984).

The white boxes represent the transposable element. The small black boxes represent the target site.

The thin line is the donor replicon and the thicker lines the recipient replicon. The dotted line represents DNA been synthesized.

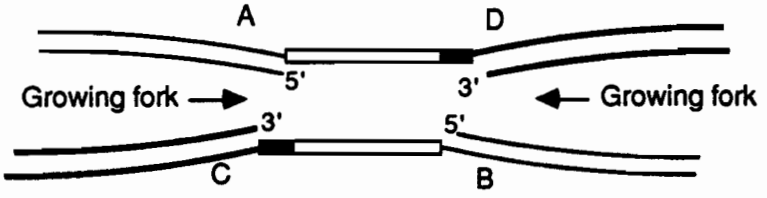
A)



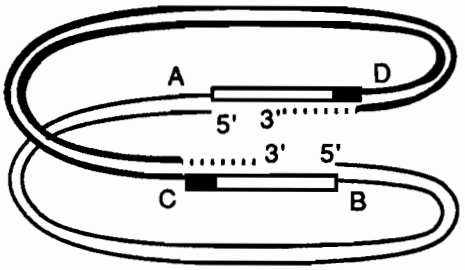
Donor plasmid containing transposon

Recipient plasmid containing target sequence

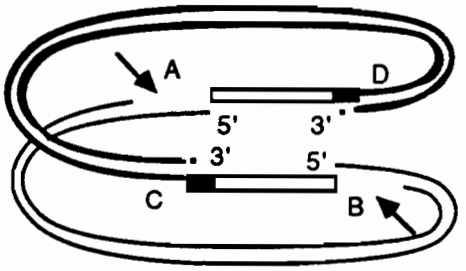
B)



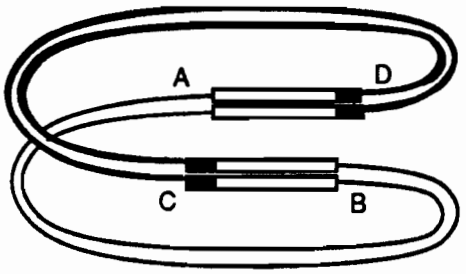
C1)



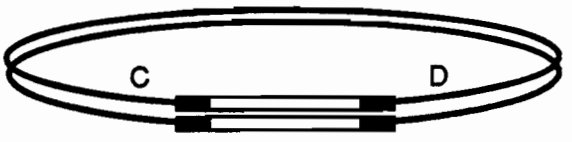
C2)



D1)



D2)



fails to replicate or if replication starts but then terminates prematurely (Biel and Berg, 1984; Kleckner et al., 1984).

Simple insertion is suggested to involve the following events (Berg et al., 1984; Mizuuchi et al., 1984): at the time of the initial strand transfer step, the junctions between the transposon sequences and the old flanked sequences are cut to produce a simple insert with a pair of single stranded gaps (Figure 10). A gap repair step will complete the simple insertion process. The possibility that simple insertion and replicative transposition processes have a common intermediate synaptic joint and that the processing of the synapse follows different pathways can be envisioned (Biel and Berg, 1984; Mizuuchi, 1984) (Figure 10).

D-3. The ends of transposable elements and Mu.

D-3.a. The ends of transposable elements.

In transposable elements, the ends are important for transposition. All autonomously transposing elements have two ends which define the DNA which can hop; all DNA found between the two ends jumps as a single unit. The ends are most likely the reactive sequences which interact with the transposase during the integration of the transposable element into the target DNA.

The ends of transposable elementsusually contain short (9 to 40 bp) inverted repeated sequences (Kleckner, 1981). These repeated sequences are perfect or nearly perfect. The nature of such repeats reflects a functional symmetry between the two ends. The inverted repeats probably function as sites at which the transposase can bind and carry out

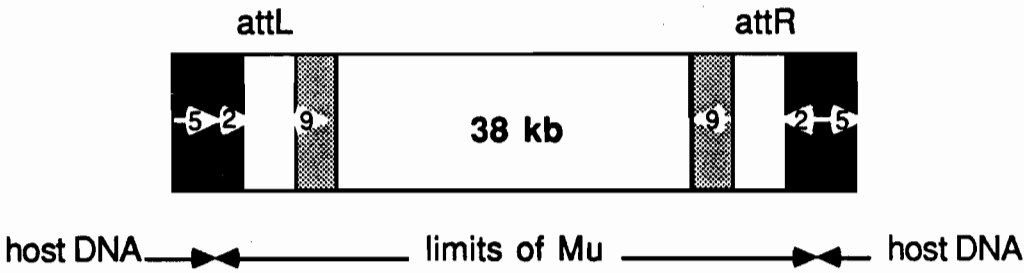
symmetrical enzymatic reactions with both ends of the transposon.

The ends of transposable elements must be intact for transposition to occur. Deletion of one or both ends produces a non-complementable transposition defect. The ends of a transposable element are required in *cis*. Furthermore, there is evidence that the terminal inverted repeats are structurally required for transposition and that only a small sequence at each end of the element is important. In the case of Tn*10*, the outer 27 bp are absolutely required for Tn*10* transposition; however, base pairs 27-70 seem to encode auxiliary sites directly facilitate, supplement, or modify interactions in the essential 1-27 bp (Way and Kleckner, 1984). It has been demonstrated by exonuclease Bal31 deletion analysis that the terminal outermost 19 bp, close to two helical turns, are essential for Tn*5* transposition (Johnson and Reznikoff, 1983; Sasakawa and Berg, 1983). Tn*3* contains identical 38 bp inverted repeats at each end. Mutations that delete the terminal inverted repeats of Tn*3* or a deletion that removes an internal part of one of the inverted repeat produces a non-complementable defect (Gill et al., 1979).Moreover, the terminal repeat sequences are highly conserved in the Tn*3* family. In spite of the close similarity, the ends of Gamma Delta and Tn*3* will not complement each other for transposition.

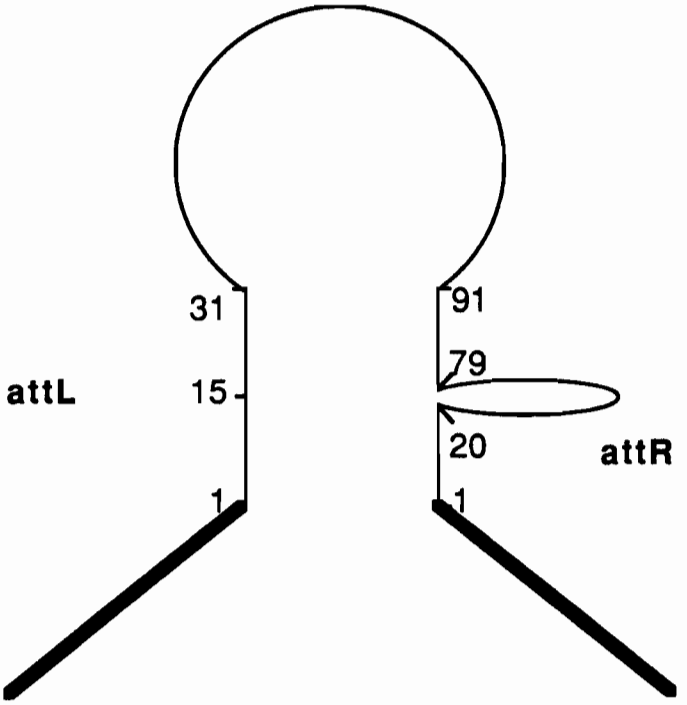
D-3.b. The ends of Mu.

To reflect the physical and functional state of the two extremities of the Mu genome, they have been designated *att*L and *att*R, respectively (for attachment site left and attachment site right). The *att* sites of Mu are not homologous and therefore they differ from the principal structures of the

A)


attL
attR
5
2
9
38 kb
9
2
5
host DNA
limits of Mu
host DNA

B)


31
91
79
attL
15
20
attR
1
1

ends of transposable elements (Kahmann and Kamp, 1979). The first and the last 40 bp of Mu show almost no homology except for the two first nucleotides at their extreme ends 5' TG...CA 3' and a 9 bp repeat near the ends (Figure11). However, the first 31 nucleotides of attL are present in attR and contain two sets of nearly homologous sequences (Figure 11). The first 14 bp from attL are found at position 79-92 in attR; nucleotides 15-31 in attL are found in the first 20 nucleotides in attR. Asymetry of the sequence complex could affect the reactions taking place at the two att sites. For example, it is known that the frequency of DNA replication is higher at attL than at attR.

Within the terminal repeated sequences of Tn3-like transposons Gamma Delta, Tn951, IS101 and bacteriophage Mu there is a specific heptanucleotide sequence 5'ACGAAAA3' that is conserved. All of these elements generate a 5 bp duplication at the target DNA upon insertion. This may suggest a common progenitor For Tn3-like transposons and Mu (Reed et al., 1979). Limited homology is also present at the ends of Mu and IS5 where 11 out of 16 nucleotides of Mu attL and 10 out of 16 nucleotides of Mu attR are homologous to the terminal sequences of IS5 (Kroger and Hobom, 1981). D108 and Mu are completely homologous for nucleotides 1 to 30 at attL. In addition, nucleotides 32 to 54 at attL share extensive homology (Priess et al., 1983). Moreover 13 out of the first 16 bp at the left end of Mu and D108 are identical with the first 13 bp at the very end of IS30 (Bukhari et al., 1985).

Both ends of Mu and Mu gene product A are necessary for the first integration after infection. Mu phage particles lacking the S end were obtained through packaging of a mutant that carries an insertion longer

Figure 12.

Mu terminal sequences involved in transposition.

The nucleotides sequences of attL and attR are taken from Groenen et al., 1985 and Craigie et al., 1984.

Data from these two works are compiled.

The sequences in boxes show the strong binding sites of Mu gene A product (Craigie et al., 1984). attL: 3' L1 --->, L2 --->, L3 ---> 5'.

L1, L2 strong binding, L3 weaker binding.

attR: 5' R3 --->, R2 <---, R1 <--- 3'.

R3 strong binding, R2, R3 weaker binding.

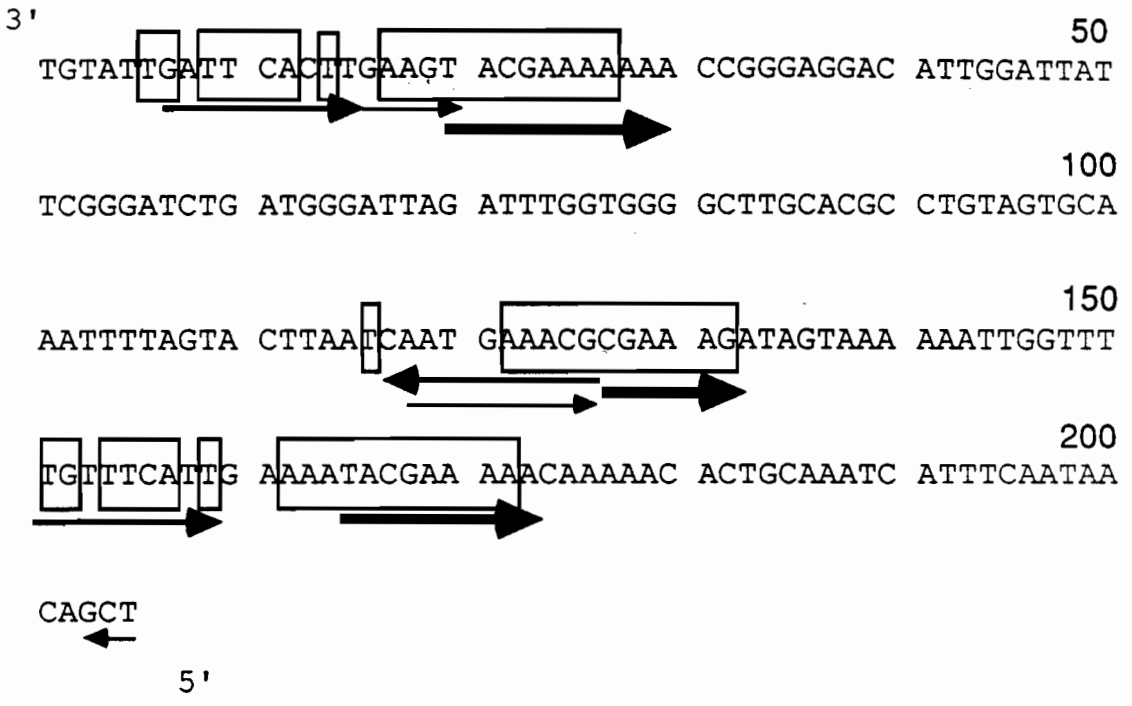
The arrows illustrate the Groenen et al., 1985 essential consensus sequences (the straight arrows) sub-consensus sequences (the dashed arrows) and some sequences that are in form of repeats (dotted arrows).

Straight arrows PyPuCGAAAA

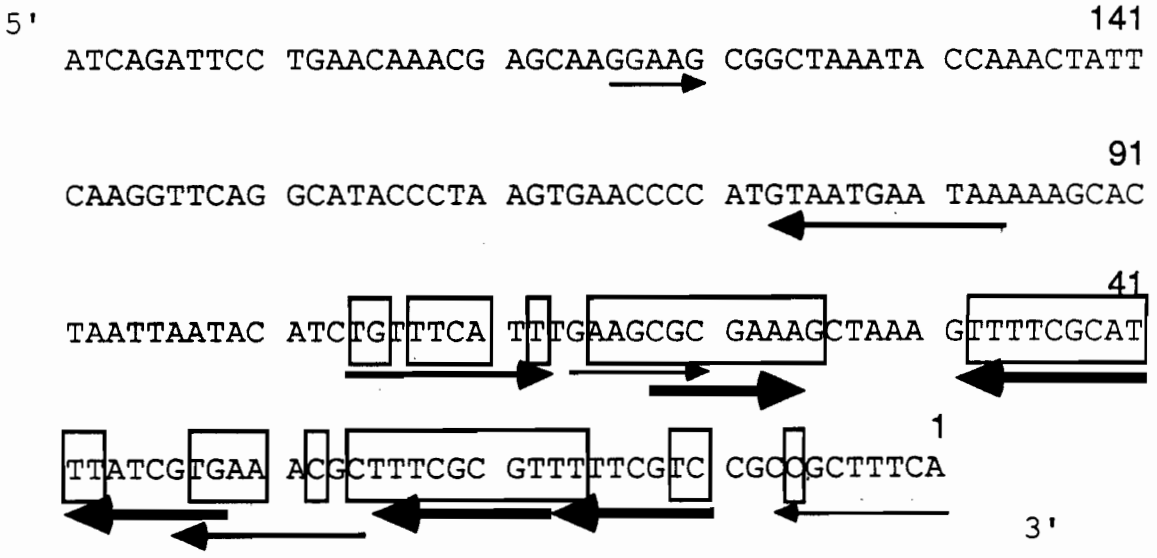
Dashed arrows PyGTTTCAPyT

Dotted arrows TGAAGCG

AttL


3'
50
TGTATTGATT CACTTGAAGT ACGAAAAAAA CCGGGAGGAC ATTGGATTAT
100
TCGGGATCTG ATGGGATTAG ATTTGGTGGG GCTTGCACGC CTGTAGTGCA
150
AATTTTAGTA CTTAATCAAT GAAACGCGAA AGATAGTAAA AAATTGGTTT
200
TGTTTCATTG AAAATACGAA AAACAAAAAC ACTGCAAATC ATTTCAATAA
CAGCT
5'

AttR


5'
141
ATCAGATTCC TGAACAAACG AGCAAGGAAG CGGCTAAATA CCAAACTATT
91
CAAGGTTCAG GCATACCCTA AGTGAACCCC ATGTAATGAA TAAAAAGCAC
41
TAATTAATAC ATCTGTTTCA TTTGAAGCGC GAAAGCTAAA GTTTTCGCAT
1
TTATCGTGAA ACGCTTTCGC GTTTTTCGTC CGCCGCTTTCA
3'

than the size of the S variable end (i.e., greater than 2 kb). These deleted phages were unable to complete a lytic cycle after infection (Howe and Bade, 1975; Bukhari, 1976; Toussaint et al., 1977). It is known that 116 bp of attR are sufficient because the phage MudII 301 in which most of the G-beta region has been substituted for by a segment of bacterial DNA and retains only 116 bp of the attR region, integrates normally after infection (Castilho et al., 1984).

Craigie et al. (1984) doing in vitro nuclease protection experiments with the Mu A gene product (the transposase) and the left and the right end of Mu defined three strong binding site for the A protein. Two of these cover the left end regions 0-30 bp and 120-160 bp and one at the right end (Figure 12). Groenen et al. (1985) studied the transposition of Mini-Mu with different sizes of the Mu ends upon provision of the products of the A and B genes cloned on the same plasmid under the control of the strong lambda pL promoter. In agreement with the in vitro data (Craigie et al., 1984),they have also defined two regions ending 25 and 166 bp from the left end and one region ending 50 bp from the right end that appear to be essential for the transposition of Mu (Figure 12). From a closer look at the DNA sequences involved in transposase binding and the transposition cis-requirement, it appears that consensus sequences overlaps these regions, the orientation of which recalls the typical inverted repeats structure of transposons (Craigie et al., 1984; Groenen et al., 1985). The consensus sequences appear as two direct repeats in attL, and in attR one consensus sequence is inverted with respect to those at the left (Figure 12).

The importance of the terminal nucleotides at the left end was stressed at the 1984 Cold Spring Harbor bacteriophage meeting by the

finding of a mutant having suffered a transition T to C at the terminal T of the Mu genome (Burligame, R., Lynn, D.L., Obukowicz, M.G., Howe, M.M., 1984).

D-4. Transposase.

D-4.a. Role of the transposase.

Most transposons code for a single protein, commonly called a transposase, that is essential for transposition. The detailed mechanism of the reaction of transposition and the role of transposase in the process is not well known. An early step in the transposition reaction involves nicks at each end of the transposons ends and a staggered cut at the target site, coupled with DNA strand transfers between the donor and the target molecules generating a replication fork at each end of the element (Please see models above) (Figure 10).

The role of transposases might be limited to recognition of the ends of the transposon with a requirement for host proteins to complete the strand transfer reaction. Alternatively, transposases may have the activity to carry out the complete strand transfer reaction without the involvement of host proteins. Experiments with Tn10 transposition provide evidence for transposase promoted break/join events at the transposon termini in vivo and for selective interactions of transposase for strong sites at the termini to the exclusion of interaction with weaker possible sites (Morisato et al., 1983). It has been shown in vivo for Tn10 that the Tn10 transposase promotes double-stranded breaks and single-stranded joints at the Tn10 termini (Morisato and Kleckner, 1984).

Efficient transposition of bacteriophage Mu *in vivo* requires a donor DNA containing the two ends of the phage (indication of them being the reactve sites of the process) and two phage coded proteins, the A and B gene products. The A protein is essential for the transposition process of Mu, A^- mutants of bacteriophage Mu are incapable of integrative-replication and do not show the characteristic Mu-mediated genomic rearrangements. Thus gpA is considered as the transposase of Mu (O'Day et al., 1978). The B gene product is essential for Mu replication and is thought to be involved in the initial steps of Mu transposition. It has been shown to enhance the efficiency of the transposition reaction of 100 fold. Other phage products, such as the *arm* gene product enhances the transpositon efficiency 10 fold (Waggoner et al., 1981; Goosen et al., 1982), however its role in the process may be indirect. A number of host proteins also are expected to be involved in the process (Toussaint et al., 1974). A cell-free reaction system used for the study of transposition of bacteriophage Mu requires a donor carrying the two ends in their proper relative orientation, extracts containing the A and B gene products and host factors (Mizuuchi, 1983).

Purified Tn*3* transposase has been shown *in vitro* to bind tightly to single stranded øX174 DNA and poly(dt) (Fennewald et al.,1981). However site-specific DNA binding to the inverted repeats at the ends of the transposon is observed in the presence of ATP (Wishart et al., 1985).The precise biological role of Tn*3* transposase in the transposition of Tn*3* *in vivo* is largely unknown.

In the case of elements integrating at 'hot-spot' sites, it has been proposed that the transposase participates in the selection of the target site. The protein is proposed to contain two distinct DNA-binding domains,

one responsible for the recognition in cis of the transposon termini, and a second for the recognition of distinct sequences in the target DNA (Weinert et al., 1984).

D-4.b. Preferential cis activity of the transposase.

The transposases appear to work predominantly in cis i.e. on the DNA molecule encoding them. Inefficient complementation of the transposase of mutants transposons has been observed with IS50, IS10 and Tn903 (Kleckner, 1981). There is preferential action of the IS10 transposase on the transposon ends located near the gene from which it is synthesized over ends located far away (Morisato et al., 1983). Many experiments have suggested that Mu transposase is less efficient in trans then in cis (Chaconas et al., 1981; Pato, 1982; Pato and Reich, 1984). Mu transposase was shown to work stoichiometrically and support only a single round of transposition. After the first round is completed, new synthesis of the transposase is required for subsequent events (Pato and Reich, 1982; 1984). Morisato et al. (1983), suggested a possible mechanism for the searching process of the transposase:

1) The transposase might bind immediately to the DNA in a tight and specific complex close to its gene of origin and then move by an active, energy requiring and processive translocation process such as DNA unwinding.

2) the transposase might first contact the DNA near its gene of origin and then move in either direction by one dimensional diffusion such as the mechanism proposed for the Escherichia coli RNA polymerase. However the long distance has to be accomplished by using other processes

such as three dimensional movement.

3) The transposase might first contact the DNA near its gene of origin and then move through the cell in three dimension by simple or facilitated diffusion. However the transposase being unstable (Pato and Reich, 1984), might encounter DNA near its gene of origin but never move more than a few kilobases before becoming inactive. Once the transposase finds one terminus by preferential cis action and locates the second terminus by one of the mechanisms proposed above, it then can assume its configuration for cleavage at both termini.

D-4.c. Regulation of transposition.

Most prokaryotic transposons promote transposition at a frequency of 10^{-4} to 10^{-7} per generation (Kleckner, 1981). These low frequencies are attributed to stringent regulation and for inefficient translation/transcription signals. Many transposons are self-regulated. In addition, the activity of transposons may be sensitive to signals from the environment and/or the host signals (Syvanen, 1984). Although the number of copies of a transposable element change in the Escherichia coli chromosome, many transposable elements are present in small numbers. This indicates some constraint on indiscriminate accumulation of transposable elements. The work of Shapiro and Brinkley (1984) indicates that Mucts can be induced at unique times during the growth of single colony populations, even at low temperature. It was pointed out that the frequency of Tn3 transposition was the same whether it was 20 or 200 doublings of the cultures. Strategies of negative transcriptional control of

transposition and genetic rearrangements might be one of the numerous factors which modulate the tendency of the mobile elements to accumulate in high numbers in a genome (Biel et al., 1984; Ahmed, 1983). Responses might be mediated also by programmed changes in the frequency of transcription impinging on a particular mobile element. Furthermore, many composite transposons (Tn*5*, Tn*10*, Tn*903*) are found to carry one functional IS element and one non-functional IS element. Retention of only one functional IS in each transposon could ensure the maintenance of the integrity of the composite element (Ahmed, 1983). Thus, the frequency of transposition and excision of transposable elements may be balanced to avoid lethality to the cell.

Elements have evolved different strategies for controlling their transposition functions. Some transposable elements are regulated by *trans*-acting repressors (Mu, Tn*5*, the Tn*3* family members). The regulation of Tn*10* called multicopy inhibition, acts at the translational level and not at the level of transcription of the transposase. In Tn*5*, the transposase activity may be normally inhibited by a related polypeptide. It does not affect either transcription or translation and probably controls transposition through direct interactions with either the transposase or with sites at which the transposase acts (Berg et al., 1984). In Tn*3*, transcription of the transposase gene (*tnpA*) and the repressor gene (*tnpR*) starts within a region located between the *tnpA* and *tnpR* coding sequences and proceeds in opposite directions (Wisbard et al., 1983). *tnpR* acts within the *tnpA*/*tnpR* regulatory region to negatively control both its expression and that of *tnpA* (Chow et al., 1979; Kitts et al., 1981).

The organization of repressor and transposition functions in bacteriophage Mu seems to be comparable to that in Tn*3*. Synthesis of Mu repressor, the product of gene *c*, is essential for the establishment of the lysogenic state. Gene *c* is transcribed from the *l* strand; while transcription of the regulatory *ner* gene and the transposition early genes *A* and *B* occurs from the *r* strand. The regulation of Mu seems to follow the bacteriophage lambda paradigm in which a repressor protein negatively controls the synthesis of the transposase and other functions that may be involved in the process and also because of the presence of a second negative regulator *ner* (analogous to gene *cro* of lambda) and the presence of two repressor promoters (Chapter on Mu life cycle).

D-5. The host target site.

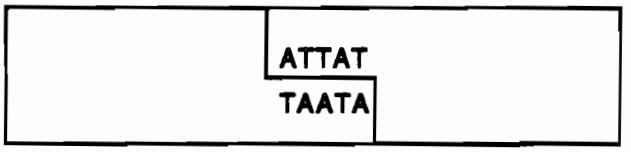
D-5.a. Duplication of the sequences at the target site.

Insertion of the transposable element is accompanied by a duplication of a short 3 to 12 bp target DNA sequence, with the transposable element integrated between the duplicated segment (Figure 13). The short target DNA repeat indicates that the target molecule is broken at staggered position on the two DNA chains (Grindley and Sherratt, 1978) prior to the integration of the element. The length of each repeat is characteristic of each element. Five base pairs repeats of host DNA are found to flank Mu and D108 prophages (Kahmann and Kamp, 1979; Allet, 1979; Szatmari et al., 1986) as well as IS*2*, Tn*3* and Gamma Delta. Comparison of the duplications generated by various Mu and D108 insertions reveals no general consensus, although the middle base of the duplication is always a G or a C (Szatmari et al., 1986).

Figure 13.

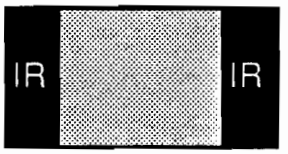
Duplication of host DNA.

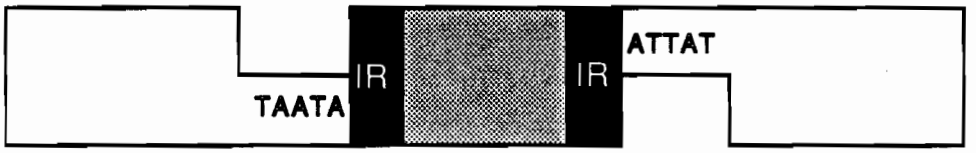
A model of transposon insertion. The host chromosome sequences flanking the transposon are usually found to be identical, implying that a staggered cut is involved in the insertion of the transposable element.

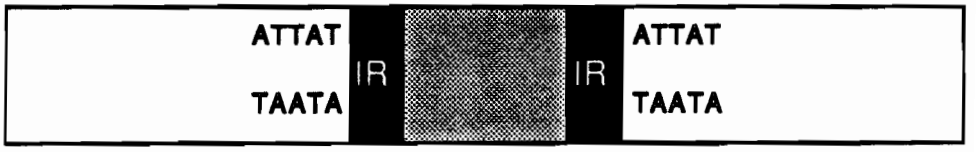


Staggered cut within host chromosome.

Transposon







D-5.b. Specificity of the target site.

Transposable elements show surprising differences in their specificity of insertion, i.e. the site at which the transpositon battery recognizes and cleaves the target molecule. Transposons such as bacteriophage Mu, Tn3 and Tn9 appear to have little insertional specificity. They show a regional preference for integration but no local specificity. Tn10, Tn5, IS2, IS4 and IS5 show significant insertional preference. The appear to insert at 'hot spots' sites of the target molecule. Tn554 (Phillips and Novick, 1979) and Tn7 (Lichtenstein and Brenner, 1982) always insert at the same location.

Regional specificity.

Bacteriophage Mu was shown to insert at a great many different sites even within a region as short as a single gene such as lacZ (Bukhari and Zipser, 1972; Daniell et al., 1971). Although from these latter experiments Mu was considered to insert randomly, detailed genetic mapping and DNA sequencing revealed that insertions are not distributed completely at random. Emr and Silhavy (1980) located 13 of 25 Mu insertions in lamB in a 12 bp interval. These results could be considered as showing regional preference for integration. Studies from a large collection of Tn3 insertions have shown that insertions are clustered in some parts of the recipient molecule but occur infrequently in others. Even within a cluster, their distribution is not random. A detailed map of Tn3 insertions has suggested that many insertions can take place

preferentially into an A+T rich DNA region and near a sequence that is homologous with the ends of Tn3 (Tu and Cohen, 1980). Preference for A+T rich sequences may suggest that local denaturation of DNA must take place before the transposable element inserts. The frequent insertion near a sequence related to the end of the transposable element may reflect a preference of the element tranposase for sequences related to its normal substrate. This indicates that close matches of the target DNA with the transposable element (Tu and Cohen, 1980; Galas et al., 1980), possibly through base pairing, may occur to stabilize an early intermediate in transposition. Alternatively recognition of similar sequence determinants in the target site and element DNA by the transposase protein may occur. Regional preferences for Tn9 insertion in lacZ and similar homology between a sequence in IS1 were observed (Miller et al., 1980). In addition, the presence of GC's at the ends of the sequence duplicated by IS1 insertion suggests a GC cutting preference (Galas et al., 1980).

'Hot spot' specificity.

The distribution of Tn10 insertions in the Salmonella typhimurium histidine operon suggests that Tn10 inserts at very high efficiency into favored sites but can recognize other sites less efficiently and less specifically (Kleckner et al.,1979). A particular 6 bp symmetrical consensus sequence within the 9 bp duplication sequence that is homologous to the ends of the element is responsible for this insertion specificity. For Tn5, 8 out of 11 bp of the target DNA matched to the sequence between the first and the second helical turn of the ends of the element (Sasakawa et al.,1983; Johnson and Reznikoff, 1983; Lupski et al.,

1984). It seems likely that once the affinity between the transposon ends and the target DNA is established, that transposase cleaves within one helical turn, and insertion into target DNA occurs.

D-6. Length dependence.

Length dependence is likely to be a property of transposition by many IS-type transposons and bacteriophage Mu. Thus, the rate of transposition decreases logarithmically as the length of the transposon increases. Length dependence was originally observed in a conjugation assay for a series of IS flanked tranposons (Chandler et al., 1982). Tn*10* transposition exhibits a dependence on transposon length similar to that observed for IS*1* flanked transposons (Morisato et al., 1983). Length dependence was also demonstrated for IS*1*, IS*10* and IS*903* derived transposons. The observed length dependence may reflect some aspect of transposition that acts over the entire length of the moving segment; one aspect proposed was the need to bring together two IS termini and the transposase into close proximity (Chandler et al., 1982; Weinert et al., 1984).

In bacteriophage Mu it was observed that the distance between the Mu termini and its replicon affects the final outcome of the transposition reaction but not its transposition frequency (DuBow and Lalumière, in press). During Mu lytic growth, the majority of Mini-Mu pMD861 outcomes are cointegrates; the outcomes of Mini-Mu's with the ends separated by 407 bp are simple inserts. This evidence suggests that the cointegrate/simple inserts ratio in Mu is proportional to the distance in between the two Mu ends (DuBow and Lalumière, in press).

D-7. Adjacent sequences.

Sequences adjacent to transposable elements can have a strong influence on their transpositional activity (Weinert et al., 1983; Sasakawa et al.,1982; Machida et al., 1982). Transcription from outside promoter was shown to impinge on IS*50* and IS*1* also decreases transposability (Sasakawa et al., 1982; Chandler and Galas, 1983; Biel and Berg, 1984; Machida et al., 1982; Ahmed, 1983). It is likely that the mobility of many elements is affected by transcriptional activity near their insertion sites. The mechanisms by which the external transcription exerts its effect on transcription remain obscure. Possible mechanisms include distortion of the DNA helix, displacement of the transposase, direct inhibition of transposase synthesis and the disruption of the protein-DNA interaction complex that is formed at the ends of the transposable element prior or during initiation of transposition by inward directed transcription (Chandler and Galas, 1983; Biel and Berg, 1984).

Factors associated with the nucleotide sequences surrounding the element in the donor plasmid also play a role in the efficiency of transposition of an element. There are indications for Tn*9* and IS*1* that the proportion of A-T base pairs in the neighborhood of the element modulates their activity as donor (Chandler and Galas, 1983). A+T density could exert its effect through alteration of the DNA conformation near the transposable element.

It was also shown that the *yup* mutation in *Escherichia coli* increases Tn*5* excision (excision is associated with the transposition of some transposable elements) at different degrees depending on the locus from which Tn*5* excises (Hopkins et al., 1983). One explanation is that the excision machinery recognizes nucleotide sequences adjacent to the transposon and has different affinities for different sequences. Topology of the DNA also has an influence of DNA transposition. It has been shown for Tn*5* that DNA supercoiling was the proximal effector of its modulation of transposition (Isberg and Syvanen, 1982). The excision frequency of Tn*5* from the same *lacZ* locus was different depending on whether the gene was on the bacterial chromosome or the F factor. In this case, chromosomal structure, folding, or replication forks were invoked to explain the phenomenom (Hopkins et al., 1983). Transposition of many elements is influenced in a host whose topoisomerases are affected (Kleckner, 1981).

E- Experimental tools.

E-1.a. Mini-Mu.

Only the ends of the Mu genome and the product of the Mu A gene are essential for Mu transposition and Mu mediated chromosomal rearrangement (Faelen et al., 1979). The Mu B gene product increases the frequency of all these events. Internally deleted Mu's in which most of the maturation functions are removed were called Mini-Mu's. Some were constructed *in vitro* by cloning the left and the right end of the Mu DNA on a plasmid or Lambda phage genome (Maynard-Smith et al., 1980; Chaconas et al., 1981; Schumm and Bade, 1979; Van Ledeerman et al., 1981; Kamp and Schummann, 1980). Others were made *in vivo*, screening for deletions induced by transposon insertion near one or the other end of the Mu genome (Faelen et al., 1978; Maynard-Smith et al., 1980; Resibois et al., 1981). The Mini-Mu systems seem to be useful in unraveling the molecular mechanisms of transposition. These Mini-Mu's can be transposed, hence the ends of the Mu genome were demonstrated to be the primary determinants in *cis* for Mu transposition. If the early genes necessary for transposition of the Mini-Mu's are not present on the plasmid molecules, the transposition functions can be provided in *trans* by a helper phage. The Mini-Mu plasmids offer some advantageous ways of examining the transposition problem:

1) Their behaviour in response to Mu induction can be examined by differentiating their forms on agarose gels.

2) Selectable markers inserted within the Mini-Mu can be used to follow the transposition of the Mini-Mu's genetically.

3) The plasmid molecules can be isolated after Mu induction and analyzed by restriction enzyme pattern or by electron microscopy (Chaconas et al., 1980).

However the Mini-Mu systems are a variation of the normal Mu situation. they may not be reliable indicators for use in defining the type of transposition events that can normally occur during the Mu life cycle (Chaconas et al., 1984). Studies in a number of Mu laboratories using of a variety of molecules carried on several different donor replicons under different conditions reported widely varying distributions of cointegrates and simple insertions (Toussaint and Resibois, 1983). Whereas Mu replication mainly initiates from its left extremity Mini-Mu replication is initiated from both ends (Resibois et al., 1982; Hershey et al., 1983).The Mini-Mu derivatives can also be used for the study of Mu DNA packaging since Mini-Mu's serve as substrate for the Mu packaging reaction (Faelen et al., 1978; Chaconas et al., 1980).

E-1.b. pMD861.

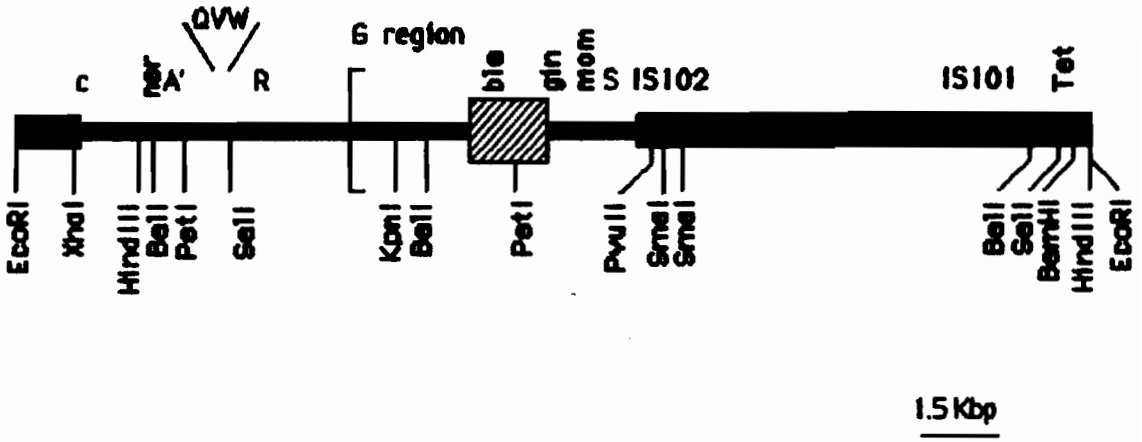
Part of this research used pMD861 as a starting plasmid. pMD861 was constructed from a prototype pSC101 plasmid carrying a whole Mu prophage, pMC321 from which the middle of Mu DNA was removed <u>in vitro</u>, generating an internally deleted Mu referrred to as a Mini-Mu. The ampicillin resistance gene (<u>bla</u>) was inserted within the Mini-Mu in pMD861. The plasmid pMC321 was constructed <u>in vivo</u> by insertion of a thermoinducible Mu prophage into a low copy number plasmid, pSC101. A Mini-Mu derivative pSC101 was constructed from pMC321 by deleting 27

Figure 14.

Map of pMD861.

A linearized map of pMD861 is presented with its EcoR1 site at each extremity.

The thin line represents Mu DNA; the thick line represents pSC101 DNA; the hatched box is the ampicillin resistance gene. (Chaconas et al., 1981).


QVW
G region
C
ger A'
R
bio
gin
mom
S
IS102
IS101
Tet
EcoRI
XhoI
HindIII
BclI
PstI
SalI
KpnI
BclI
PstI
PvuII
SmaI
SmaI
BclI
SalI
BamHI
HindIII
EcoRI
1.5 Kbp

kb of Mu DNA from between the ends of phage Mu. pMC321 (pSC101::Mu) DNA was cleaved with PstI which cleaves in the A gene and to the left of the Q gene of Mu (Schumann and Bade, 1979) and then the DNA was ligated. This Mini-Mu plasmid called pGC121 contains 1.65 kb of DNA from the left end of Mu and 7.7 kb of DNA, including the G region from the Mu right end. An in vivo recombination between Mucts62pAp1 and Mini-Mu pGC121 generated the Mini-Mu Amp plasmid pMD861 (Figure 14). This recombinant DNA was found to contain 1.3 kb of the right end of Tn3 (Leach and Symonds, 1979) and resides in the G region of the Mini-Mu. The presence of the Beta-lactamase gene of Tn3 on pMD861 introduces a second PstI site between the two ends of the Mini-Mu. The bla gene can be utilized as selective marker to follow Mini-Mu behavior during transposition and packaging.

Mini-Mu DNA replication can be catalyzed by an induced Mu helper prophage.The Mini-Mu of pMD861 can transpose when complemented by a Mu helper prophage. Approximately one out of every 10 F' factors transferred from one strain carrying a mini-Mu and an induced helper prophage carries a Mini-Mu insertion. The Mini-Mu replication can be estimated as well by measuring the increase in Beta-lactamase activity which is the reflection of the copy number of the Mini-Mu present at a given time after prophage induction (Chaconas et al., 1980; DuBow and Bukhari, 1981).

Association of the Mini-Mu plasmid with the host is indicative of its cointegrate transposition process. Plasmid-host association can be observed on a low concentration agarose gel where Mini-Mu plasmids migrate with Escherichia coli DNA. The interactions underlying the plasmids and the host genome imply that Mini-Mu plasmids appear

covalently integrated into the host chromosome. However only a portion of the Mini-Mu plasmids are associated with the host DNA (Chaconas et al., 1980; DuBow and Lalumière,in press). This may result from the presence of the Mini-Mu in 4-6 copies whereas there is only one helper prophage. The production of the A and B gene products may therefore be limiting. In addition the A transposase was shown to act preferrentially in cis (Pato, 1982).

Mini-Mu's can be used as well to study Mu DNA packaging since they serve as a substrate for Mu packaging upon provision of proteins of an induced helper prophage. pMD861 displays immunity to superinfecting Mu phage. It has been shown that the left end PstI fragment can synthesize repressor, however in the absence of the Mini-Mu right end it is no longer immune to Mu superinfection. This suggested an immunity control on the right end of Mini-Mu designated cimII (Van de Putte et al., 1980).

Mini-Mu plasmid pMD861 did not express the mom locus. Growth of the Mini-Mu containing strains at 43^{0}C resulted in plasmid DNA which was unmodified. However, when a Mucts mom^{-} prophage is induced in the presence of a Mini-Mu plasmid, mom modified phage DNA is recovered. Indicating the necessity of a trans-acting factor encoded from the phage (called dad) for mom expression (Hattman et al., 1984).

pMD861 offers many advantages to isolate and characterize cis^{-} dominant mutants defective in transposition. The clone is also suitable to study intramolecular transposition both in vivo and in vitro.

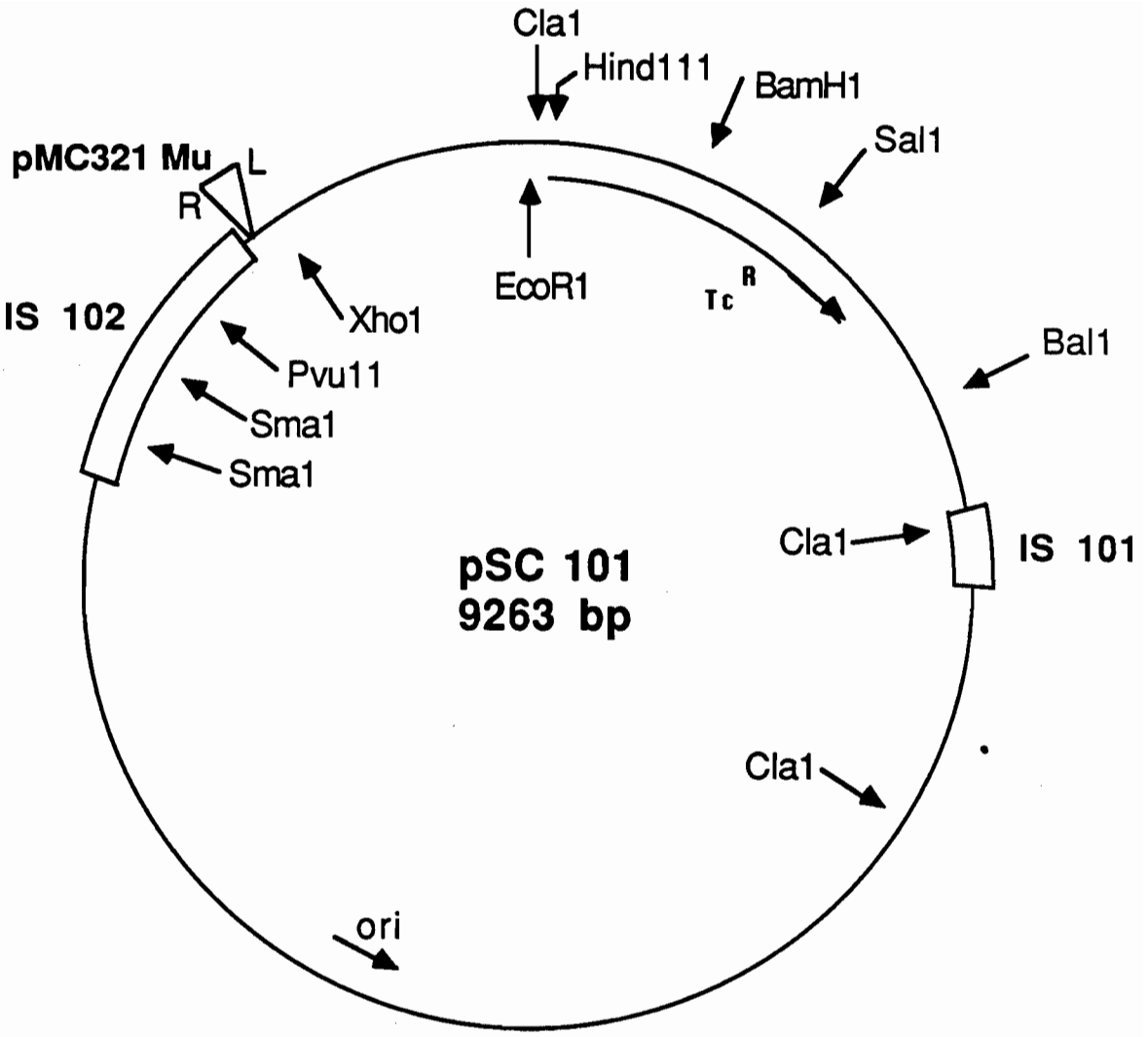
Figure 15.

Maps of pSC101 and IS102.

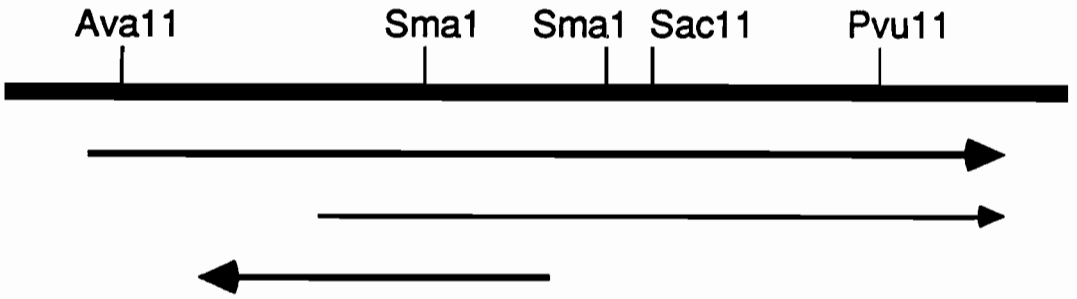
A. Circular map of pSC101(Bernardi and Bernardi, 1984).

B. Map of IS102.

The putative open reading frames deduced from the DNA sequences in IS102 are shown by arrows. (Bernardi and Bernardi, 1982).



IS102
1057 bp



100bp

E-2.a. pSC101.

pSC101 is a low copy number plasmid vector isolated from Salmonella (Cohen and Chang, 1977) which confers tetracycline resistance to the recipient cell (Tait and Boyer, 1978) (Figure 15). It has a stringent mode of replication and requires the dnaA gene product for replication (Hasunuma and Sekiguchi, 1977). Replication of pSC101 is unidirectional (Churchward et al., 1983). A function rep, required for pSC101 replication, is encoded by a segment of DNA adjacent to the origin of replication of pSC101. Another function responsible for the accurate partitionning of plasmids to daughter cell at the division is located to the other side of the origin of replication. pSC101 is a nonconjugative plasmid but is mobilizable at a variable frequency according to the R factors used for the transfer. pSC101 is 9.263 kb long (Figure 15) (Bernardi and Bernardi, 1984). The presence of two insertions elements IS101 (Fischoff et al., 1980) and IS102 (Ohtsubo et al., 1980; Bernardi and Bernardi, 1981) has been revealed by the biological activities displayed by the elements.

E-2.b. IS101.

IS101 is 209 bp long . It is dependent on the transposon Gamma delta of the sex factor F, which is a member of the Tn3 family, for both its transposase and its resolvase (Heffron, 1983).

E-2.c. IS102.

IS102 is 1004 bp long (Bernardi and Bernardi, 1981) (Figure 15).

IS102 generates mainly deletions. Most IS elements create deletions in adjacent sequences. These deletions are believed to occur as the result of an intramolecular transposition event (Grindley and Sherratt, 1978; Shapiro, 1979; Galas and Chandler, 1981). IS102 belongs to a group of elements IS2, IS4, IS5, IS903 that shares a common organization: one large open reading frame running in one orientation and a second open reading frame in the opposite orientation which is in codon-codon register with the larger one (Figure 15). Little is known about the biological role of these polypeptides.

Bernardi and Bernardi (1984) have created in vitro deletions in IS102 affecting only the larger open reading frame. The ability of the mutated IS102 to produce deletions was reduced. Moreover, it was observed that IS associated deletions are characterized as having one fixed end point located at one end of the element and the other endpoint exhibit the same specificity as target sites used in transposition. However, in the case of the IS102, mutants where deletions alter the carboxyl end of the larger polypeptide, show that the ends of the element are no longer recognized as fixed endpoints. It was speculated by the authors (Bernardi and Bernardi, 1984b) that the carboxy termini of the larger polypeptide was involved in the recognition of the ends of the element. This recognition step is involved in the deletion formation by the element.

F. Definition of the project and outline of the thesis.

F-1. Definition of the project.

The transposition of mobile DNA segments of bacteria is mediated by element-specific proteins termed transposases that act by binding to sequences at both ends of their cognate elements. The cis-acting nature of mutations at the extremities of the elements demonstrated that their ends are recognized by the proteins involved in transposition.

Not only are the extremities of Mu the reactive sequences involved in Mu DNA transposition but they are multifunctional, playing a preponderant role in all steps of the Mu cycle. Throughout Mu's lytic cycle and lysogeny the ends remain intact and always covalently linked to host DNA.

1) The ends of Mu are the attachment sites of the Mu genome during Mu's integration as a linear non-permuted genome in the host chromosome.

2) Initiation of replication starts predominantly at the left extremity of the Mu genome and replication proceeds unidirectionnally towards the right end.

3) The repressor gene lies within the extreme left end and the intricate regulatory regions of the early operons resides as well in the left extremity of the Mu genome.

4) Bacteriophage Mu packages its DNA by an headful mechanism, starting from the left end of the Mu genome. A packaging recognition site, called pac, is nestled in the extreme left end of the Mu genome.

The goal of my work was to analyze the cis-acting DNA sequences

which are required, or may influence, Mu DNA transposition as well as the relationships between the Mu extremities and the transposition and maturation properties of Mu. We dissected the different functional domains located in the ends of the Mu genome and related them to their specific roles and interactions during Mu transposition and maturation. We have developed two strategies to study the cis requirements of bacteriophage Mu, first by the creation of a variety of transposition mutant Mini-Mu's and second by the cloning of minimal regions of the extremities of the Mu genome. We found a cis dependence of Mu transposition upon adjacent sequences and we have defined borders in the extremities of Mu for transposition as well as for maturation of Mu DNA. Therefore, the thesis is divided into two main themes, the first one is devoted to the influence of adjacent sequence on transposition activity and the second one concentrates on defining the minimal domains of the Mu genome required in cis for Mu transposition and maturation.

During the course of experiments with exonuclease III and S1 to create deletions at the Mini-Mu extremities of plasmid pMD861, we selected a class of transposition mutants in which small deletions downstream from the Mini-Mu genome influenced the rate of transposition of the Mini-Mu. Furthermore, we found that these alterations in the neighboring sequences near the right extremity of a Mini-Mu in plasmid pMD861 exerted an influence on the extent of Mini-Mu DNA transposition only when the helper prophage contains a polar insertion in its semi-essential early region (SEER). The semi-essential early region is known to encode several functions that may affect DNA transposition and our results suggest that some functions in the semi-essential early region of the Mu genome modulates the transposition activity of Mini-Mu in

conjunction with the *cis*-acting sequences adjacent to the Mini-Mu genome. It is possible that one or several of these functions may act on adjacent sequences to allow maximal Mu DNA transposition from virtually any location during the short lytic cycle of bacteriophage Mu.

We have defined the domains for transposition and packaging of Mu's 37 kilobase pair genome located at the extremities of Mu DNA. Our experimental system consists of monitoring the *in vivo* transposition and maturation behavior of pSC101 and pBR322 plasmids containing various sized Micro-Mu's in the presence of an induced helper prophage to provide the transposition functions and packaging functions. We have found that nucleotides 0 to 55 of the Mu left end define the essential domain for transposition and that sequences bordering nucleotides 126 and 203 define an auxiliary domain that stimulates transposition *in vivo*. At the Mu right extremity the essential sequences for transposition require no more than the first 62 nucleotides. Moreover, using pBR322::Micro-Mu *Kan* derivatives in the presence of an induced helper prophage, we have delineated the *pac* recognition site for Mu DNA maturation to reside within the leftmost 55 bp of the Mu genome. Thus, different functional domains have been integrated within the extremities of Mu during the evolution of this transposable bacteriophage.

CHAPTER II. MATERIALS AND METHODS.

A- Media conditions.

All bacteriological culture media were prepared according to Miller (1972). All bacteriological culture components were purchased from Difco (Michigan, USA) or Maknur (Ottawa, Canada).

Media and buffers for the propagation and dilution of phage lysates have been described by Bukhari and Ljungquist (1977). The antibiotics used were ampicillin (Bristol) (40 µg/ml), tetracyclin (Sigma) (10 µg/ml), spectinomycin (UpJohn) (100 µg/ml), kanamycin (Boeringher Mannheim Company) (50 µg/ml). They were prepared monthly and stored at 4^0c.

Strains were routinely grown in LB broth (Miller, 1972) plus the appropriate antibiotic(s). LB broth consisted of 10 g/l of bactotryptone, 5 g/l of yeast extract and 10 g /l of NaCl.

All bacterial strains used in this study are shown in Table 2. Phages and plasmids used in this study are shown in Table 3.

After characterization of the strain, an overnight culture in LB plus the appropriate antibiotic(s), was stored in 15 % glycerol, in small vials at -20^0C. Viable bacteria were recovered by a small inoculum (0.1 ml) of the glycerol cell suspension. Bacterial cells were also kept in stab cultures containing 3 ml of LB agar. The presence of a plasmid, in the harboring strain, was checked by antibiotic resistance of the strain, and the DNA content of the strain by "cracking" the cells harboring the plasmids on an electrophoresis agarose gel.

Table 3.

Bacterial strains	Genotype	References
DH1	F⁻,*recA1*,*endA1*,*gyrA96*,*thi*-1, *hsdR17*(r^-_k,m^-_k),*supE44*,λ^-.	Low, 1968.
HB101	F⁻,*hsdS20*(r-,m-),*recA13*,*ara*-14,*proA2*, *lacY1*,*galK2*,*rpsL20*(Sm^r),*xyl*-5,*mtl*-1, *supE44*.	Boliver and Backmann, 1968.
RR1	F⁻,*hsdS20*(r-,m-),*ara*-14,*proA2*, *lacY1*,*galK2*,*rpsL20*(Sm^r),*xyl*-5,*mtl*-1, *supE44*. λ^-	Boliver et al., 1977.
40	F⁻,Δ*pro*-*lac*,*rpsL*,*trp*,Sm^r	Bukhari and Metlay,1973
Bu5029	F⁻,Δ*pro*-*lac*,*rpsL*,*trp*,*recA*,Sm^r	Bukhari and Metlay,1973
FPL5014	F'*pro*-*lac*/Δ*pro*-*lac*, *SupE*,*nalA*,*rpsE*..	Miller,1972.
LF110	F'*pro*-*lac*/Δ*pro*-*lac*,*rpsL*,*trp*, *xyl*⁻::*Muc*ts62.	This study.

Bacterial strains	Genotype	References
LF113	F'pro-lac/Δpro-lac,rpsL,trp. xyl⁻::Mucts62pf7701Δ445-3.	This study.
LF1037	F'pro-lac/lacY1,hsdS20(r-,m-),recA13, ara-14,proA2,galK2,rpsL, xyl-5,mtl-1,supE44, λ⁻, mal⁻::Mucts62pf7701Δ445-3.	This study.
LF1040	F⁻,Δpro lac, rpsL,trp,Mu^r, rpsE,Spc^r	This study.
LF1004	F⁻,Δpro-lac,sulI⁺,thi⁻, Spec^r, Mu^r(character unstable).	This study.
HM8305	F'pro lacZ::Mucts62/Δpro lac,his, met, rpsL.	Bukhari et al., 1978.
LF249	F⁻,hsdS20(r-,m-),ara-14,proA2, lacY1,galK2,rpsL20(Sm^r),xyl-5,mtl-1, supE44, λ⁻,sor⁻::Mucts62.	This study.
CT152	gal,su,lac, rpsL^r,Kan^r, Mucts62pf7701Δ445-3.	Martha Howe

Table 3.

Phage and plasmid list.

Phages.	Description or phenotype	Reference or source.
Mucts62	thermoinducible.	M.Howe, 1973.
Mucts62pf7701 Δ445-3	thermoinducible, Kan^r(insertion of defective Tn5 between B and C gene	M.Howe.

Plasmids	Description or phenotype	Reference or Source
pMD861	pSC101::Mini-Mu bla, Tet^r,Amp^r.18.9 kb non-amplifiable.	Chaconas et al., 1981a.
pJK43	pBR322::1.0kb attL-1.2kb attR of Mu (ClaI-HindIII from pMD186), Amp^r.	J.S.Kahn

Plasmids	Description or phenotype	Reference or Source
pSC101	low copy number vector, non-amplifiable. Tet^r, 9.2 kb.	Cohen and Chang, 1977.
pBR322	ColE1, relaxed replicon, Tet^r, Amp^r, 4.3 kb.	Sutcliffe, 1979.
pUC71K	pUR der:: Kan gene from Tn903 flanked by polylinker sequences, Amp^r, Kan^r.	S.Brown.
pSAS1206	Sm^r, Sulf^r, amplifiable, 5.9 kb.	F.Shareck et al., 1983.
pBR322(BamHI$^-$)	pBR322 derived, BamHI^r, Amp^r, Tet^s.	This study.
PJo501	pSAS::bla (bla inserted at SstI)	This study.
pJo7	pSC101 PvuII site changed into SstI site.	This study.
pJoA	pJo7::bla (bla gene inserted at SstI) Tet^r, Amp^r, 9.9 kb.	This study.
pJo2A	pJoA derived, BamHI$^-$, Amp^r, Tet^s.	This work.

B- Phage lysates.

Phage lysates were prepared by thermo-induction of strains lysogenic for Mucts62 using the procedure of Ljungquist and Bukhari (1977). Cultures of lysogenic strains were grown in LB broth overnight at 32°C with aeration. The cultures were then diluted 1/25 in LB broth containing 10mM $CaCl_2$ and 1mM $MgSO_4$ and further incubated at 32°C until an A_{550}: 0.3-0.4. The cultures were then shifted to 43°C until lysis. Once lysis occured, a few drops of chloroform were added and incubation continued for 5 minutes. The cells were then collected at 4000 x g for 20 minutes at 4°C. The supernatant fluid was removed, several drops of chloroform were added, and the lysate was stored at 4°C.

C- Phage titration.

In order to titer the phage lysate, dilutions (10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}) of the lysate in 1X Mu buffer (Ljunquist and Bukhari, 1977) are prepared. 0.1 ml of each dilution was added to 2.5 ml of 0.5% soft agar (10g bactotryptone, 5 g yeast extract, 10 g NaCl, 5 g agar per liter) at 45°C containing 2 X 10^8 cells of Mu^S Escherichia coli, and poured immediately on a TCMG plate (10g BBL trypticase, 5 g NaCl, 8.5 g agar, 10 ml $MgSO_4$ per liter). The plates were incubated at 37°C for 16 hours. The phage titer was expressed by the number of plaque forming units per ml of the lysate (pfu/ml).

D- Plasmid transduction.

When a Mu prophage is induced in the presence of a Mini-Mu plasmid containing the pac site and a phage lysate is prepared, the Mini-Mu sequences are found packaged in Mu phage particles (Chaconas et al., 1981; Toussaint et al., 1979). Since the Mini-Mu plasmids are often too small to be packaged into Mu particles, they are attached to larger molecules as demanded by the headful packaging mode of Mu DNA (Bukhari and Taylor, 1975). The Mini-Mu containing phage particles can be injected into Mu^S cells upon infection and transductants containing Mini-Mu's that harbor an antibiotic resistance marker can be obtained. It should be noted that the transduction frequencies are relatively low in comparison to the total number of Mini-Mu particles present in the lysate (Chaconas et al., 1981) and these particles are unstable (our observations).

To test for the presence of the Mu pac site, recipient cells were infected with phages grown in pBR322::Micro-Mu Kan carrying strains. Phage adsorption was allowed for 15 minutes at 32^0C, or at room temperature, and the cells were then plated on LB kan agar plates and incubated overnight at 32^0C to allow the colony formation. Cotransduction of Kan^r and Amp^r markers (indication of plasmid cointegration) was examined by replica-plating the Kan^r transductants on LB amp agar plates (Figure 16).

The transduction frequencies were given as transductants per plaque forming units. The values were the average of at least three independent experiments.

Figure 16.

The transduct test.

The strain harbors oligomeric forms of plasmid::Micro-Mu Kan, in which lie the pac recognition site, as well as a thermoinducible Mucts62 prophage.

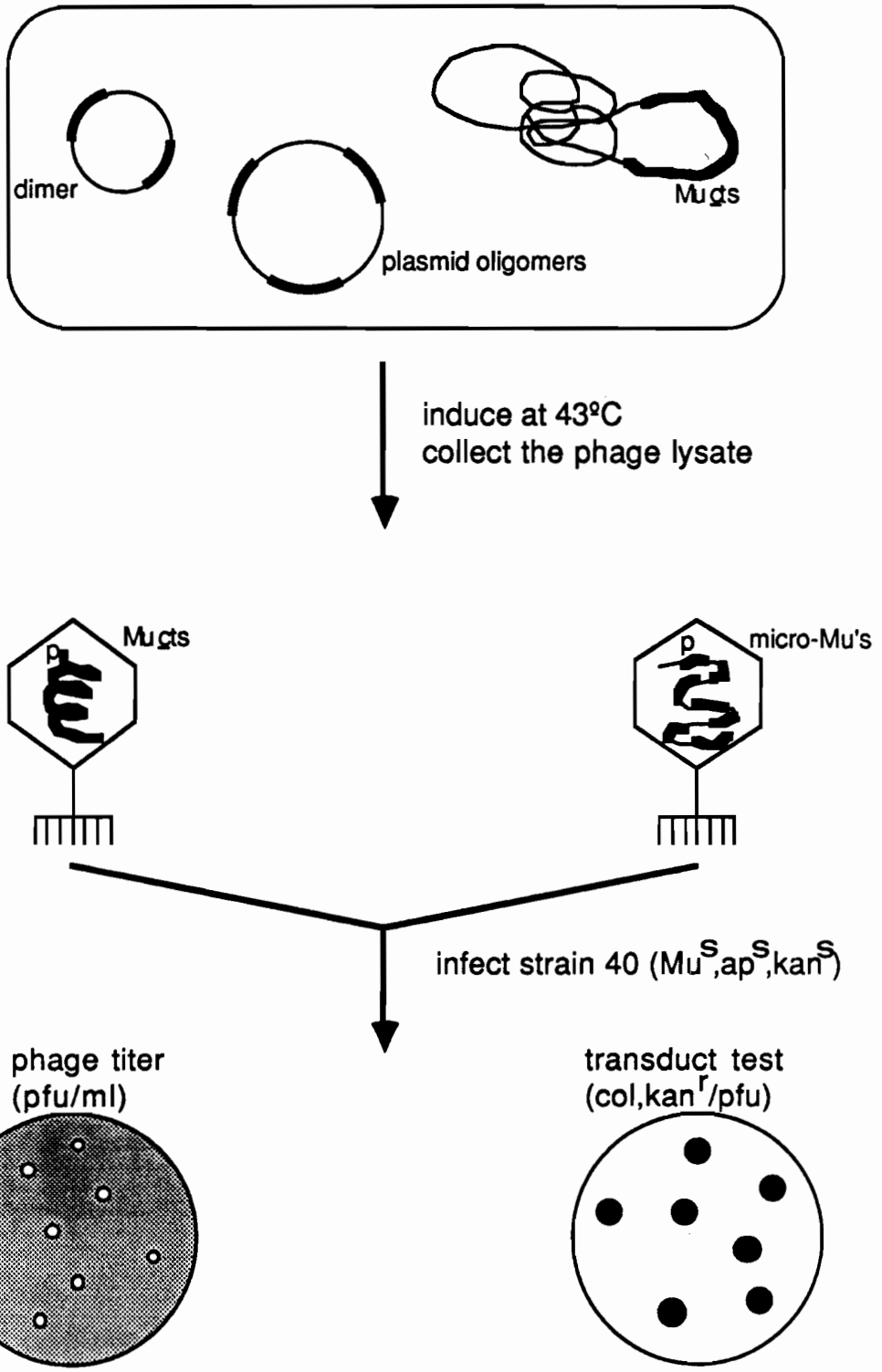
Upon thermoinduction, the phage will provide in trans the transposition function as well as the packaging system. to the plasmid containing Mu DNA

The lysate contains a mixture of packaged Mu DNA and plasmids::Micro-Mu Kan.

The lysate is titrated by infecting a Mu sensitive lawn of cells and estimated as plaque forming units per ml (pfu/ml).

The transduction efficiency of the plasmid::Micro-Mu Kan is evaluated as Kan^r colonies (that have received a plasmid::Micro-Mu Kan) per plaque forming unit per ml.

Transduct test



E- Assay of Mu immunity.

Strains were assayed for their immunity to superinfection by Mucts62 by the procedure of Tolias and DuBow, 1986. The strains were grown overnight in LB broth containing the appropriate antibiotics and 0.1 ml of the culture is added to 2.5 ml of 0.5% LB agar at 45^0C. The inoculated top agar was spread over an LB plate. Different dilutions of a Mucts62 phage lysates (10^{10}, 10^9, 10^8, 10^7, 10^6 pfu/ml) were spotted on the lawns and the plates were incubated at 37^0c for 16 hours (Chaconas et al., 1980). The level of immunity was defined by the final dilution that produces a cleared phage spot (plaque) on the lawn of cells. Immunity was arbitrarly chosen as the level of the phage dilution that no longer produces clearing on the lawn of cells.

F-Transposition assay.

Transposition of Mini-Mu or Micro-Mu DNA was monitored using a conjugation assay which measured Mini-Mu or Micro-Mu insertions into an F' pro$^+$lac$^+$episome.

Transposition of pSC101::Mini-Mu Amp and pSC101::Micro-Mu Kan genomes to an F'pro-lac episome was monitored by a conjugation assay during which the helper prophage Mucts62 lytic growth was thermoinduced at 43^0C(Chaconas et al., 1981). The recipient strain, LF1040, was Δ pro-lac, thi, rpsL, trp, Spc^r, Mu^r (Figure 17).

The donor strains were grown at 32^0C in LB broth containing 2.5mM $CaCl_2$ and 2.5mM $MgCl_2$ (plus 40 ug/ml ampicillin or 50 ug/ml kanamycin .

Figure 17.

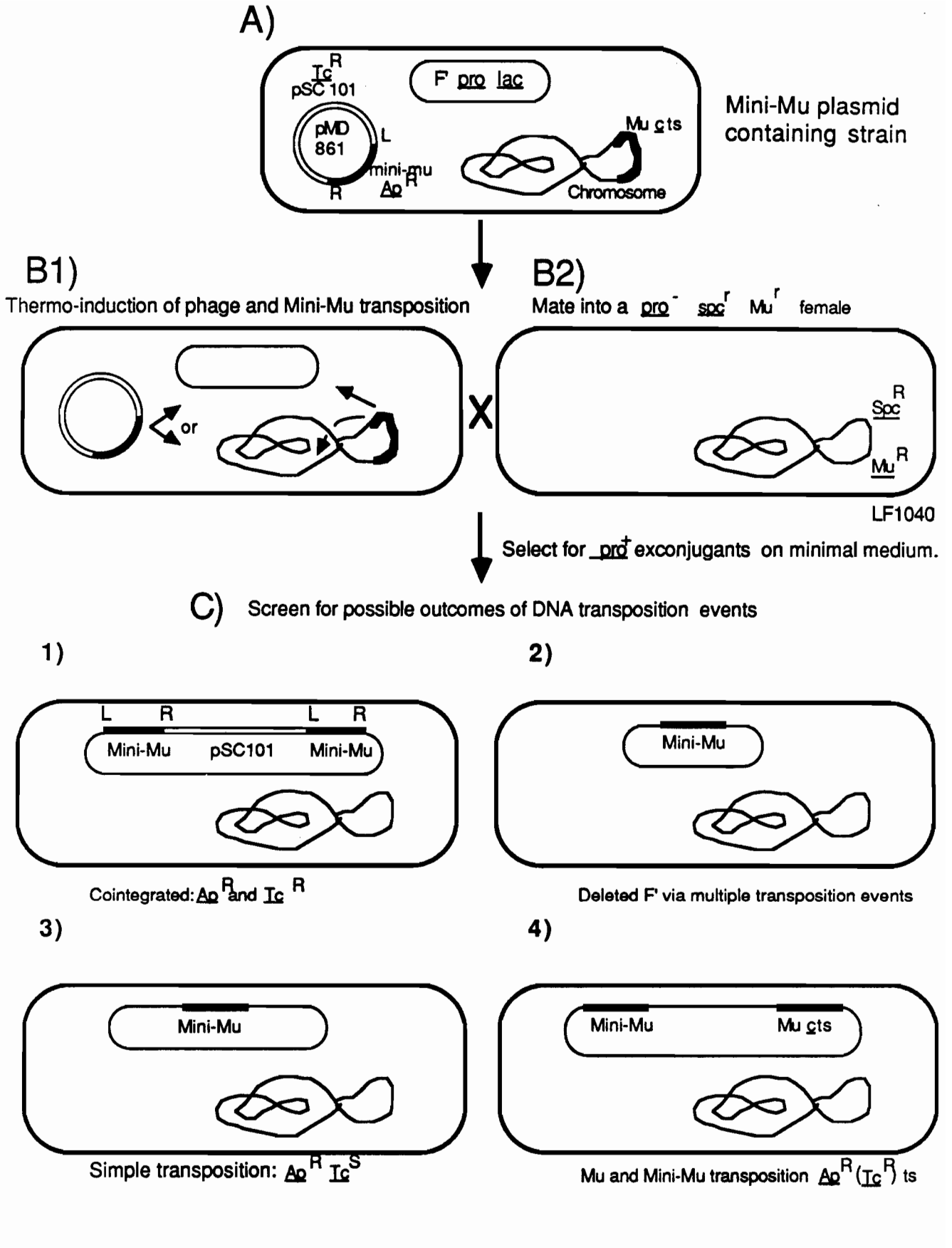
Transposition test.

A- The donor strain contains a pSC101::Mini-Mu Amp plasmid, an F'Δpro-lac conjugative plasmid and a thermoinducible helper prophage in the chromosome.

B1- Thermoinduction of the prophage in the donor strain allows expression of its transposition functions and its transposition. Upon provision of the transposition functions in trans, the Mini-Mu genome can transpose.

B2- The thermoinduced donor strain is mated into a pro^-, Spc^r, Mu^r female.

C- the pro^+, Spc^r exonjugants are screened for the Mini-Mu transposition events that have occured onto the F'Δpro-lac conjugation factor by analysis of the Amp^r outcome that are or are not Tet^r.


A)
Tc R
pSC 101
F' pro lac
pMD 861
L
mini-mu
Ap R
R
Mu cts
Chromosome
Mini-Mu plasmid containing strain
B1)
Thermo-induction of phage and Mini-Mu transposition
or
X
B2)
Mate into a pro - spc r Mu r female
Spc R
Mu R
LF1040
Select for pro + exconjugants on minimal medium.
C) Screen for possible outcomes of DNA transposition events
1)
L R L R
Mini-Mu pSC101 Mini-Mu
Cointegrated: Ap R and Tc R
2)
Mini-Mu
Deleted F' via multiple transposition events
3)
Mini-Mu
Simple transposition: Ap R Tc S
4)
Mini-Mu
Mu cts
Mu and Mini-Mu transposition Ap R (Tc R) ts

The recipient strains were grown to 3 X 10^8 cells per ml in LB (without spectinomycin).

Transposition was induced by shifting the exponentially growing donor culture to 43^0c for 20 minutes to induce Mu lytic growth. The mating, at a ratio of 10 females to 1 male, was incubated statically for 1 hour at 32^0c. After the mating, the cells were collected by centrifugation at 5000 x g for 10 minutes. The cells were washed once in sterile saline (0.9% w/v NaCl) and then resuspended in 2 ml of saline. 200 µl of various dilutions (10^{-5}, 10^{-6}, 10^{-7}) in saline were spread on minimal A glucose plates containing spectinomycin to select for Spc^r pro$^+$ exconjugants. Dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) were also spread on minimal A glucose plates containing the appropriate antibiotics to select for Spc^r Kan^r(or Amp^r) pro$^+$ exconjugants to measure transposed Mini-Mu's (or Micro-Mu's) onto the transferred the F'pro-lac episome.

The number of exconjugants was generally between 10^6 and 10^7 per/ml in this conjugation assay. The proportion of F' episomes containing Mini-Mu or Micro-Mu insertions was determined by dividing the number of Amp^r (or Kan^r), pro$^+$ exconjugants (episomes which contained Mini-Mu or Micro-Mu insertions) by the total number of pro$^+$ exconjugants (total number of transferred episomes). The transposition frequency was expressed as the percentage of Spc^r exconjugants that are Amp^r (or Kan^r).

The proportion of the transferred episomes containing Mini-Mu or Micro-Mu insertions which also contained the pSC101 plasmid donor molecule was determined by replica-plating the Amp^r or the Kan^r exconjugants onto plates containig 10 µg/ml tetracycline.

G- Rapid visualization of DNA in induced cultures.

The fate of pSC101::Mini-Mu or Micro-Mu plasmids during transposition is an indication of the Mini-Mu transposition behavior. Mini-Mu or Micro-Mu plasmid-association with the host chromosome during Mu lytic growth is an indication that PSC101 sequences are transposed via the Mu sequences of the Mini-Mu or Micro-Mu plasmid, presumably through cointegrate formation (Chaconas et al., 1980).

Rapid visualization of plasmid and maturing phage DNAs was performed by first growing a Mucts62 lysogenic strain containing a pSC101::Mini-Mu or Micro-Mu to early log phase, after which the cultures was transferred to a 42^{0}C water bath shaker. 1.0 ml aliquots were removed at 10 minute intervals, until complete lysis had occured. The aliquots were centrifuged for 5 minutes at 4^{0}C in a Brinkmann microcentrifuge. The supernatant fluid was removed, and the cell pellet was resuspended in lysis buffer (Chaconas et al., 1981), left at room temperature for 10 minutes to allow gentle lysis, and subjected to centrifugation for 10 minutes in a Brinkmann microcentrifuge at 4^{0}C. The supernatant fluid was subjected to electrophoresis on 0.35% horizontal agarose gels for 16 hours in Tris acetate buffer (Maniatis et al., 1982). The gels were run for 16 hours at 30 mA until the bromophenol blue dye had migrated about 15 cm from the origin. The total nucleic acids were visualized by staining the gels in 2.5 µg/ml ethidium bromide, followed by photography under 260 nm UV illumination. The gels were transferred to Genescreen (Dupont) membranes according to Southern (1977) and hybridized using pSC101 probe (Chaconas et al., 1980) labelled with alpha-^{32}P-dNTP's (Dupont or ICN) by nick translation (Rigby et al., 1977).

After washing, the blots were dried and exposed to X-ray film (Kodak XAR or Afga Curix RPI) under Dupont Cronex intensifying screens at -70^{0}C.

H- Transformation.

Transformations were carried out using a modified procedure described by Cohen et al. (1972). Cells were grown to a density of 1x10^8 cells/ml and were pelleted and resuspended in 1/2 volume of 10mM $MgSO_4^+$-2mMHepes, pH:7.5. Following incubation at 0^{0}C for 15 minutes the cells were pelleted by centrifugation at 3000 X g for 10 minutes at 4^{0}C and resuspended in 1/2 the volume of 10mM $CaCl_2$-2mM Hepes, pH:7.5 for 25 minutes. Following incubation, the cells were centrifuged and resuspended in 1/10 the original volume in 10mM $CaCl_2$-2mM Hepes, pH:7.5. At this step the cells were competent.

DNA was added to the competent cells and incubated at 4^{0}C for 30 minutes and then heatshocked for 30 minutes at 37^{0}C. 5 volumes of LB broth was added and the cells were incubated at 32^{0}C for 2 hours.

The transformation mixture (1ml) was spread onto LB plates containing the appropriate antibiotic(s), using 0.1-0.25 ml per plate. The plates were incubated at 32^{0}C for 24 hours. For cloning purposes, the strain DHI was used.

I- Large scale DNA preparation.

Large scale preparations of pSC101-derived plasmids were obtained by growing 2 liter cultures to saturation in LB broth and purifying the plasmid DNA using the cleared lysate procedure of Clewell and Helinski (1969). Overnight cultures of 20 ml with the appropriate antibiotic(s) were inoculated into 1 liter of LB broth with the appropriate antibiotic(s) in a 2.8 liter Fernbach flask and grown to an A_{550} of 2.0-2.5 in a shaker at 32^0C or 37^0C at 200 rpm.

Large scale preparations of pBR322-derived plasmids were done according to Maniatis et al. (1982). Overnight cultures of 20 ml were inoculated into 1 liter LB broth with the appropriate antibiotic (s) in a 2.8 liter Fernbach flask. Plasmid pBR322 and its derivatives were selectively amplified by the addition of chloramphenicol (50 µg/ml) to a logarithmically-growing culture.

The non-amplifiable plasmids as well as the amplifiable plasmids were collected in 1 liter plastic bottles by centrifugation at 8,000 X g for 20 minutes at 4^0C in a Sorvall RC3B centrifuge. The cells pellet was then washed with saline (0.9% NaCl) or PBS and recentrifuged at 10,000 X g for 15 minutes at 4^0C in a Beckman J2-21centrifuge in a JA-20 rotor.

Per liter of cells, the cell pellet was resuspended in 20 ml resuspension buffer (25% sucrose, 50mM Tris-HCl, pH:8.0, 40mM EDTA) at 4^0C. Subsequently the lysate was treated with 2.0 ml of lysozyme (BMC) (10 mg/ml in 10mM Tris-HCl, pH:8.0) and 2.0 ml of RNAaseA (Sigma)(1 mg/ml in T.E.) for 10 minutes at 4^0C. This treatment was followed by the addition of 8 ml of an EDTA solution (0.5M, pH:8.0). The lysate was then incubated with 2.0 ml of pronase (Sigma) (20 mg/ml of 10mM

Tris-HCl,pH:7.5) for 15 minutes. Lysis buffer was added (50mM Tris-HCL, pH:7.8, 0.05M EDTA, 0.3% (v/v) Triton X-100) and the tubes were gently mixed to lyse the spheroplasts. These series of incubations were done on ice at 4^0C. The RNAseA had been incubated at 100^0C for 10 minutes to inactivate any DNAase activity and the pronase had been autodigested at 37^0C for 2 hours.

The lysate was subjected to centrifugation at 30,000 X g in the J2-21 Beckman centrifuge for 1 hour at 4^0C. The supernatant fluid was removed and the volume was measured. If the volume exceeded 20 ml, the supernatant was isopropanol precipitated with a half volume of cold isopropanol at 0^0C for 2 hours. The mixture was centrifuged in a Beckman J2-21 at 25,000 xg for 30 minutes at 4^0C and the DNA pellet was resuspended in 7.5 ml of 1X TE (10 mM Tris-HCl, pH:7.5, 1mM EDTA). The DNA solution was mixed with CsCl (7.5 g/8 ml of solution) and ethidium bromide (0.3 ml of a 5 mg/ml /8 ml of DNA solution). 12 ml polyallomer heat-sealable tubes were filled with this solution. The density gradients were obtained by centrifugation at 39,000 rpm, at18^0C, for 48 hours in a type 40 rotor in an L8-70 Beckman ultracentrifuge.

The DNA bands were visualized by ultraviolet light illumination (254 nm) and the lower (plasmid-containing) band was collected through a 21" gauge needle. The ethidium bromide was removed by 4 extractions with equal volumes of isopropanol saturated with 40X SSC (6M NaCl, 0.6M sodium citrate, pH:7.6).

The DNA was dialysed against 1X TE at 4^0C, for 48 hour with a few changes of 1X TE buffer. The DNA could then be concentrated by ethanol precipitation if necessary and resuspended in 1X TE and stored at 4^0C.

J- Rapid plasmid isolation.

Rapid plasmid isolation were performed according to the procedure of Holmes and Quigley (1981). The cells were grown in 10 ml of LB with the appropriate antibiotic(s) overnight. The cells were centrifuged at 15,000 X g for 10 minutes at 4^0C. The cells were then resuspended in 100 µl of 25% (w/v) sucrose in 10mM Tris-HCl, pH:8.0 and incubated for 10 minutes. 50 µl of lysozyme (10 mg/ml, prepared fresh in 10mM Tris-HCl, pH:8.0) is then added to the mixture for 20 minutes. Then 600 µl of lysis buffer (50mM Tris-HCl, pH:7.5, 50mM EDTA, 1% (v/v) Triton X-100, 5% (w/v) sucrose) was added for 10 minutes. These incubations were done at 0^0C. After boiling for 1 minute and returning to ice for 2 minutes, the viscous mixture was then centrifuged in a Brinkmann microcentrifuge for 15 minutes at 4^0C.

The supernatant fluid was collected and treated with previously boiled RNAaseA (Sigma) for 20 minutes at 4^0C. The supernatant fluid was then phenol extracted, chloroform isoamyl-alcohol (24:1) extracted, and ether extracted. An equal volume of cold isopropanol was added and after 15 minutes at -70^0C, the DNA was centrifuged in a Brinkmann microfuge for 15 minutes at 4^0C. The DNA precipitate was dried under vacuum and then resuspended in 100 µl of 1X TE (10mM Tris-HCl, pH:7.5, 1mM EDTA) and kept at 4^0C.

K- Restriction endonuclease digestion and enzyme conditions.

All restriction enzymes were purchased from Boehringer Mannheim Canada Ltd. (BMC) or Bethesda Research Laboratories (BRL) Inc. T4 DNA ligase, T4 polynucleotide kinase and the large fragment (Klenow) of DNA polymerase I were purchased from Pharmacia P-L Biochemicals. Bacterial alkaline phosphatase, E.coli DNA polymerase I, and Exonuclease III were obtained from BRL. Calf intestinal alkaline phosphatase was obtained from NEN.

All restriction endonuclease hydrolyses were performed at 37^0C in 6mM Tris-HCl, pH:7.5, 6mM $MgCl_2$, 6mM 2-mercaptoethanol, 75mM NaCl, 125 µg/ml Bovine serum albumin (Pentex fraction V, Miles). However ,the reactions using SmaI, ThaI, Sau3A and TaqI were performed according to the manufacturer's specifications.

Nuclease S1 reactions were performed at 45^0C in 60mM sodium acetate, pH:4.6, 100mM NaCl, 2mM $ZnCl_2$.

L- Ligations.

The ligations were carried out on DNA which had been hydrolysed with a restriction endonuclease(s) and subsequently phenol, choroform isoamylalcohol (24:1), ether extracted and then ethanol precipitated. Ligations were performed with T4 DNA ligase (Pharmacia P-L Biochemicals) in a buffer consisting of 60 mM Tris-HCl, pH:7.4, 10mM $MgCl_2$, 15 mM dithiothreitol, 1mM spermidine, 0.75 mM ATP, 50 µg/ml

autoclaved gelatin at 15^{0}C, for 16 hours. The DNA concentration in the reactions varied depending upon the desired end-products as described by Dugaiczk et al. (1975).

M- Extraction of DNA.

An equal volume of distilled phenol equilibrated with 1X TE (10 mM Tris-HCl, pH:7.6, 1 mM EDTA) was added to the solution of DNA. After vortexing the mixture and centrifugation in a Brinkmann microcentrifuge for 5 minutes, the aquous phase was then reextracted with an equal volume of chloroform-isoamyl alcohol (24:1).

After centrifugation, the upper layer was mixed with ether. Then, 1/10 volume of ammonium acetate (2.5 M, pH:8.0) or sodium acetate (2.5 M, pH:8.0) was added. 2.5 volumes of absolute ethanol was then added to the solution and then incubated at -70^{0}C for at least 15 minutes, in a dry-ice ethanol bath or -20^{0}C overnight. The DNA was centrifuged in a Brinkmann microcentrifuge for 15 minutes at 4^{0}C. The DNA pellet was dried by dessication. The DNA was then resuspended in 1X TE and stored at 4^{0}C or at -20^{0}C.

Extraction of total Escherichia coli DNA. Logarithmically growing cells were havested at 10,000 X g for 10 minutes at 4^{0}C and resuspended in 5 ml of 10mM Tris HCl, pH: 8.0, 1mM EDTA, 0.5% SDS and treated with 0.5 ml of pronase (10 mg/ml, predigested) at 37^{0}C for 4 hours. The DNA solution was then extracted twice with phenol, twice with chloroform isoamyl alcohol and ether extracted. The DNA solution was then dialysed

against 1 X TE for 24 hours. The DNA was treated with RNAaseA (1mg/ml, previously boiled) at 37^{0}C for 2 hours. The DNA solution was then phenol extracted twice and ether extracted. A final step includes dialysis against 1 X TE. The purified chromosomal DNA was kept at 4^{0}C.

N- Gel electrophoresis.

N-1. Agarose gels.

Horizontal agarose gel electrophoresis of DNA was carried out on slab gels in 1X E buffer (40mM Tris, 20mM acetic acid, 2mM EDTA, pH:8.1). Loading dye (25% (w/v) sucrose, 1 X E buffer, 2.5 ug/ml bromophenol blue) was added to DNA samples to be loaded onto the gel.

DNA bands were visualized by staining the gels with ethidium bromide (2.5 µg/ml). The gels were photographed under shortwavelenght UV light with a Polaroid camera, using type 57 polaroid film. The DNA fragment size was calculated using molecular weight standards.

N-2. Acrylamide gels.

A solution of 40 % acrylamide-bis acrylamide was diluted to the needed concentration with 1X TBE buffer (0.16M Tris-HCl, pH:8.0, 0.16 M boric acid, 20mM EDTA). The solution was deaerated under vacuum for 10 minutes. Ammonium persulfate and a solution of TEMED were added for a final concentration of 0.04% and 0.01% respectively before pouring the gel between glass plates of 20X20X1.5 cm. The gel was run at 40mA.

N-3. DNA extraction from gels.

N-3.a. From agarose gels:

DNA fragments were purified from agarose gels by electroelution into dialysis bags (Maniatis et al., 1982). The DNA band on an agarose gel was stained with ethidium bromide (2.5 µg/ml) and was visualised by 254 nm UV light illumination. The DNA band of interest was excised from the gel with a razor blade and put into a dialysis bag with1X E electrophoresis buffer previously washed with 50 µg/ml BSA in 1X E buffer. The dialysis bag was subjected to 100 volts for 2-3 hours. The electrical current was then reversed for 2 minutes in order to detach the DNA from the dialysis bag. The DNA was recuperated by phenol, chloroform-isoamylalcohol extraction of the buffer within the dialysis bag and then ethanol precipitated.

N-3.b. From acrylamide gels:

The DNA band was visualised after staining the acrylamide gel with ethidium bromide followed by detection with UV light, or by autoradiography, if the fragment was radioactive. The band was excised from the gel with a razor blade and then cut into little pieces. The pieces were soaked into a solution of 500mM ammonium acetate, 10mM magnesium acetate, 1mM EDTA, 0.1% SDS, and then incubated at 37^0C for 20 hours to allow the DNA to diffuse from the gel. The DNA was recuperated from the mixture by passage on a column of siliconized fiberglass. The DNA was then phenol, chloroform-isoamyalcohol extracted

and ethanol precipitated. The DNA pellet was washed with 70% ethanol (Maxam and Gilbert, 1980).

O. Cracking cells.

This simple technique of "cracking" cells allows one to visualize plasmids and episomes by cell lysis and subsequent gel electrophoresis using a modification of the method of Barnes (1977).

From a Master petri dish containing single colonies, 0.5cm^2 patches were scraped off with a toothpick. The cells were resuspended in 75 µl of cracking buffer (50mM Tris-HCl, pH:8.0, 1.0% SDS, 2mM EDTA, 0.4M sucrose and 0.01% bromophenol blue) in 1.5 ml Eppendorf conical tubes (Chaconas et al., 1981). The cells were incubated in this solution for 10 minutes at room temperature. The lysate was then spun in a Brinkmann microcentrifuge for 15 minutes and the supernatant fluid was removed and loaded directly onto 0.35% (pSC101-derivatives) or 0.6% (pBR322-derivatives) horizontal agarose electrophoresis gels run in 1X E buffer and subjected to electrophoresis as above.

P- Linkers.

The source of synthetic linkers was Collaborative Research (Waltham, Mass.). Phosphorylated linkers were ligated to purified DNA fragments having blunt ends dephosphorylated by calf intestinal alkaline phosphatase. 5 µg of DNA was incubated with 3 units of calf intestinal alkaline

phosphatase (NEN) in 25 µl of 10mM Tris-HCl, pH:8.0, for 30 minutes at 60^0C.

The linkers (0.5µg) were kinased with T4 polynucleotide kinase (10 units) in 0.07M Tris-HCl, pH:7.5, 0.01M $MgCl_2$, 5mM dithiothreitol and 20 µCi of γ-^{32}P ATP (ICN) at 37^0C for 15 minutes, followed by a chase with unlabelled ATP (Maniatis et al., 1982). The ligation on a fraction of the phosphorylated linkers, and the enzymatic endonuclease digestion on a sample of this fraction, were performed to verify the efficiency of the phosphorylation reaction. The reactions were checked by autoradiography of a polyacrylamide gel.

Phosphorylated linkers were ligated to the DNA in 25 µl of 1X ligation solution and 10 units of T4 ligase are added. The ligation reaction was stopped after 16 hours incubation at 15^0C. The ligated material was then subjected to the appropriate restriction endonuclease digestion to eliminate the extra linkers and leave a single restriction enzyme site at the end of the fragment. The DNA was then phenol extracted, ether extracted.

Q- Southern blotting and hybridization.

Q-1. Gel preparation.

After agarose electrophoresis and gel staining, the DNA was denatured in a solution of 0.4M NaOH, 1.0M NaCl for 30 minutes and neutralized with a solution of 0.7M Tris-HCl, pH:7.5, 2M NaCl for 30 minutes. The gel was then placed onto Whatman 3MM paper soaked in a

solution of 20X SSC (1X SSC: 0.15M NaCl, 0.015M sodium citrate) and allowed to transfer onto a presoaked Genescreen sheet (NEN) overnight. The Genescreen filter was then rinsed in 20 X SSC and baked for 3-4 hours at 80^{0}C under vacuum, to fix the DNA to the filter.

The Genescreen filter was incubated in a prehybridization solution at 40^{0}C overnight. The prehybridization solution contained: 5 X Denhardt solution [1 X Denhart is composed of 0.02% polyvinylpyrollidone, 0.02% BSA (Pentex fraction V)] plus 50 % (V/V) deionised formamide, 50 µg/ml calf thymus DNA, 5 X SSC). The prehybridization solution was then replaced by the hybridization solution (containing: 1 X Denhart solution plus 50 % (V/V) deionised formamide, 50 µg/ml calf thymus DNA, 5X SSC, 0.5% SDS, 25µM ATP,1 mM EDTA) containing 2 to 4 X10^6 cpm of the ^{32}P-labelled probe. The ^{32}P labelled probe was denatured for 5 minutes at 100^{0}C, rapidly cooled, and then added to the hybridization solution. The hybridization reaction was incubated at 40^{0}C for 16 hours.

The filter was washed with a solution of 0.5X SSC and 0.2 % SDS twice for 30 minutes at room temperature. The filter was then dried at room temperature and exposed at -70^{0}C with a Kodak R-P X Omat or Afga Curix RPI film using Dupont Cronex "lighting plus" screens.

Q-2. Probe preparation by nick-translation.

1 µg of DNA was added to a solution of 0.05M Tris HCl, pH:7.5, 5mM $MgCl_2$, 10mM beta-mercaptoethanol, 25 µg/ml BSA, 0.005mM of 3 dNTP's. 25 µci of α-^{32}P dNTP (3 000 µci/mmole) (ICN). 1/10 volume of freshly prepared DNAase I (100 ng/ml) was added to the solution and incubated for 10 minutes at 43^{0}C and 1 unit of DNA polymerase I (BMC) was then added.

The one hour incubation at 15^0C was stopped by phenol extraction. The separation between labelled DNA and unincorporated nucleotides was done through a Sephadex G-50 column. The fractions containing the radiolabelled DNA were then pooled and stored at 4^0C.

R- Labelling of the DNA extremities.

R-1. With DNA polymerase I Klenow fragment.

To label the 3' end of the DNA, the Klenow fragment of DNA polymerase I (P.L. Pharmacia) was used to backfill 3' recessed ends generated by restriction enzymes with radiolabelled nucleotides. The DNA (0.1 to 1 µg) was mixed with a solution of 0.05M potassium phosphate, 5mM magnesium chloride, 0.01M dithiothreitol, 0.05mM dNTP's plus the appropriate 15 µci α-^{32}P dNTP (3000 Ci/mmole) (ICN) and 1 unit of the DNA polymerase Klenow fragment was added. After incubation for 30 minutes at 20^0C, the reaction was stopped with 0.025M Tris-HCl, pH:7.5, 0.01M EDTA, 0.3M sodium acetate. The DNA was phenol and ether extracted and ethanol precipitated.

Mutagenesis by DNA polymerase I Klenow fragment backfilling was done in 70mM Tris HCl, pH:7.6, 70mM $MgCl_2$, 500mM NaCl and 10mM DTT at 20ºC for 30 minutes.

R-2. With T4 polynucleotide kinase.

The DNA was first dephosphorylated with 1 unit of bacterial alkaline phosphatase (BMC) in 10mM Tris-HCl, pH:8.0, at 37^{0}C for 1 hour (Maniatis et al., 1982). The DNA was then phenol extracted twice, chloroform-isoamylalcohol and ether extracted, then ethanol precipitated.

The DNA was then labelled at its 5' ends with T4 polynucleotide kinase (Pharmacia P.L. inc). Using 150 µCi of γ ^{32}P ATP (5000 Ci/mmole) (Amersham) as well as 2 units of T4 polynucleotide kinase (Maniatis et al., 1982). The reaction was allowed to proceed for one hour at 20^{0}C. The reaction was stopped with 0.01M EDTA and the DNA was phenol and ether extracted and ethanol precipitated.

S- Exonuclease III.

Exonuclease III (Linxweiler and Horz, 1982) had become an important tool on research on DNA and DNA-protein interactions mostly because of its ability to hydrolyse sequences of DNA at a fairly constant and predictable rate.

To generate deletions in DNA, linearized DNA was treated with exonuclease III and S1. The exonuclease III reaction was performed in 50mM Tris-HCL, pH:8.0, 2mM $MgCl_2$, 10mM 2-mercaptoethanol (Smith, 1979).

S1 Nuclease digestions following the exonuclease treatment (to remove the single-stranded DNA generated by the exonuclease digestion) were performed at 45^{0}C in 60mM sodium acetate, pH:4.6, 100mM NaCl, 2mM $ZnCl_2$.

Figure 18.

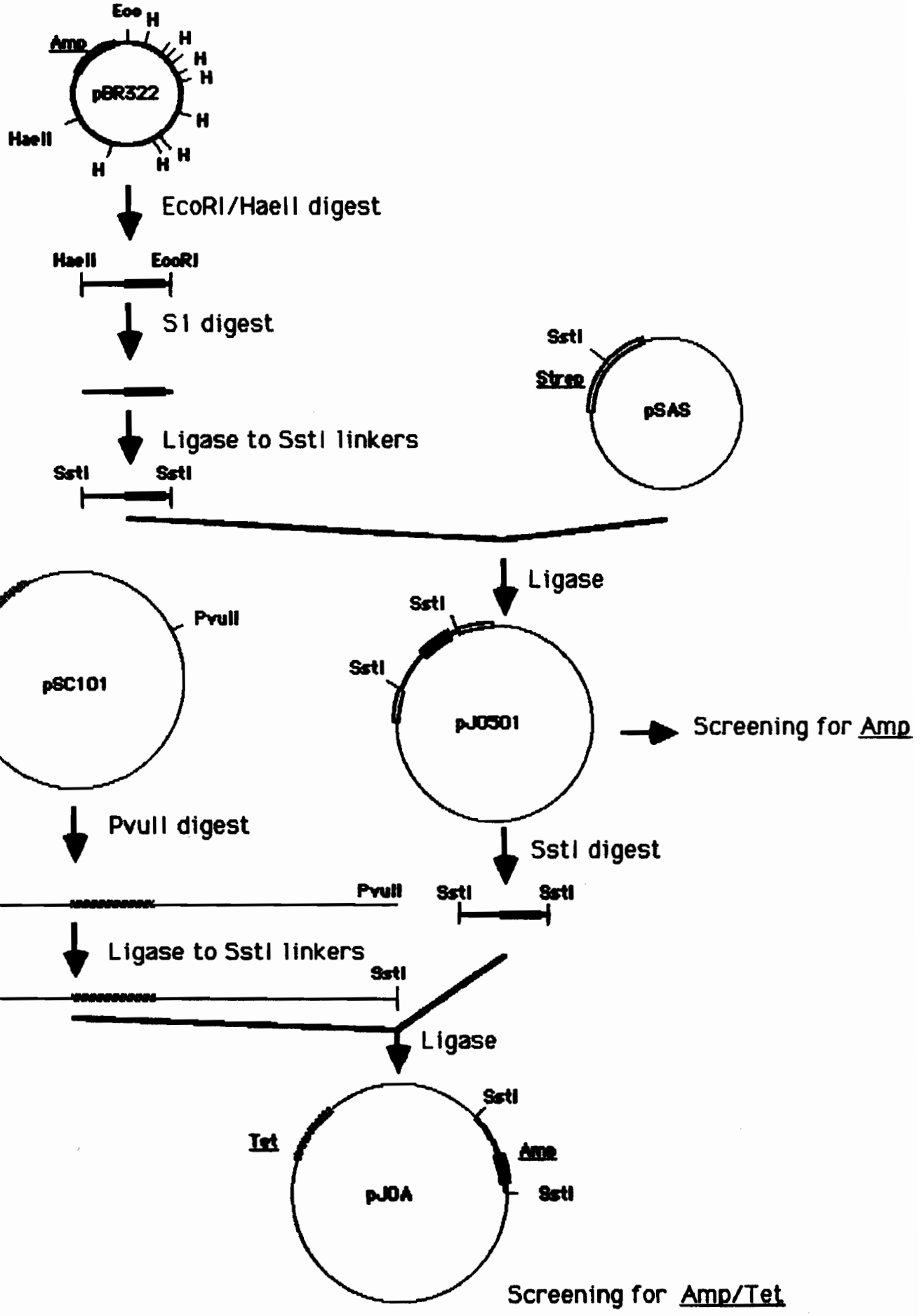
Construction of pJoAmp.

The HaeII fragment from pBR322, containing the ampicillin resistance determinant, was blunt ended by SI nuclease treatment. SstI linkers were added to the HaeII fragment.

The Amp fragment was then inserted into the SstI site of pSAS1206. Transformants were screened for their ampicillin and streptomycin resistances.

The Sst1 fragment of pJo501 containing the Amp gene was then inserted into pSC101 that has acquired SstI site by linker ligation in its former PvuII site. Transformants were screened for their ampicillin and tetracyclin resitance.

The plasmid pJoAmp is a pSC101 derivative, now having an ampicillin determinant.


Eco H
Amp
pBR322
HaeII
EcoRI/HaeII digest
HaeII
EcoRI
S1 digest
Ligase to SstI linkers
SstI
SstI
SstI
Strep
pSAS
Ligase
Tet
PvuII
pSC101
SstI
SstI
pJ0501
Screening for Amp
PvuII digest
SstI digest
PvuII
PvuII
SstI
SstI
Ligase to SstI linkers
SstI
SstI
Ligase
SstI
Tet
Amp
pJDA
SstI
Screening for Amp/Tet

T- DNA sequencing.

DNA sequencing. was performed according to Maxam and Gilbert (1977).

U- Construction of pJoA (Figure 18).

We have created a pSC101derivative containing the bla gene from Tn3. The HaeII-EcoRI fragment of pBR322 containing the ampicillin gene was cloned into PSC101. This bla fragment was first cloned into the SstI site of plasmid pSAS1206 (Shareck and Sasarman, 1983). This was inserted into pSC101 at its former PvuII site that had been changed for an SstI site by linker addition.

The 1.5 kb HaeII-EcoRI fragment containing the bla gene of pBR322 was treated with S1 nuclease in order to trim the protruding single-strands from the restriction reaction. The blunt-end DNA was resuspended in 20µl of linker ligation buffer and 10 units of T4 ligase plus 0.5 µg of ^{32}P-labeeled SSTI linkers (Collaborative Research, Waltham, Mass.), previously phosphorylated using T4 polynucleotide kinase were added. The ligation reaction was allowed to proceed at 15^{0}C for 16 hours, terminated by incubation at 60^{0}C for 10 minutes, and then diluted to 100 ul with restriction endonuclease buffer. 100 units of Sst1 were added and the reaction to "trim" the linkers was incubated at 37^{0}C for four hours. The DNA was then phenol, Chloroform-isoamylalcohol (24:1) and ether extracted and ethanol precipitated.

The DNA was resuspended in 50 µl ligation buffer with pSAS1206 plasmid linearized at its SstI site. 10 units of T4 ligase was added and the

DNA was allowed to ligate at 15^0C for 16 hours. The reaction was terminated by incubation at 65^0C for 10 minutes. The DNA was transformed into DHI competent cells and the clones were selected for ampicillin resistance and streptomycin sensitivity. The selected clone was analysed by SstI and PstI digestion and named pJo7.

SstI linkers at the PvuII linearized pSC101 were added in the same manner described above. pSC101 containing a unique SstI site was screened for the loss of its PvuII site and the gain of an SstI site and was named pJo7.

10 µg of pSC101($PvuII^r$,$SstI^s$) plasmid was linearized with SSt1, phenol and ether extracted, and ethanol precipitated and resuspended in 10 µl of 10 mM Tris-HCl, pH:7.5, 1mM EDTA, and added to a reaction containing the SstI bla fragment along with 10 units of T4 ligase. The ligation was performed and terminated as above. The DNA was transformed into E.coli strain DHI. Ampicillin-resistant transformants were selected and subsequently screened for the presence of an 11 Kb plasmid. Potential candidates were then analyzed using several restriction endonucleases such as SstI flanking the Amp fragment and PstI within the Amp fragment, and plasmid pSC101::Amp (pJoA or pJo101) was isolated.

CHAPTER III. MODULATION OF MINI-MU TRANSPOSITION EXERTED BY NEIGHBORING SEQUENCES.

A- Introduction.

The DNA of bacteriophage Mu embodies the properties of both a transposable element and a phage. Its 37 kb linear, double stranded DNA is capable of catalysing and undergoing the full spectrum of DNA rearrangements characteristic of prokaryotic genetic elements (Toussaint and Resibois, 1983). Mu DNA transposition requires the presence of the Mu-encoded A and B gene products as well as enzymes encoded in the "semi-essential" early region of the phage plus many Escherichia coli enzymes (O'Day et al., 1978; Faelen et al., 1973; Waggoner et al., 1981; Goosen et al., 1982). These enzymes act upon sequences at or near the extremities in cis, in the proper orientation, in order to transpose the Mu genome. It has been previously demonstrated that the efficiency of a mobile element transposition can be influenced by sequences adjacent to them (Machida et al., 1982; Weinert et al., 1983; Chandler et al., 1983).

We have been studying the cis-acting DNA sequences which affect DNA transposition using the plasmid pMD861 (Chaconas et al., 1981a), a derivative of pSC101 carrying an internally deleted Mu prophage called Mini-Mu. This Mini-Mu plasmid contains all the necessary DNA sequences required for DNA transposition but requires the transposition proteins from an induced helper prophage in order to transpose (Chaconas et al., 1981a; Chaconas et al., 1980). We report here that alterations in the neighboring plasmid sequences near the Mini-Mu extremities exert an influence in the frequency of transposition of the Mini-Mu DNA when the helper prophage contains a polar insertion in its semi-essential early

region. This region of the Mu genome is expressed early during the Mu lytic cycle along with the A and B genes required for DNA transposition and encodes for several functions that may affect DNA transposition (Waggoner et al., 1981; Goosen et al., 1982; Waggoner et al., 1984). It is possible that one or several of these functions may act on the adjacent sequences to allow maximal Mu DNA transposition from virtually any location during the short lytic cycle.

B- Cis requirement and cis influence of adjacent sequences on transposition, gene regulation, transcription and replication.

B-1 Influence of adjacent DNA on transposition.

The DNA sequences in cis required for transposition are not solely dependent on the DNA regions at the ends of mobile elements. The DNA environment i.e base composition, DNA homology, DNA topology, DNA orientation at the site of insertion as well as at the target site may influence the transposition of the transposable element. Thus, the sequences at the termini of transposable elements are not the sole consideration in a discussion on cis requirement for transposition:

a) Sequence homology of segments inside the transposable element with the target DNA segments, may serve to align the element with its target site during integration.

b) The presence of the A-T plastic sites and GC anchors may affect the orientation of the element in a cis fashion by influencing the

transposition requirement (Tu and Cohen, 1980).

c) A preferred orientation may expose the DNA to a more pliable section of the element.

d) Defects in supercoiling DNA affect DNA transposition.

B-1.a) Host target specificity.

Host site specificity has been discussed in chapter I, section C. The general concept is that some elements, like Tn10, show hot-spot specificity, while others, such as Tn3, show regional specificity. Partial homology between the target DNA and the transposable element ends may be responsible for the insertion specificity. Many transposable elements integrate preferentially into A+T rich region.

Mu shows relaxed target host sequence preference. This can be attributed to its large size that may permit a greater flexibility of the molecule i.e. the flexibility in a long chain of nucleotides is greater than that of that small chain (Benham, 1982). This can also be one of the reason why Mu has a high frequency of transposition.

B-1.b) DNA plasticity.

A-T bases at the target site can confer elasticity on the molecule and lower energy requirement for insertion (due to a lower number of hydrogen bonds that must be broken for insertion to occur). A-T rich regions can be important in the regulation of transposition because, as in

transcription, it can provide thermolabile regions and thus facilitate the melting of the DNA duplex and enzyme entry. It can also provide transient single-stranded regions (bubbles) which, under the torsional strength of supercoiling, are recognized by the transposition enzyme (s). Breathing of the two DNA strands as a result of the relative "looseness" of AT base pairing may influence the specifity of insertion.

B-1.c) Spatial orientation of the transposable element.

Spatial orientation of the sequences in relation to each other and to the host chromosome are important cis factors. Tn4 insertions often occur with the same pattern. The inverted repeat (IR L) left was brought into close proximity with homologous DNA sequence of the target, and thus a preferred orientation of the element was observed (Hyde and Tu, 1982).

Sites and orientation of insertion of Tn3 are at least partly determined by the primary nucleotide sequences of the recipient genome. This suggests that insertional specificity may result from the combined effects of AT richness plus homology of the recipient genome with the terminal sequences of Tn3 (Tu and Cohen, 1980). Base pairing of the Tn3 terminus with homologous sequences would hold together the two region of DNA to be recombined. The occurence of A-T rich sequences at the vicinity of the target would facilitate strand displacement by allowing the two DNA strands to separate more easily.

B-1.d) DNA topology and transposition.

A deficiency in DNA supercoiling caused a defect in Tn*5* transposition. The absence of supercoils caused an apparent deficiency in single-stranded DNA. It is suggested that single strands provided by supercoiled DNA may be necessary for strand invasion during transposition (Isberg et al., 1982). The host mutations in *top*, *gyrA*, *gyrB* and *polA* are the best characterized mutations affecting transposable elements.

Inverted repeat sequences of transposable elements may be sites of precisely regulated and delicately interacting cruciform structures (Benham, 1982). It is proposed that cruciform structures are formed by intrastrand base pairing and consists of two arms. It can be formed at suceptible sites when the DNA is negatively coiled.

Substrate superhelicity is known to influence activities involved in DNA replication, recombination, transcription and repair (Wang, 1983). It is well documented that, *in vivo*, if DNA gyrase activity is blocked, chromosomal replication and some forms of recombination are halted and the rate of transcription from some promoters are also altered. This control may be exerted through the creation of local sites of altered secondary structures (Benham,1982).

B-2. Effect of transcriptional readthrough on transposition.

The two IS*1* elements in Tn*9*, designated IS*1*L and IS*1*R, are functionally different. One IS*1* element is ten fold more active than the

other.It was shown that the inactivity of IS1R is not due to an altered sequence but due to inhibition of transcription from the chloramphenicol acetyl transferase (CAT) gene promoter encoded in the internal part of the Tn9.

Readthrough message transcribed from a gene into an IS1 sequence inhibits its ability to mediate plasmid cointegration. The removal of the promoter region of the CAT gene in transposon Tn9 or the introduction of the terminator of the phage T7 transcription downstream of the CAT gene increase the cointegrate ability of the downstream IS1. Analysis of transcripts synthesized in vitro demonstrated that the CAT gene transcripts are readthrough into the IS1 located downstream in either orientation. The transposition activity is inversely correlated with the strength of upstream promoters in the vector molecule (Biel et al., 1984).

Transposition may require secondary structures formed by IS1 to facilitate the molecular processes involving the two ends of the IS1 at the target site. Transcriptional readthrough into an IS1 may cause structural and topological alterations inside the IS sequences and around the terminal inverted repeat region, unwinding their DNA strand. These alterations can result in the disturbance of molecular events which may include binding of the IS1 transposase at its termini.

Transposition of IS50 from a site is also inhibited by transcription impinging on it from nearby promoters (Sasakawa et al., 1982). The transpositional movement of IS50 and IS1 are inhibited by transcription from outside promoters to the same extent despite their lack of homology and apparent basic differences in transposition mechanism. IS50, unlike

IS*1*, does not generate transpositional cointegrates and can move without replication. Transcriptional inhibition is thus due to interference with either the synthesis or the action of the transposase.

Transcription could interfere with transposase action. The passage of an RNA polymerase molecule might dislodge transposase complexes during their assembly on a recogntion site. Alternatively, the disruption of DNA structure by transcription might obscure the transposase binding site.

Tn*10* is thought to protect itself from fortuitous activation by external promoters by adopting a secondary RNA structure that could prevent translation of inappropriate readtrough messages initiated from outside the element. The outside ends of IS*10* elements contains symmetries such that a transcript entering IS*10* from the outside can form a stable secondary structure where the initiation codon ATG could be sequestered (Kleckner et al., 1984).

B-3. Influence of adjacent sequences on transposition.

The *uup* mutation was found to increase precise excision of Tn*5* (Hopkins et al., 1983). The *uup* mutation affects different insertions to different degrees. Precise excision of a *cys*::Tn*5* insertion was stimulated 625 fold whereas a *pyr*::Tn*5* insertion showed only a 20 fold stimulation. One explanation is that the gene product involved in excision recognizes nucleotide sequences adjacent to the transposon and has different affinities for different sequences. However, using the same *lacZ*::Tn*5* residing in the chromosome and on the F factor, it was found that the

chromosome excision is 200 fold, and in the episome, was only 5 fold. Thus, specific nucleotide sequences are not the sole influencial criteria for the modulation of excision, chromosomal structure such as folding or the movement of the replication fork might also be involved.

IS1 transposition activity is modulated by the transcription activity of adjacent sequences in the donor plasmid. Transcription directed into an IS1 inhibits its activity. Deletion of adjacent promoters relieves the inhibitory effect on IS1 cointegrate activity (Ahmed, 1983; Chandler and Galas, 1983). Chandler and Galas (1983) have proposed that additional factors associated with the nucleotide sequences surrounding the element also can influence the transposition activity of the element. They raise the possibility that A-T base pairs in the neighborhood of the element may modulate its activity.

They argued that since Tn9 and IS1 are known to prefer A-T rich regions as sites of insertion, that the A-T rich region could play a role as a preferred donor site in transposition. In such cases, the A-T density could exert its effect through alteration of the DNA conformation near the transposable element as was invoked in one of the explanations on the negative transcriptional effect on transposition.

Sequences between the left end of IS903 and the EcoRI site of pBR322 affect IS903 transposition (Weinert et al., 1983; Grindley and Joyce, 1980). In regard to the possibility that adjacent DNA sequences could affect the accessibility of the recogntion sites within the transposon to its own transposase, Weinert et al., (1983) observed that sequences to the left of IS903 exerted a strong influence on its transpositional activity.

B-4. Effect of neighboring sequences on DNA expression and regulation.

A transposon, gamma-delta, was shown to alter the expression of the cloned gene ebgA (evolved beta-galactosidase) activity through a mechanism that involves neither insertional inactivity nor promoter occlusion but may alter the local supercoiling in the region of the ebgA promoter is such a way as to inhibit its transcription (Stokes and Hall,1983). This acts at a distance to reduce expression, and requires the element gamma-delta in cis with respect to the ebg gene. The effect is independent of the orientation of the position of gamma-delta but depends upon the orientation of the ebgA gene. This effect is suggested to be topologically mediated. The ebgA gene may thus be more suceptible to local changes in DNA supercoiling.

The cryptic bgl operon can be activated by insertion of either insertion sequences IS1 or IS5 upstream from it. The authors proposed that the IS elements alter the local supercoiling since neither of these elements is known to contain a promoter sequence (Dinardo et al., 1984).

Several insertion elements have been shown to prevent expression of the bop gene of Halobacterium halobium when the element was inserted as much as 1.4 kb from the bop gene.

The nic-bom cis-acting sequence in pBR322 was shown to reduce the ability of the hybrid plasmid pBR322-SV40 (Simian Virus) to retransform Escherichia coli recovered from transfected simian cells with an

SV40-pBR322 plasmid (Lusky and Botchan, 1981). The modification of the nic-bom site of SV40-pBR322 may occur in the simian cells during transfection thus influencing the establishment of the chimeric DNA in bacteria.

In a case of gamma-beta thalassemia, it was found that the inactivation of the beta-globin gene was due to a long range cis effect. Sequences far from the beta-globin gene influenced in cis the expression of the gene (Kioussis et al., 1983).

B-5. Cis requirements in transcription.

The role of thermal and conformational properties of DNA in gene regulation is important. The information for a promoter function is provided directly by the DNA sequence: its structure is its signal. Promoters have three conserved features: a GC rich discriminator region close to the transcription startpoint within the region of DNA melted by RNA polymerase, an entry region near the -35 box, and a -10 region essential for RNA polymerase binding. For promoter sequences that react with the RNA polymerase, the context within which it is located may also influence the ability to initiate transcription (Klein and Wells, 1982a,b). Some promoters are found in G-C and A-T rich blocks. Structural features of a stringently regulated promoter, such as the GC rich discriminator close to the transcription startpoint and for a poor entry region may inhibit interaction with RNA polymerase by impeding the crucial DNA

strand separation necessary for the initiation of transcription. In many stable RNA promoters, the sequence separating the -10 and -35 region may impose a similar constraint. A+T blocks can provide a thermolabile region and thus facilitate the melting of the DNA duplex and enzyme entry.

B-6. Cis Requirements for DNA replication.

The origin of replication is a cis-acting site able to affect only that molecule of DNA of which it is physically apart. Instead of lying in the regular duplex structure, the DNA at the origin of many organisms can assume a distinctive secondary structure. Cis requirements for replication must be assisted by factors such as supercoiling, stabilizing proteins, sequence environments such as A-T and G-C blocks.

In Escherichia coli and lambdoid phages, the sequences of the origin can assume a single-stranded secondary structure resembling a clover leaf. It is possible that the cis-acting site required for replication lies within this secondary structure (Kornberg, 1982).

B-7. DNA notches in regulatory regions.

It was suggested that DNA in regulatory regions may have notches, or "molecular dents", like notches on the balance beam of a scale. When proteins slide along the DNA searching for the regions where they bind, they may be stopped by these dents (Kolata, 1983). It is proposed that

local alterations may provide information to the proteins that are sliding down the DNA to slow down and indicate where to bind. The investigators found that short DNA sequences seem to form notches in control regions of bacterial and eukaryotic DNA. A short sequence GTG/CAC in the middle of the lac operator appears to be crucial for the binding of the lac repressor protein. The sequence GTG/CAC was found in other prokaryotic control regions such as operators of lambda, the operator of the gal operon of Escherichia coli as well as in eukaryotic control regions such as enhancers and regions of the long terminal repeats of retrovirus.

DNA sequences external to the recognition site markedly enhance the rate at which endodeoxyribonuclease EcoR1 locates and leaves its recognition site (Jack et al., 1982). The external sequences are, however, without effect on the intrinsic equilibrium constant governing specific protein-DNA interactions. The kinetic parameters governing both the formation and decay of specific endonuclease-DNA complexes increases with increasing chain length.

C- Results.

C-1. In vitro enzymatic deletion of pMD861 plasmid DNA (Figure 19).

We investigated the role of cis-required sequences that influenced Mini-Mu transposition in plasmid pMD861 (Figure 14). For this purpose, we created small deletions at the PvuII site, 320 bp downstream from the right extremity of the Mini-Mu genome, in the pSC101 sequences adjacent to the attR of Mini-Mu, using exonuclease III and S1 nuclease (Figure 19). The resultant nested set of small deletions was transformed into strains harboring a thermoinducible prophage, Mucts62 pf7701 or Mucts62, and an F' pro-lac episome (Figure 17). The transposition-defective mutants were screened by a replica-plate mating method which consisted of examining the fusion of the Mini-Mu plasmids with an F' episome (Figure 20). We found that deletions of small regions outside Mini-Mu could affect the transposition frequencies of Mini-Mu, but only when the the transposition functionn were provided by Mucts62 pf7701 helper prophage.

C-1a. Linearization of pMD861.

In order to create deletions with exonuclease III and S1, we first linearized the plasmid pMD861 (Figure 19). 10 micrograms of plasmid pMD861 were hydrolyzed to completion with 25 units each of either XhoI (87 bp upstream from attL) or PvuII (320 bp downstream from attR) and

Figure 19.

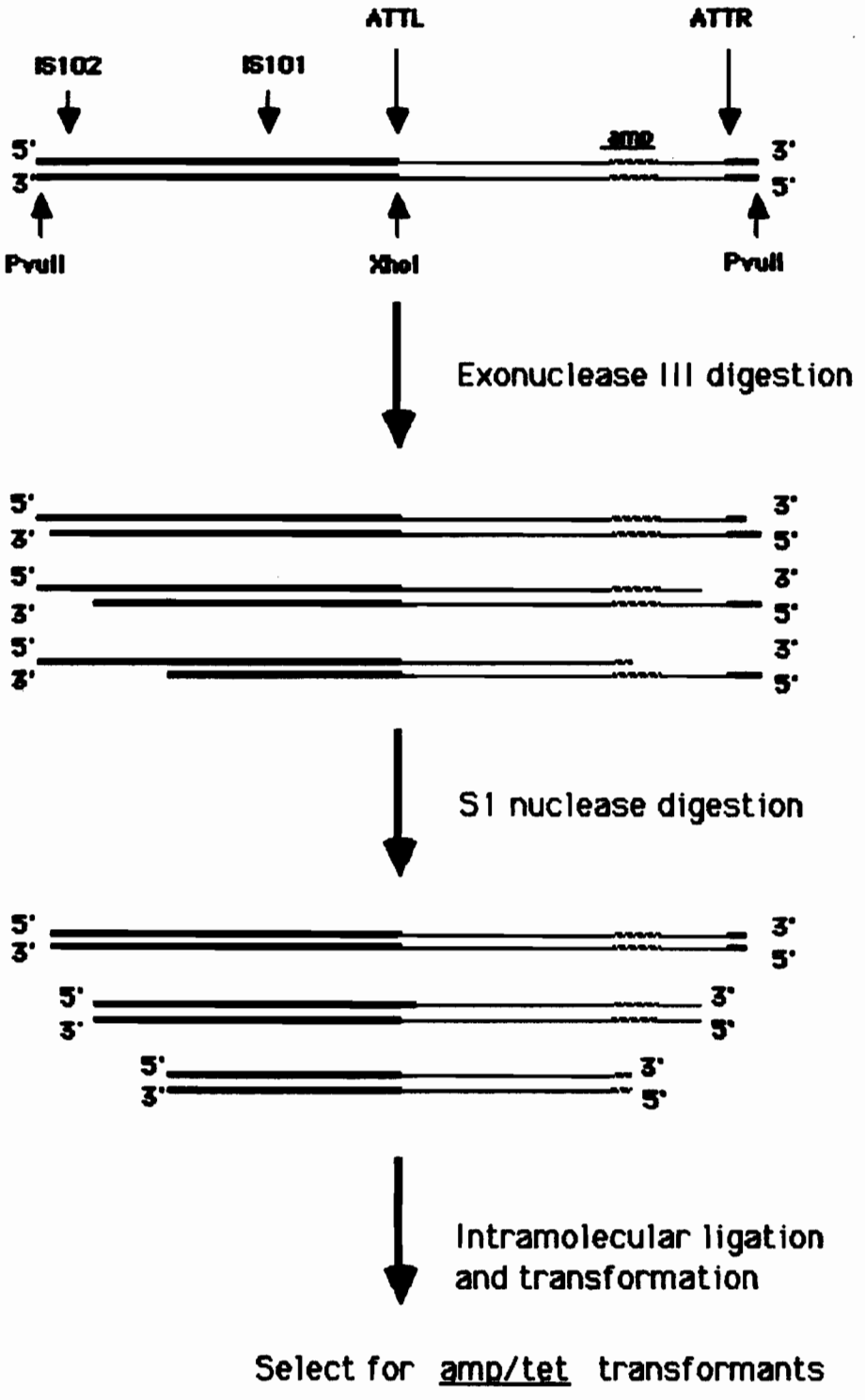
Schematic representation of the mutagenesis treatment of pMD861.

1- pMD861 is linearized at its PvuII site.

2- The linear DNA molecules at exposed for different periods of time to exonuclase III.

3- Pool DNA molecules are SI nuclease treated to remove sinlge-stranded regions.

4- DNA molecules are intermolecularly ligated and transformed into strain LF1037. The transformed clones are selected for ampicillin and tetracyclin resistance.


ATTL
ATTR
IS102
IS101
5'
3'
3'
5'
PvuII
XhoI
PvuII
Exonuclease III digestion
S1 nuclease digestion
Intramolecular ligation
and transformation
Select for amp/tet transformants

the 200 µl reactions were extracted twice with an equal volume of distilled phenol saturated with T.E. (10 mM Tris-HCl, pH:7.5, 1mM EDTA) from and once with chloroform iso-amyl acohol (24:1) , and then once with an equal volume of ether. One tenth volume of 2.5 M ammonium acetate, pH:8.0, and 2.5 volumes of absolute ethanol were added to the reaction fluid and, after mixing and incubation at -70^0C for 30 minutes, the DNA precipitate was collected by centrifugation in a Brinkman microcentrifuge at 15, 000 X g for 15 minutes at 4^0C.

C-1.b. Deletion treatment of the linearized plasmid.

To generate deletions in DNA, linearized DNA was treated with exonuclease III and S1. The exonuclease III reaction was performed in 50mM Tris-HCL, pH:8.0, 2mM $MgCl_2$, 10mM 2-mercaptoethanol (Smith, 1979). The DNA was exposed to exonuclease III (BRL) at 0.25 units/µg of DNA for the XhoI restricted DNA and 0.5 units/ µg of DNA for the PvuII restricted DNA. The exonuclease concentration in the reaction was calculated on the basis of its nucleolytic activity according to Smith, 1979. The degradation rate is about 800-1000 nucleotides/ hour in an exonuclease reaction with a concentration of 0.5 units of exonuclease per 0.5-0.25 pmole of ends.The incubation of the reactions was done at 30^0C for 2 minutes, 3 minutes, 4 minutes, 5 minutes, 10 minutes and 12 minutes. The reactions of each time points were pooled and further processeced together. Phenol extraction as well as chlorofom iso-amyl alcohol and ether extractions followed the procedure.

S1 nuclease digestion followed the exonuclease treatment to remove the single-stranded DNA regions generated by the exonuclease III hydolysis. The ethanol precipatated DNA's were resuspended in a solution containing 30 mM sodium acetate, pH: 4.5, 0.1 mM $ZnSO_4$, 0.15 M NaCl, 5% glycerol. Nuclease S1 (BMC) (0.2 units/µg of DNA) reactions were performed at 45^0C for 30 minutes. Then The DNA's were phenol, chloroform-isoamyl alcohol (24:1) and ether extracted and ethanol precipitated.

C-1.c. Ligation and transformation.

A ligation reaction of the DNAs was performed in a 15^0C waterbath for 16 hours. In some experiments, the exonuclease and S1 treated and ligated DNA's were digested with the initial restriction enzyme that the DNA's had been digested with, that is XhoI or PvuII, in order to eliminate intact DNA's and enhance for the population of trimmed plasmids.

Transformation of plasmid DNA's was performed by the method of Cohen and Chang (1977) into LF1037 competent cells. The transformed LF1037 clones were selected for their tetracyclin and ampicillin resistance transformants. The clones were screened for their transposition phenotype by the replica-plating transposition test that we developed. The clones which exhibited an altered transposition behavior different than from the parental LF1037 harboring pMD861 were further analyzed.

C-2. Replica-Plating transposition test.

We have developed a primary screening test for transposition that allowed us to select, from a master plate, clones carrying transposition defective Mini-Mu's. The transposition defective Mini-Mu plasmids were screened by a replica-plating assay that selects for Mini-Mu transposition onto an F' pro-lac episome (figure 20). Instead of examining genetically Mini-Mu fusions with a conjugative plasmid in liquid culture (Chaconas et al., 1980) (Figure 17), we modified this conjugation assay to fit plate screening (Figure 20).

Briefly, a master plate was containing clones of bacteria with putative altered Mini-Mu plasmids. The transposition of these clones was induced by incubating the plate ar 43^0C, for 24 hours. The transposition events of the Mini-Mu Amp from the plasmid were rescued by replica plating the induced master plate onto the "mating plate", that contained a lawn of recipient strain. A selective medium that inhibited both the growth of the donor and recipient strains and allowed only the growth of sexductants, was used. The sexducdants that received Mini-Mu Amp via transposition onto the F'pro-lac factor were screened for their ampicillin resistance phenotype, provided from Mini-Mu Amp.

The mutagenized Mini-Mu plasmid DNAs were transformed into strain LF1037. Strain LF1037 harbors a thermoinducible Mucts62 pf7701 (Waggoner et al., 1984) helper prophage and an F'pro-lac episome and is Spc^s. Master plates of transformants containing 50 clones/plate were incubated at 43^0C for 12 to 24 hours to partially induce Mu lytic growth. a

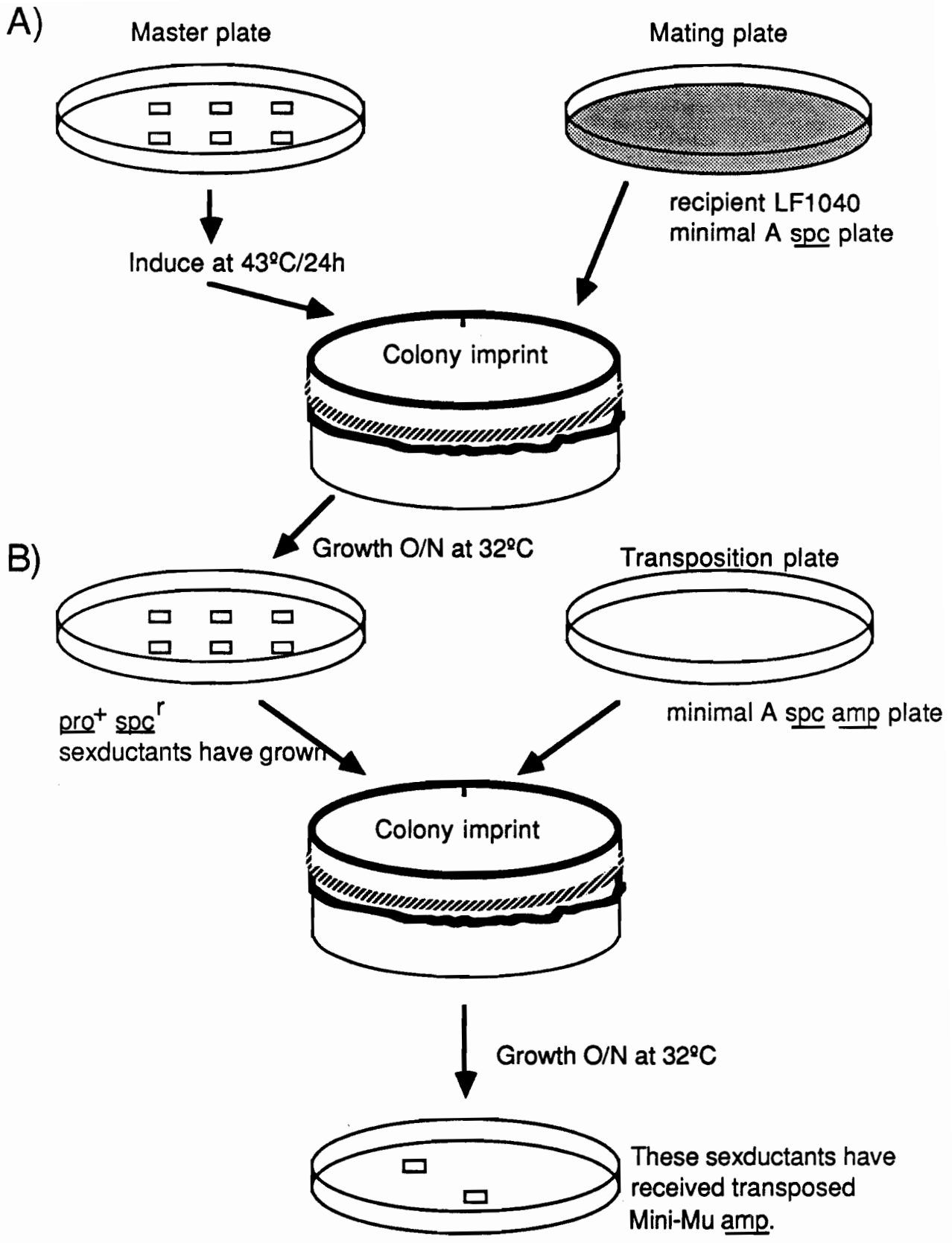
Figure 20.

Replica-plating transposition test.

A) LF1037 transformed clones with mutagenized pMD861 are induced at 43^{0}C for 24 hours.

The induced plate is replicated onto a Minimal media spectinomycin plate containing a lawn of strain LF1004, the recipient strain.

B) The Minimal A spectinomycin plate containing growing *pro*$^{+}$*Spc*^{r} exconjugants clones is replicated onto a minimal A spectinomycin, ampicillin plate in order to select for the sexductants that have received an F' with a transposed Mini-Mu *Amp* genome.


A)
Master plate
Mating plate
recipient LF1040
minimal A spc plate
Induce at 43ºC/24h
Colony imprint
B)
Growth O/N at 32ºC
Transposition plate
pro+ spcr
sexductants have grown
minimal A spc amp plate
Colony imprint
Growth O/N at 32ºC
These sexductants have
received transposed
Mini-Mu amp.

The plates were then replica-plated onto a minimal medium 0.75% soft agar lawn of a female Spc^r pro$^-$,lac$^-$ recipient strain, such as strains LF1004 or LF1040, on a minimal agar plate.

The pro$^+$ Spc^r exconjugant plates were then replica-plated onto a minimal agar plate with spectinomycin plus ampicillin. pro$^+$Spc^{r}Amp^r exconjugants, which had received an F'pro-lac episome on which Mini-Mu Amp has transposed, could grow on the minimal media plus spectinomycin and ampicillin and thus form a patch of colonies.

Putative transposition mutants which failed to form a patch or formed only faint patch (indicative of a different transposition behavior than the parental pMD861) were picked for further study in a more detailed and quantitative manner (Chaconas et al., 1980).

The size of the plasmids of the putative transposition mutants clones was analyzed by cracking the clones (Materials and Methods, Section O) and electrophoresing their DNA on 0.35% agarose gels. We choose clones harboring plasmids with small deletions, or no apparent deletions, in order to confine the transposition studies on Mini-Mu to minimal deletions affecting its transposition. Big deletion-containing clones were kept as well for comparative studies.

C-2. Transposition characterization of some of the class I and class 2 plasmids.

A more detailed and careful study of the putative transposition mutants was performed by first using quantitative transposition analysis and analysis of the plasmidic DNAs from these clones.

The transposition frequencies of the putative transposition mutants in LF1037 transformants was studied by analysing the frequency of Mini-Mu transformed onto an F' pro-lac episome, using the conjugation assay in liquid culture (Materials and Methods, Section F) (Chaconas et al., 1980). The transposition mutants were then divided into two classes.

The class 1 had a transposition frequency diminished 5 to 8 fold compared with pMD861, and their sizes were barely different from that of pMD861 (Class 1, pJoE14, pJoE32, pJoE43) (Table 4, Table 5).

One class (Class 2, pJoB2, pJoD31) exhibited a very low transposition frequency (0-4% of the frequency of the parental pMD861) (Table 4, Table 5). The plasmidic deletions were, in general, big (2 kb or bigger).

Transposition of pMD861 was detected at a frequency of about 0.3-0.5% of the transferred F' episomes with a Mucts62 pf7701 helper phage (Table 4). Class 2 large deletion plasmids (pJoB2, pJoD31) showed almost no transposition activity whether the deletion started from attL (pJoD31) or from attR (pJoB2) (Table 4). However, the class 1, pJoE14, pJoE32, pJoE43 mutants exhibited 10-30 % of the transposition frequency as compared to the frequency of the parental pMD861 in the original

Table 4.

Frequency of transposition of pMD861 and pMD861 derivatives in strains LF110, LF113, LF1037.

The transposition frequency was monitored by a conjugation assay during lytic growth of helper phage Mucts62 or Mucts62 pf7701 harbored in LF110 and LF113 (or LF1037) respectively.

LF1040 was the recipient strain. (LF1040 is spec^r Mu^r Δ pro-lac).

Transposition was induced by shifting the exponentially growing donor cultures at 43^{0}C for 40 minutes to induce Mu lytic growth. The mating at a ratio of 5-10 females to one male was done at 32^{0}C for 1 hour. After conjugation, the cells were washed in saline. The cells were resuspended in saline and 100 µl of diluted cell suspensions were plated onto minimal A glucose plates containing spectinomycin with or without ampicillin.

The transposition frequency was first evaluated by percent of pro$^+$ Spc^r Amp^r exconjugants/ pro$^+$ Spc^r exconjugants. The transposition frequency of the plasmids was then standardized taken the transposition frequency of the control pMD861 as 100% transposition as reference.

Table 4.

Transposition frequencies.

	LF110	LF113	LF1037
PMD861	100%	100 %	100%
CLASS 1:			
pJoE14	94%	27%	24%
pJoE32	95%	19%	8.3%
pJoE43	78 %	30%	7.3%
pJo64	82 %	29%	16.5%
pJo65	78%	36 %	21.5%
pJo66	100%	26%	23.7%
pJo67	93%	46%	45.3%
CLASS 2:			
pJoB2	0.5 %	3%	0.8%
pJoD31	0%	ND	2%
KAN INSERTION:			
pJo02	13 %	17 %	ND
pJo03	10 %	16 %	ND
pJoA	0 %	0%	ND
pSC101	0 %	ND	0%

Table 5.

List of plasmids studied in chapter III.

Plasmids	Phenotype, approximate size,	description, source.
pJoE14	Amp^r, Tet^r, class 1. 20.5kb	pMD861 opened at PvuII exoIII/SI treated
pJoE32	Amp^r, Tet^r, class 1. 18.9 kb	pMD861 opened at PvuII exoIII/SI treated
pJoE43	Amp^r, Tet^r, class 1. 18.9 kb	pMD861 opened at PvuII exoIII/SI treated
pJo64	Amp^r,Tet^r, 18.7 kb	ΔSmaI in pMD861
pJo65	Amp^r,Tet^r, 18.7 kb	ΔXmaI in pMD861.
pJo66	Amp^r,Tet^r, 18.7 kb	ΔSmaI-PvuII in pMD861
pJo67	Amp^r,Tet^r, 18.5 kb	ΔSmaI-PvuII in pMD861

Table 5.

Plasmids	Phenotype, approximate size,	description, source,
pJoB2	Amp^r, Tet^r, class 2 16.5 kb	pMD861 opened at PvuII exoIII/SI treated
pJoD31	Amp^r, Tet^r, class2 16.0 kb	pMD861 opened at XhoI exoIII/SI treated
pJo02	Amp^r,Tet^r, Kan^r,20.1 Kb	pMD861 PvuII::Kan
pJo03	Amp^r,Tet^r,Kan^r, 20.3 kb	pMD861 XhoI::Kan
pJo(ΔXI-RI)	Amp^r,Tet^r, 18.0 kb	ΔXhoI-EcoRI in pMD861
pMD861	Amp^r,Tet^r, pSC101::Mini-Mu blo 18.9 kb.	Chaconas et al, 1981a.
pSC101	Tet^r, 9.2 kb	Cohen and Chang, 1977
pJoA	Amp^r,Tet^r , 10.kb	this work pSC101 der.

transformed strain LF1037 (Table 4). Their plasmid size of plasmids pJoE32 and pJoE43 from class 1 was similar to that of pMD861(18.9kb) on a cracking gel except that pJoE14 appeared slightly bigger (20.5 kb).

The transposition activity of these classes of plasmids was tested with the transformed strain LF110 (harboring a wild-type Mu<u>c</u>ts62 helper prophage). Class 2 plasmids (pJoB2 and pJoD31) showed the same low frequency of transposition, whereas the pJoE14, pJoE32 and pJoE43 plasmids behaved similarly to pMD861 (Table 4).

Thus, This class 1 series of mutants displayed different transposition frequencies when compared to the parent Mini-Mu plasmid, pMD861, in the presence of different Mu helper prophages: The transposition frequency of plasmids pJoE14, pJoE32 and pJoE43 was reproducibly reduced 3 to 5 fold in strain LF1037 as compared to the transposition activity of pMD861 (Table 4), but they exhibited a similar transposition frequency in strain LF110 compared with the transposition of the parental plasmid pMD861.

Similar results to the ones obtained with strain LF1037 were observed when using strain LF113, a strain isogenic to LF110 but containing the Mu<u>c</u>ts62 pf7701 prophage (Table 4). This suggested that the modulation of the frequency of transposition was not attributable to the host strain background in these experiments.

Mu<u>c</u>ts62 pf7701 (Waggoner et al., 1984) contains a polar insertion of Tn5 just beyond the <u>B</u> gene and is deleted for Mu sequences from 4.4 kb to 7.2 kb (Figure 31, C-B.a.). Thus this phage is defective for the semi-essential early region (SEER) which encodes several functions which

aid Mu to transpose but, as the name states, are not essential for Mu lytic growth and transposition.

We concluded that plasmids pJoE14, pJoE32 and pJoE43 were deficient in their ability to transpose in the absence of a function(s) encoded by Mucts62 pf7701. Good candidates for an enhancing transposition function within the semi-essential early region of the Mu genome such as the function expressed by the arm gene of Mu (amplification of replication of Mu).

In this study we divided into two classes the plasmids that we have created so far. Class1 series included plasmids that transposed at normal frequency in the presence of an induced Mucts62 prophage, but transposed at a reduced rate when the induced helper prophage used was Mucts62 pf7701. The class 2 series included plasmids with very low transposition frequencies, regardless of the helper prophage used.

C-4. Mapping of class 1 and class 2 plasmids.

C-4. a. Restriction enzyme pattern analysis.

Preliminary mapping indicated that pJoE14, pJoE32, pJoE43 and pJoB2 had lost their PvuII site, the site from which the exonuclease III treatment was initiated. Moreover, pJoE32 and pJoE43 and pJoB2 did not lose their SmaI sites, while in pJoE14 the two SmaI sites were missing (Figure 21,22).

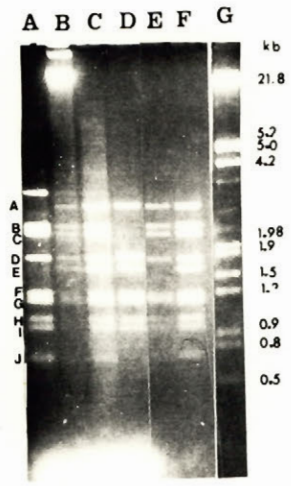
Figure 21.

HincII mapping of pMD861 and its derivatives.

1- HincII digestions of E series plasmids and class1 plasmids are run on a 0.7% agarose gel: pJoE14 (A), pJoE32 (B), pJoE43 (C), pJoD31 (D), pJoB2 (E), pMD861 (F). Lambda c1857 cut with EcoRI/HindIII (G) is used as molecular weight markers.

2-The gel is Southern blotted and hybridized with a Mu specific probe.The HincII Mu-specific fragments are indicated.

1


A B C D E F G
kb
21.8
5.2
5.0
4.2
A
B
C
D
E
F
G
H
I
J
1.98
1.9
1.5
0.9
0.8
0.5

2

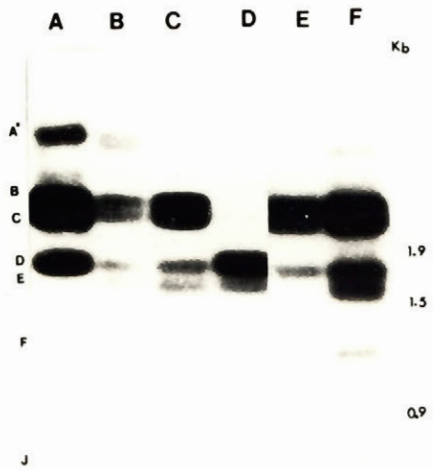

A B C D E F
Kb
A
B
C
D
E
F
J
1.9
1.5
0.9

Figure 22.

Mapping of the pMD861 derivated mutants.

The region of pMD861 from the EcoRI site to 900 bp inside the IS102 element is presented.

pJoE14, pJoE32, pJoE43 and pJoB2 are pMD861 deletion transposition mutants generated by exonuclease III and SI treatment of pMD861. During the course of the mutagenesis, pJoE14 had been subjected to a deletion and insertion event. This DNA insertion is represented by a black line with white dots.

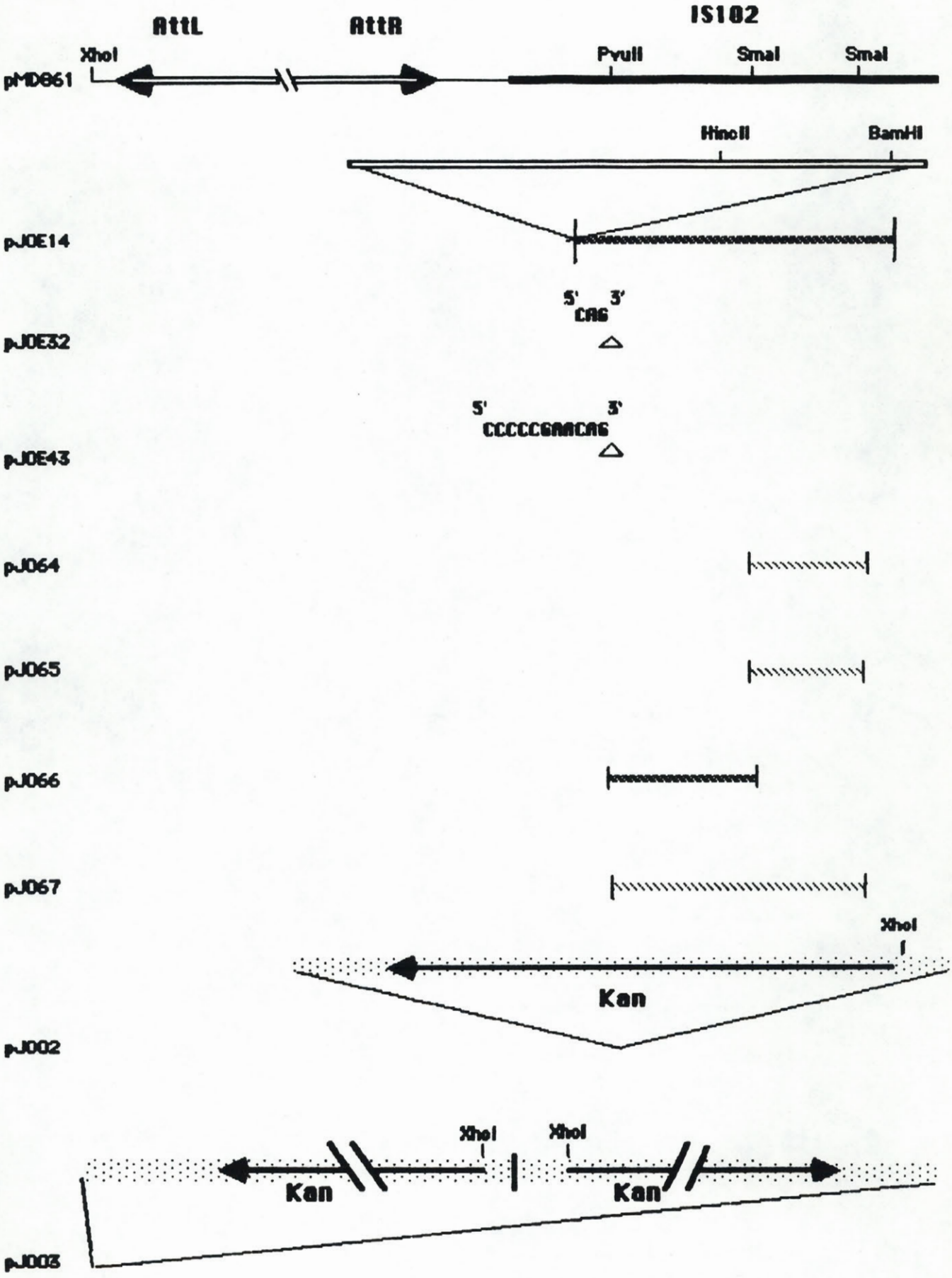
Site-specific deletion plasmids of pMD861 were created. pJo64, by SmaI deletion, pJo65 by XmaI deletion, pJo66 and pJo67 by SmaI-PvuII deletions.

In pJo02, The HindII fragement containing the Kan gene from pUC71K was inserted into the pMD861 PvuII site. In pJo03, the SalI fragment containig the Kan gene of pUC71K was inserted into the pMD861 XhoI site.

Large deletions are represented by hatched lines; small deletions are represented by a Δ sign and the deleted nucleotide sequences are indicated 5'-3'; the arrow in the Kan gene indicates the direction of its transcription.

B, BamHI; H, HindII; P, PvuII, S, SmaI; X, XhoI.

Bla, beta-lactamase gene; attL and attR, Mu attachment sites left and right, respectively.


AttL
AttR
IS102
XhoI
PvuII
SmaI
SmaI
pMD861
HincII
BamHI
pJOE14
5'
3'
CAG
pJOE32
5'
3'
CCCCCGAACAG
pJOE43
pJ064
pJ065
pJ066
pJ067
XhoI
Kan
pJ002
XhoI
XhoI
Kan
Kan
pJ003

Finer mapping with HincII on a 0.7% agarose gel was done on these plasmids. Preliminary HincII mapping on pMD861 and hybridization with a Mu specific probe allowed us to complete an HincII map of pMD861 (figure 21). pJoE32 and pJoE43 had a HincII digestion pattern indistinguishable from pMD861. It appeared that the class1 plasmids, pJoE14, pJoE32, pJoE43, had no grossly altered Mu right extremities.

pJoE14, however, acquired a bigger A fragment (about 0.8 kb) and lost the E fragment. Specific hybridization of HincII-cleaved pMD861 with Mu DNA revealed that pJoE14 had acquired extra DNA sequences within its A' fragment corresponding to the pSC101-attR junction. The A' fragment of E14 had also acquired new BamHI site. The precise deletion lenght of pJoE14 was difficult to evaluate since the DNA insertion origin is not yet well defined (see C-4.c.).

Class 2 plasmid, pJoB2, appeared to have lost the rightmost HincII fragment covering the flanking region of Mini-Mu attR and pSC101. Plasmid pJoB2 was deleted for about 1.5 kb at the attR region of the Mini-Mu within the HincII E fragment and did not transpose in the presence of either wild type or SEER negative induced helper prophages (table 4).

In plasmid pJoD31, another class 2 plasmid, was deleted for the entire HincII B and K Mu specific fragment. In this instance, the exonuclease III deletion went almost unidirectionally from XhoI 2.5 kb inward toward Mu DNA, removing the whole left Mu DNA portion.

Thus, the E series plasmids were deleted for sequences within the pSC101 genome adjacent to the Mini-Mu genome and were deficient in their ability to transpose in the absence of a function(s) encoded by Mu

(Figure 22; table 4).

C-4.b. DNA sequencing of pJoE32 and pJoE43.

Finer mapping with HaeIII and TaqI on 5% polyacrylamide gels (Maniatis et al., 1982) on class 1 plasmids, pJoE32 and pJoE43 revealed different bands compared to the pMD861 pattern. These corresponded to increases of 213 bp for TaqI (858-1058 bp) and 190 bp for HaeIII (746-936 bp) which corresponded to the region surrounding the PvuII site in the pSC101 plasmid (Bernardi and Bernardi, 1981) (Figure 23).

End labelling of these DNA fragments, fragment separation, chemical cleavages and sequencing were done using the procedure of Maxam and Gilbert (1980). The polymorphic band produced by TaqI was dephosphorylated with bacterial alkaline phosphatase and kinased at its 5' ends with T4 Kinase (Pharmacia P.L.) and γ^{32}P-ATP. Subsequent digestion with HaeIII and polyacrylamide gel purification of the two fragments (122 bp A fragment and 112 bp B fragment for pMD861) (figure 24) allowed us to perform the sequencing of the PvuII region from which the exonuclease digestion had started in pJo32 and pJo43.

Sequencing of the polymorphic TaqI-HaeIII B fragments revealed that pJoE32 and pJoE43 have small deletions of 3 bp and 11 bp respectively (Figure 24). These occured from nucleotide 7794 to nucleotide 7797, and from nucleotide 7794 to nucleotide 7805 respectively in pSC101, according to the pSC101 DNA sequence chart published by Bernardi and

Figure 23.

TaqI and HaeIII digestion pattern of E series plasmids.

In 1) pJoE32 (B), pJoE43 (C), pMD861 (D) and pSC101 (F) are digested with Taq1. pMD861 is double digested Taq1/PvuII (E).

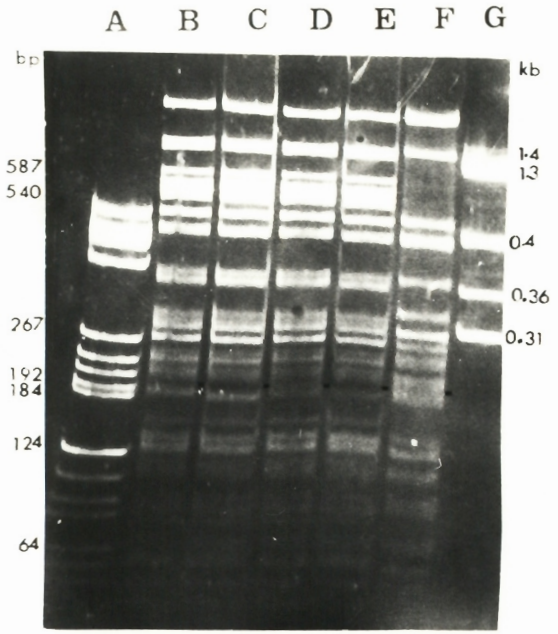
The digestions are run on a 5% polyacrylamide gel and HaeIII digested pBR322 (A) and TaqI digested pBR322 (G) are used as molecular weight markers.

In 2) pJoE32 (B), pJoE43 (C), pMD861 (D), pSC101 (F) are digested with HaeIII. pMD861 was double digested HaeIII/PvuII (E).

The digestions are run on a 5% polyacrylamide gel and HaeIII digested pBR322 (A) and TaqI digested pBR322 (G) are used as molecular weight markers.

The polymorphic bands are pointed with a dot.

1


A B C D E F G
bp
587
540
267
192
184
124
64
kb
1·4
1·3
0·4
0.36
0.31

2

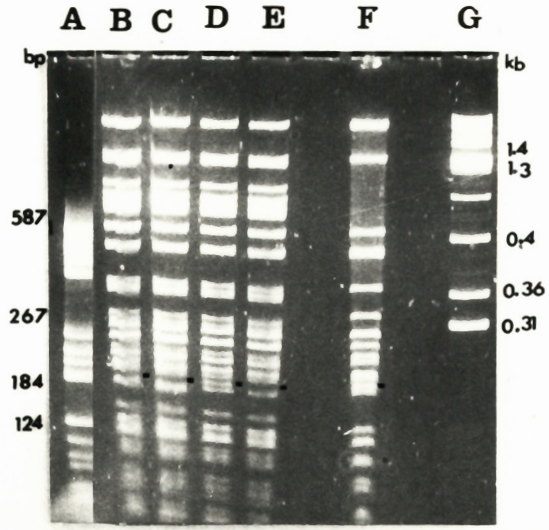
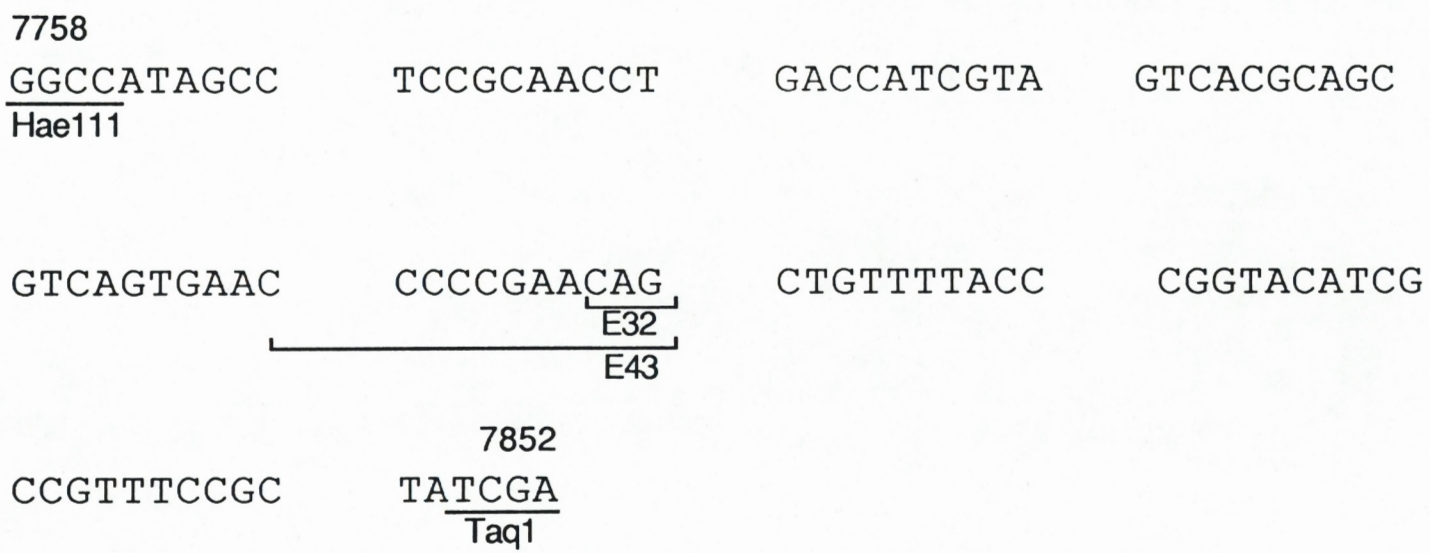

A B C D E F G
bp
587
267
184
124
kb
1·4
1·3
0·4
0.36
0.31

Figure 24.

The DNA sequence of the TaqI-HaeIII fragment.

DNA sequence of the polymorphic TaqI- HaeIII fragment containing the PvuII site in pMD861 is shown as well as the nucleotides deletions found in pJoE32 and pJoE43 within this region.


7758
GGCCATAGCC
TCCGCAACCT
GACCATCGTA
GTCACGCAGC
Hae111
GTCAGTGAAC
CCCCGAACAG
CTGTTTTACC
CGGTACATCG
E32
E43
7852
CCGTTTCCGC
TATCGA
Taq1

Bernardi (1984). These deletions occured in the IS102 sequences of pSC101 from 878 nucleotide to 881 nucleotide for pJoE32 and from 878 nucleotide to 889 nucleotide for pJoE43 according to Bernardi and Bernardi (1981) sequencing data for IS102 (Figure 15).

C-4.c. Characterization of pJoE14 insertion.

Class 1 plasmid, pJoE14, however, contains both a deletion, and insertion of an unknown foreign DNA sequence at its PvuII region. However, the right extremity of the Mini-Mu genome appeared to remain intact because of its biological similarity with the parental pMD861 except when tested with a Mucts62 pf7701 helper prophage background. This insertion might have occured prior to mutagenesis or might have been the result of some DNA rearrangements after mutagenesis and transformation.

The A' HincII (4.2 kb) fragment containing the insertion and a deleted region, contained an extra BamHI site and missing the PvuII site and one SmaI site. Sites BglI, EcoRI, HindIII, PstI were not observed within the A' HincII fragment of pJoE14 (Figure 25, Figure 22).

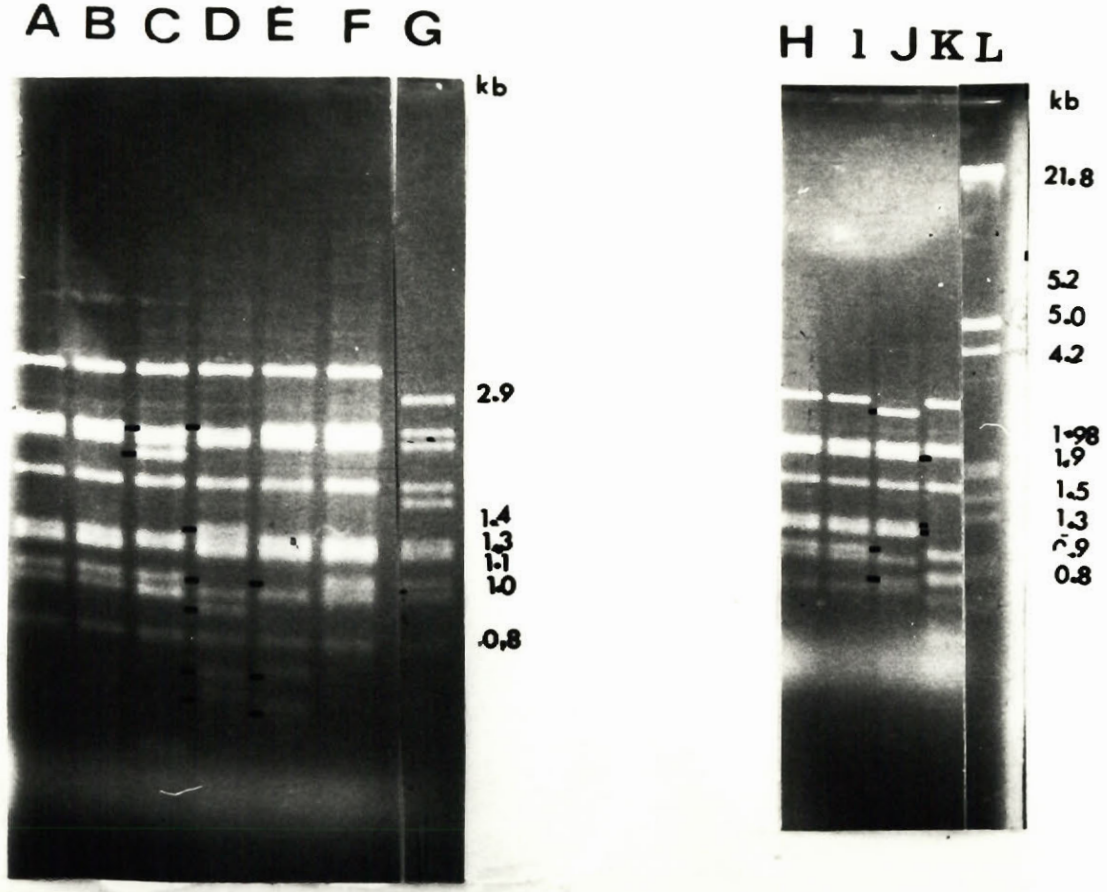
Escherichia coli carries a number of different IS elements in its chromosome. One can estimate that 1-2% of the Escherichia coli K12 chromosome represents IS sequences (Iida et al., 1983). Each of these elements IS1, IS2, IS3, IS4, IS5, IS30 and gamma-delta is carried in 1 to 10 copies on the chromosome of Escherichia coli. These elements may transpose to new locations at a frequency estimated in the order of 10^{-3} to 10^{-6} per IS element per cell per generation. The IS elements vary in

Figure 25.

Restriction enzyme pattern of pJoE14.

pJoE14 was cut with HincII (A,H), HincII/PvuII (B), HincII/PstI (C), HincII/HindIII (D), HincII/EcoRI (E), HincII/BamHI (F), HincII/BglI (I), HincII/BamHI (J), HincII/SmaI (K).

HincII digested pMD861 (G) and EcoR1/HindIII digested lambda c1857 (L) were used as molecular weight standarts. The digests were run on a 0.7% agarose gel.


A B C D E F G
kb
2.9
1.4
1.3
1.0
0.8
H I J K L
kb
21.8
5.2
5.0
4.2
1.98
1.9
1.5
1.3
0.9
0.8

length between 700 and 1800 bp, except for gamma delta which is around 5800 bp. Gamma delta (5.8 kb) from the F factor and and an uncharacterized Fθ element (1.5 kb) could be likely candidates based on restriction maps (Iida et al., 1983; Szybalski, 1980). The insertion is at most 2 kb. However, the region of insertion and the inserted sequences might have transposed near the PvuII site and have been subjected to deletion during the in vitro mutagenesis.

To detect if the foreign sequences were present in Escherichia coli, we performed hybridization with pJoE14 plasmid as a probe to various strains of Escherichia coli. Purified F factor (a kind gift from A.Sasarman) and strain 40 Escherichia coli chromosomal DNA digested with BamHI and EcoRI did not give convincing evidence on the origin of this DNA (Figure 26). A 16 kb fragment with EcoRI and a 23 kb fragment with BamHI for the F factor; a 3 kb fragment with HincII and a 25 kb with BamHI and 16 Kb with EcoR1 for Escherichia coli K12 chromosomal DNA appeared to hybridize slighlty with the A' fragment of pJoE14. However, the origin of this foreign DNA remains unknown at this time.

C-5. Biological characteristics of some of class 1 and 2 plasmids.

C-5.a. Chromosomal Association.

There is an intracellular phase during the transposition cycle of Mu plasmids and Mini-Mu plasmids when the physical integrity of the Mu DNA is lost as would be expected if cointegrate formation with the host

Figure 26.

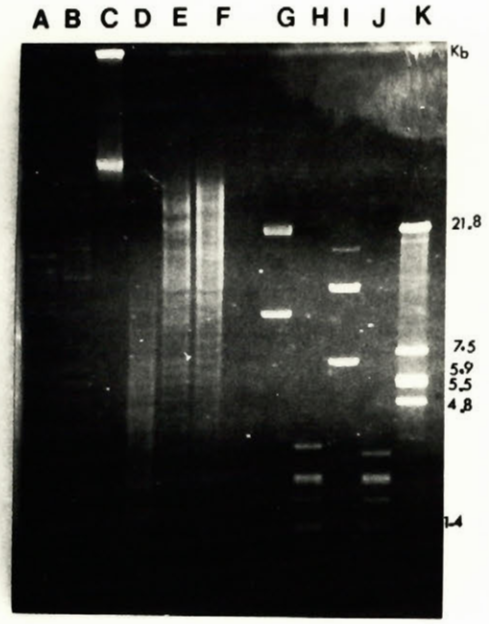
Hybridization of F and E.coli DNA with pJoE14 A' fragment.

1- Purified F' factor cleaved with EcoRI (A) and BamHI (B), and purified E.coli strain 40 chromosomal DNA undigested (C) and cleaved with HincII (D), with BamHI (E) and EcoRI (F) as well as pJoE14 plasmid (G), pJoE14 cleaved with HincII (H), pJoe14 cleaved with BamHI (I) , pJoE14 cleaved with HincII/BamHI (J) were run on a 0.35% agarose gel. The photograph of the ethidium bromide stained gel is shown in 1).

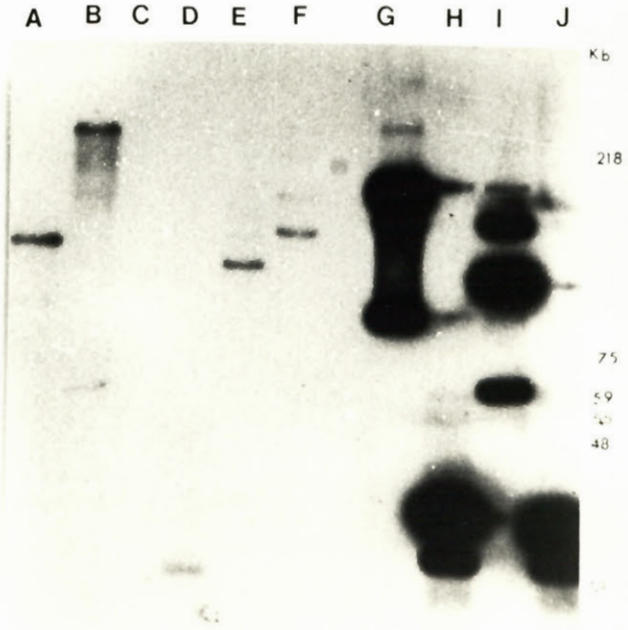
2- The gel was Southern blotted and hybridized with gel purified A' fragment probe of HincII of pJoE14.

EcoRI digested fragments of lambda c1857 were used as molecular weight markers.

1



2



chromosome occurs. We can follow the fate of Mu-containing plasmid DNA after prophage induction and follow its association with the bacterial chromosome on an agarose gel and "tagging" the plasmid sequences by Southern blot hybridization with labelled pSC101 DNA. Chromosomal association is thus a physical test of transposition events by Mini-Mu plasmids. In Figure 27, chromosomal association tests with pMD861 as a positive example and pSC101 as a negative example are shown.

We performed chromosomal association tests on class 1 plasmids (pJoE14, pJoE32 and pJoE43) and class 2 plasmid (pJoB2). However, in this test, we used an LF110 host strain containing these plasmids because during cell lysis was more complete then when the LF1037 derivative were used (and more presumably lytic growth; see C-8.a. and Figure 32).

The class 2 plasmid (pJoB2) did not indicate any chromosomal association, confirming the low transposition activity observed by the transposition test in conjugation assays (Figure 29; Table 6). LF110 containing pJoD31 presented a very quick lytic cycle in comparison with pMD861 and other plasmids; since pJoD31 was a plasmid with a large deletion from attL, we could suspect that the repressor gene was missing and thus was no longer acting to repress Mu early functions (Not shown) (Table 6).

The class 1 plasmids, pJoE14, pJoE32 and pJoE43, did chromosomally associate; thus confirming that in LF110 background these class1 plasmids had a transposition frequency as efficient as pMD861 (Figure 28; Table 6).

Table 6

Biological characterizations of class 1 and class 2 plasmids.

(A) The transduction frequency was defined as number of Amp^r transductants per plaque forming-unit. The transduction numbers were standardized with parental pMD861 transduction frequency within the same experiment.

(B) The relative immunity properties of pMD861 derivatives were defined according to the relative immunity of these plasmids in E.coli strain 40 to dilutions of superinfecting Mucts62.

1, indicates no lysis in a spot test of phage at up to 10^9 pfu/ml.

5, indicates no lysis in a spot test of phage at up to 10^6 pfu/ml.

(C) The chromosomal association of pMD861 derivatives was visualized by Southern blot analysis of strain LF110 containing the plasmids and an induced Mucts62 prophage. Hybridization with ^{32}p-pSC101 DNA within the host DNA during the lytic cycle is an indication of chromosomal association.

Detection of chromosomal association is indicated by (+) sign. No detection of chromosomal association is indicated by (-) sign.

Table 6.

plasmid	Freq. of transduction(%)[A]	Relative immunity[B]	chromosomal association[C]
pMD861	100	1	+
pJoE14	62.3	1	+
pJoE32	120	1	+
pJoE43	76	1	+
pJoB2	0	2	_
pJoD31	0.3	5	_

C-5.b. Transduction.

When a Mu prophage is induced in the presence of a Mini-Mu and phage lysates are prepared, The Mini-Mu sequences can be found packaged into Mu particles (Faelen et al., 1979; Chaconas et al., 1981). The packaging of Mu sequences requires a pac specific recognition sequence for packaging known to reside within the leftmost part of the Mu genome and a total of 37 kb of DNA in order to perform headful packaging of the Mu capsid.It was noticed by Chaconas et al. (1981) that only Mu right end plasmids are not transduced significantly. However, Mu left end plasmids also showed little transduction, presumably because these pSC101 plasmids are too small and cannot olgomerize to fill a Mu capsid.

The attL deleted plasmid from class 2, pJoD31, was not tranduced presumably because they were missing the pac site. The attR deleted plasmid, pJoB2, transduced at a lower frequency (1000 fold less) than pMD861 (Table 6).

The class 1 plasmids, pJoE14, pJoE32 and pJoE43 were transduced at a high frequency, indicating that the packaging features for transduction of these plasmids remained intact (Table 6).

C-5.c. Immunity test.

A Mu lysogen can block the expression of a superinfecting Mu phage because of the function of the Mu repressor expressed by Mu prophage. Mu immunity was reported to be positively controlled by the cim I gene

function in the semi-essential early region. In the right end of Mu, a cimII function was also described as a second positive effect or allowing a Mu phage to establish immunity (Van de Putte et al., 1981).

The immunity strains containing the class1 (pJoE14, pJoE32 and pJoE43) and class 2 plasmids (pJoB2 and pJoD31) were tested (Table 6). The left end deleted pJoD31 plasmid, from class 2 series, did not show immunity to the incoming phage, suggesting that the repressor gene had been altered. The attR deleted plasmid, pJoB2, was 10 fold less immune than pMD861. Although the function of cimII is not well documented, the immunity data on pJoB2 could be indicative that cimII action was altered.

The class 1 plasmids, pJoE14, pJoE32 and pJoE43, were as immune as the parental plasmid pMD861. This showed that the immunity functions of these class1 plasmids were not altered.

C-6. pMD861 site-specific deletions mutants.

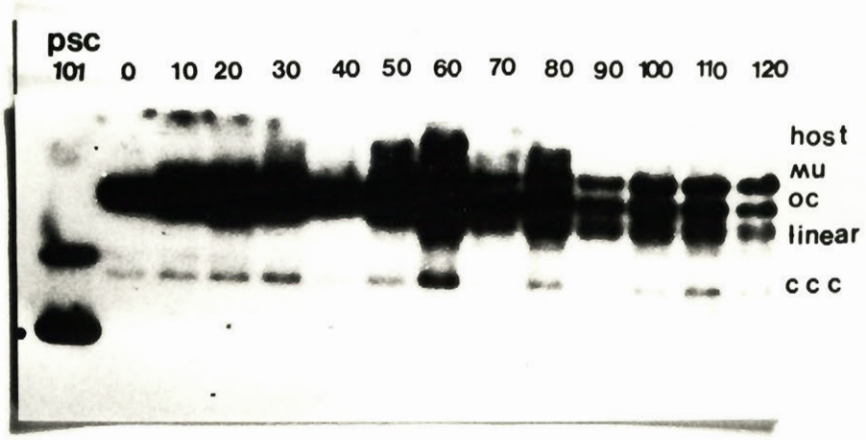
To understand the role of the pSC101 plasmid sequences in the modulation of Mini-Mu plasmid transposition activity, we created site-specific deletions in pMD861 by removing 196 bp between the SmaI (pJo64) or the XmaI (pJo65) region from nucleotides 7327 to nucleotides 7523 according to the pSC101 DNA sequencing chart, and the PvuII-SmaI region from nucleotide 7327 to 7794 (pJo67) and from nucleotide 7523 to 7794 (pJo66), near the attR of the Mini-Mu genome (Figure 14). Deletion plasmids were analysed with HincII digestion (Figure 30; Figure 22).

Figure 27.

Chromosomal association with pMD861 and pSC101 plasmids.

The numbers over each slot represent the time (in minutes) after induction of Mu lytic growth at 43^{0}C from which each sample was taken. The samples were removed, the cells lysed and the DNA electrophoresed, blotted and hybridized versus ^{32}P-pSC101 DNA as described in Materials and Methods. Southern blots represent Mu-infected cells containing plasmid pMD861 in top panel, and pSC101 in bottom panel.

The position of Mu and host DNA as well as the various plasmid forms are indicated.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0
10
20
30
40
50
60
70
80
90
100
110
120
host
Mu
oc
linear
ccc

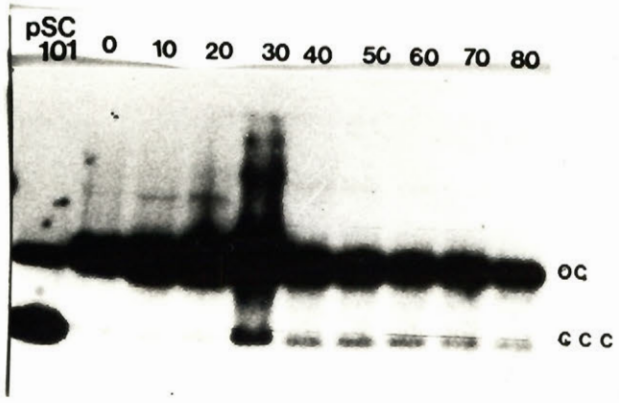

pSC
101
0
10
20
30
40
50
60
70
80
oc
ccc

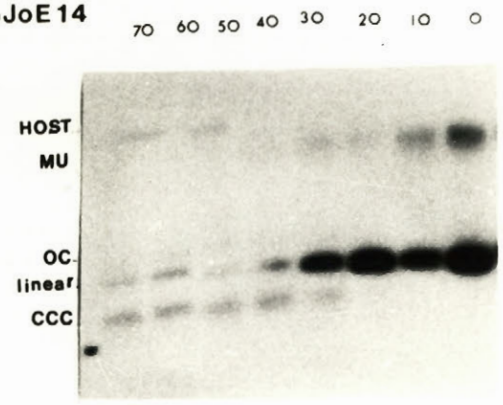
Figure 28.

Chromosomal association with class 1 plasmids.

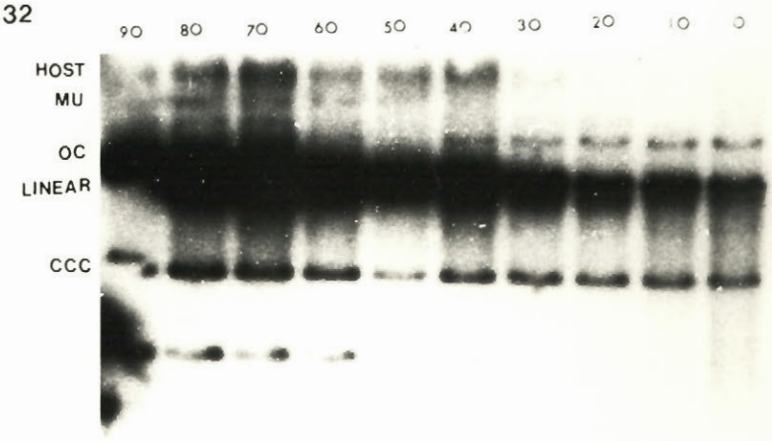
The numbers over each slot represent the time (in minutes) after induction of Mu lytic growth at 43^0C from which each sample was taken. The samples were removed, the cells lysed and the DNA electrophoresed, blotted and hybridized versus ^{32}P-pSC101 DNA as described in Materials and Methods. Southern blot represent Mu-infected cells containing plasmids pJoE14 in top panel, pJoE32 in the middle panel and pJoE43 in the bottom panel.

The position of Mu and host DNA as well as the various plasmid forms are indicated.

pJoE14



pJoE32



pJoE43

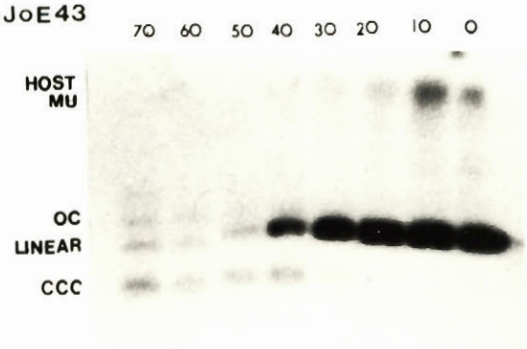


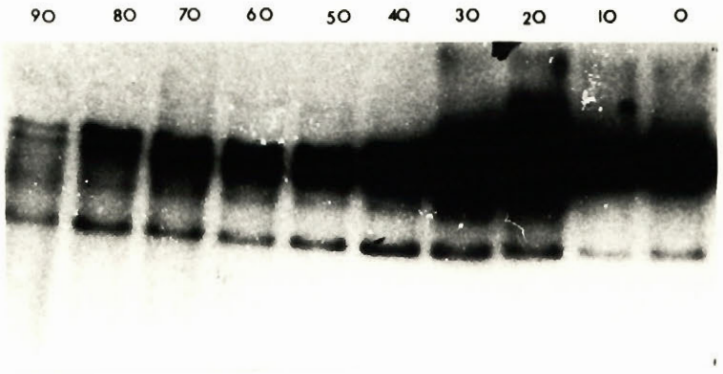
Figure 29.

Chromosomal association of plasmid pJoB2 AND PMD861.

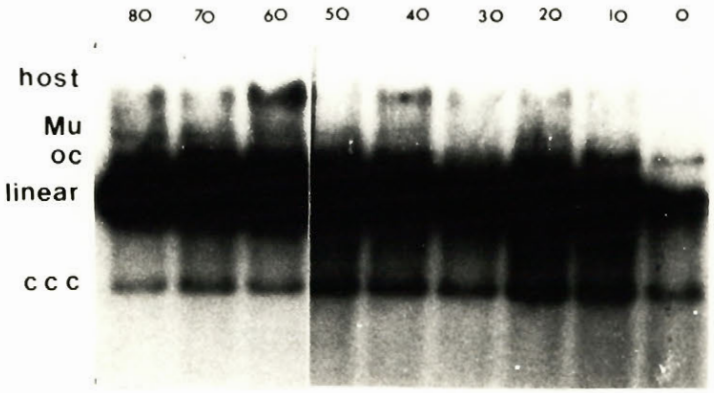
The numbers over each slot represent the time (in minutes) after induction of Mu lytic growth at 43^0C from which each sample was taken. The samples were removed, the cells lysed and the DNA electrophoresed, blotted and hybridized versus ^{32}P-pSC101 DNA as described in Materials and Methods. Southern blot represent Mu-infected cells containing plasmids pJoB2 in top panel and pMD861 in bottom panel.

The position of Mu and host DNA as well as the various plasmid forms are indicated.

pJoB2



pMD861



These deletions were located within the IS102 element of pSC101 (Figure 15). The SmaI-PvuII deletion would affect the transcription of the small and the large putative open reading frames of IS102 that have the same carboxy terminus (Figure 15). The SmaI, XmaI, as well as the large SmaI-PvuII deletions, would affect or abort the transcription of all the putative open reading frames in IS102 (pJo64, pJo65, pJo66, pJo67). It was shown by Bernardi and Bernardi (1984b) that deletions in this SmaI-PvuII region affected the transposition behavior and frequencies of IS102-mediated events.

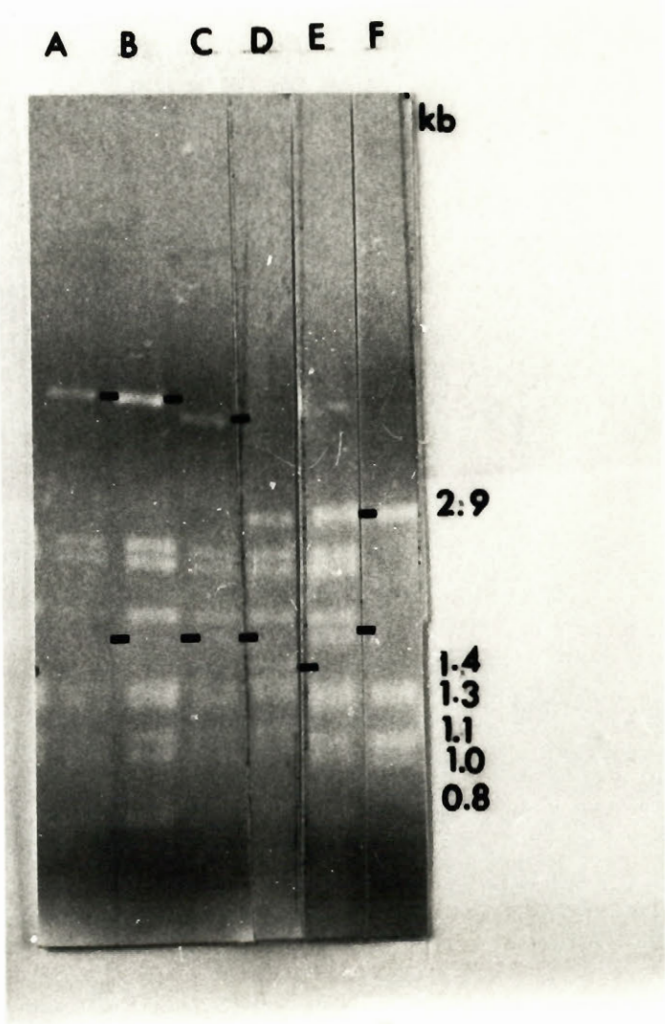
Similar transposition frequencies to those of the PJoE14, pJoE32 and pJoE43 plasmids were observed with pJo64, pJo65, pJo66, pJo67, in Mucts62 and Mucts62 pf7701 background (Table 4). The transposition frequencies of these site-specific deleted plasmids were reduced 3 to 5 fold compared with the parental plasmid pMD861 if the helper prophage was Mucts62 pf7701. Thus, the decreased transposition was also observed for the site-specific deletion plasmids as it was observed for the pJoE series plasmids with a SEER$^-$ helper prophage. These plasmids, pJo64, pJo65, pJo66, pJo67, were classified into the class1 series.

It seems unlikely that the deleted IS102 within the class1 plasmids derived from pMD861 would have a direct positive influence on Mini-Mu transposition when the induced transposition functions are provided by Mucts62 pf7701, because Mini-Mu plasmids with site-specific deletions within IS102 (PJo64, pJo65, pJo66, pJo67) still behaved like pJoE14, poJoE32 and pJoE43 plasmids. The DNA sequence composition upstream from the Mini-Mu, or DNA topology of this region could have played a role

Figure 30.

Restriction pattern analysis of pMD861 derivatives submitted to site-specific deletions.

HincII digestion of pJo63 (A), pJo64 (B), pJo65 (C), pJo66 (D), pMD861 (E) and pSC101(F). The digests were run on a 0.7% agarose gel.


A
B
C
D
E
F
kb
2.9
1.4
1.3
1.1
1.0
0.8

in the transposition activity of our Mini-Mu plasmids.

C-7. Insertion of of the Kan gene in pMD861.

We tested the hypothesis of transcriptional readthrough from the IS102 sequences into the Mu right extremity. To test this hypothesis, we inserted the HindII fragment containing the kanamycin resistance (Kan) gene of Tn903 from plasmid pUC71K into the PvuII site of pMD861 (Figure 14; Figure 19). The Kan gene of Tn903 is responsible for the decrease of transposition efficiency of the IS903 element because of the influence of the transcriptional readthrough inward towards the IS903 element (Grindley and Joyce, 1982).

Analysis of the insert within the PvuII site of pMD861 with XhoI and HindIII enzymes that cleave within the Kan gene asymetrically indicated that the Kan gene is present in only one copy and that the direction of Kan transcription is oriented toward the right extremity of the Mini-Mu genome (Figure 22).

As a control, a double insert of the SalI fragment of the Kan gene from pUC71k was placed into the XhoI site of pMD861. The XhoI site is located in the pSC101 sequences 87 bp upstream from the left extremity of the Mini-Mu. The double insertion of the Kan gene contains the genes in opposite orientation with one Kan gene having its transcription orientated towards the leftmost extremity of the Mini-Mu genome; as revealed by analysis with HindIII and XhoI (figure 22).

With these plasmids, regardless of the Mu helper prophage (either

Mucts62 or Mucts62 pf7701), the transposition frequency was decreased dramatically 10 to 20 fold (table 4). These latter results indicated that inhibition of transposition by strong transcriptional readthrough cannot be overcome by function(s) from the semi-essential early region of the induced Mu genome.

However, we could not reject the hypothesis that the transcriptional readthrough of IS102 could affect transposition of the Mini-Mu, though this latter is probably weaker than the kan gene. The hypothesis also states that this effect, being mild, could be overcome by functions within the semi-essential early region of the Mu genome. Placing a strong terminator sequence of transcription within the PvuII site of pMD861 would indicate if transcription plays a definitive role in pMD861 transposition.

C-8. Characterization of phage Mucts62 pf7701.

C-8.a. Mapping of Mucts62 pf7701.

Mucts62 pf7701 was a kind gift of Martha Howe. Mucts62 pf7701 originates from strain CT152 harboring Tn5 (Kan^r) inserted into a Mucts62/445-3 prophage in the lacI gene of an F'lac plasmid. The locations of the Tn5 insertion had been determined by marker rescue from lambda.p.Mu transducing phages, and by electron microcopic observation of DNA heteroduplex. Mucts62 pf7701 was described as a kil^- phage and

Figure 31.

Restriction maps of Mucts62 and Mucts62 pf7701.

Restriction maps of the genomes of Mucts62, in panel A), and Mucts62 pf7701, in panel B), are presented.

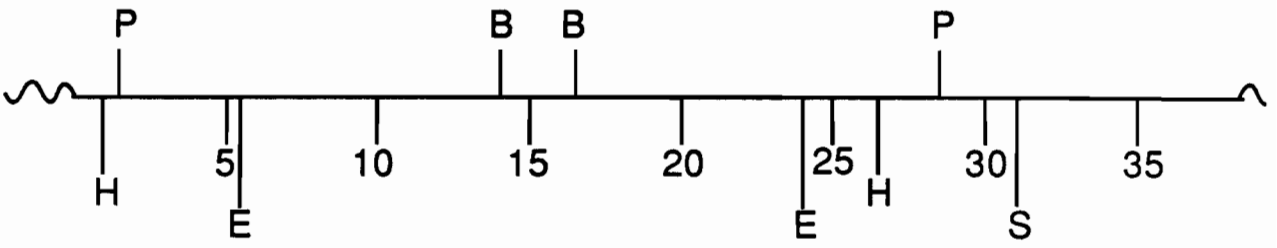
The thin line represents Mu DNA.

The thicK black line represents Tn5 DNA.

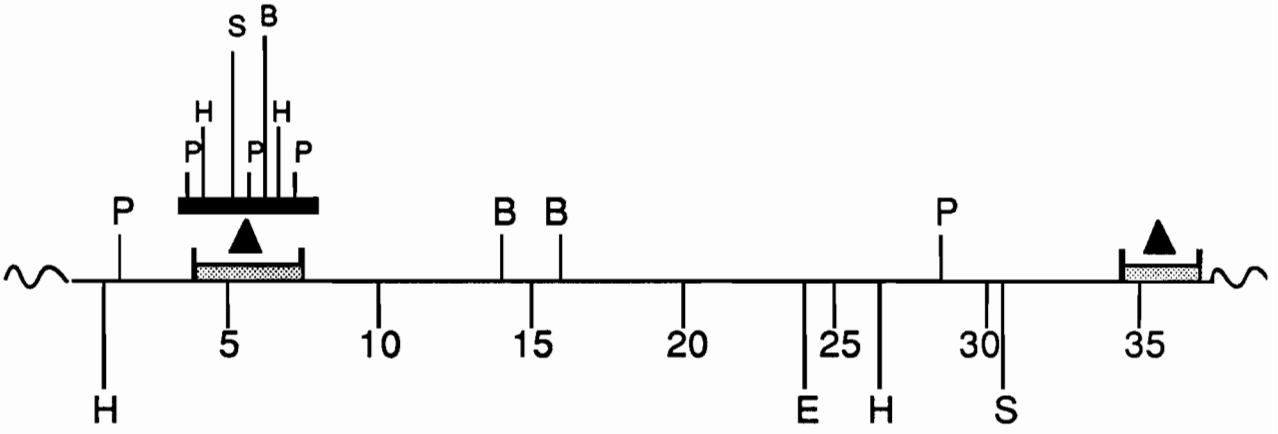
The broken line represents Host DNA.

The gray line indicates deletion of DNA.

A)


P
B
B
P
H
5
E
10
15
20
25
E
H
30
S
35

B)


S
B
H
H
P
P
P
P
B
B
P
H
5
10
15
20
25
E
H
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S
35

perhaps also as a gam$^-$ phage. Tn5 was found to be inserted between the B and C genes, in the alpha region of Mu genome. The initial kil$^-$ kan^r phage was defective in plaque formation, presumably due to the loss of the beta end of Mu DNA during packaging. Plaque forming kil$^-$kan^r phage were isolated as pseudorevertants of phages containing the inserted Tn5. The phage Mucts62 pf7701 has lost part of the Tn5 but remains Kan^r. Mucts62pf7701 has deleted part of Tn5 and part of Mu. DNA heteroduplex studies indicated that Mucts62 pf7701 has lost 2.8 Kb between Mu genes B and C and 2.09 kb at its right end (Figure 31). Tn5 is about 3.3 kb and was shown to have lost its ability to transpose.

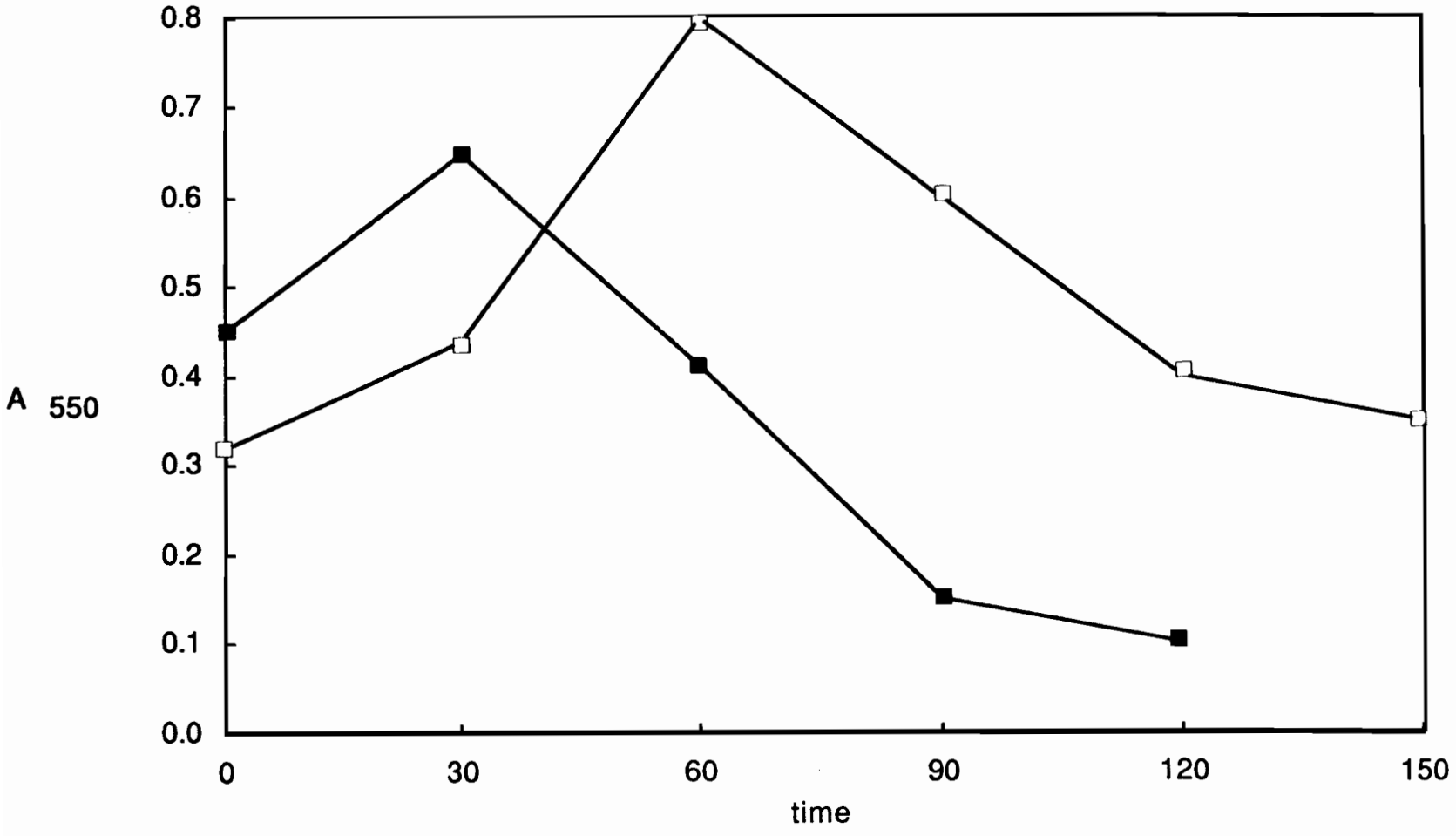
Two different strains were lysogenized with Mucts62 pf7701. Mucts62 pf7701 was inserted into the Xyl locus of strain Bu5029 and Mucts62 pf7701 was inserted into one of the Mal loci of HB101.We characterized the prophage Mucts62 pf7701 in strain LF113 and LF1037 by its themoinducible properties and restriction enzyme analysis. It is interesting to note that thermal induction of Mucts62 pf7701 did not yield complete lysis of the cells due to its kil$^-$ (and presumably arm$^-$) phenotype in comparison with a Mucts62 prophage containing strain. Whereas Mucts62 containing strains lysed after about 60 minutes after thermoinduction, in Mucts62 pf7701 containing strains (Figure 32) cell lysis was not drastic. In strains containing an induced Mucts62 pf7701, although Mu early and late functions were induced and Mu DNA was replicating, some functions in the semi-essential early region of Mu (e.g. arm) were not expressed due to the polar effect of the inserted Tn5 in between the B and the C genes. Moreover, the phage yield from a 'lysate' of

Figure 32.

Lytic profile graphs of strains HM8305■ and CT152□.

The X axis represents the time in minutes after induction of the strain at 43ºc.

The Y axis represents the OD A_{550} of the culture.


A 550
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
30
60
90
120
150
time

Muct62 pf7701 was very low (10^3-10^4 pfu/ml).

The mapping of the Muts62 pf7701 DNA as well as the positioning of the truncated Tn5 DNA was done by restriction enzymes analysis of chromosomal DNA from strains LF113 and LF1037 containing Muts62 pf7701 lysogens. Specific Mu bands were detected by hybridization with a Mu probe from the plasmid RP4::Mu. RP4::Mu was grown in Proteus mirabilis to avoid cross-hybridization with Escherichia coli chromosomal DNA, and to hybridize specifically with Mu bands of the lysogens.

The number of prophage copies in the chromosomal DNA was determined by SacI digestion. SacI is a "no-cut" enzyme for Mu as well as for Tn5. Both LF113 and LF1037 strains harbored only one copy of Muts62 pf7701 as revealed by the presence of one Mu specific band by SacI analysis as well as by genotypic determination (figure 33).

The sites of HindIII, BamHI, PstI and SalI cleavages within Mu and within Tn5 allowed agarose gel electrophoretic determination of the Tn5 insertion site.

Analysis of the restriction pattern of Muts62 pf7701 and comparison with Muts62 (figure 33) revealed that the Tn5 insertion is within the leftmost 4.5 kb of Mu DNA; Mu had lost its leftmost EcoRI site at 5.1 Kb and that the beta end of Mu DNA was shorter by about 2.5kb within the rightmost 10 kb of Mu DNA. The HindIII, BamHI and PstI sites within Mu and Tn5 revealed that the deletion of Mu was from about 4 to 7 kb within the leftmost end of the Mu genome. 2.5 kb of the SalI rightmost Mu DNA fragment was also absent. A Tn5 specific probe would have helped us to determined more precisely the DNA sequences and location of Tn5 within

Figure 33.

Restriction map of Mucts62pf7701.

Chromosomal DNA from LF1037 in (2) and LF113 in (3) lysogenic strains for Mucts62pf7701 compared with chromosomal DNA from LF110 lysogenic for Mucts62 in (1) are cut with SacI (S), BamHI (B), EcoRI (E), HindIII (H) and PstI (P) and run on a 0.7 % agarose gel.

The gels were Southern blotted and hybridized with RP4::Mu ^{32}P labelled DNA.

HindIII Lambda c1857 is used as the molecular weight marker.

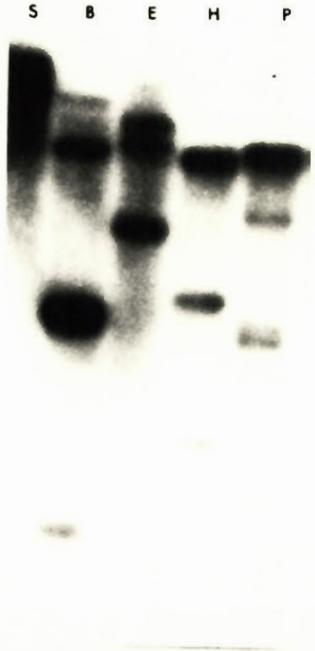
1


S
B
E
H
P
Kb
21,8
5.0
3,4
1,9
1,0

2


S
B
E
H
P
Kb
21,8
5.0
3.4
1.9
1.0

3


S
B
E
H
P
Kb
21.8
5.0
3.4
1.9
1.0

the Mu genome. In a recent paper, Waggoner et al (1984) have localised precisely the Mu leftmost deletion from 4.4 kb to 7.8 kb of its leftmost DNA.

C-8.b. Influence of Mucts62 pf7701 on transposition of pMD861 and cointegrate formation.

Mucts62 pf7701 has lost a region between 4.4 kb and 7.8 kb of its genome (Waggoner et al., 1984) that is part of its semi-essential early region. The Tn5 insertion within the semi-essential early region of Mucts62 pf7701 was also described to have a polar effect on transcription downstream from its point of insertion (Figure 31).

The transposition of pMD861 in strains such as LF113 and LF1037 containing Mucts62 pf7701 compared with the transposition frequency of pMD861 in strain LF110 (Mucts62 prophage) was found to be in many of the transposition frequency experiments lower 2 to 8 fold in Mucts62 pf7701 strains (Table 7). This indicated that functions in the semi-essential early region of Mu involved in the efficiency of transposition of Mu and Mini-Mu (such as arm) described by Waggoner et al. (1982) as positively influencing the efficiency of transposition of Mu 3 to 10 fold, were absent.

We tested the influence of Mucts62 pf7701 on the cointegrate transposition end-products of Mini-Mu plasmids by replica-plating Amp^r/Spc^r exconjugants onto a tetracyclin containing plate (Table 8).

Table 7.

Transposition frequencies of pMD861 in E.coli strains LF110 and LF113.

LF110	LF113	A*
$pro^+/pro^+ amp^r$	$pro^+/pro^+ amp^r$	
5.7×10^{-2}	0.75×10^{-2}	7.6 x
8×10^{-2}	2.3×10^{-2}	3.4 x
7.9×10^{-2}	1×10^{-2}	7.9 x

The transposition frequency was expressed as the number of pro^+ sexductants that are pro^+, Amp^r.

Each line is representing the transposition of pMD861 assayed within the different E.coli strains LF110 and LF113.

A*- Differential ratio of transposition frequencies of pMD861 in LF110 versus LF113.

Table 8.

Cointegrate frequencies of pMD861 in strains LF110 and LF113.

LF110	LF113
91%	44%
88%	57%

The cointegrate frequency was expressed in the percentage of Amp^r pro^+ exconjugants that were Tet^r, later verified by plasmid analysis of the exconjugants.

Although no dramatic difference was observed, the general pattern was that for pMD861, as well as for other plasmids in the class 1 plasmids, cointegrate formation of Mini-Mu transposition was reduced 1.5 to 2 fold.

The interpretation of these results remains unclear since there is not a general trend for Mini-Mu cointegrates/simple inserts ratios; the results vary from laboratory to laboratory (Toussaint and Resibois, 1982; 1984). Under our conditions, however, it seems that cointegrate formation is lower in a Mucts62 pf7701 background than in a Mucts62 background. It can be proposed that some functions involved in the transposition of Mu and that help the cointegrate formation of Mu are absent in Mucts62 pf7701, such as gam that protects Mu DNA from recBC nuclease or perhaps arm whose real activity is unknown.

C-9. DNA composition of sequences adjacent to Mini-Mu.

The region of pMD861 surrounding the PvuII and the SmaI sites was analysed using a computer analysis program that calculates the % of GC per 50 base pairs intervals (Lussow and DuBow, in preparation), from nucleotides 6916 to nucleotide 8316 according to the pSC101 sequences (Bernardi and Bernardi, 1984,a). From this analysis, it appeared that the SmaI-PvuII region is rather GC rich (Figure 34). The DNA composition of regions surrounding the Mini-Mu genome could influence the topology of Mini-Mu to transpose. Local alteration in DNA structure might affect Mini-Mu transposition in this DNA transposition topology or because of the presence of notche sequences, could orientate the transposition proteins

Figure 34.

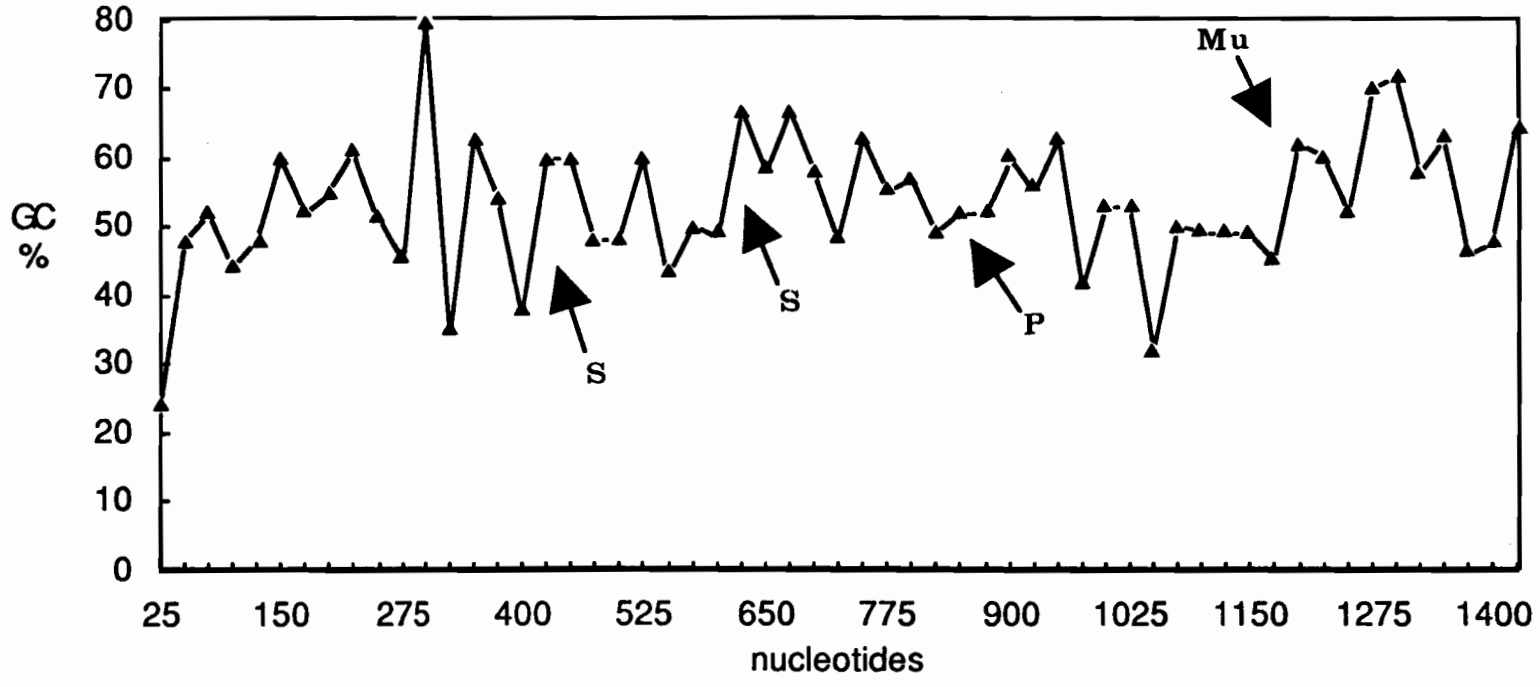
GC content of pSC101 from nucleotide 6916 to nucleotide 8316.

The region of pSC101 containing the restriction sites for PvuII and SmaI was scanned for its GC content. Each point represents the average percentage of GC contents per 50 bp intervals.

The arrow above the curve indicates Mu insertion site.

The arrows below the curve indicate the restriction sites of SmaI (S) and PvuII (P).

Map of pSC101



with respect to the Mini-Mu genome. A computer search for GTGCAC notch sequences (Kolata, 1983) in pSC101, however, indicated that no such sequence existed within pSC101.

D- Discussion.

Mu and other transposons show a cis dependence on their extremities in order to transpose. Moreover, the adjacent DNA sequence environment can have a large influence on the relative activity of transposons.

In an effort to understand the cis-acting sequences requirements of transposable bacteriophage Mu DNA transposition, we have created a class Mini-Mu plasmids (class 1) in which alterations in the neighboring plasmid sequences of the Mini-Mu exert an influence on the frequency of transposition of the Mini-Mu DNA. However, this phenomenom is seen only when the helper prophage contains a polar insertion in its semi-essential early region (Mucts62pf7701).

Our results indicate that some function(s) absent from this phage may modulate Mini-Mu DNA transposition activity through by DNA sequences adjacent to the Mini-Mu genome. Bacteriophage Mu uses multiple rounds of DNA transposition to amplify its DNA rapidly and efficiently from virtually any site in the Escherichia coli genome to another. Thus, it is reasonable to propose that some function(s) in the semi-essential early region of the Mu genome have evolved to enable Mu to transpose from any location and from any potentially poor site into which Mu may have integrated.

Mucts62pf7701 contains an insertion of Tn5 in the region beyond the B gene and a consequent deletion of 2.8 kb of this region (Waggoner et al., 1984). The insertion of Tn5 within this region exert a polar effect on the transcription of the downstream semi-essential early region.

Mucts62pf7701 exhibits a kil$^-$ phenotype. The semi-essential early region encodes several modulatory functions for Mu DNA transposition such as cim (control of immunity), kil (host cell killing), arm (amplification of Mu replication), gam (anti-recBC nuclease), sot (stimulation of transfection) and lig (DNA ligase) (Zipser et al., 1978; Schumann et al., 1982; Waggoner et al., 1982; Goosen et al., 1982; Schaus and Wright, 1980; Waggoner et al., 1984; Van Vliet et al., 1978; Van de Putte et al., 1977; Ghelardini et al., 1980). It is not known precisely where these functions map nor is it known if these represent multiple functions of a smaller set of genes. One candidate for a function that would participate to the enhaced rate of transposition is the arm locus, as arm was defined as a locus which stimulates Mu DNA transposition 3-10 fold during lytic growth (Waggoner et al., 1981; Goosen et al., 1982).

There are several possibilities to explain why deletions outside the Mini-Mu sequences can affect Mini-Mu transposition only when the helper prophage is Mucts62 pf7701. One mechanism to account for this cis-acting repression is transcription into the attR of the Mini-Mu genome from a promoter within the IS102 element on the pSC101 (Bernardi and Bernardi, 1981). Our deletions in the adjacent IS102 could alter genetic signals that allow enhanced or reduced transcriptional readthrough of IS102 into the Mini-Mu genome. It has been demonstrated that for IS1 and IS903, the efficiency of transposition and cointegrate formation is influenced by the sequences adjacent to them (Weinert et al., 1983; Machida et al., 1982; Chandler et al., 1983;Grindley et al., 1980). The most probable cause of

this is transcription that is directed into the IS elements from the neighboring sequences (Ahmed, 1984; Biel et al., 1984).

In that perspective, a 1.2 kb Kan resistance gene from Tn903 was inserted into the PvuII site and the XhoI site of pMD861. The transposition of the Mini-Mu genome containing the Kan gene was affected in either prophage background, presumably due to constitutive transcription from the Kan gene into the right and the left extremity of the Mini-Mu genome, repectively (Biel et al., 1984; Ahmed, 1984; Machida et al., 1981). Most probably, the Kan gene transcription proceeds into attR or attL of the Mini-Mu genome and this transcriptional readthrough inhibition of transpositon cannot be overcome by products from the semi-essential early region.

The mechanisms by which external transcription exerts its effects on transposition remains obscure. It is possible that the protein-DNA complex that is formed at the ends of the transposable element prior to and during initiation of transposition is disrupted by inward directed transcription (Bick et al., 1972). In this regard, it is interesting to note that no transcription proceeds outwards from Mu during lytic growth (DuBow and Bukhari, 1980). Although initiation of replication of Mu was shown to preferentially initiate at the c left end, initiation of Mini-Mu DNA replication was observed to occur at either end of the Mini-Mu genome (Toussaint et al, 1984). Since the presence of the kan gene at either end of the Mini-Mu genome alters its transposition, this phenomenom favors the concept of transcriptional readthrough of the kan gene inhibiting the transposition of the Mini-Mu genome. Moreover, it suggests that the

possible transcriptional inhibition from the IS102 sequences can be overcome by semi-essential early region products. This hypothesis would be strengthened by data on IS102 transcription.

Another possibility is that the product(s) of IS102 open-reading frame(s) is (are) presumably involved in IS102 transposition (Bernardi and Bernardi, 1981; 1984b). The deletions in pJoE14 and pJoE32 and pJoE43 occur in two hypothetical open reading frames of IS102. Although the three base pair deletion in pJoE32 does not change the reading frame, it can be hypothesized that some IS102 gene product may affect Mini-Mu transposition in cis or in trans, either by acting in conjunction with the transpositional apparatus of Mu or by its own properties of transposition acting in parallel with the Mu transpositional apparatus. In this regard, however, IS102 is known to have a very low transposition frequency compared with an induced prophage or a Mini-Mu (Bernardi and Bernardi, 1981; 1984b; Ohtsubo et al., 1980). The presence of some product of the Mu semi-essential early region may, thus, compensate the effect of the altered putative function of the IS102. This is indicative that the role of some polypeptide from IS102 could be minimal.

One other explanation proposes that the long-range cis effect of the adjacent sequence environment may influence the Mini-Mu transposition. Many studies have assessed the role of neighboring DNA sequences in gene regulation, replication, repair, and the influence on DNA topology or base composition. DNA topology and sequence environment such as AT richness can modulate transposition activity. It has been shown that DNA supercoiling affects the transposition frequency of Tn5 (Isberg and

Syvanen, 1982). The frequency of precise excision of Tn*5* in an *uup*$^-$ *Escherichia coli* differs depending on the location of the original Tn*5* as well as the DNA state of the replicon (Hopkins et al., 1983). A *cis* influence of DNA sequences located at great distances from the structural gene has been described in the beta-globin system (Kioussis et al., 1983) and in exhaustive studies on enhancers (Yaniv, 1982; Boss, 1983; Khoury and Gruss, 1983). Expression of cloned *ebgA* gene in *Escherichia coli* is drastically altered by insertion of a transposon 1.4 kb upstream from the gene (Stokes et al., 1984). Reports on local alteration in notches in DNA structures that could affect the protein binding have been made (Kolata, 1984).

In the case of pMD861 derivatives, a modulation of transposition is seen in the absence of the expression of the semi-essential early region from the helper prophage when the sequences between 300 bp and 600 bp downstream from the *attR* of the Mini-Mu genome are deleted. This area is found to be more GC rich than the rest of the sequences near the right extremity of the Mini-Mu. This base composition may account for some of its effect when deleted.

If the Mini-Mu is sensitive to local changes in DNA topology, base composition, or long range *cis*-acting sequences then this may reinforce the notion that Mu is an efficient viral transposon that has evolved functions that allows rapid and efficient transposition from any site. Genes from the semi-essential early region of the Mu genome are difficult to localize and study because of their semi-essential nature. The use of Mini-Mu plasmids such as the class1 plasmids could enable one to more

precisely define the role of the semi-essential genes involved in the modulation of the transposition phenotype, in order to ultimately to characterize these genes and determine their functions and analyze their interaction in Mu transposition.

CHAPTER IV. THE MU SPECIFIC *cis*-REQUIRED SEQUENCES FOR TRANSPOSITION AND MATURATION.

A- Introduction.

The temperate bacteriophage Mu can be regarded as a giant transposon with a molecular weight of 25 megadaltons (Taylor, 1963). The transposition of bacteriophage Mu is an obligatory feature in the life cycle of the virus (Bukhari, 1976; Toussaint and Resibois, 1983). From in vivo and in vitro studies of the transposition reaction, two phage encoded proteins , the A and B gene products, are thought to be directly involved in the process (O'Day et al., 1978; Craigie et al., 1984; Coelho and Symonds, 1982; Resibois et al., 1983). Other phage-encoded gene products in the semi-essential early region have been suggested to play a role for efficient transposition of Mu (Chapter III). Several host proteins are required for Mu DNA transposition (Toussaint and Resibois, 1974; Teifel and Greding, 1983; Craigie et al., 1984).

The transposition process of Mu requires the ends of Mu in the correct orientation in a donor DNA. The ends of the transposon must be intact for transposition to occur, as deletion of one or both ends produces a non-complementable transposition defect (Chaconas et al., 1981; Faelen et al., 1979; Schumm and Howe, 1982). Hence the ends of Mu harbor cis required sequences for Mu transposition.

In contrast to other transposons, the ends of Mu do not have long terminal inverted repeats (Kahmann and Kamp, 1979). Homologies between the ends do occur but are not symetrically disposed. This structural asymetry may reflect an underlying functional asymetry. The ends of Mu play a continuous role throughout the viral cycle of Mu (Toussaint and Resibois, 1983). The fact that the ends, upon integration and throughout lytic replication, remain intact indicates the importance of their integrity

(Kamp and Kahmann, 1980; Chaconas et al., 1981; van de Putte et al., 1978). Moreover, the direction of replication proceeds from the left toward the right end (Goosen et al.,1978). Finally the packaging site has been found for Mu headful packaging (Bukhari and Taylor, 1975) to be located in the left extremity of the phage genome (Teifel-Greding, 1984; Goodchild et al., 1985; Groenen and van de Putte, 1985).

It was previously shown that there is an essential Mu sequence for transposition form nucleotides 68 for the right end of the Mu genome (Castilho et al., 1984). In a recent study (Groenen et al., 1985) where a system consisting of the A and B genes cloned on the same muticopy vector as the Mu ends to be tested under lambda pL control, two regions essential for Mu transposition at attL ending 25 and 160 bp from the left extremity and one region of 50 bp from the right extremity of Mu were found. Moreover using an in vitro assay, Craigie et al. (1984) demonstrated regions of specific binding and nuclease protection of the gpA of Mu at the extremities of the Mu genome.

To dissect the different domains at the extremities of Mu genome in relation to their biological roles, we used an experimental system in which Micro-Mu's are harbored by a plasmid vector and are dependent upon the expression of a thermoinducible Muc ts62 helper prophage functions to transpose. We have constructed various sized Micro-Mu's, containing the gene conferring kanamycin resistance (kan) cloned either onto a low copy vector (pSC101) derivative or onto a high copy vector (pBR322). Their transposition properties were tested with the low copy vector carrying the Micro-Mu's in a strain where the transposition functions are provided in trans by an induced Muc ts62 helper prophage. The packaging capacities

of the various Micro-Mu's were checked by screening linearization of pSC101::Micro-Mu plasmids late in the lytic cycle. Furthermore their maturation was monitored by transduction of oligomerized Micro-Mu plasmids using pBR322::Micro-Mu's in a strain harboring an induced Mucts62 phage.

Different biological functions inherent to the Mu phage life-style throughout its cycle were correlated with DNA sequences at the Mu ends. We found that the transposition domains are lying within a necessary region (1-55 bp) and an auxiliary region (126-203 bp) at Mu attL and a necessary region in the first 62 bp at Mu attR. The pac site, the site required for Mu maturation into virions, is lying within the 55 leftmost nucleotides of the Mu genome. The arrangement of these different functional domains suggests that the cis functions have been integrated into the Mu genome during the evolution of this transposable bacteriophage.

B-Results.

B-1. Construction and characterization of Micro-Mu derivatives.

B-1.a.Initial cloning.

In order to investigate the sequence requirements for Mu transposition and packaging, we varied, by subcloning, the lengths of the sequences in attL and attR of the Mu genome. Figure 35 gives an overall picture of the cloning strategy we undertook with the details of the constructions in order to obtain a collection of Micro-Mu's.

We started with a pBR322 derivative plasmid, pJK43. pJK43 contains the terminal Cla1-HindIII fragment of the Mu genome from the plasmid pMD186 (DuBow and Lalumière, in press) (Figure 36). The unique aspect of pMD186, derived from plasmid pMD861, is that the two ends of Mu are facing each other and are separated by 407 bp of pSC101 (from nucleotides 378 to 1272 in pSC101 sequence map according to Bernardi and Bernardi, 1984). Inside the 407 bp region that separates the two ends is an EcoR1 site.

The Cla1-HindIII fragment of the Mu genome (originated from pMD186) in pJK43 consists of 1.2 kb of the terminal sequences of attR and 1.0 kb of the terminal attL sequences.

Figure 35.

Outline of the Micro-Mu constructions.

1- BamHI-EcoRI fragments of Mu attR and attL are ligated together then cleaved with EcoRI in order to obtain fragments ligated in the correct orientation with the Mu extremities facing outward.

2- The EcoRI ligated attL-attR fragment is inserted into the EcoRI site of pBR322 (BamHI$^-$). The E.coli DHI transformants are selected for ampicillin resistance.

3- pBR322 (BamHI$^-$)::Micro-Mu is opened at its unique BamHI site in between Micro-Mu attL and attR and the BamHI fragment, containing the Tn903 Kan gene, is inserted. Transformants are selected for ampicillin and kanamycin resistance.

4- pBR322 (BamHI$^-$)::Micro-Mu Kan is transformed into strain LF249 to study Micro-Mu maturation and packaging properties.

5- The EcoRI fragment containing Micro-Mu Kan from pBR322 (BamHI$^-$)::Micro-Mu Kan is inserted into the pJo2A EcoRI site. pJo2A::Micro-Mu Kan is transformed into strain LF110 to study Micro-Mu transposition properties and maturation capabilities.

The construction of the sets of Micro-Mu's carrying various lengh of attL and attR is described in Results.

Mu attL and attR are shown with thin arrows.

Filled thick segments are pBR322 sequences.

Medium thick segments are pSC101 sequences.

The dotted line represents Amp^r gene.

The open line represents Kan^r gene.

B* indicates that the plasmid has lost is BamHI site because the BamHI site was backfilled and is therefore Tet^s.

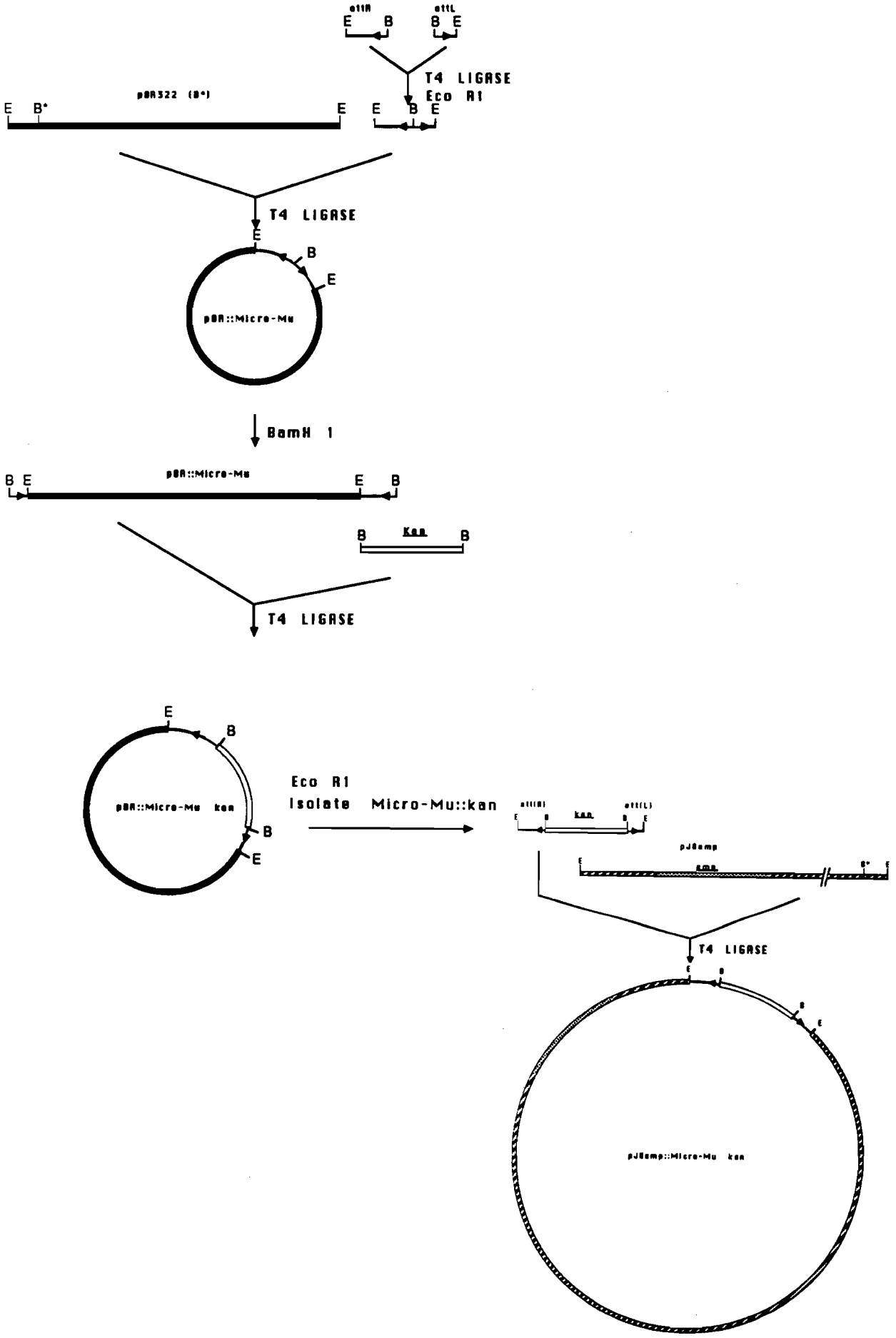

attR
attL
T4 LIGASE
Eco R1
pBR322 (B*)
T4 LIGASE
pBR::Micro-Mu
BamH 1
pBR::Micro-Mu
Kan
T4 LIGASE
pBR::Micro-Mu kan
Eco R1
Isolate Micro-Mu::kan
pJBamp
amp
T4 LIGASE
pJBamp::Micro-Mu kan

Figure 36.

Initial cloning of Mu extremities from pJK43.

This diagram shows the Mu fragment ClaI-HindIII from pJK43, and the Mu-specific fragments derived from pJK43 when pJK43 is cleaved with Sau3A, Tha1, Hinf1,Alu1 restriction enzymes.

BamHI linkers were added inside the extremities of the Mu fragments.

The thick line represents Mu sequences.

The thin line represents pSC101 sequences.

B, BamHI; C, ClaI; E, EcoRI; H, HindIII.

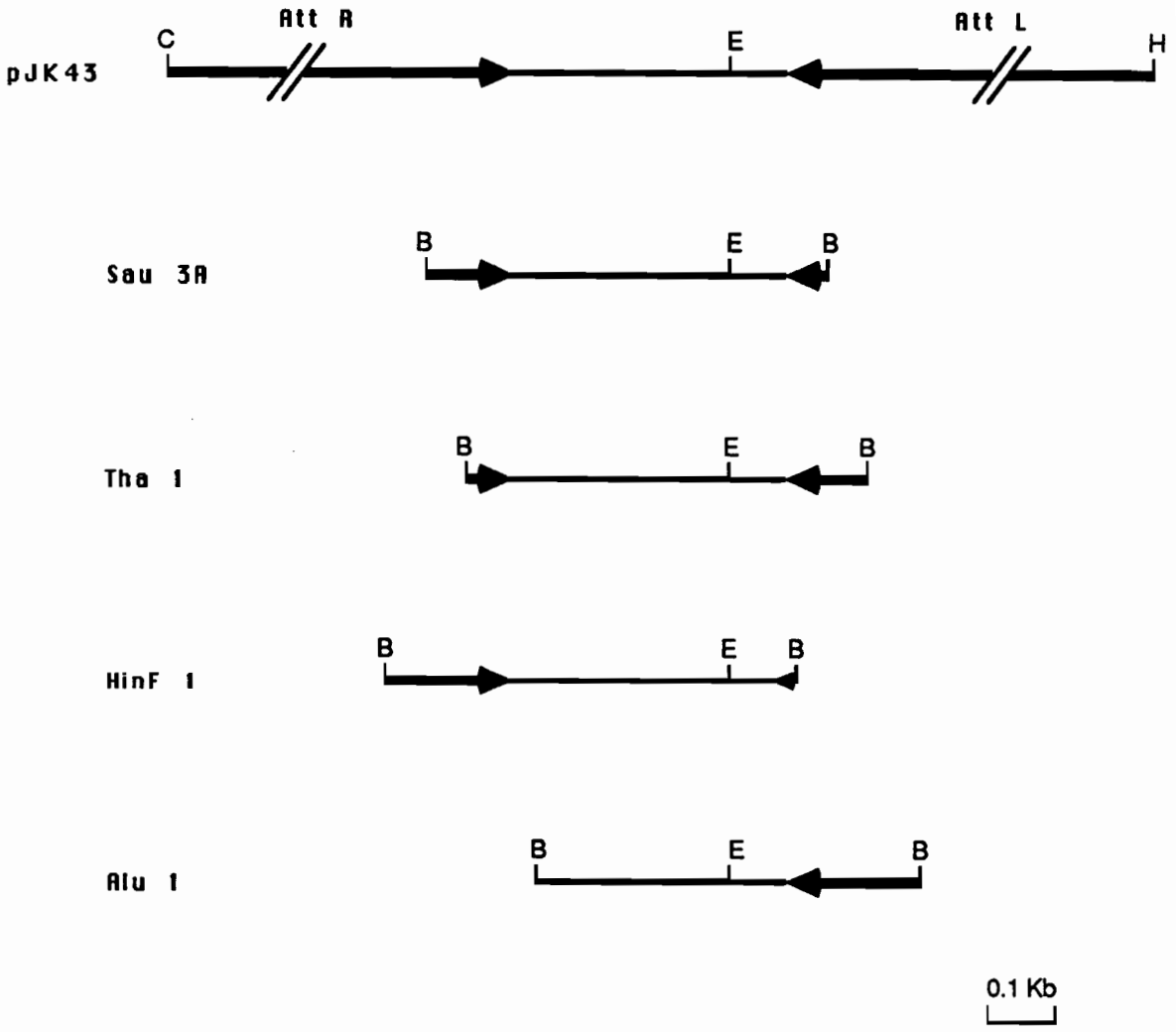

pJK43
C
Att R
E
Att L
H
Sau 3A
B
E
B
Tha 1
B
E
B
HinF 1
B
E
B
Alu 1
B
E
B
0.1 Kb

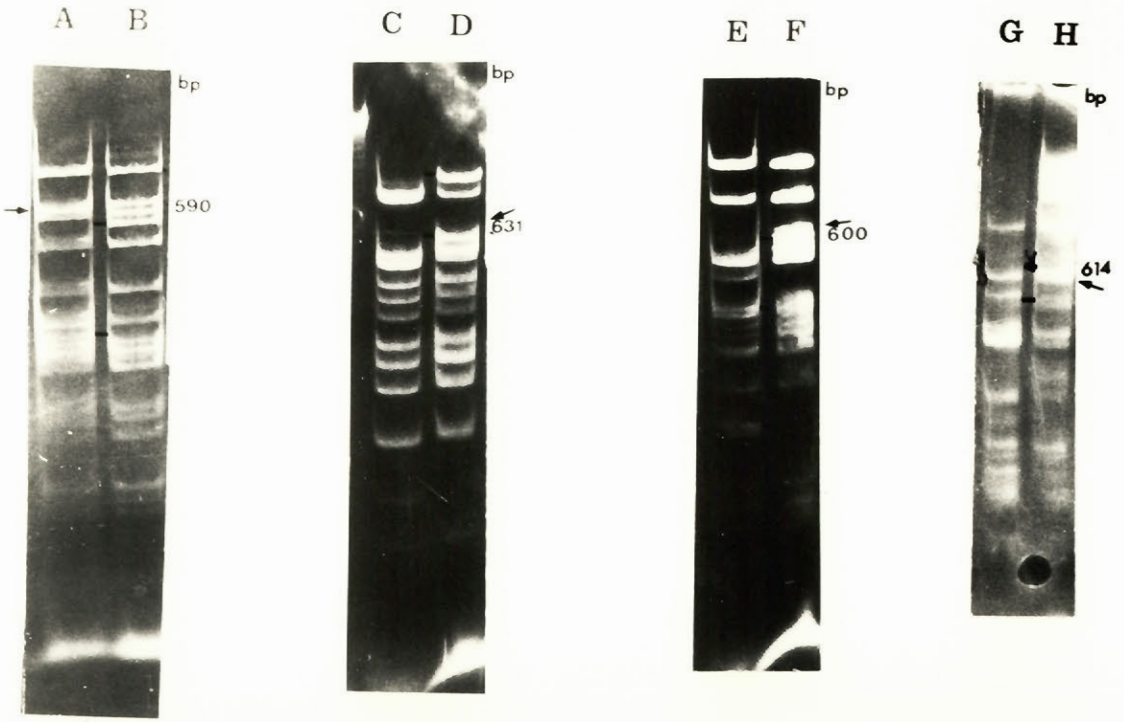
Figure 37.

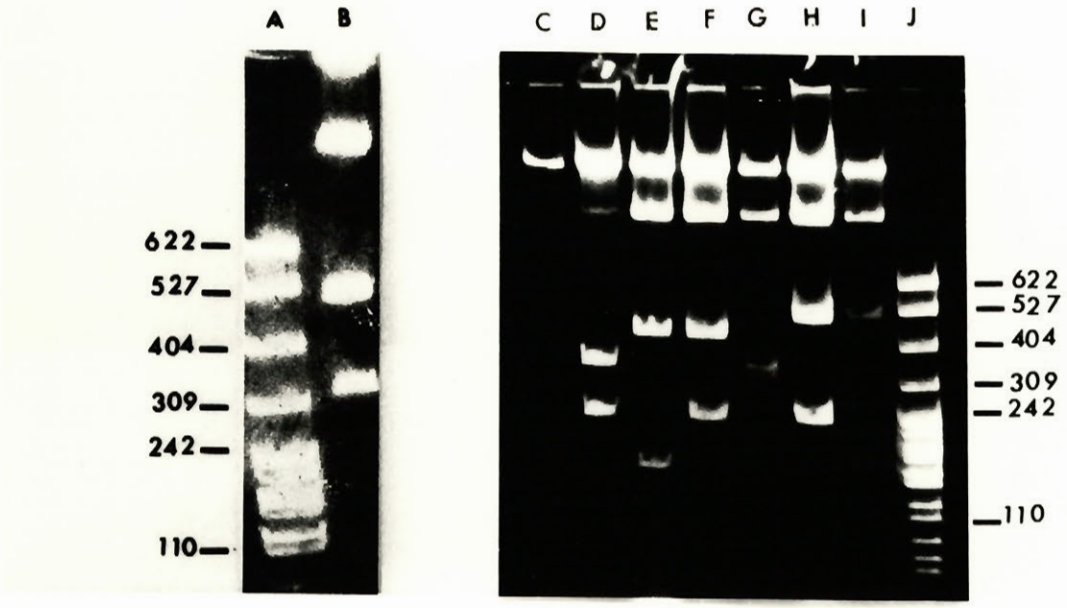
Polymorphic pattern of pJK43 cleaved with different enzymes.

pJK43 was cleaved with AluI (A), AluI/EcoRI (B), HinfI/EcoRI (C), HinfI (D), Sau3A/EcoRI (E), Sau3A (F), TaqI/EcoRI (G) TaqI (H).

The digests were run on a 5% polyacrylamide gel. HpaII fragments of pBR322 served as molecular weight markers.

The Mu bands containing the EcoR1 site are indicated by an arrow.


A
B
bp
590
C
D
bp
631
E
F
bp
600
G
H
bp
614


A
B
C
D
E
F
G
H
I
J
622
527
404
309
242
110
622
527
404
309
242
110

We have isolated various lengths Micro-Mu's using the HinfI, ThaI, AluI, Sau3A fragments of the Mu genome from pJK43 (Figures 36, 37). HinfI, ThaI, Sau3A were initially choosen because they did not cleave in the 407 bp between the two ends of Mu. These HinfI, ThaI and Sau3A fragments contain different lengths of attL and attR of the Mu genome separated by 407 bp of pSC101 DNA.

HinfI cleaves 7 bp into attL and 185 bp into attR.

ThaI cleaves 126 bp into attL and 62 bp into attR.

Sau3A cleavess 55 bp into attL and 117 into attR.

AluI cleaves 203 bp into attL but also into the 407 sequences of pSC101. The AluI fragment contains only 203 bp of attL sequences and 301 bp of pSC101 (Figure 38).

The DNA fragments harboring the Mu extremities and the EcoRI site in between them were identified on 5% polyacrylamide gel after hydrolysis of pJK43 with the appropriate restriction enzyme (HinfI, ThaI, Sau3A or HinfI). The restriction fragment polymorphism of the pattern created by one of the above enzyme plus EcoRI is compared with the pattern with that of the enzyme alone. EcoRI cleaves pJK43 twice, once in the pBR322 sequences, and once in between the two ends of Mu (Figure 39).

DNA fragments harboring attL and attR, from pJK43, after identification on 5% polyacrylamide gels were purified by the "crush and soak" procedure (Maxam and Gilbert, 1980). The DNAs were ^{32}P end-labelled by T4 DNA kinase plus $\delta^{32}P$-ATP, cleaved with EcoRI, and repurified on a polyacrylamide gel. Maxam and Gibert sequencing on these DNA fragments confirmed that they were Mu att specific.

Figure 39.

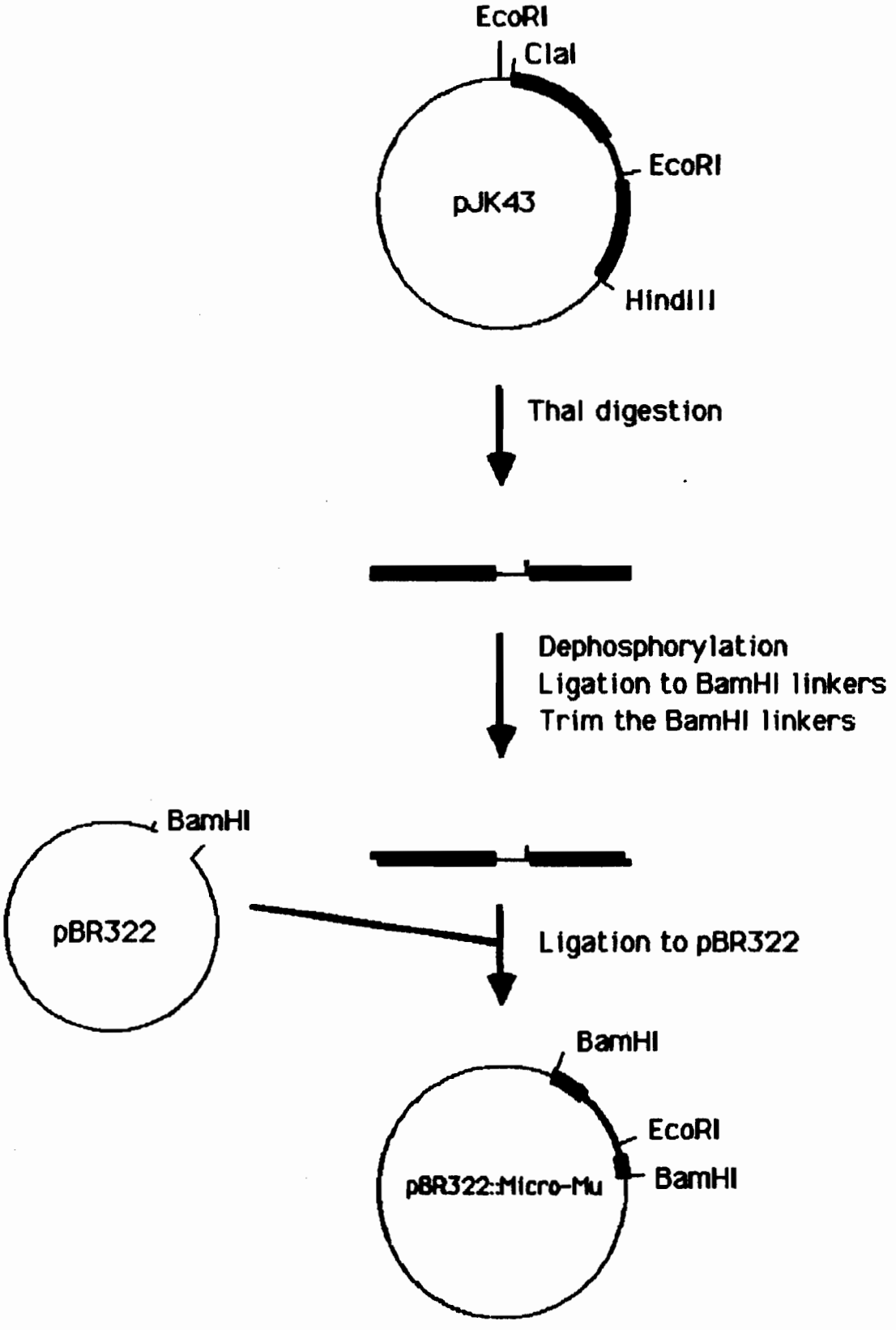
Schematic representation of the initial cloning of the Micro-Mu DNA's.

1- pJK43 is digested with an enzyme such as ThaI. A Mu ThaI specific fragment contains both ends of Mu of different sizes separated by 407 bp of pSC101 sequences, from pMD861 (DuBow and Lalumière, in press).

2- The ThaI fragment containing both ends of Mu as previously determined by the restriciton fragment pattern of ThaI/EcoRI compared to the pattern of ThaI is isolated. ^{32}P-labelled BamHI linkers are added to the ends of the fragment.

3- The Micro-Mu fragment flanked by BamHI sites at its Mu-interior extremities, is inserted into the dephosphorylated BamHI site of pBR322. The chimeric plasmid is transformed into DHI. Transformants are selected for ampicillin resistance and tetracyclin sensitivity. The att sites within this initial cloning in pBR322::Micro-Mu are facing each other and are separated by 407 bp of pSC101 in which is an EcoRI site is located 87 bp from attL.

BamHI-EcoRI digestion and polyacrylamide electrophoresis allows one to isolate the att sequences separately.



Select transformed cells $\underline{amp}^r$ $\underline{tet}^s$

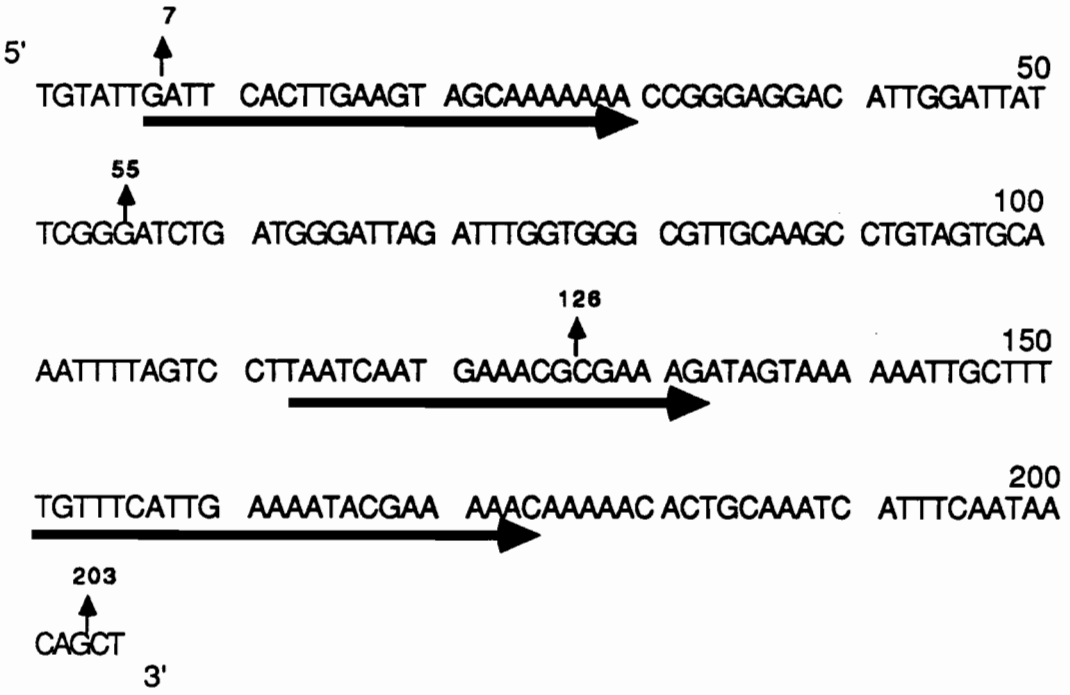
Figure 38.

DNA sequences of attL (Priess et al. 1982) and attR (Kahmann, 1983; Plasterk et al., 1984) of the Mu genome.

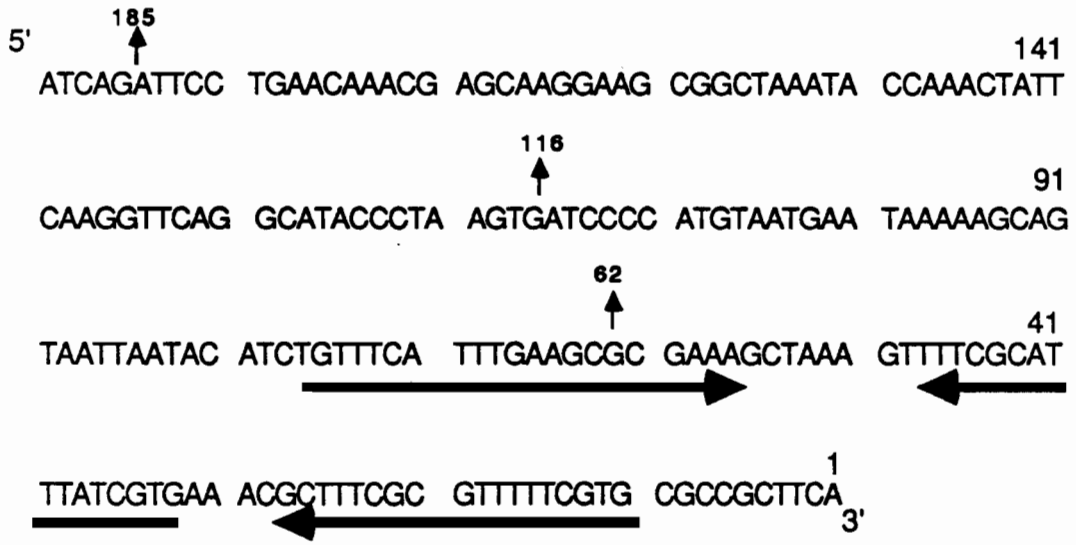
The initial cloning of Micro-Mu's fragments from pJK43 was done using AluI, HinfI, ThaI, Sau3A.The position of Mu segments generated by these restriction enzymes are indicated by a vertical arrow, and are numbered.

The thick arrows underline the consensus sequences PyNNNNNAAPuPyPuCGAAAPu (Craigie et al., 1984; Groenen et al., 1985).

Att L


7
5'
50
TGTATTGATT CACTTGAAGT AGCAAAAAAA CCGGGAGGAC ATTGGATTAT
55
100
TCGGGATCTG ATGGGATTAG ATTTGGTGGG CGTTGCAAGC CTGTAGTGCA
126
150
AATTTTAGTC CTTAATCAAT GAAACGCGAA AGATAGTAAA AAATTGCTTT
200
TGTTTCATTG AAAATACGAA AAACAAAAAC ACTGCAAATC ATTTCAATAA
203
CAGCT
3'

Att R


185
5'
141
ATCAGATTCC TGAACAAACG AGCAAGGAAG CGGCTAAATA CCAAACTATT
116
91
CAAGGTTCAG GCATACCCTA AGTGATCCCC ATGTAATGAA TAAAAAGCAG
62
41
TAATTAATAC ATCTGTTTCA TTTGAAGCGC GAAAGCTAAA GTTTTCGCAT
1
TTATCGTGAA ACGCTTTCGC GTTTTTCGTG CGCCGCTTCA
3'

As a previous step, the Hinf1 fragments were made blunt end by S1 treatment prior to the addition of BamHI linkers. BamHI linkers were added to their inside extremities and the fragments were inserted into the BamHI site of pBR322 (Figure 36, Figure 37). The cloning of the Sau3A fragment into the BamHI site of pBR322 restored BamHI sites at each end.

Phosphorylated BamHI linkers were ligated to purified (HinfI, ThaI or AluI) fragments containing flush ends that had been dephosphorylated with calf intestinal alkaline phosphatase. The blunt-end DNA was resuspended in 20 µl of linker ligation buffer and 10 units of T4 ligase plus 0.5 µg of γ^{32}P-labelled BamHI linkers (Collaborative Research, Waltham, Mass.), previously phosphorylated using T4 polynucleotide kinase, were added. The ligation reaction was allowed to proceed at 15^0c for 16 hours, terminated by incubation at 60^0c for 10 minutes, and then diluted to 100 µl with restriction endonuclease buffer. 100 units of BamH1 were added and the reaction to "trim" the linkers was incubated at 37^0c for four hours. The DNA was then phenol, chloroform-isoamylalcohol (24:1) and ether extracted and ethanol precipitated as above.

The DNA was resuspended in 50 µl ligation buffer with pBR322 plasmid linearized at its BamHI site and previously dephosphorylated with calf intestinal alkaline phosphatase. 10 units of T4 ligase were added and the DNA was allowed to ligate at 15^0c for 16 hours. The reaction was terminated by incubation at 65^0c for 10 minutes.

The DNAs were transformed into competent bacteria (DHI). Bacterial clones were screened for ampicillin resistance and tetracyclin sensitivity as well as the presence of extra DNA of about 0.8 kb in size by rapid screening of a "cracking" gel (see in Materials and Methods, section

O). Plasmids obtained by rapid DNA purification of the putative clones were analysed by BamHI, EcoRI digests as well as BamHI-EcoRI double digests; The pBR322 HpaII pattern served as a molecular weights standard. These gels were also hybridized with Mu DNA specific probes to eliminate false positive clones. The DNA of the positive clones and then purified were examined in more details.

The Micro-Mu extremities in these initial clones were facing each other, separated by 407 bp of pSC101 DNA. The length of the extremities of these various Micro-Mu's ranges between 203 bp to 7 bp for attL of Mu and 185 bp to 62 bp for attR of Mu (Figure 36, Figure 38). The plasmids clones were called HinfI (185R-7L), TaqI (62R-126L), AluI (203L) and pJK5 (116R-55L) and they served as DNA source of Micro-Mu BamHI-EcoRI extremities (Figure 35).

B-1.b. Construction of Micro-Mu's in the correct orientation.

By the use of the BamHI restriction site at the inside ends of the Micro-Mu's and the EcoRI site in between the outside ends, we have been able to construct various combinations of attL and attR Micro-Mu's in their correct orientation as subsets of BamHI-EcoRI extremity fragments (Figure 35). Various combinations of Micro-Mu's were created.

The BamHI-EcoRI fragments containing, separately, attL and attR were allowed to ligate together. The ligated DNA is then cleaved with EcoRI in order to obtain Micro-Mu attL-attR monomers with the Mu extremities in the correct orientation. Ligated EcoRI digested Micro-Mu DNA is inserted into the dephosphorylated EcoRI site of pBR322 (BamHI$^-$).

The DHI Amp^r clones were screened for the correct plasmid size by gel "cracking". Mini-preparations of plasmids DNA's of the putative clones were analysed with BamHI, EcoRI and BamHI-EcoRI digestions. Micro-Mu DNA in the correct orientation in pBR322 (BamHI$^-$) were purified and analyzed thoroughly (Figure 40).

B-1.c. Insertion of the Kan^r gene within the Micro-Mu's.

pBR322 was created BamHI$^-$ for the subsequent DNA manipulations of the Micro-Mu's in order to be able to insert DNA such as the BamHI containing the Kan^r gene fragment into a unique BamHI site between the two Micro-Mu ends of the PBR322 (BamHI$^-$)::Micro-Mu plasmids. The BamHI site of PBR322 was "backfilled" with the Klenow fragment of Escherichia coli DNA polymerase. The pBR322 became then BamHI resistant as well as sensitive to tetracyclin.

The BamHI fragment containing the kanamycin resistance determinant (Kan) of Tn903 (Oka et al., 1982) from the plasmid pUC71K (a kind gift from Dr S. Brown) was inserted at the inside BamHI site of the Micro-Mu's in the various combinations of Micro-Mu's cloned in the correct orientation (Figure 35). The pBR322 containing Micro-Mu Kan plasmids were transformed into strain LF249 to test their packaging properties.

B-1.d. Micro-Mu::kan orientation within pBR322.

The orientation of attL and attR within the plasmid was determined

Figure 40.

<u>BamHI/EcoRI digests of pBR322 (BamHI^{-}):: Micro-Mu Kan</u>.

The digests were run on a 5% polyacrylamide gel. <u>Hpa</u>II fragments of pBR322 served as molecular weight markers (A,J).

pBR::185(R)-203(L) (B), pBR322 B* (C), pBR::63(R)-126(L)<u>Kan</u> (D), pBR::116(R)-55(R)<u>Kan</u> (E), pBR::116(R)-126(L)<u>Kan</u> (F), pBR::61(R)-203 (R)<u>Kan</u> (G), pBR:: 185(R)- 126(L)<u>Kan</u> (H), pBR:: 185(R)-7(L)<u>Kan</u> (I).

by the BamHI-HindIII digestion pattern of the pBR322::Micro-Mu Kan plasmids on 5% polyacrylamide gel (Figures 41, 42). BamHI cuts in the Micro-Mu's att inside ends, while HindIII cleaves within the Kan gene but also 29 bp to the left of the EcoRI Micro-Mu insertion site (Examples are presented in Figure 42).

Once the att orientation within the plasmid was determined, the transcription orientation of the Kan gene within each attL-attR pBR322 derivative was determined by the pattern of DNA bands on a the 5% polyacrylamide gel after HindIII digestion of the plasmids (Examples are presentented in Figure 42). HindIII cuts within the pBR322 sequences 29 bp upstream from the left attL-attR EcoRI insertion site and cuts assymetrically within the Kan gene (Oka et al., 1982). XhoI also cleaves assymetrically within the Kan gene (Oka et al., 1982).

We created the following pBR322 (BamHI$^-$)::Micro-Mu Kan clones (Figure 43):

pJo185(R)-203(L),pJo185(R)-126(L),pJo185(R)-55(L), pJo185(R)-7(L).
pJo116(R)-203(L),pJo116(R)-126(L),pJo116(R)-55(L),pJo116(R)-7(L).
pJo62(R)-203(L),pJo62(R)-126(L), pJo62(R)-55(L).
pJo203(L)-203(L).

B-1.e. Cloning of Micro-Mu:: Kan into pJo2A.

The EcoRI fragment of pBR322::Micro-Mu Kan plasmids, that carried the Micro-Mu Kan DNA, was subcloned into pJo2A (pSC101 amp plasmid). In pJo2A, the BamHI site was mutagenized by "backfilling" with the Klenow

Figure 41.

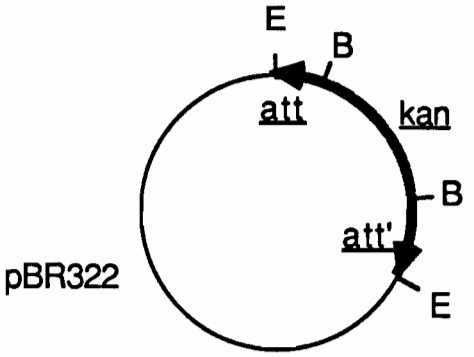
Schematic representation of the strategies used to orientate Micro-Mu fragments.

A) The orientation of Micro-Mu attL and attR with respect to the vector is deduced from BamHI/HindIII digestion pattern.

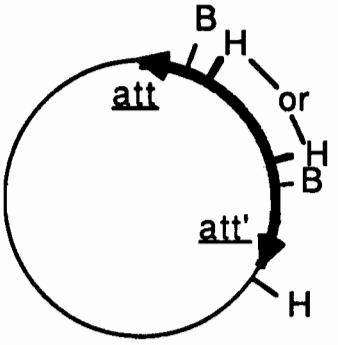
B) Once the att orientation is determined, the orientation of Kan is deduced by the HindIII digestion pattern, since HindIII cuts assymetrically within the Kan gene and 29 bp downstream the EcoRI Micro-Mu insertion.

Thick black lines are Micro-Mu's att.
Thin black line represents the vector sequences.
Dotted lines represent the Kan gene.

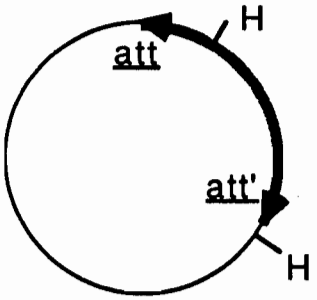
B, BamHI; E, EcoRI; H, HindIII.



A) Orientation of att by a BamH1/Hind111 digestion.



B) Once theatt orientation is determined, the orientation of the kan gene can be found by a Hind111 digestion.



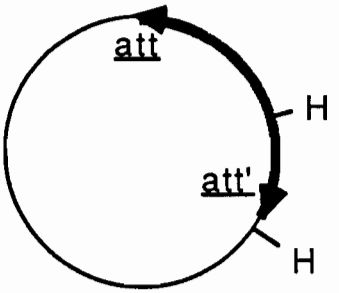


Figure 42.

Orientation of Micro-Mu att and Kan gene within Micro-Mu's.

Examples of digestion pattern obtained for the orientation of Micro-Mu's and the Kan gene of Micro-Mu's are given.

1) pBR322 (BamHI$^-$) plasmids containing Micro-Mu Kan were digested with BamHI/HindIII.

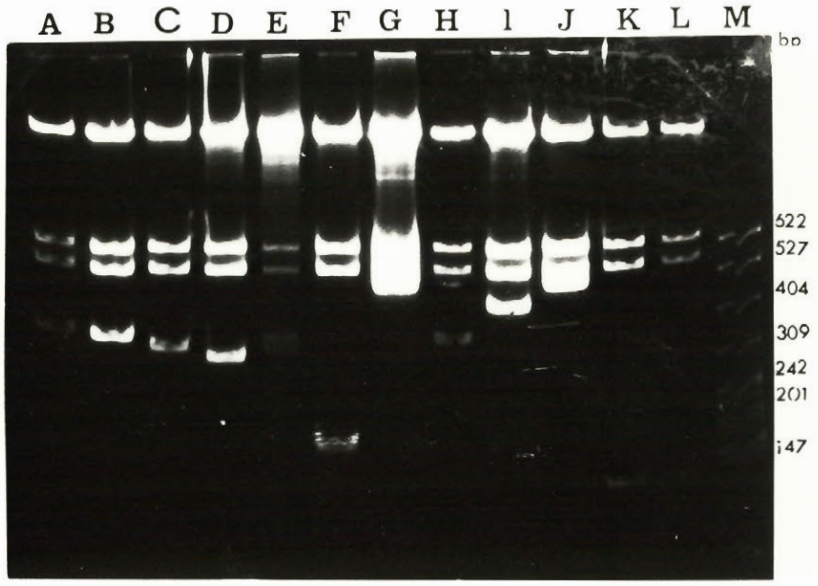
63(R)-203(L) (A), 116(R)-203(L) (B), 185(R)-203(L) (C), 203(R)-203(R) (D), 63(R)-55(L) (E),116(R)-55(L) (F), 185(R) -55(L) (G), 63(R)-126 L) (H), 116(R)-126(L) (I). 185(R) -126(L) (J), 116(R) -7(L) (K), 185(R)-7(L) (L).

2) pBR322 (BamHI$^-$) plasmids containing Micro-Mu Kan were digested with HindIII.

116(R)-203(L) (B), 185(R)-203(L) (c), 203(R)-203(R) (D), 63(R)-55(L) (E), 116(R)-55(L) (F), 185(R)-55(L)(G), 63(R)-126(L) (H), 116(R)-126(L) (I), 185(R)-126(L) (J), 116(R)-7(L) (K), 185(R)-7(L) (L).

The digests were run on a 5% polyacrylamide gel. HpaII fragments of pBR322 served as molecular weight markers (in 1-M; in 2-A).

1


A B C D E F G H I J K L M
bp
622
527
404
309
242
201
147

2

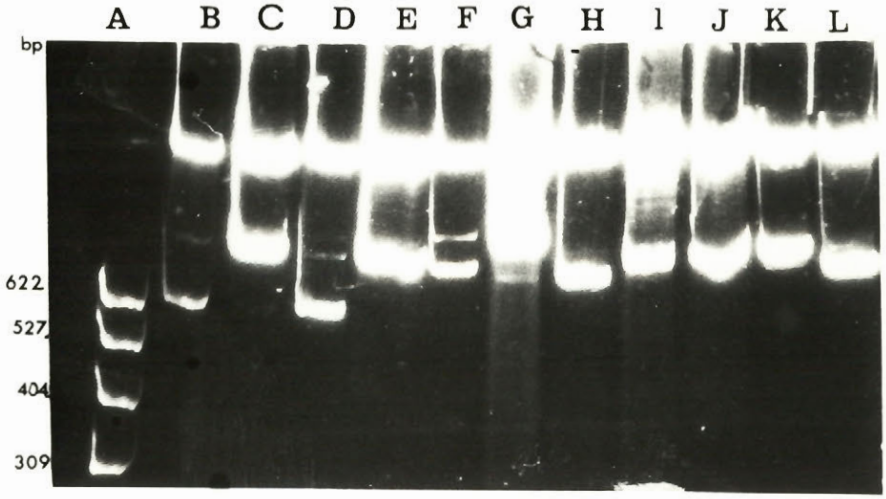

A B C D E F G H I J K L
bp
622
527
404
309

Figure 43.

Mapping and orientation of Micro-Mu's in its pBR322(B^-) vector.

The orientation of att of Micro-Mu's with respect to its vector HindIII site is shown, as well as the transcription direction of the inserted Kan gene.

The thick arrows represent attL and attR respectively.

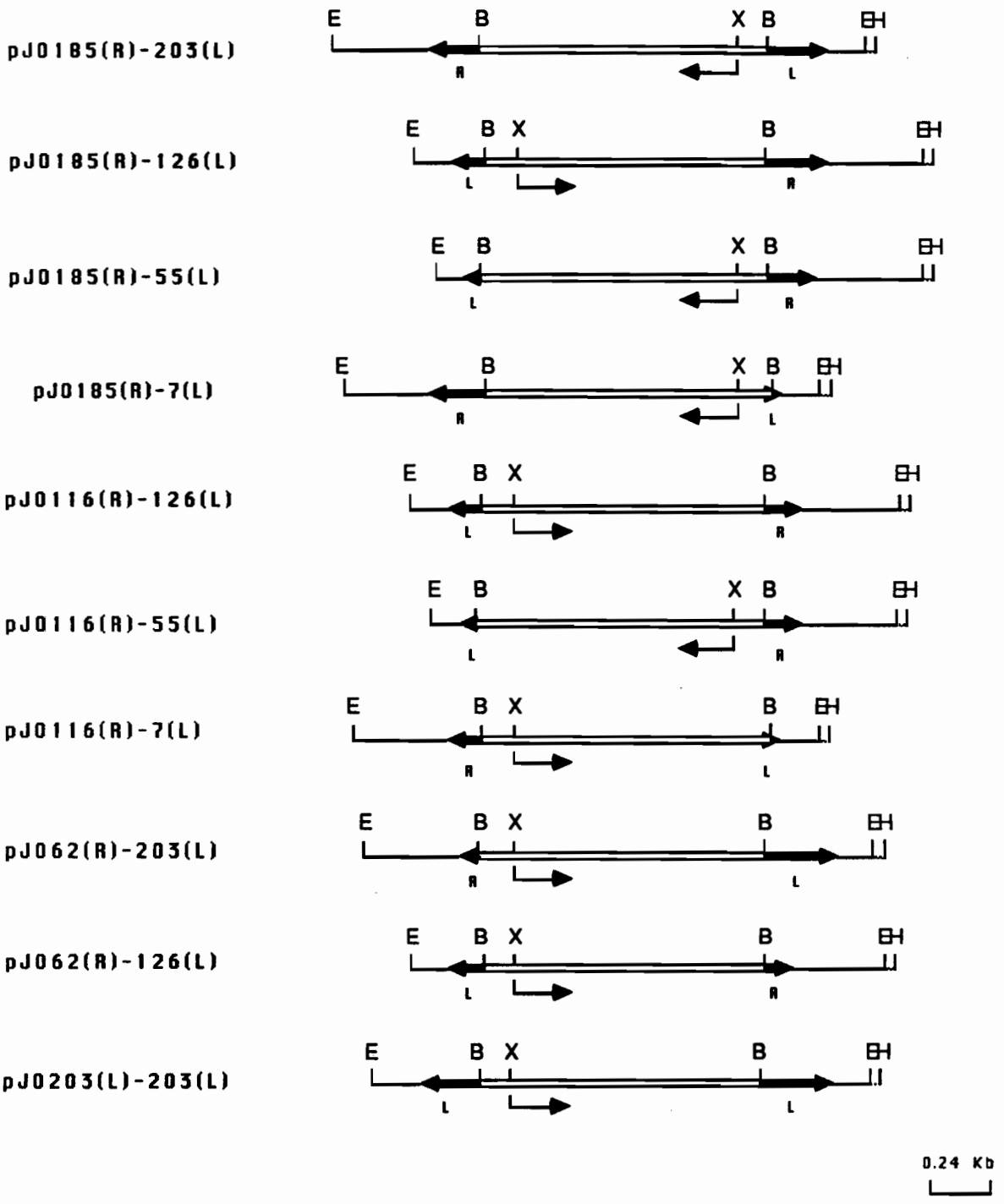
The white line represents the Kan gene. The thin arrows indicate the direction of transcription of the Kan gene.

The thin line represents some pSC101 sequences, construction vestiges of the original source of the Micro-Mu's.

The dotted line represents the actual vector sequences.

B, BamHI; E, EcoRI; H, HindIII; X, XhoI.

Cloning of Micro-Mu into pBR322.



fragment of Escherichia coli DNA polymerase in a similar manner to that of pBR322 BamHI$^-$. pJo2A containing Micro-Mu Kan derivatives were transformed into strain LF110 to study the Micro-Mu's transposition and packaging properties. The orientation of the Micro-Mu ends in pJo2A were analysed by HindIII digestion.

We obtained and characterized the following pJo2A::Micro-Mu Kan clones (Figure 44):

pJo2A185(R)-203(L),pJo2A185(R)-126(L),pJo2A185(R)-55(L), pJo2A185(R)-7(L).

pJo2A116(R)-203(L),pJo2A116(R)-126(L),pJo2A116(R)-55(L), pJo2A116(R)-7(L).

pJo2A62(R)-203(L),pJo2A62(R)-126(L), pJo2A62(R)-55(L).

pJo2A203(L)-203(L).

B-2. Transposition of plasmids pJo2A::Micro-Mu::Kan onto F'episome.

To determine which sequences at the ends of the Mu genome are required for transposition, Micro-Mu Kan derivatives containing various lengths of attL and attR in plasmid pJo2A were tested. The end-products of Micro-Mu transposition onto an F' episome during induced helper Mu lytic growth were examined after mating the lysogenic cells with a suitably marked female recipient. The Spc^r sexductants that were also Kan^r are the result of Micro-Mu Kan that have transposed onto the F' episome.

The transposition results with various pJo2A::Micro-Mu Kan derivatives allowed us to dissect the sequences requirements for

Figure 44.

Mapping and orientation of Micro-Mu's in its pJoA vector.

The orientation of att of Micro-Mu's with respect to its vector HindIII site is shown, as well as the transcription direction of the inserted Kan gene.

The thick arrows represent attL and attR respectively.

The white line represents the Kan gene. The thin arrows indicate the direction of transcription of the Kan gene.

The thin line represents some pSC101 sequences, construction vestiges of the original source of the Micro-Mu's.

The dotted line represents the actual vector sequences.

B, BamHI; E, EcoRI; H, HindIII; X, XhoI.

Cloning of Micro-Mu into pJ02A.

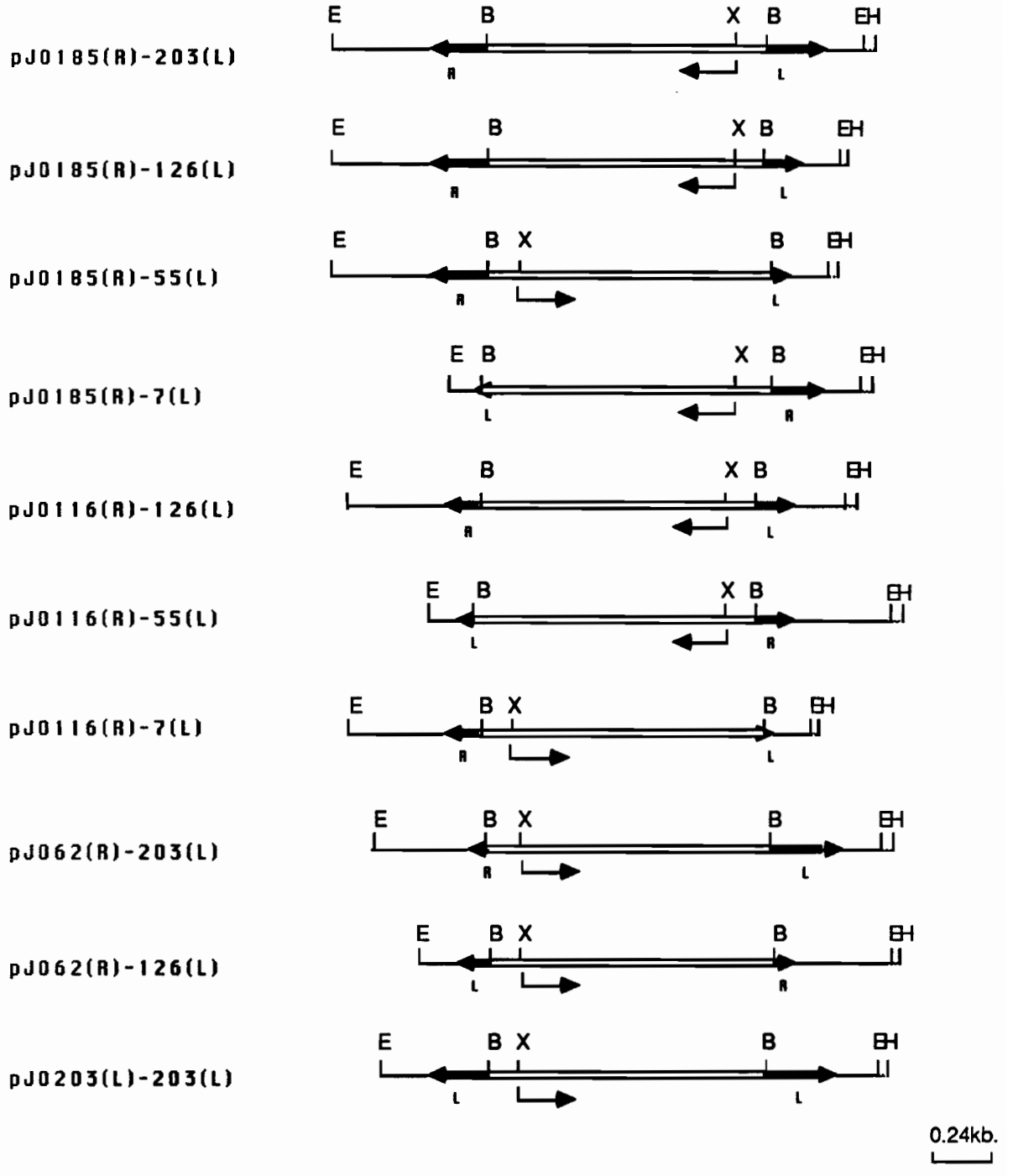


Table 9.

A- Transposition.

The transposition of Micro-Mu plasmids was tested using pJoA::Micro-Mu derivatives. The transposition frequency was expressed as the percentage of pro$^+$, Spc^r exconjugants that are Kan^r. The number of exconjugants was between 10^6 and 10^7 per conjugation experiment. The values were the average of 3 independent experiments.

B- Transduction.

The transduction frequencies of pBR322 (B$^-$)::Micro-Mu derivatives were defined as the number of Kan^r, Amp^r transductants per plaque forming units (pfu/ml).

C- Chromosomal association.

The physical behavior of pJoA::Micro-Mu plasmids during Mu lytic growth observed by Southern blotting with ^{32}P-pSC101 probe throughout the lytic Mu helper cycle was described in Materials and Methods.

The observation of chromosomal association or no chromosomal association was indicated by +, +/-, -.

The observation of linearized plasmid in this same set of experiments was also evaluated by +, +/-, -.

Table 9.

Characteristics of Micro-Mu's

Plasmid	Frequency of transposition[a]	Chromosomal association[b]	Transduction $Kan^r/10^{10}$ pfu[c]	Linear[b]
pJ0185(R)-203(L)	0.042	+	17.75	+
pJ0185(R)-126(L)	0.0075	(+)	14	+
pJ0185(R)-55(L)	0.0059	(+)	7.1	+
pJ0185(R)-7(L)	ND	-	0	-
pJ0116(R)-203(L)	ND	ND	20	ND
pJ0116(R)-126(L)	0.0059	+	6.8	+
pJ0116(R)-55(L)	0.0081	+	4.3	+
pJ0116(R)-7(L)	0	-	0	-
pJ062(R)-203(L)	0.0008	-	3.06	+
pJ062(R)-126(L)	0.0004	-	2.3	+
pJ062(R)-55(L)	ND	ND	7.5	ND
pJ0203(L)-203(L)	0.037	+	39.4	+
pJ062(R)-62(L)	ND	ND	0	-
pMD861	0.29	+	ND	ND
pJ0A	0	-	ND	ND

Micro-Mu transposition in our particular system (Table 9).

attL: We have found that Micro-Mu's with 203 bp of attL and 185 bp of attR or a pair of 203 bp of attL could transpose at the same rate (0.04). Plasmids pJo2A185(R)-126(L) and pJo2A116(R)-126(L) did transpose, but at a reduced rate, from 5 to 8 fold compared to the transposition rate of plasmids pJo2A185(R)-203(L). Micro-Mu's harboring 55 bp of attL and either 185 bp of attR or 116 bp of attR could transpose but at the same reduced efficiency as plasmids pJo2A185(R)-126(L) or pJo116(R)-126(L) (Table 9). Plasmids pJo2A185(R)-203(L) and pJo2A203(L)-203(L) showed a distinct enhancement of Micro-Mu transposition rate as compared to plasmids pJo2A183(R)-126(L), pJo2A116(R)-126(L), pJo2A185(R)-55(L) or pJo2A116(R)-55(L) of about 10 fold. Plasmids pJo2A185(R)-7(L), pJo2A117(R)-7(L) and pJo2A62(R)-7(L) did not transpose. This indicates that 7 bp of attL was not sufficient for Micro-Mu transposition.

attR: 62 nucleotides of attR with either 203 bp or 126 bp of attL allow minimal transposition rate. An increased transposition frequency of Micro-Mu::Kan requires at least 116 bp of attR to transpose in comparison with the situation when 62 bp of attR is used. There is no significant change in the transposition of Micro-Mu's having 185 or 116 bp of attR with 126 bp of attL: 0.006% to 0.007% of the sexductants had received a Micro-Mu Kan.

The following conclusions can be drawn: In the region from 0-55 bp of attL there are sequences that can be defined as an essential region for Micro-Mu transposition; while in the region spanning from 126 bp to 203 bp of attL, there are sequences that serve as an auxiliary domain which

augments the transposition frequency of the Micro-Mu's. In the region from 0-62 bp of attR, there are sequences that can be defined as an essential region for Micro-Mu transposition, while in the region spanning from 62 bp to 116 bp of attR, there are sequences that serve as an auxiliary domain which augments the transposition frequency of the Micro-Mu's.

The transposition frequency of the plasmids pJo2A185(R)-203(L) and was 5 to 10% lower than expected for a Mini-Mu genome (e.g. pMD861) in the same experimental condition as which usually occur at 0.3 to 0.5 transposition events per transferred episome, during Mu lytic growth (Table 9). It can be hypothesized that the distance between the ends of Mu separated by only the length of the Kan^r gene might reduce the efficiency of transposition. The close proximity of the two extremities might have some steric hindrance for the transpositon reaction to occur efficiently. The transcriptional readthrough effect from the Kan^r gene might reduce the efficiency of transposition as found for other Tn systems (please see chapter III on effect of adjacent sequences on transposition).

It is possible that although all the required minimal sequences for transposition are found within 203 nt of attL and 116 nt of attR that other auxiliary sites, distances and their orientation may contribute to maximize transposition. The transposition test with similar plasmids containing bigger DNA lengths of attL and attR would determine if 203 bp of attL and 116 bp of attR can be considered as maximized transposition substrates in this system or if they showed transposition frequencies that are not maximal .

On average, 90 to 95 % of the transposition events of all the Micro-Mu's were found to result in cointegrate end products (Kan^r and

Amp^r exconjugants). These results correlated with our earlier observations that most end products with pSC101 derivative Mini-Mu's were mostly cointegrates (DuBow and Lalumière, in Press; Chaconas et al., 1981).

B-3. Physical behavior of pJoA2::Micro-Mu during lytic growth.

B-3.a. Chromosomal association of pJoA::Micro-Mu Kan plasmids.

During the Mu lytic growth cycle, the transposing Mu and Mini-Mu plasmids are covalently associated with the Escherichia coli chromosome (Chaconas et al., 1980). We have examined the fate of pJo2::Micro-Mu Kan plasmids after thermoinduction of a Mucts62 prophage in Escherichia coli strain LF110 harboring these plasmids.

Comparisons were done with the behavior of the prototype Mini-Mu Amp in pMD861. The lytic cycle of LF110 derivatives containing pJOA2::Micro-Mu Kan plasmids was approximatively 60 minutes after temperature shift to 43^{0}C, while pMD861 containing LF110 strains required 90 minutes of induction (Figure 45). A longer time of induction for pMD861 was most likely necessary because of the production of the negative regulatory function such as the product of the c and ner genes present in pMD861 (Chaconas et al., 1981) and absent in the Micro-Mu's.

Samples of crude lysates were taken every 10 minutes post-induction (43^{0}C) throughout the lytic cycle and subjected to electrophoresis on a horizontal agarose gel followed by Southern blotting and hybridization. To determine if there is chromosomal association and attachment of the

Figure 45.

Lytic profile graphs of strain LF110 containing different pJo2A::Micro-Mu Kan derivatives.

1- LF110 containing plasmids pJo2A 185(R)-203(L)▲ ,pJo2A 185(R)-126(L)■, pJK43□ pJo2A 185(R)-7(L)△

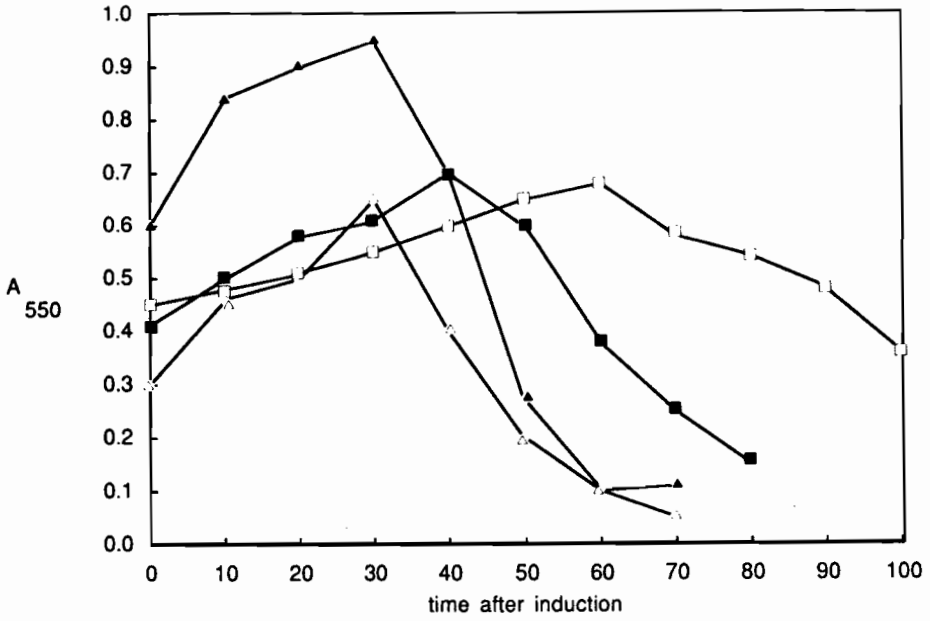
2- LF110 containing plasmids pJo2A 116(R)-203(L)△ ,pJo2A 116(R)-126(L)□, pJo2A 116(R)-55(L)▲, pJo2A 116(R)-7(L)■.

3- LF110 containing plasmids pJo2A 62(R)-203(L) □ ,pJo2A 62(R)-126(L) ■ .

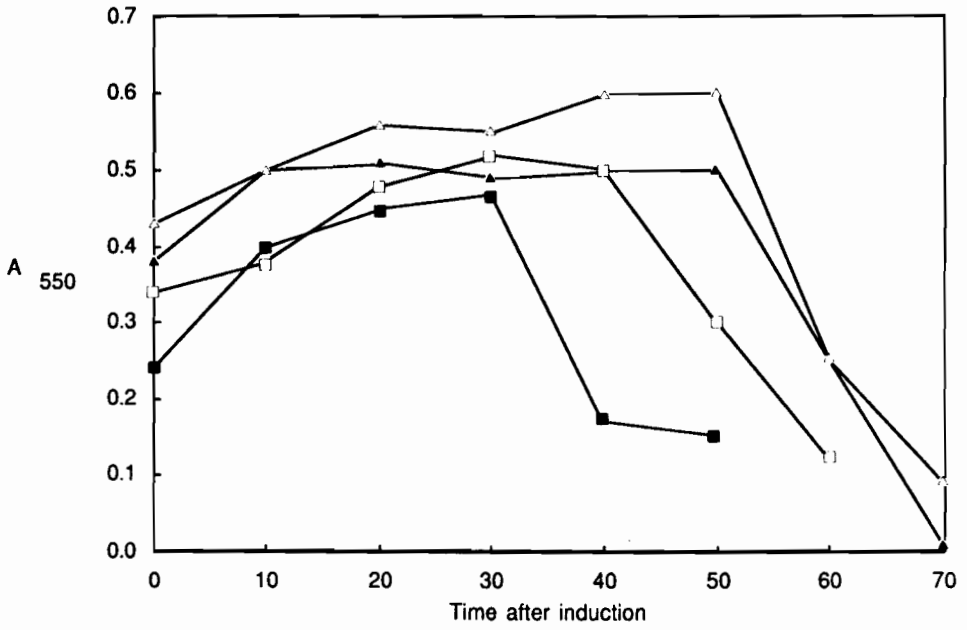
The X axis represents the time in minutes after induction of the strain at 43ºc.

The Y axis represents the OD A_{550} of the culture.

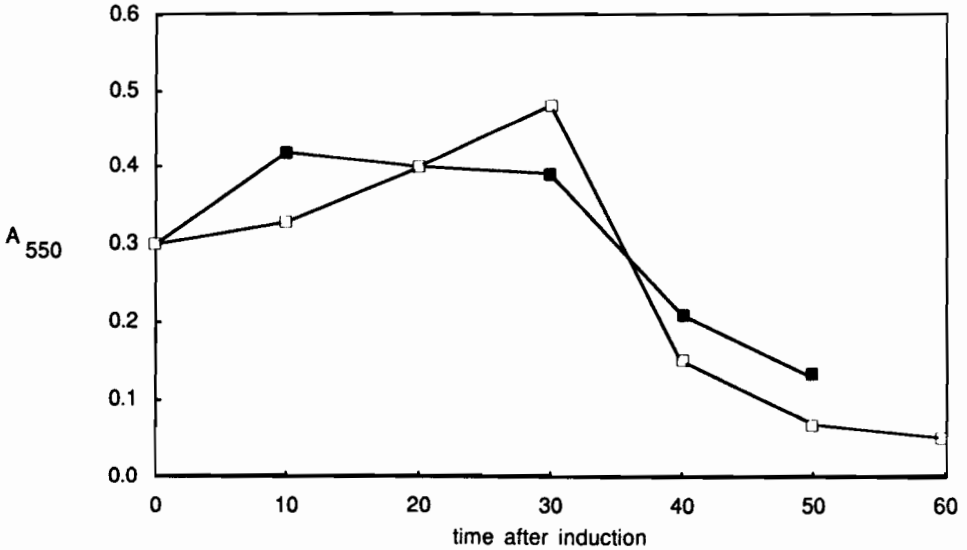
1.


A 550
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 10 20 30 40 50 60 70 80 90 100
time after induction

2.


A 550
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 10 20 30 40 50 60 70
Time after induction

3.


A 550
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 10 20 30 40 50 60
time after induction

Mini-Mu plasmid, the blots were hybridized with ^{32}P-labelled pSC101 DNA. All of the transposition-positive (as determined genetically) Micro-Mu's were found to be associated chromosomally (figure 46,47) (Table 9).

It was observed that with pMD861 and similar Mini or Micro-Mu plasmids, only a portion of the total plasmid population associated with the host chromosome (Chaconas et al., 1981; DuBow and Lalumière, in press) in contrast with a pSC101 derivative harboring the whole Mu genome such as pMC321. Explanations invoked the fact that the transposition proteins, such as the A and B gene products, were provided in trans and the A protein was shown to be unstable and to act stoichiometrically and to work preferentially in cis (Pato, 1982; Pato and Reich, 1984).

Thus, the transposition activity of Mini or Micro-Mu pSC101 derivatives plasmids which are in 5 copies per cell, in a cell harboring one Mu prophage, could be reduced because of the limiting avalability of the A gene product and because the A gene product has to act in trans to transpose these Mini-Mu and Micro-Mu plasmids. The chromosomal association procedure is not a quantitative test for transposition, but rather a complementary test that can only confirm the transposition results obtained by mating tests.

Nevertheless, the transposition positive Micro-Mu's were found to chromosomally associate. Chromosomal association could be detected with Micro-Mu plasmids such as pJo2A185(R)-203(L). However, in some Micro-Mu plasmids that transposed at lower rates than pJo2A62(R)-203(L) such as pJo2A62(R)-126(L) the detection of chromosomal association was ambiguous. Because the transposition frequencies of the positive Micro-Mu

Figure 46.

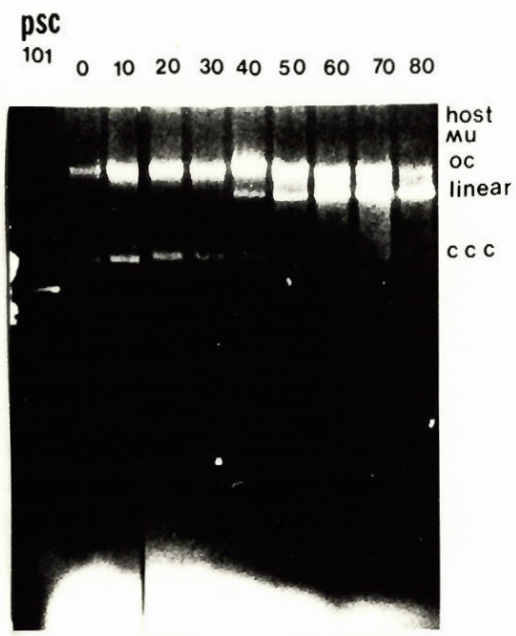
Chromosomal association with pJo185(R)-55(L)

In the top panel is a photograph of the ethidium bromide stained the 0.35% agarose gel prior to blotting.

In the bottom panel is a Southern blot of DNA from Mu-infected cells containing plasmid pJo185(R)-55(L).

The numbers over each slot represent the time (in minutes) after induction of Mu lytic growth at 43^0C from which each sample was taken. The samples were removed, the cells lysed and the DNA electrophoresed, blotted and hybridized versus ^{32}P-pSC101 DNA as described in Materials and Methods.

The position of Mu and host DNA as well as the various plasmid forms are indicated.


psc
101
0 10 20 30 40 50 60 70 80
host
Mu
oc
linear
c c c

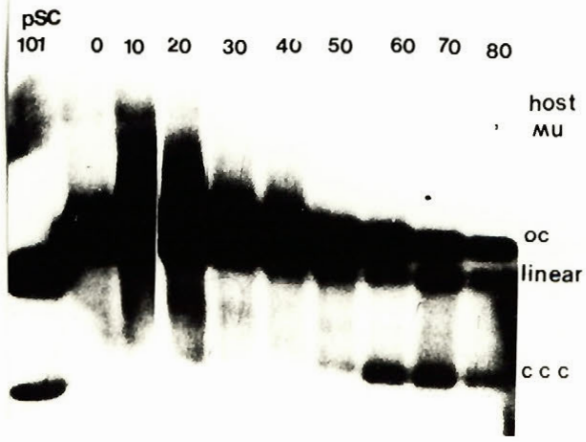

pSC
101
0 10 20 30 40 50 60 70 80
host
Mu
oc
linear
c c c

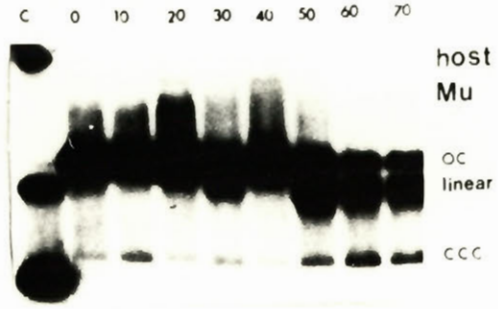
Figure 47.

Southern blot of DNA from Mu-infected cells containing plasmids pJo 185(R)-203(L) (a), pJo63(R)-203 (L) (b), pJo 185(R)-7(L) (c).

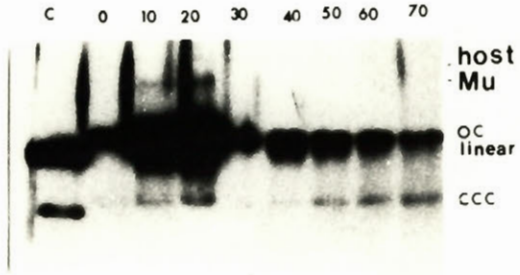
The numbers over each slot represent the time (in minutes) after thermoinduction of Mu lytic growth at 43^0C from which each sample was taken. The samples were removed, the cells lysed and the DNA electrophoresed, blotted and hybridized versus ^{32}P-pSC101 DNA as described in Materials and Methods.

The position of Mu and host DNA as well as the various plasmid forms are indicated.

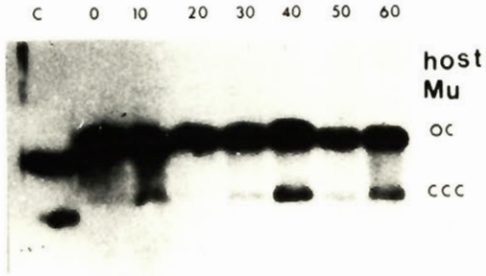
a


C 0 10 20 30 40 50 60 70
host
Mu
OC
linear
CCC

b


C 0 10 20 30 40 50 60 70
host
Mu
OC
linear
CCC

c


C 0 10 20 30 40 50 60
host
Mu
OC
CCC

Kan plasmids are 5-10 fold lower compared to the transposition frequency of pMD861 (Table 9), some Micro-Mu plasmids that were positive by quantitative transposition assay were not detected to chromosomally association.

The visualization of chromosomal associate of some Micro-Mu's, such as pJo2A185(R)-126(L), pJo2A185(R)-55(L), and pJo2A116(R)-55(L), pJO2A62(R)-126(L) that had lower transposition rates than the positive pJo2A185(R)-203(L), was barely detectable (Examples are shown in Figure 47).

B-3.b. Maturation of DNA and linearization of plasmid.

It is noteworthy to observe the linearization of the Micro-Mu Kan plasmids late in the lytic cycle (Figures 46, 47) in the chromosomal association test. Linearization of plasmids may represent nonspecific linerization of the plasmids as a consequence of the lytic cycle, or specific cleavage due to attempted maturation of Micro-Mu DNA because of the pac site harbored by some Micro-Mu's at the extreme left end of their genome. The appearance of a new band comigrating with the linear plasmid could be an intermediate product or aborted attempt of Micro-Mu plasmid packaging into Mu virion.

Linearization of the plasmids and appereance of an extra band of approximatively 10.5 kb was specific to the pSC101-based plasmids bearing at least 55 bp of attL (Table 9). This most-likely represented linearization and attempted maturation of the Micro-Mu DNA. In Micro-Mu plasmids with only 7 bp of attL no extra band appeared separated from plasmid DNA. This suggested that the Mu specific pac site is in 55 bp of

attL. The minimal attL length of Micro-Mu DNA required for Mu packaging was subsequently confirmed by transduction assays using these plasmids.

Another striking point was that Micro-Mu plasmids such as pJo2A62(R)-55(L), pJo2A62(R)-203(L), pJo2A62(R)-126(L), that did not appear at low rate were still able to be linearized and packaged. Plasmids pJo2A185(R)-7(L), pJo2A116(R)-7(L) did not transpose, and did not chromosomally associate, nor were they packaged into virions.

B-4. Maturation and transduction of Micro-Mu's plasmids.

Maturation of Mu DNA occurs by a "headful" packaging mechanism from host-phage intermediates (Bukhari and Taylor, 1975; Bukhari et al., 1976) (Chapter I. B-3.g.). To gain more insight into the functional domains for transposition and maturation of the Mu ends, two experimental systems were used. The first consisted of following the physical behavior of pJoA::Micro-Mu Kan in an induced Muc ts62 strain as explained above (B-3.b.). Linearization of the plasmid was indicative of attempted maturation.

In order to confirm these results, we analyzed the transduction behavior of pBR322::Micro-Mu Kan derivatives. We took advantage of the fact that pBR322 plasmids are often found in oligomeric form. Headful packaging of a pBR322::Micro-Mu Kan requires a 39 kb oligomer consisting of about 7 pBR322::Micro-Mu Kan (7 X 6.2 kb) plasmids. Though rare, these oligomers can be packaged into virions to provide efficient transducing particles. Once in a rec$^+$ strain, recombination between theses plasmids in in the linear oligomer will occur establishing the Micro-Mu plasmid in the

transductant.

A single lytic cycle was allowed to occur after prophage Mucts62 thermoinduction, and crude lysates, containing approximatively 10^{10} plaque-forming units per ml (pfu/ml), were assayed for the presence of Micro-Mu Kan by the virtue of their ability to act as kanamycin resistance particles. The lysates were used to infect a Mu sensitive rec^+ strain such as Escherichia coli strain 40 and the infected cells were plated on Kanamycin containing plates at 32^0c to allow the Kan^r colony formation.

In the oligomeric plasmid the putative pac signal was present at least 7 times, this might affect the transduction efficiency. One indirect evidence was that pJK43 which had bigger ends (1.0 kb attL and attR 1.2 kb) of Mu distanced by 6 kb of the pBR322 plasmid had a 10 fold higher transduction rate.

The Kan^r transductants were analyzed on gels by "cracking" the cells (Materials and Methods, section O) and analyzing for the plasmids presence and their molecular weight. Most of the Kan^r transductants were found to contain of approximately 6 kb plasmid; moreover, the transductants were capable of growth at 43^0c and were Amp^r, indicative of the presence of pBR322 sequences. This implied that the packaging of the Mini-Mu Kan is not due to the concommitent packaging of the Mu helper, from the Mu helper pac site, but rather due to the packaging of the Micro-Mu sequences carried on pBR322 ($BamHI^-$) plasmids.

The transduction results confirmed the observations of linearized plasmids during lytic growth (Table 9). Micro-Mu substrates possessing at least 55 bp of the Mu attL could package. It was interesting to note that pJo185(R)-203(L) and pJo116(R)-203(L) displayed an higher rate of transduction of 5-8 fold compared to other Micro-Mu's that were packaged.

A correlation with their better efficiency of transposition might explain a better source of Micro-Mu Kan to be packaged. When these plasmids transposed to the chromosome, they could be excellent substrates for maturation as chromosomal DNA adjacent to attR could be used to fill-up the Mu capsid during morphogenesis. The minimal domain for packaging was found to reside within the leftmost 55 bp of the Mu genome; and 7 bp of attL was insufficient for packaging.

C- Discussion.

The transposition of mobile genetic DNA segments of bacteria is mediated by element-specific proteins termed transposases that are believed to act by binding to sequences at both ends of their cognate elements. The cis acting nature of mutations at the extremities of these elements demonstrates that their ends are recognized by the protein(s) involved in transposition (Kleckner, 1981).

A pair of these terminal recognition sites in the correct orientation probably constitutes the only irreplaceable component of a functional transposable element. The ends of transposable elements must be intact for transposition to occur. Deletion of one or both ends produces a non-complementable transposition defect. The ends of a transposable element are required in cis. The DNA sequences found repeated in opposite orientations at the ends of insertion elements are thought to contain sites at which the transposase acts. Many elements have inverted repeats of at least 15 bp. There is evidence that those repeats are structurally required for transposition and that only a small sequence at each end of the element is important.

In the case of Tn10, the outer 27 bp are absolutely required for Tn10 transposition. However, base pairs 27-70 seem to be auxiliary sites that directly facilitate, supplement, or modify interactions in the essential 1-27 bp (Way and Kleckner, 1984). The terminal outermost 19 bp, close to two helical turns, are essential for Tn5 transposition (Johnson et al., 1983; Sasakawa et al., 1983). Tn3 contains identical 38 bp inverted repeats at each end. Mutations that delete the terminal inverted repeats of

Tn*3* or a deletion that removes an internal part of one of the inverted repeats produces a non-complementable defect (Gill et al., 1979).

Bacteriophage Mu is a temperate phage which replicates its 37 kb double-stranded DNA by a process that involves multiple rounds of duplicative DNA transposition (Toussaint et al., 1983). Mu requires only the terminal DNA sequences in *cis* for transposition to occur. This was demonstrated by the construction of plasmids containing the ends of Mu with different selectable markers and following the fate of these so-called Mini-Mu plasmids upon provision of the transposition enzymes in *trans*. Segments of the Mu genome can be deleted without affecting the intracellular transposition if functions involved in transposition are complemented by a non-defective helper Mu or by cloned transposition genes as long as the DNA sequences at each end are maintained intact (Toussaint et al., 1981).

Not only are the ends of Mu the presumptive recognition sites for transposition but, in the left end, the recognition signal for the commencement of headful packaging of Mu DNA is located. Moreover, initiation of replication occurs preferentially at the left end of the Mu genome and many regulatory sites for Mu gene expression are located within the left extremity of the Mu genome; thus, demonstrating the functional and sequence heterogeneity of the Mu extremities. An asymetric organization of inverted repeats in Mu DNA might be required for the process of replication and packaging to occur in the correct direction; the two processes show polarity initiated at *att*L and proced toward the right end of the genome.

To examine the cis-acting sequences that are required by a Mu phage genome, we have undertaken studies to define the functional domains of the extremities of Mu DNA in vivo with the phage functions provided in trans by an induced helper prophage.

To characterize the regions necesssary for transposition, we used a low copy vector in which various lengths of attR and attL of the Mu genome were cloned. The transposition of these Micro-Mu's was dependent on the transposition products from an induced Mu helper prophage. Our results suggested that the outer 55 bp of attL of the Mu genome contains a site that is essential for Mu transposition. Moreover the region from base pairs 126 to 203 represents an auxiliary site that enhances the transposition of these Micro-Mu's. We found that the extreme 62 bp of attR of the Mu genome contains a site that is essential for Mu transposition. Moreover the region from base pairs 62 to 116 represents an auxiliary site that enhances the transposition of these Micro-Mu's. The cooperative nature of the role of the auxiliary regions is not known. It could be involved in facilitating or modifying the interaction of the transposition functions and the essential domain lying from 0-55 bp in attL. In Tn10, sequences needed for transposition have been classified into region absolutely required for transposition and auxiliary sites that enhance the transposition frequency (Way and Kleckner, 1984).

Our results are in agreement with the recent published research by Groenen et al. (1985) who used a Mini-Mu transposition system in which genes A and B were expressed artificially under pL promoter and cloned on the same multicopy plasmid as the Mini-Mu tested. They found a strict transposition dependence on the leftmost 25 bp and that addition of the

region from 25 to 160 bp enhanced the efficiency of transposition. From *in vitro* data, Craigie et al.,(1984), have defined specific strong binding sites of Mu gpA in regions 0-30 bp and 150-160 bp and a weaker binding site from 122 bp to 132 bp in the same orientation. A finer detailed analysis of these regions, as well as site-specific mutations in conjunction with DNA protein interaction studies, will help us to unveil the basic functional and structural nature of these domains.

In our system, the transposition requirements for attR is at least 62 bp of the rightmost attR, but the minimal 116 bp of the left end enhances the transpostion frequency. However, the addition of the region from 116 bp to 185 bp does not increase the transposition frequency. Castilho et al., (1984), observed that 97 bp of attR was able to transpose and they could detect some transposition events having just 69 bp of attR present. However, Groenen et al., (1985), found that a minimum of 52 bp from attR allowed a reduced level of transposition. Our system, where the transposition functions are provided in *trans* and under Mu control, detects inefficiently low-level transposition events to occur, probably because gpA is more efficient *in cis* (Pato and Reich, 1984) and works stoichiometrically. In the system used by Groenen et al., (1985), the transposition functions are provided in *cis* and under the stronger lambda pL promoter. *In vitro* nuclease protection experiments on the right extremity of Mu performed by Craigie et al., (1984), showed a continuous block of protection until 90 bp and stronger binding in the region covering 53 to 82 bp, which may be indicative of the relative importance of this region. It may explain the increase in transposition efficiency when 116 bp of the extreme right is used in comparison with the minimal 62 bp attR. It is interesting to note that Mu and D108, despite the fact that their attR

sites are interchangeable (Toussaint et al., 1981), have within their extreme 75 bp a region of non-homology between nucleotides 32 to 55 bp (Szatmari et al., 1986).

From comparisons of the DNA sequences involved in the transposase binding regions (Craigie et al, 1984; Groenen et al., 1985) and sequences required in transposition a consensus sequence was proposed. The consensus sequences appear as two direct repeats in attL and, in attR, one consensus sequence is inverted with respect to those at the left (Figure 12; Figure 38). The orientation of the consensus sequences recalls typical inverted repeat structure of transposons. The 22 bp common consensus sequence which is composed of PyNNNNNAAPuPyPuCGAAAPu, is found in the regions essential for Mu transposition in vivo and in vitro (Groenen et al., 1985; Craigie et al., 1984; our observations). The role of this sequence, as well as it relative spatial arrangement and orientation, could be required for efficient transposase binding for Mu DNA transposition. Site-specific mutagenesis, further subcloning of these sequences in different orientation and spatiual organizations, as well as in vitro experiments with the transposition enzymes, will help to study these domains in the transposition of Mu DNA.

These 22 bp sequences are composed of two consensus sequences Py Pu C G A A A A and Py G T T T T C A Py T as inverted repeats or direct repeats. Py Pu C G A A A A is found in the ends of Tn3 and related transposons, like Mu catalyse 5 bp duplications at the target site during integration. In the Groenen et al.(1985) experiments, the removal of this particular sequence did not affect transposition. When the sequence Py G T T T C A Py T was absent, transposition was abolished. Thus, this sequence

is considered essential for transposition. It is interesting to note that in our hands, a pair of 203 bp of Mu extreme left end in the correct orientation could transpose as well as a Micro-Mu containing 185 of attR and 203 of attL.

Inverted repeats seem to play an important role in transposition. The difference however between Mu and the other transposable elements is that the inverted repeats in Mu are not at the very end except for the last 5'TG...CA 3'. The importance of the terminal nucleotides at the left end was stressed at the 1984 Cold Spring Harbor bacteriophage meeting by the finding of a mutant having suffered a T to C transition, at the right terminal T of the Mu genome (Burligame et al., 1984). It would be interesting to investigate what are the minimal sequence requirements within the 55 bp of attL and where do the transposition domains lie.

Mu DNA packaging is highly specific to Mu phage DNA. Packaging proceeds from the left end toward the right end and terminates when the phage head is full (headful packaging) (Bukhari and Taylor, 1975). The pac site was known to reside in the extreme left end of Mu and was further localized within the last 100 bp of the Mu genome (Teifel-Greding,1983; Goodchild et al., 1985; Groenen and Van de Putte, 1985).

We used a transduction assay with pBR322 plasmids containing different lengths of Micro-Mu Kan to define the minimal pac site of Mu. An oligomer of at least 7 pBR322::Micro-Mu Kan plasmids is required for testing the packaging capability of a particular Micro-Mu in these

plasmids. pBR322::Micro-Mu Kan derivatives are transduced as head to tail oligomers (Teifel-Greding, 1984). The transduction efficiency was dependent on the presence of a minimum of the leftmost 55 bp of the Mu genome. The transduction assays revealed that the region from 0 to 55 bp at attL of Mu was found to have the recognition site for Mu packaging. Analysis of the physical behavior of pJo2A::Micro-Mu Kan derivatives confirmed these findings. It is interesting to note that attL of Mu and D108 share perfect homology up to 55 bp and are able to package each other (Toussaint et al. 1983; Gill et al., 1981; Craigie et al., 1984). An interesting observation is that Mu packaging does not require a priori transposition of Mu sequences. Moreover, the pac signal is nestled in the cis-acting transposition sequences.

The asymetric organization of the inverted repeats might represent functional and structural requirements for a transposable bacteriophage such as Mu. Mu, being a viral transposon, must have evolved sequences with multifunctional roles to efficiently accomplish its life cycle.

The cloning of different regions and site-specific mutagenesis within the last 203 bp of attL and 116 bp of attR will prove crucial to classify and separate the different domains that play a role in the Mu life cycle. Not only transposition domains and packaging sites could be pinpointed, but the interactions with the enzymatic apparatus involved in these specific events should be further studied. The dissection of these various functions and comprehension of the mechanisms attached to them would be a step further to decipher the mystery of bacteriophage Mu evolution as a viral transposon.

CHAPTER V. CONCLUSIONS.

Mu, like other transposons, requires sequences at or near its extremities in *cis* in order to transpose. These extremities are multifunctional, and play a preponderant role throughout Mu cycle. To reflect their physical and functional state, the two extremities of the Mu genome are designated *attL* and *attR*, respectively (for attachment site left and attachment site right). The Mu *A* gene product (transposase) is expected to play a key role in the transposition reaction, the Mu *B* gene product is essential for the high efficiency of the reaction and product(s) from the semi-essential early region of the Mu genome enhance(s) the efficiency of the reaction. In addition, several host *Escherichia coli* DNA replication functions have also been shown to participate to the transposition of Mu, as well as a large number of host proteins.

The DNA sequences required in *cis* for transposition are not solely dependent on the DNA regions at the ends of mobile elements, The DNA environment i.e. base composition, DNA homology, DNA topology, DNA orientation at the sites of insertion as well as at the target site may influence the transposition of the transposable element.

We have undertaken an analysis of the *cis*-acting DNA sequences which are required for Mu DNA transposition, or may influence its transposition, as well as the relationships between the functional domains at the extremities of the Mu genome and their specific role and interactions during Mu transposition and interactions. In order to accomplish this, we used two strategies:

1- The creation of a variety of transposition mutants Mini-Mu's.

2- The cloning of the minimal regions of the extremities of the Mu genome.

1- Transposition mutants.

We have been studying the *cis*-acting DNA sequences which affect DNA transposition using plasmid pMD861, a derivative of pSC101 carrying an internally deleted Mu prophage called Mini-Mu. This Mini-Mu plasmid contains all the necessary *cis*-acting DNA sequences for DNA transposition but requires the transposition proteins from an induced helper prophage in order to transpose.

We have created a class of Mini-Mu plasmids in which alterations such as deletions in the neighboring plasmid sequences exert an influence on the frequency of transposition of Mini-Mu DNA. Studying these plasmids, we have determined that alterations in the neighboring plasmid sequences near the Mini-Mu extremities exert an influence in the frequency of transposition of the Mini-Mu DNA when the helper prophage contains a polar insertion in its semi-essential early region (SEER). This region of the Mu genome is expressed early during the Mu lytic cycle along with the *A* and *B* genes required for DNA transposition and encodes several gene products that may affect DNA transposition. It is not known precisely where these functions map nor is it known if these represent multiple functions of a smaller set of genes. It is possible that one or several of these functions may act on adjacent host sequences to allow maximal Mu DNA transposition (and thus its replication) from virtually any location during the short lytic cycle.

Bacteriophage Mu uses multiple cycles of DNA transposition to amplify its genome during lytic growth. To accomplish this task within the one hour lytic cycle, Mu must be able to transpose rapidly and efficiently from virtually any site to another in the *E.coli* chromosome. Thus, it is reasonable to propose that some function(s) in the semi-essential early

region may have evolved to enable Mu to transpose from any location, in particular from any potentially poor site into which Mu may have integrated. One potential candidate for this function is the *arm* locus, as *arm* is defined as a locus that stimulates Mu DNA transposition 3-10 fold during lytic growth.

The alterations of the Mini-Mu plasmids can be used as tools to devise identification and characterization of the expressed gene product from the semi-essential early region of the Mu helper genome, involved in transposition enhancement. Because of the semi-essential nature of the genes in this region of the Mu genome, gene characterization has been difficult.

There are several possibilities to explain why deletions outside the Mini-Mu sequences can affect Mini-Mu transposition only when the prophage is defective for its semi-essential early region.

a) One mechanism to account for this *cis*-acting repression is transcription into the right end of Mini-Mu genome from a promoter within the IS*102* element on the pSC101 plasmid. Our deletions in the adjacent IS*102* element could alter genetic signals that allow enhanced or reduced transcriptional readthrough of IS*102* into the Mini-Mu genome.

As a control for this potential transcriptional effect, the 1.2 kb kanamycin resistance gene from Tn*903* was inserted into the *Pvu*II site and the *Xho*I site of pMD861, near the right and left extremities, respectively, of the Mini-Mu genome. The transposition of the Mini-Mu plasmids containing the kanamycin resistance gene was depressed 10 to 20 fold in the presence of either a Mu *SEER*$^{+}$ or *SEER*$^{-}$ induced helper prophage, presumably due to the constitutive transcription from the kanamycin resistance gene into the Mu extremities. The mechanisms by

which externally derived transcription directed into the ends of a transposon exerts their effects on DNA transposition remain obscure. It is possible that the protein-DNA complex that is formed at the ends of the transposable element prior to and during initiation of transposition is disrupted by inwardly directed transcription. In this regard it is interesting to note that there is no extensive internal transcription from Mu that proceeds through the extremities into adjacent host DNA during lytic growth and DNA transposition. Since the presence of the kanamycin resistance gene at either end of the Mini-Mu genome alters its transposition, this phenomenon favors the concept that transcriptional activity by the Kan gene at the Mini-Mu extremities inhibits markedly the transposition efficiency of the Mini-Mu under SEER$^+$ and SEER$^-$ background.

b) The product(s) of IS102 open reading frame is (are) presumably involved in IS102 transposition and deletion activities. In plasmids pJoE14, pJoE32 and pJoE43 the deletions that we have created occur in the open reading frames of IS102. It can be hypothesized that the IS102 gene product(s) may affect Mini-Mu transposition in cis or in trans by acting either in conjunction with the Mu enzymatic transposition apparatus or in parallel with Mu transposition functions to increase Mu transposition efficiency. When the putative gene product(s) of IS102 was (were) altered, normal transposition could be restored by product(s) from the semi-essential early region of the Mu genome. However, there is a low level occurrence of deletions and mobilization of pSC101 via IS102 element, thus minimizing a major involvement of IS102 polypeptide(s) in Mini-Mu transposition. A better understanding of the expression and role of the putative IS102 polypeptides in IS102 and perhaps Mini-Mu

transposition is necessary. Studies on the frequencies of transposition of Mini-Mu inserted into different sites within the pSC101 vector would allow one to study the influence of the region from which Mini-Mu transposes out on the Mini-Mu transposition.

1-c) One other explanation is that a long-range cis effect of the adjacent sequence environment may influence transposition of Mini-Mu. Many studies have assessed the role of neighboring DNA sequences, such as DNA topology or base composition, in gene regulation. A cis influence of DNA sequences located great distances from a structural gene has been described in the beta-globin system and in exhaustive studies on enhancers. Thus, expression of the cloned gbgA gene in E.coli was decreased by the insertion of a transposon located 1.4 kb upstream from the gene. Moreover, experiments on how local alterations in DNA structure (notches) could affect the protein-DNA interactions have been described. In the case of pMD861 derivatives, modulation of transposition in the absence of expression of the semi-essential early region of an induced helper prophage is observed when the sequences between 340 bp and 600 bp downstream from the right extremity of the Mini-Mu genome are deleted. This area was found to be more GC rich than the rest of the sequences near attR of Mini-Mu, which may account for some of its effects when deleted. If Mini-Mu transposition is sensitive to local changes in DNA topology as in the case of Tn5 or in base composition then this may reinforce the notion that Mu is an efficient viral transposon that has evolved functions that allow rapid and efficient transposition from any site during its short lytic cycle. Exhaustive studies of transposition efficiency in different host strain background, such as mutants for enzymes acting on DNA topology, would allow comparative studies on the

effect of DNA conformation on the transposition frequencies of Mini-Mu with that of the altered Mini-Mu plasmids.

2-Cloning of the extremities of Mu DNA:

Not only are the ends of Mu the presumptive recognition site for transposition, but the left end contains the recognition signals for the commencement of the headful packaging mechanism. To examine the sequences that are required for these processes by the Mu genome, we have undertaken studies to define the functional domains of the extremities of Mu in vivo in which the Mu functions necessary to accomplish these processes were provided by an induced helper prophage.

2-a) In order to characterize the regions required for transposition, we used a low copy number vector, pSC101 derivative, into which various lengths of attR and attL of the Mu genome were cloned. The transposition of these Micro-Mu's was dependent upon the provision of the transposition products from an induced helper prophage. We have found that nucleotides 0 to 55 of the Mu left end define an essential domain for transposition and that sequences between nucleotides 126 and 203 define an auxiliary domain that stimulates transposition in vivo. At the Mu right extremity the essential sequences for transposition require not more than the first 62 nucleotide pairs; sequences between nucleotides 62 and 117 define an auxiliary domain that stimulates transposition in vivo.From all of these results, that is in vivo and by nuclease protection of the Mu extremities by gpA, it appears that a common consensus sequence which is composed of Py N N N N N A A Pu PY PU C G A A A Pu is found in the regions essential for Mu transposition. The role of these sequences seems to be tightly linked to Mu transposition.

Subcloning of the Micro-Mu processively deleted as well as site-specific mutagenesis such as base substitutions or base methylation might bring up the real importance of these domains as well as the relative importance of the spatial arrangement and orientation of the various consensus sequences. It would pinpoint the interactions between the transposition domains and allow one to understand the interactions between attL and attR during transposition. In order to understand the interaction between the transposition machinery enzymes, one could take advantage of a DNA-footprinting technique which displays DNA sequences protected from DNAase I digestion by crude bacterial (or cell-free) extracts, we can physically identify the transposition binding site. Our Micro-Mu DNAs are an excellent source of specific DNA substrate for the band competition assay as well as DNA footprinting. It will be useful to characterize the DNA-binding activity of the transposase protein (A gene product) as well as the proteins that are involved in enhancing the efficiency of transposition such as B and arm. It would allow a better understand the molecular interaction between the transposition machinery and the DNA recognition sequences.

2-b) Mu phage DNA maturation and packaging proceeds from the left end toward the right end and terminates when the phage head is full (headful packaging). We used a transduction assay with pBR322 plasmids containing different lengths of Micro-Mu Kan DNA to define the minimal pac site of Mu. Using transduction assays and analysis of the physical behavior of our Micro-Mu plasmids during lytic growth, we concluded that the region of 0-55 bp of Mu attL has the recognition site for Mu packaging. Of note is that attL of Mu and D108 share perfect homologies up to 54 bp

and are able to package each other's genomes. Of note, Mu packaging does not require a priori transposition of Mu. The pac signal of Mu is thus nestled in the sequences required for Mu DNA transposition. Resection of the leftmost 55 bp of the Mu genome as well as site-specific mutagenesis of these sequences would define the necessary sequences required for packaging, as well as provide a source of DNA in the study of interactions between Mu leftmost DNA and packaging enzymes such as the DNAaseI protection experiments, and as it would provide a system to isolate the enzymes involved in packaging. Ultimately, this would permit understanding of how the leftmost 55 bp of bacteriophage Mu contributes to the packaging of about 39 kb of DNA.

In summary, the asymetric organization of the Mu extremities might reflect functional requirements for a transposable bacteriophage such as Mu. Mu, being a viral transposon, might have evolved sequences with multifunctional roles to efficiently accomplish its life cycle.

ORIGINAL CONTRIBUTIONS TO KNOWLEDGE.

In the first part of the study, we found a class of Mini-Mu transposition mutants in which alterations in the neighboring plasmid sequences of pMD861 near the Mini-Mu extremities exert a modulatory influence in the extent of transposition of Mini-Mu when the helper prophage contains a polar insertion in its semi-essential early region.

Mu transposition is clearly influenced by sequenced in its proximity.

We provided evidence of a role for genes in the semi-essential early region of the Mu genome in helping Mu to transpose out from non-favorable sites.

Transcriptional readthrough of the kanamycin gene from Tn903 does reduce transposition frequency. And, this effect is not overcome by functions from the semi-essential early region of Mu.

In the second part of this study, by subcloning the extremities of the Mu genome, we provided evidence for different domains important for transposition and maturation of the Mu DNA.

First, using an experimental system that consists of monitoring the *in vivo* transposition and maturation behavior of pSC101 and pBR322 plasmids containing various sized Micro-Mu's in the presence of an induced helper prophage to provide the transposition and DNA maturation functions, we have found that nucleotides 0 to 55 of the Mu left end define an essential domain for transposition and that sequences between nucleotides 126 and 203 define an auxiliary domain that stimulates transposition *in vivo*. At the Mu right extremity the essential sequences

for transposition require not more than the first 62 nucleotide pairs; sequences between nucleotides 62 and 117 define an auxiliary domain that stimulates transposition *in vivo*.

Second, using pBR322::Micro-Mu derivatives in the presence of an induced helper prophage, we have delineated the *pac* recognition site for DNA maturation to reside within the leftmost 55 bp of the Mu genome.

We found that the transposition domains at the left extremity of the Mu genome are composed of a necessary sequences within the region from 1 bp to 55 bp and auxiliary sequences within the region 126 bp to 203 bp. The transposition domain at the right extremity of the Mu genome reside within the rightmost 62 bp. The packaging recognition site was found to be located within the leftmost 55 nucleotides of the Mu genome.

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