Regulation of phage Mu transposition

C.M. van Drunen

VAN DRUNEN - REGULATION OF PHAGE MU TRANSPOSITION

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Promotor:	Prof. dr. ir. P. Van de Putte
Co-promotor:	Dr. N. Goosen
Referent:	Dr. E. Vijgenboom
Overige leden:	Prof. dr. P.J.J. Hooykaas
	Prof. dr. C.W.A. Pley
	Prof. dr. D. Bedeaux

Contents

General introduction

"Many hereditary traits of bacteria are determined by a special class of genetic elements termed episomes. Episomes usually determine nonessential characters not ordinarily represented in the bacterial gene pool. Some episomic elements, e.g., transducing phages and the sex factor of Escherichia coli strain K12, are instrumental in providing means for the intercellular transfer of bacterial genes. It has been postulated that episomes may also regulate the biosynthetic activities of bacteria at the genetic level by stimulating, inhibiting, or otherwise modifying the phenotypic expression of specific genes. Recent discoveries indicate that episomes can in fact stimulate the activity of bacterial genes under special conditions, but total suppression of the normal genetic functions by an episome has not been observed in Eubacteria.

This report describes a novel temperate bacteriophage, designated phage Mu1, that causes severe genetic modifications in its natural host E.coli K12. The prophage of Mu1 abolishes normal phenotypic expression of many host genes located at (or adjacent to) the chromosomal sites of phage integration. The over-all effect of Mu1 on bacteria is superficially indistinguishable from that of a mutagen. Hence, the phenomenon described here will be referred to as phage-induced mutation."

Taylor, 1963

Introduction

The opening sentences from the original article by Austin Taylor (121) describe the discovery of the temperate phage Mu and explain the rationalism for giving the name to this new phage as Mu is short for mutator. It was later realized that the observed mutations in *Escherichia coli* were not solely due to the insertion of the Mu prophage in a particular gene but also to Mu induced rearrangements of the bacterial genome (37). In lytic development of Mu about 100 copies are formed by successive rounds of transposition where duplicates of Mu are integrated at random in the host genome without the original copy ever being excised from the chromosomal site. This process can result in the formation of deletions and inversions in the host DNA which accounts for the observed mutator phenotype.

The Mu life cycle starts when the Mu phage infects a susceptible host. The DNA is injected into the host as a nucleo-protein complex (24,54,106). The attachment sites are protected from nuclease degradation through the formation of a DNA semi-circle mediated by the Mu *N* gene product (41). The following integration of Mu is at a non-specific site in the host genome (21) and is, in contrast to the later discussed transposition mechanism, of a conservative nature (3,21,52,53). There was some controversy about the host DNA replication forks being a preferred integration site for the injected Mu DNA, but this idea has now been abandoned by Mu workers (38,95).

This thesis focuses on the transposition reaction of phage Mu. In this general introduction a detailed description will be presented of both DNA and protein factors that are directly involved in the transposition reaction. As the main theme of the work in this research is the involvement of Fis (Factor of inversion stimulation) from *E.coli* in the regulation of Mu transposition, a review of processes which are known to be dependent on Fis is presented.

Regulation of bacteriophage Mu transposition

The Mu A and the B protein were identified as the principle proteins involved in the transposition reaction through isolation of amber mutations (am) in the corresponding genes (36,37,100). These *A*am and *B*am phages showed a reduced burst size upon induction or were not able to produce any new phages at all. Early work on transposition however was mainly concentrated on the elucidation of the regulation of transposition rather than on the molecular mechanism of the reaction (7,42,43).

The regulatory region of Mu (Fig.1) is located in the left attachment site (*att*L) and comprises of two converging promoters expressing either the repressor from Pc or Ner, A and B from Pe (44). In a stable lysogen of Mu only Pc is active and therefore only repressor is produced. The repressor binds to three operator sites (O1, O2 and O3) in the regulatory region (85). As a consequence of repressor binding to O2 the overlapping early promoter Pe is

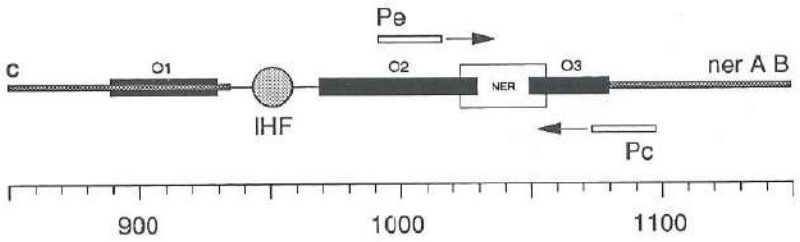

Figure 1. Organization of the regulatory region of Mu. Depicted is the region around position 1000 in *att*L with the promoters Pe that drives the expression of the operon *nerAB* and Pc that drives the expression of repressor c. The reading frames of these genes are represented by the shaded bars. The regulatory region furthermore contains binding sites for IHF (shaded circle), repressor (filled boxes O1-O3 with O1 in the reading frame of the *c* gene) and Ner (open box).

shut off, preventing the synthesis of the transposase A. This mantains lysogeny. The third binding site (O3) has also a clear role in the lysogenic state of Mu. At high concentrations of the repressor protein the weak binding site O3 will be occupied which will block further transcription from Pc. This autoregulation mechanism results in a steady state level of repressor. The binding site O1, which like O2 has a high affinity for repressor, was thought to overlap with a weak promoter for the *c* gene (42). Later results showed that this promoter was situated in the coding region of repressor and could, as a consequence, not be involved in the synthesis of active repressor (cited in 93). The role of the repressor binding site O1 in Mu development will be discussed in a later section.

Spontaneous induction of a Mu lysogen is very low (10^{-4}) and can not be increased by UV irradiation or treatment with other stress inducing agents. To circumvent this problem in the study of transposition, mutants of Mu have been isolated which encode a thermosensitive repressor protein. In these mutants Mu transposition can be readily induced at 42°C whereas Mu remains quiescent at 32°C (16). At the nonpermissive temperature repressor is inactivated and the repression of Pe is relieved. When Pe becomes active the operon comprising of *A*, *B* and *ner* is transcribed. The Ner protein acts as anti-repressor by binding to a site that overlaps with Pc (Fig.1) thereby preventing repressor synthesis (43). In this way a firm decision is made to enter the lytic pathway.

A search for factors that induce lytic development has not been successful

although a protein that might influence the choice between lysogeny and lytic development has been identified. In the operator region a binding site for Integration Host Factor (IHF) is situated adjacent to Pe (Fig.1). In fact it was shown that binding of IHF to this site stimulates transcription from both Pe and Pc **(127,128)**. This shows that IHF is probably not a discriminating factor but that it helps to establish either lysogeny or lytic development. Interestingly IHF is thought to stimulate the two promoters through different mechanisms. This was deduced from the observation that the stimulation of Pe is dependent on the relative orientation on the DNA helix of the *ihf* box with respect to Pe whereas Pc stimulation by IHF is orientation independent **(128)**. The molecular mechanisms for both modes of action however remain to be clarified although protein-protein interaction in the case of Pe and bending in the case of Pc have been proposed **(128,129)**.

Mechanism of transposition

Replication and transposition of Mu are linked and are both dependent on the A and B proteins. Analysis of the replication process revealed unidirectional replication of Mu specific DNA sequences **(131)**. This strong tendency for left to right replication, which is in the same direction as overall Mu transcription **(5)**, is remarkable in the light of the latest model for Mu transposition described in this section which proposes a transposition intermediate with two

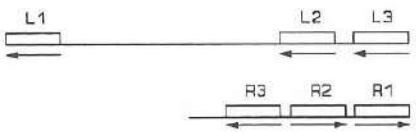

Figure 2. Organization of the attachment sites. Both attachment sites have three transposase A binding sites of which localization and relative orientation are indicated.

potential replicationforks at either end of Mu. The mechanism of unidirectionality in Mu is unknown but could reside in differential and specific binding of A to the attachment sites of Mu.

Identification of DNA sites involved in the Mu transposition reaction came from deletion and mutation studies using a plasmid born mini-Mu system **(18,23,46,47,50)** and from the development of an *in vitro* assay for transposition **(20,27,91)**. The attachment sites of Mu, *att*L and *att*R share three 22 base pairs repeats which are in footprint experiments protected from DNaseI digestion by Mu A (Fig.2) **(32,48)**. The binding of A to the attachment sites is mediated through a helix-turn-helix motive common to other DNA binding proteins (Fig. 3) **(96)**. Through comparison of the binding sites the A binding consensus (YGtTTCAYtNNAA RYRCGAAAR) was deduced which in fact is comprised of two different moieties **(47)**. The relative weak transposase

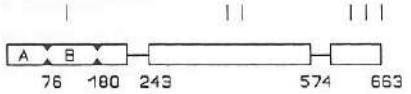

Figure 3. Schematic representation of the structure of the A protein. The Mu transposase has two protease sensitive sites dividing A into three domains (I, II and III) of which the borders are marked with the numbers of the aminoacids. Domain I has two distinct DNA binding regions (borders marked by the black triangles), IA that interacts with the enhancer and IB that interacts with the attachment sites. The central domain II has a general DNA binding capacity but no defined function. Domain III possibly contains the endonuclease activity and the ability to interact with Mu B.

binding site L2 in *att*L only contains the 3'-part of this sequence (AARYRC GAAAR). Interestingly this sequence is also conserved in the transposons D108, Tn3, and IS30 (Fig.4) **(17,19)**. These transposons have further in common that they produce a five base-pairs duplication of the target DNA upon transposition **(4,107)**. Evidently these transposons are evolutionary related and the 3'-part of the A binding consensus probably represents a rudimentar binding sequence of their common transposase ancestor.

The transposition reaction proceeds through the formation of successive nucleo-protein complexes (Fig.5) which eventually lead to the formation of a second Mu copy by replication (Fig.5) **(22, 28, 92, 114, 117)**. Through the

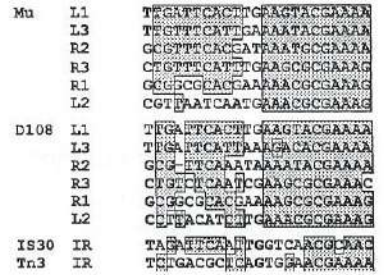

Figure 4. Comparison of the A binding sites. Homology (shaded box) between L1-L3 and R1-R3 of Mu, the related phage D108 and the IR sequences of IS30, Tn3 and γδ of the Tn3 class to which also Mu belongs.

binding of A molecules to multiple sites within the attachment sites and the action of the *E.coli* protein HU the first complex in the transposition reaction is formed (cleaved donor complex or CDC) in which the two nicked Mu ends are held together. HU can be efficiently removed from the CDC by increasing the salt concentration. This process is completely reversible as an immunological analysis of this complex showed **(86)**. The correct alignment of the A binding sites could require an altered helical pitch or the formation of a nucleosome-like complex which could explain the role of HU in the formation of the CDC **(14,110)**. In the CDC there are two nicks at the 3'-boundaries of Mu which are created by the A protein **(28,30,101)**. The domain responsible for this cutting reaction has been assigned to the C-terminal part of the protein **(8,9,34,55)**. Supercoiling is a prerequisite for the formation of the CDC either as driving force for the cutting

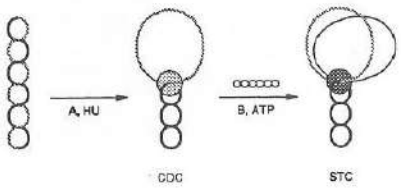

Figure 5. Model for phage Mu transposition. Indicated are Mu DNA (thick line), plasmid DNA (shaded line), target DNA (thin line), the CDC- (light circle) and STC- (dark circle) nucleo-protein complexes.

reaction or as a means to facilitate the paring of the attachment sites (**29, 31,116**). This dependency on a high level of supercoiling can be alleviated by the *E.coli* protein IHF (**116,118**). The role of IHF in this dependency is not yet known, but is mediated through the IHF binding site situated in the regulatory region of Mu. The supercoils in the Mu part of the CDC remain trapped by protein-protein interactions that hold the attachment sites together whereas the flanking DNA regions are relaxed. This complex and also the complex depleted of HU can be isolated through gel filtration methods and can be chased to form the STC (strand transfer complex) by the addition of a target substrate and the Mu B protein (Fig.5) (**86**). In this complex the 3'-ends of Mu are connected to the 5'-ends of the target site. Apparently the role of HU could be restricted to the formation of the CDC. Since HU is required for replication of the *E.coli* chromosome a role of HU in the replication step of transposition is not excluded (**35**). As even linear DNA is also an efficient target it seems that there are no topological restrictions for the target DNA this in contrast to the supercoiling dependency of donor DNA. A gyrase binding site situated midway the Mu genome could be important for increasing the supercoil density of Mu as deleting this sequence leads to a drop in the burst size (**102**).

In contrast to the specific binding of A, the B protein (**39,90**) has a general DNA binding capacity. The function of B seems to be to present target DNA to the CDC by interacting with the C-terminal part of A thereby facilitating the transposition reaction (**88**). An additional role for B is the stabilization of the interaction between A molecules in the CDC. This stimulation only requires binding of B to the A protein but not to the DNA (**6,119**).

The bias for intermolecular transposition in the presence of B is probably caused by transposition immunity (**1,2**), where a replicon containing Mu sequences is not an efficient target for Mu transposition. This immunity is dependent on the ATPase activity of B which can be activated by A bound to one of the attachment sites. After ATP hydrolysis B dissociates from the DNA ren-dering this molecule a less efficient target as B bound to DNA is the principle target for the CDC. Not all A

binding sites mediate this transposition immunity as the sequences involved only coincide with the region L2-L3 in *att*L and R3 in *att*R (**33**).

The cellular localization of B in the membrane suggests an additional role for B in replication of Mu (**11,12, 113**). By association with the membrane B might facilitate replication as replication of the bacterial genome itself is also associated with the membrane. The STC has two replicationfork-like structures. However only the fork formed at the left attachment site is used leading to unidirectional replication (**108,131**). It is not known why the other potential fork is not functional. The replication step leads to the formation of a cointegrate between the Mu and target replicon (**51**).

The regulatory region of Mu which has a major role in the choice between lysogeny and lytic development (see previous section), was also shown to have a direct function in Mu transposition. The region of the repressor binding sites O1 and O2, in the absence of repressor, acts as an enhancer-like sequence for transposition (**87,93,118**). This internal activating sequence or IAS is orientation dependent and it only stimulates transposition when located between the Mu attachment sites. These last restrictions are topological rather than functional restrains as a small oligo DNA fragment containing the IAS can stimulate transposition *in trans* (**120**). The action of the IAS is mediated through binding of A to O1 and O2. Domain analysis of A showed that the first 80 amino acids of the protein form a domain that specifically interacts with the IAS. This region of the transposase has a considerable similarity with the DNA binding domain of Mu repressor (**56,94**) which accounts for the affinity of both proteins for the same sites in the IAS. When the IAS binding domain of the transposase protein is deleted, the IAS can no longer stimulate the transposition reaction. This domain is distinct from the classical helix-turn-helix motive of A that interacts with the A binding sites in the attachment sites. Noteworthy is that the A proteins from Mu and the related phage D108 can only mediate their own transposition although they are able to bind each others attachment sites. This would suggest that the D108 transposase is specific for a D108 enhancer and the Mu transposase for the Mu IAS (**124**). Although the transposases from Mu and D108 are largely conserved the IAS binding domains have major differences. The mechanism of action of the IAS has not yet been resolved but it has indirectly been shown that only the formation of the CDC is stimulated by the IAS whereas the formation of the STC is not dependent on the IAS (**95**). The involvement of three DNA sequences in the transposition reaction, i.e. *att*L, *att*R and IAS, suggests the formation of a three site complex (Fig.6). Such a structure was first shown for an invertasome where two recombination sites and an enhancer form the functional complex (**57,76**). No such complex has been observed between the IAS and

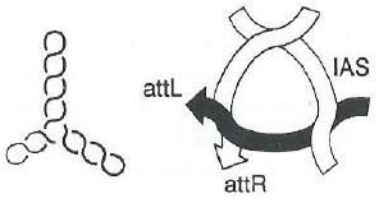

Figure 6. Proposed structure of the three-site-complex. Supercoiled DNA molecule with branch point (left panel, Mu DNA thick line) and detail (right panel) of proposed complex between *att*L, *att*R and IAS.

one or both ends of Mu. This means that either such complexes are too unstable to be detected in gels or by electronmicroscopy or that it is immediately converted into the CDC. The IAS could function as a hit-and-run enhancer only required to bring the Mu ends in a correct conformation to form the CDC.

Factor of inversion stimulation

In the previous paragraph we described the involvement of the HU and IHF in the transposition reaction. Through the work of M.A.M. Groenen (45) in our lab and M. Bétermier (10) the possible involvement of an other host protein, factor of inversion stimulation (Fis) in Mu transposition was suggested. Fis is a small 12 kDa highly basic DNA binding protein present in *E.coli* and related bacteria from the enterobacter group (69,80). The structure of the Fis dimer has now been resolved and was shown to comprise four tightly intertwined α-helices (25,81). The two C-terminal helixes form the DNA binding domain like theoretically was predicted. The remaining two helixes are thought to be involved in dimerization of Fis. Besides Mu trans-position three other systems have been described where Fis acts as an auxiliary protein which will be described in the following sections.

The role of the N-terminus of Fis whose structure could not exactly be determined is not fully understood as not all processes stimulated by Fis require this part of the protein (115).

The role of Fis in G-loop inversion in phage Mu

Fis was discovered as host factor for the Gin-mediated inversion of the G-loop in bacteriophage Mu region (70). This is a genetic switch that determines the host range of Mu (15,49,58,59,72, 125). In this region different sets of tail fibre genes can be expressed depending on the orientation of the G-loop (Fig.7) (40,60,111,112). The region that is inverted by the action of the invertase Gin (77,78,89) is flanked by inverted repeats or *gix* sites which are the binding sites for this protein (105). The inversion reaction is stimulated by a third DNA sequence that is situated within the coding region of Gin (61,70, 75,79). This site of inversion stimulation (*sis*) was shown to comprise two binding sites for Fis that binds to these

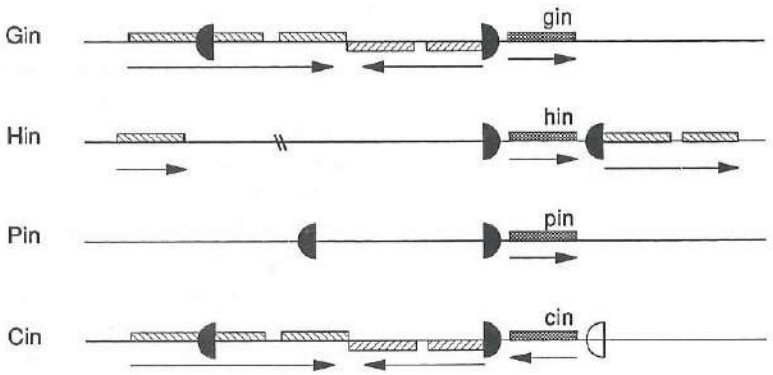

Figure 7. Organization of the invertible regions. The direction and localization of the reading frames of *gin* (phage Mu), *hin* (Salmonella), *pin* (phage e14) and cin (phage P1) are indicated by the arrows under the shaded boxes. The black half-circles depict the recombination sites on which the invertases act whereas the white one depicts a pseudo-site in the Cin system. The reading frames that form the functional part of the particular systems are represented by the hatched boxes with their direction of transcription indicated by arrows.

sites as a symmetrical dimer (62). This allows stimulation of the inversion reaction independent of the orientation or localization of the enhancer (73). Gin belongs to a class of invertases which can complement each other in the inversion reaction (66,82,104,126). These invertases Gin, Hin, Pin and Cin although from different organisms (65, 71,83) have a high degree of homology at the protein level, share a similar genomic organisation (26,63,64,84,103) and are probably all stimulated by Fis bound to an enhancer (68,83). It has been shown that the enhancer together with the *gix* sites forms a three site complex at the base of a branch point in a supercoiled DNA molecule (Fig.6) (67,74,76).

The role of Fis in excision of the lambda prophage

Excision from the host genome is the first step in the lambda life cycle after induction of the prophage. *In vitro* this excision reaction was shown to be dependent on Xis which acts on two adjacent binding sites in lambda *att*P (Fig.8). *In vivo* however there is an additional dependency on Fis which had been missed in previous *in vitro* experiments. At physiological concentrations of Xis the reaction can not proceed in the absence of Fis, which *in vitro* can be circumvented by an increased level of the excisionase Xis. Footprint experiments showed that binding of Fis to a site that partly overlaps with the

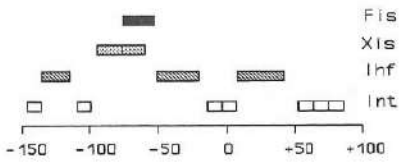

Figure 8. Organization of protein binding sites in lamoda attP. The localization of the protein binding sites for Fis (factor of inversion stimulation), Xis (excisionase), IHF (integration host factor) and Int (integrase) around the site of λ recombination are indicated.

right Xis binding site strongly stimulates the binding of Xis to the left site (123). The left Xis binding site can be considered to be the active site in excision. Fis can stimulate Xis binding to the left site either by direct protein-protein interaction or indirectly through the formation of a for binding more favourable conformation of the DNA through the introduction of a 90°-bend in the DNA upon binding (122).

The role of Fis in transcription of stable RNA operons in *E.coli*

The promoters of several stable RNA operons have an enhancer sequence (Upstream activating sequence or UAS) that stimulates transcription from the promoter both *in vivo* and *in vitro* (97, 109). The stimulatory activity of this enhancer is mediated through binding of FIS to this sequence (13,98). The promoters of three stable RNA operons have now been shown to bind Fis and sequence analysis predicts that nearly all stable RNA operons will be stimulated by Fis bound to the UAS (130). Careful measurements of growth rates under several environmental conditions of wild type and mutant Fis strains showed that the wild type can respond faster to more favourable growth conditions than the mutant strain (99). There is no insight into the mechanism by which Fis stimulates transcription but bending of the promoter region has been suggested. This was concluded from the observation that *in vivo* in the absence of Fis the UAS, which has an intrinsic bend, can stimulate transcription threefold by itself.

Outline of the thesis

The basis of the work presented in this thesis was the discovery of a host protein that protected a specific region in the left attachment site of Mu. As this site is situated adjacent to the for transposition important transposase binding site L2 we were interested in both the identification and possible function of this protein. The protein was first observed by M.A.M. Groenen during the purification of the A protein of Mu and was later identified as Fis by Bétermier and co-workers. This thesis describes experiments aimed at the determination of the role of Fis in Mu transposition.

Chapter 2 bears on the function of Fis binding to *att*L and shows that Fis in a concerted action with Mu repressor inhibits transposition in a manner that is dependent on the IAS. We subsequently studied the effect of mutations in the Fis binding site in *att*L on the observed inhibition of transposition.

In chapter 3 we address the question of transposition stimulation by the Mu enhancer and identify sequences within the attachment sites that are important for the stimulatory activity of the IAS. We show that a potential interaction of the IAS with the attachment sites is mediated through L2 in *att*L and R2 in *att*R. From these results a model emerges were *att*L interacts with the IAS to form a docking-complex that allows *att*R to correctly align with *att*L.

The role of Fis in Mu development was studied with an *in vitro* transposition assay which will allow to determine the level of mini-Mu transposition under various conditions. In chapter 4 we show that the absolute level of transposition is dependent on what F-factor is used as target for mini-Mu transposition.

In chapter 5 we investigate the distribution of Fis throughout several genera of bacteria species. Fis-like proteins are detected in the enteric group of gram-negative facultative anaerobic bacteria but not in less related species which indicates that *fis* is a relatively recent gene. Interestingly these close relatives of *E.coli* also are the natural hosts of Mu.

In chapter 6 we share some thoughts concerning the presented work. Here we discuss the proposed models for negative and positive regulation of transposition and present an evolutionary frame of thought in which *att*L and the IAS can be seen as the remains of an ancestral IS-like element.

Literature cited

(1) **Adzuma, K., and Mizuuchi, K.** (1988). Target immunity of Mu transposition reflects a differential distribution of Mu B protein. *Cell 53*:257-266.

(2) **Adzuma, K., and Mizuuchi, K.** (1989). Interaction of proteins located at a distance along DNA: Mechanism of target immunity in Mu DNA strand-transfer reaction. *Cell* 57:41-47.

(3) **Akroyd, J., and Symonds, N.** (1983). Evidence for a conservative pathway of transposition of bacteriophage Mu. *Nature (London) 303*:84-86.

(4) **Allet, B.** (1979). Mu insertion duplicates a 5 base pair sequence at the host inserted site. *Cell 16*:123-129.

(5) **Bade, E.G.** (1972). Asymmetric transcription of bacteriophage Mu-1. *J. Virol. 10*:1205-1207.

(6) **Baker, T.A., Mizuuchi, M., and Mizuuchi K.** (1991). Mu B protein allosterically activates strand transfer by the transposase of phage Mu. *Cell 65*:1003-1013.

(7) **Barlach, S., and Schumann, W.** (1983). Mapping of binding sites for Mu repressor and *ner* product within the left-end *Eco*RI.C fragment of the Mu genome. *FEBS Lett. 157*:119-123.

(8) **Bétermier, M., Alazard, R., Lefèvre, V., and Chandler, M.** (1989). Functional domains of bacteriophage Mu transposase: properties of *C*-terminal deletions. *Mol. Microbiol. 3*:1159-1171.

(9) **Bétermier, M., Alazard, R., Raguet, F., Roulet, E., Toussaint, A., and Chandler, M.** (1987). Phage Mu transposase: Deletion of the carboxy-terminal end does not abolish DNA binding activity. *Mol. Gen. Genet. 210*:77-85.

(10) **Bétermier, M., Lefèvre V., Koch C., Alazard, R., and Chandler, M.** (1989). The *Escherichia coli* protein Fis: Specific binding to the ends of phage Mu DNA and modulation phage growth. *Mol. Microbiol. 3*:459-468.

(11) **Boeckh, C., Middendorf, A., and Bade, E.G.** (1983). Expression of early genes of bacteriophage Mu inhibits cell division concomitantly with the insertion of protein B into the membrane of *E.coli* K12. *Hoppe-Seyler's Z. Physiol. Chem. 364*:1102.

(12) **Boeckh, C., Bade, E.G., Delius, H., and Reeve, J.N.** (1986). Inhibition of bacterial segregation by early functions of phage Mu and association of replication protein B with the inner cell membrane. *Mol. Gen. Genet. 202*:461-466.

(13) **Bosch, L., Nilsson, L., Vijgenboom, E., and Verbeek, H.** (1990) Fis-dependent *trans*-activation of tRNA and rRNA operons of *Escherichia coli*. *Biochim. Biophys. Acta 1050*:293-301.

(14) **Broyles, S.S., and Pettijohn, D.E.** (1986). Interaction of the *Escherichia coli* HU protein with DNA: Evidence for formation of nucleosome-like structures with altered DNA helical pitch. *J. Mol. Biol. 187*:47-60.

(15) **Bukhari, A.I., and Ambrosio, L.** (1978). The invertible segment of bacteriophage Mu DNA determines the adsorption properties of Mu particles. *Nature (London) 271*:575-577.

(16) **Bukhari, A.I., and Curtin, P.** (1974). Temperature-sensitive mutants of bacteriophage Mu. *J. Virol. 14*:1615-1616.

(17) **Bukhari, A.I., Lupski, J.R., Svec, P., and Godson, G.N.** (1985). Comparison of left-end DNA sequences of bacteriophage Mu and D108. *Gene 33*:235-239.

(18) **Burlingame, R.P., Obukowicz, M.G., Lynn, D.L., and Howe, M.M.** (1986). Isolation of point mutations in bacteriophage Mu attachment regions cloned in a λ::mini-Mu phage. *Proc. Natl. Acad. Sci. (USA) 83*:6012-6016.

(19) **Cameron, R.K., Jarjour, A.M., Tolias, P.P., and Dubow, M.S.** (1988). The bacteriophage Mu transposase protein can form high-affinity protein-DNA complexes with the ends of transposable elements of the Tn3 family. *FEBS Lett. 229*:283-288.

(20) **Chaconas, G., Gloor, G., and Miller, J.L.** (1985). Amplification and purification of the bacteriophage Mu encoded *B* transposition protein. *J. Biol. Chem. 260*:2662-2669.

(21) **Chaconas, G., Kennedy, D.L., and Evans, D.** (1983). Predominant integration end-products of infecting bacteriophage Mu DNA are simple insertions with no preference for integration of either Mu DNA strand. *Virology 128*:48-59.

(22) **Chaconas, G., Harshey, R.M., Sarvetnick, N., and Bukhari, A.I.** (1981). Predominant end-products of prophage Mu DNA transposition during the lytic cycle are replicon fusions. *J. Mol. Biol. 150*:341-359.

(23) **Chaconas, G., de Bruin, F.J., Casadaban, M.J., Lupski, J.R., Kwoh, T.J., Harshey, R.M., DuBow, M.S., and Bukhari, A.I.** (1981). *In vitro* and *in vivo* manipulations of bacteriophage Mu DNA: Cloning of Mu ends and construction of mini-Mu's carrying selectable markers. *Gene 13*:37-46.

(24) **Chase, C.D., and Benzinger, R.H.** (1982). Transfection of *Escherichia coli* spheroplasts with a bacteriophage Mu DNA-protein complex. *J. Virol. 42*:176-185.

(25) **Choe, H.-W., Labahn, J., Itoh, S., Koch, C., Kahmann, R., and Saenger, W.** (1989). Crystallization of the DNA-binding *Escherichia coli* protein Fis. *J. Mol. Biol. 208*:209-210.

(26) **Chow, L.T., and Bukhari, A.I.** (1976). The invertible DNA segments of coliphage Mu and P1 are identical. *Virology 74*:242-248.

(27) **Craigie, R., and Mizuuchi, K.** (1985). Cloning of the *A* gene of bacteriophage Mu and purification of its product, the Mu transposase. *J. Biol. Chem. 260*:1832-1835.

(28) **Craigie, R., and Mizuuchi, K.** (1985). Mechanism of transposition of bacteriophage Mu: Structure of a transposition intermediate. *Cell 41*:867-876.

(29) **Craigie, R., and Mizuuchi, K.** (1986). Role of DNA topology in Mu transposition: Mechanism of sensing the relative orientation of two DNA segments. *Cell 45*:793-800.

(30) **Craigie, R., and Mizuuchi, K.** (1987). Transposition of bacteriophage Mu: Joining of Mu to target can be uncoupled from cleavage at the ends of Mu. *Cell 51*:493-501.

(31) **Craigie, R., Arndt-Jovin, D.J., and Mizuuchi, K.** (1985). A defined system for the DNA strand-transfer reaction at the initiation of bacteriophage Mu transposition: Protein and DNA substrate requirements. *Proc. Natl. Acad. Sci. (USA) 82*:7570-7574.

(32) **Craigie, R., Mizuuchi, M., and Mizuuchi, K.** (1984). Site-specific recognition of the bacteriophage Mu ends by the Mu A protein. *Cell 39*:387-394.

(33) **Darzins, A., Kent, N., Buckwalter, M., and Casadaban, M.** (1988). Bacteriophage Mu sites required for transposition immunity. *Proc. Natl. Acad. Sci. (USA) 85*:6826-6830.

(34) **Desmet L., Faelen, M., Gama, M.-J., Ferhat, A., and Toussaint, A.** (1989). Characterization of amber mutations in bacteriophage Mu transposase: a functional analysis of the protein. *Mol. Microbiol. 3*:1145-1158.

(35) **Dixon, N.E., and Kornberg, A.** (1984). Protein HU in the enzymatic replication of the chromosomal origin of *E.coli*. *Proc. Natl. Acad. Sci. (USA) 81*:424-428.

(36) **Faelen, M., and Toussaint, A.** (1973). Isolation of conditional defective mutants of temperate phage Mu-1 and deletion mapping of the Mu-1 prophage. *Virology 54*:117-124.

(37) **Faelen, M., Huisman, O., and Toussaint, A.** (1978). Involvement of phage Mu-1 early functions in Mu-mediated chromosomal rearrangements. *Nature (London) 271*:580-582.

(38) **Fitts, R.A., and Taylor, A.L.** (1980). Integration of bacteriophage Mu at host chromosomal replication forks during lytic development. *Proc. Natl. Acad. Sci. (USA) 77*:2801-2805.

(39) **Giphart-Gassler, M., and van de Putte, P.** (1978). Early gene products of bacteriophage Mu: Identification of the *B* gene product. *J. Mol. Biol. 120*:1-12.

(40) **Giphart-Gassler, M., Plasterk, R.H.A., and van de Putte, P.** (1982). G inversion in bacteriophage Mu: a novel way of gene splicing. *Nature (London) 297*:339-242.

(41) **Gloor, G., and Chaconas, G.** (1986). The bacteriophage Mu *N* gene encodes the 64-kDa virion protein which is injected with and circularizes infecting Mu DNA. *J. Biol. Chem. 261*:16682-16688.

(42) **Goosen, N., and van de Putte, P.** (1984). Regulation of Mu trans-position. I. Localization of the presumed recognition sites for HimD and Ner functions controlling bacteriophage Mu transcription. *Gene 30*:41-46.

(43) **Goosen, N., and van de Putte, P.** (1986). Role of Ner protein in bacteriophage Mu transposition. *J. Bacteriol. 167*:503-507.

(44) **Goosen, N., and van de Putte, P.** (1987). Regulation of transcription. In *Phage Mu* (ed. N. Symonds *et al.*), p.41-52. Cold Spring Harbor Laboratory, Cold Spring Harbor.

(45) **Groenen, M.A.M.** (1987). The ends of the genome of bacteriophage Mu: active sites in transposition. *Thesis Leiden University*

(46) **Groenen, M.A.M., and van de Putte, P.** (1986). Analysis of the ends of bacteriophage Mu using site-directed mutagenesis. *J. Mol. Biol. 189*:597-602.

(47) **Groenen, M.A.M., Timmers, E., and van de Putte, P.** (1985). DNA sequences at the ends of the genome of bacteriophage Mu essential for transposition. *Proc. Natl. Acad. Sci. (USA) 82*:2087-2091.

(48) **Groenen, M.A.M., Vollering, M., Krijgsman, P., van Drunen, C.M., and van de Putte, P.** (1987). Interactions of the transposase with the ends of Mu: formation of specific nucleoprotein structures and non-cooperative binding of the transposase to its binding sites. *Nucleic Acids Res. 15*:8831-8844.

(49) **Grundy, F.J., and Howe, M.M.** (1984). Involvement of the invertible G segment in bacteriophage Mu tail fiber biosynthesis. *Virology 134*:485-504.

(50) **Harel, J., Duplessis, L., Kahn, J.S., DuBow, M.S.** (1990). The *cis*-acting DNA sequences required *in vivo* for bacteriophage Mu helper-mediated transposition and packaging. *Arch. Microbiol. 154*:67-72.

(51) **Harshey, R.M.** (1983). Switch in the transposition products of Mu DNA by proteins: Cointegrates versus simple insertions. *Proc. Natl. Acad. Sci. (USA) 80*:2012-2016.

(52) **Harshey, R.M.** (1984). Nonreplicative DNA transposition: Integration of infecting bacteriophage Mu. *Cold Spring Harbor Symp. Quant. Biol. 49*:273-278.

(53) **Harshey, R.M.** (1984). Transposition without duplication of infecting bacteriophage Mu DNA. *Nature (London) 311*:580-581.

(54) **Harshey, R.M., and Bukhari, A.I.** (1983). Infecting bacteriophage Mu DNA forms a circular DNA-protein complex. *J. Mol. Biol. 167*:427-441.

(55) **Harshey, R.M., and Cuneo, S.D.** (1986). Carboxy-terminal mutants of phage Mu transposase. *J. Genet. 65*:159-174.

(56) **Harshey, R.M., Getzoff, E.D., Baldwin, D.L., Miller, J.L., and Chaconas, G.** (1985). Primary structure of phage Mu transposase: Homology to Mu repressor. *Proc. Natl. Acad. Sci. (USA) 82*:159-174.

(57) **Heichman, K.A., and Johnson, R.C.** (1990). The Hin-invertasome: protein mediated joining of distant recombination sites at the enhancer. *Science 249*:511-517.

(58) **Howe, M.M.** (1978). Invertible DNA in phage Mu. *Nature (London) 271*:608-610.

(59) **Howe, M.M.** (1980). The invertible G segment of phage Mu. *Cell 21*:605-606.

(60) **Howe, M.M., Schumm, J.W., and Taylor, A.L.** (1979). The *S* and *U* genes of bacteriophage Mu are located in the invertible G segment of Mu DNA. *Virology 92*:108-124.

(61) **Huber, H.E., Iida, S., Arber, W., and Bickle, T.A.** (1985). Site-specific DNA inversion is enhanced by a DNA sequence element in *cis*. *Proc. Natl. Acad. Sci (USA) 82*:3776-3780.

(62) **Hübner, P., and Arber, W.** (1989). Mutational analysis of a prokaryotic recombinational enhancer element with two functions. *EMBO J. 8*:577-585.

(63) **Iida, S.** (1984). Bacteriophage P1 carries two related sets of genes determining its host range in the invertible C segment of its genome. *Virology 134*:421-434.

(64) **Iida, S., Meyer, J., Kennedy, K.E., and Arber, W.** (1982). A site-specific conservative recombination system carried by bacteriophage P1. Mapping the recombinase gene *cin* and the crossover sites *cix* for the inversion of the C segment. *EMBO J. 1*:1445-1453.

(65) **Iida, S., Sandmeier, H., Hübner, P., Hiestand-Nauer, R., Schneitz, K., and Arber, W.** (1990). The Min DNA inversion enzyme of plasmid p15B of *Escherichia coli* 15T⁻: a new member of the Din family of site-specific recombinases. *Mol. Microbiol. 4*:991-997.

(66) **Iino, T., and Kutsukake, K.** (1981). *Trans*-acting genes of bacteriophages P1 and Mu mediate inversion of a specific DNA segment involved in the flagellar phase variation of *Salmonella*. *Cold Spring Harbor Symp. Quant. Biol. 45*:11-16.

(67) **Johnson, R.C., and Bruist, M.F.** (1989). Intermediates in Hin-mediated DNA inversion: a role for Fis and the recombinational enhancer in the strand exchange reaction. *EMBO J. 8*:1581-1590.

(68) **Johnson, R.C., and Simon, M.I.** (1985). Hin-mediated site-specific recombination requires two 26 bp recombination sites and a 60 bp recombinational enhancer. *Cell 41*:781-791.

(69) **Johnson, R.C., Ball, C.A., Pfeffer, D., and Simon, M.I.** (1988). Isolation of the gene encoding the Hin recombinational enhancer binding protein. *Proc. Natl. Acad. Sci. (USA) 85*:3484-3488.

(70) **Kahmann, R., Rudt, F., Koch, C., and Mertens, G.** (1985). G inversion in bacteriophage Mu DNA is stimulated by a site within the invertase gene and a host factor. *Cell 41*:771-780.

(71) **Kamp, D., and Kahmann, R.** (1981). The relationship of two invertible segments in bacteriophage Mu and *Salmonella typhimurium*. *Mol. Gen. Genet. 184*:564-566.

(72) **Kamp, D., Kahmann, R., Zipser, D., Broker, T.R., and Chow, L.T.** (1978). Inversion of the G DNA segment of phage Mu controls phage infectivity. *Nature (London) 271*:577-580.

(73) **Kanaar, R., van Hal, J.P., and van de Putte, P.** (1989). The recombinational enhancer for DNA inversion functions independent of its orientation as a consequence of dyad symmetry in the Fis-DNA complex. *Nucleic Acids Res. 17*:6043-6053.

(74) **Kanaar, R., Klippel, A., Shekhtman, E., Dungan, J.M., Kahmann, R., and Cozzarelli, N.R.** (1990). Processive recombination by the phage Mu Gin system: Implications for the mechanism of DNA strand exchange, site alignment and enhancer action. *Cell 62*:353-366.

(75) **Kanaar, R., van de Putte, P., and Cozzarelli, N.R.** (1986). Purification of the Gin recombination protein of *Escherichia coli* phage Mu and its host factor. *Biochim. Biophys. Acta 866*:170-177.

(76) **Kanaar, R., van de Putte, P., and Cozzarelli, N.R.** (1989). Gin-mediated recombination of catenated and knotted DNA substrates: Implications for the mechanism of interaction between *cis* acting sites. *Cell 58*:147-159.

(77) **Klippel, A., Cloppenborg, K., and Kahmann, R.** (1988). Isolation and characterization of unusual *gin* mutants. *EMBO J. 7*:3983-3989.

(78) **Klippel, A., Mertens, G., Patschinsky, T., and Kahmann, R.** (1988). The DNA invertase Gin of phage Mu: formation of a covalent complex with DNA via a phosphoserine at amino acid position 9. *EMBO J. 7*:1229-1237.

(79) **Koch, L., and Kahmann. R.** (1986). Purification and properties of the *Escherichia coli* host factor required for inversion of the G segment in bacteriophage Mu. *J. Biol. Chem. 261*:15673-15678.

(80) **Koch, L., Vandekerckhove, J., and Kahmann, R.** (1988). *Escherichia coli* host factor for site-specific DNA recombination: Cloning and characterization of the *fis* gene. *Proc. Natl. Acad. Sci. (USA) 85*:4237-4241.

(81) **Kostrewa, D., Grazin, J., Koch, C., Choe, H.-W., Raghunathan, S., Wolf, W., Labahn, J., Kahmann, R., and Saenger, W.** (1991). Three-dimensional structure of the *E.coli* DNA-binding protein Fis. *Nature (London) 349*:178-180.

(82) **Kutsukake, K., and Iino, T.** (1980). A *trans*-acting factor mediates inversion of a specific DNA segment in flagellar phase variation of *Salmonella*. *Nature (London) 284*:479-481.

(83) **Kutsukake, K., and Iino, T.** (1980). Inversions of specific DNA segments in flagellar phase variation of *Salmonella* and inversion systems of bacteriophages P1 and Mu. *Proc. Natl. Acad. Sci. (USA) 77*:7238-7341.

(84) **Kutsukake, K., Nakao, T., and Iino, T.** (1985). A gene for DNA invertase and an invertible DNA in *Escherichia coli* K-12. *Gene 34*:343-350.

(85) **Kwoh, D.Y., and Zipser D.** (1979). Specific binding of Mu repressor to DNA. *Nature (London) 277*:489-491.

(86) **Lavoie, B.D., and Chaconas, G.** (1990). Immunoelecton microscopic analysis of the A, B, and HU protein content of bacteriophage Mu transpososomes. *J. Biol. Chem. 265*:1623-1627.

(87) **Leung, P.C., Teplow, D.B., Harshey, R.M.** (1989). Interaction of distinct domains in Mu transposase with Mu ends and an internal transpositional enhancer. *Nature (London) 338*:656-658.

(88) **Maxwell, A., Craigie, R., and Mizuuchi, K.** (1987). B protein of bacteriophage Mu is an ATPase that preferentially stimulates intermolecular DNA strand transfer. *Proc. Natl. Acad. Sci. (USA) 84*:699-703.

(89) **Mertens, G., Hoffmann, A., Blocker, H., Frank, R., and Kahmann, R.** (1984). *Gin*-mediated site-specific recombination in bacteriophage Mu DNA: Overproduction of the protein and inversion *in vitro*. *EMBO J. 3*:2415-2421.

(90) **Miller, J.L., Anderson, S.K., Fujita, D.J., Chaconas, G., Baldwin, D., and Harshey, R.M.** (1984). The nucleotide sequence of the *B* gene of bacteriophage Mu. *Nucleic Acids Res. 12*:8627-8638.

(91) **Mizuuchi, K.** (1983). *In vitro* transposition of bacteriophage Mu: A biochemical approach to a novel replication reaction. *Cell 35*:785-794.

(92) **Mizuuchi, K.** (1984). Mechanism of transposition of bacteriophage Mu: Polarity of the strand transfer reaction at the initiation of transposition. *Cell 39*:395-404.

(93) **Mizuuchi, M., and Mizuuchi, K.** (1989). Efficient Mu transposition requires interaction of the transposase with a DNA sequence at the Mu operator: Implications for regulation. *Cell 58*:399-408.

(94) **Mizuuchi, M., Weisberg, R.A., and Mizuuchi, K.** (1986). DNA sequence of the control region of phage D108: The N-terminal amino acid sequences of repressor and transposase are similar both in phage D108 and in its relative, phage Mu. *Nucleic Acids Res. 14*:3813-3825.

(95) **Nakai, H., and Taylor, A.L.** (1985). Host DNA replication forks are not preferred targets for bacteriophage Mu transposition. *J. Bacteriol. 163*:282-290.

(96) **Nakayama, C., Teplow, D.B., and Harshey, R.M.** (1987). Structural domains in phage Mu transposase: Identification of the site-specific DNA-binding domain. *Proc. Natl. Acad. Sci (USA) 84*:1809-1813.

(97) **Nachaliel, N., Melnick, J., Gafny, R., and Glaser, G.** (1989). Ribosome associated protein(s) specifically bind(s) to the upstream activator sequence of the *E.coli rrn*A P1 promoter. *Nucleic Acids Res. 17*:9811-9822.

(98) **Nilson, L., Vanet, A., Vijgenboom, E., and Bosch, L.** (1990). The role of Fis in *trans* activation of stable RNA operons in *E.coli*. *EMBO J. 9*:727-734.

(99) **Nilson, L., Verbeek, H., Vijgenboom, E., van Drunen, C.M., and Bosch, L.** (1991). Fis-dependent *trans* activation of stable RNA operons of *Escherichia coli* under various growth conditions. *J. Bacteriol. 174*: 921-929.

(100) **O'Day, K.J., Schultz, D.W., and Howe, M.M.** (1987). Search for integration-deficient mutants of bacteriophage Mu. In *Microbiology*. (ed. D. Schlessinger), p.48-51. American Society for Microbiology, Washington, D.C.

(101) **Pato, M.L., and Reich, C.** (1984). Stoichiometric use of the transposase of bacteriophage Mu. *Cell 36*:197-202.

(102) **Pato, M.L., Howe, M.M., and Higgins, N.P.** (1990). A DNA gyrase-binding site at the center of the bacteriophage Mu genome is required for efficient replicative transposition. *Proc. Natl. Acad. Sci. (USA) 87*:8716-8720.

(103) **Plasterk, R.H.A., and van de Putte, P.** (1985). The invertible P-DNA segment in the chromosome of *Escherichia coli*. *EMBO J. 4*:237-242.

(104) **Plasterk, R.H.A., Brinkman, A., and van de Putte, P.** (1984). DNA inversions in the chromosome of *E.coli* and in bacteriophage Mu: Relationschip to other site-specific recombination systems. *Proc. Natl. Acad. Sci. (USA) 80*:5355-5358.

(105) **Plasterk, R.H.A., Kanaar, R., and van de Putte, P.** (1984). A genetic switch *in vitro*: DNA inversion by Gin protein of phage Mu. *Proc. Natl. Acad. Sci. (USA) 81*:2689-2692.

(106) **Puspurs, A.H., Trun, N.J., and Reeve, J.N.** (1983). Bacteriophage Mu DNA circularizes following infection of *Escherichia coli*. *EMBO J.* 2:345-352.

(107) **Reed, R.R., Young, R.A., Steitz, Grindley, N.D.F., and Guyer, M.S.** (1979). Transposition of the *E.coli* insertion element γδ generates a five-base-pair repeat. *Proc. Natl. Acad. Sci. (USA) 76*:4882-4886.

(108) **Resibois, A., Pato, M., Higgins, P., and Toussaint, A.** (1984). Replication of bacteriophage Mu and its mini-Mu derivatives. In *Proteins involved in DNA replication (ed. U. Hubscher, and S. Spadari)*, p.69-76. Plenum Publishing Corp., New York.

(109) **Ross, W., Thompson, J.F., Newlands, J.T., and Gourse, R.L.** (1990). *E.coli* Fis protein activates ribosomal RNA transcription *in vitro* and *in vivo*. *EMBO J. 9*:3733-3742.

(110) **Rouvière-Yaniv, J., Yaniv, M., and Germond, J.E.** (1979). *E.coli* DNA binding protein HU forms nucleosome-like structures with circular double-stranded DNA. *Cell 17*:265-274.

(111) **Sandulache, R., Prehm, P., and Kamp, D.** (1984). Cell wall receptor for bacteriophage MuG(+). *J. Bacteriol. 160*:1226-1227.

(112) **Sandulache, R., Prehm, P., Expert, D., Toussaint, A., and Kamp, D.** (1985). The cell wall receptor for bacteriophage MuG(-) in *Erwinia* and *Escherichia coli* C. *FEMS Microbiol. Lett. 28*:307-310.

(113) **Schumann, W., Simon, V., and Lögl, C.** (1984). The bacteriophage Mu *B* product is incorporated into the inner membrane of *Escherichia coli*. *Gene 29*:167-173.

(114) **Shapiro, J.A.** (1979). Molecular model for the transposition and replication of bacteriophage Mu and other transposable elements. *Proc. Natl. Acad. Sci. (USA) 76*:1933-1937.

(115) **Spaeny-Dekking, E.H.A., Goosen, N., and van de Putte, P.** (1992). The role of the N-terminus of the Fis protein in different Fis-mediated processes. *J. Mol. Biol. 11*:91-104.

(116) **Surette, M.G., and Chaconas, G.** (1989). A protein factor which reduces the negative supercoiling requirement in the Mu DNA strand transfer reaction is *Escherichia coli* integration host factor. *J. Biol. Chem. 264*:3028-3034.

(117) **Surette, M.G., Buch, S.J., and Chaconas, G.** (1987). Transpososomes: Stable protein-DNA complexes involved in the *in vitro* transposition of bacteriophage Mu DNA. *Cell 49*:253-262.

(118) **Surette, M.G., Lavoie, B.D., and Chaconas G.** (1989). Action at a distance in Mu DNA transposition: an enhancer-like element is the site of action of supercoiling relief activity by integration host factor (IHF). *EMBO J. 8*:3483-3489.

(119) **Surette, M.G., and Chaconas, G.** (1991). Stimulation of the Mu DNA strand cleavage and intramolecular strand transfer reactions by the Mu B protein is independent of stable binding of the Mu B protein to DNA. *J. Biol. Chem. 266*:17306-17313.

(120) **Surette, M.G., and Chaconas, G.** (1992). The Mu transpositional enhancer can function *in trans*: requirement of the enhancer of synapsis but not strand transfer. *Cell 68*:1101-1108.

(121) **Taylor, A.L.** (1963). Bacteriophage induced mutation in *E.coli*. *Proc. Natl. Acad. Sci. (USA) 50*:1043-1051.

(122) **Thompson, J.F., and Landy, A.** (1988). Empirical estimation of protein-induced DNA bending angles: applications to λ site-specific recombination complexes. *Nucleic Acids Res. 16*:9687-9704.

(123) **Thompson, J.F., Moitoso de Vargas, L., Koch, C., Kahmann, R., and Landy, A.** (1987). Cellular factors couple recombination with growth phase: Characterization of a new component in the λ site-specific recombination pathway. *Cell 50*:901-908.

(124) **Toussaint, A., Faelen, M., Desmet, L., and Allet, B.** (1983). The products of gene *A* of related phages Mu and D108 differ in their specificities. *Mol. Gen. Genet. 181*:201-206.

(125) **Van de Putte, P., Cramer, S., and Giphart-Gassler, M.** (1980). Invertible DNA determines host specificity of bacteriophage Mu. *Nature (London) 286*:218-222.

(126) **Van de Putte, P., Plasterk, R.H.A., and Kuijpers, A.** (1984). A Mu *gin* complementing function and an invertible DNA region in *Escherichia coli* K12 are situated on the genetic element e14. *J. Bacteriol. 158*:517-522.

(127) **Van Rijn, P.A., Goosen, N., Turk, S.C.H.J., and van de Putte, P.** (1989). Regulation of phage Mu repressor transcription by IHF depends on the level of the early transcription. *Nucleic Acids Res. 17*:10203-10212.

(128) **Van Rijn, P.A., Goosen, N., and van de Putte, P.** (1988). Integration host factor of *Escherichia coli* regulates early- and repressor transcription of bacteriophage Mu by two different mechanisms. *Nucleic Acids Res. 16*:4595-4605.

(129) **Van Rijn, P.A., van de Putte, P., Goosen, N.** (1991). Analysis of the IHF bindingsite in the regulatory region of bacteriophage Mu. *Nucleic Acids Res. 19*:2825-2834.

(130) **Verbeek, H., Nilsson, L., Baliko, G., Bosch, L.** (1990). Potential binding sites of the *trans*-activator Fis are present upstream all rRNA operons and many but not all tRNA operons. *Biochim. Biophys. Acta 1050*:302-306.

(131) **Wijffelman, C.A., and van de Putte, P.** (1977). Assymmetric hybridization of Mu strands with short fragments synthesized during Mu DNA replication. In *DNA insertion elements, plasmids and episomes* (ed. A.I. Bukhari *et al.*), p.329-333. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Inhibition of bacteriophage Mu transposition through the concerted action of Mu repressor and Fis

C.M. van Drunen, C. van Zuylen, E.J. Mientjes, N. Goosen and P. van de Putte

In this paper we show that the *Escherichia coli* protein Fis has a regulatory function in Mu transposition in the presence of Mu repressor. Fis can lower the transposition frequency of a mini-Mu 3-80 fold but only if simultaneously the Mu repressor is expressed.

In this novel type of regulation of transposition by the concerted action of Fis and repressor also the IAS, the internal activating sequence, is involved as deletion of this site leads to the loss of the Fis effect. As the IAS contains strong repressor binding sites these are probably the target for the repressor in the observed negative regulation by Fis and repressor. However, the role of Fis and repressor is not only to inactivate the IAS since a 4 bp insertion in the IAS, which changes the spacing of the repressor binding site, abolishes the enhancing function of the IAS but leaves the repressor-Fis effect intact.

A likely target for Fis in this regulation is a strong Fis binding site which is located adjacent to the L2 transposase binding site. However when this Fis binding sequence was substituted by a random sequence and Fis did no longer show specific binding to this site, the Fis effect was still observed. Although it is still possible that Fis can function by binding to this aspecific site in a particular complex, it seems more likely that Fis is directly or indirectly involved in determining the level of the repressor.

INTRODUCTION

Bacteriophage Mu has shown to be a powerful model system (22) for studies on transposition both in bacteria and higher organisms. For instance transposons like Tn10 and retro-transposons share with Mu several steps in the molecular mechanism of the transposition reaction as these were first elucidated for Mu (4). The study of transposition in Mu was aided by the high efficiency of the transposition reaction. This efficiency is probably the result of the successive formation of several highly ordered nucleo-protein complexes.

The right and left attachment sites (*att*R and *att*L) share three 22 base pairs repeats which are the binding sites for transposase A (Fig. 1). Extensive deletion and mutation studies have indicated that all transposase binding sites with the exception of the proximal site in *att*R (R3) are important for transposition (12,13). Through binding of A to these sites and the action of the *Escherichia coli* protein HU, *att*R and *att*L are brought together. The formation of this complex initiates the transposition reaction leading to the cleaved donor complex (CDC) where A has specifically nicked the 3'-ends of bacteriophage Mu DNA (5,29). The next step in transposition is the formation of the strand transfer complex (STC) in which Mu DNA is covalently joined to the target DNA (29). This reaction is stimulated by the presence of Mu B bound to target DNA molecule

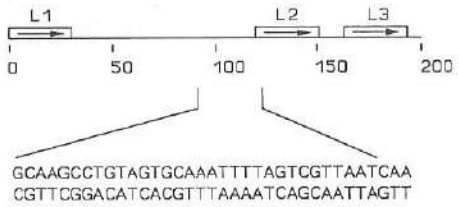

Figure 1. Structure of the left attachment site of bacteriophage Mu. Indicated are the three transposase binding sites L1-L3 and the region adjacent to L2 protected by Factor of inversion stimulation in top and bottom strand.

(1,21). The interaction between B and A bound at the attachment sites is thought to stabilize the intermolecular complex thereby facilitating the formation of the STC. The final step in Mu transposition is independent of any Mu proteins and comprises solely of replication of the STC.

Part of the operator region of Mu has been identified to act as an enhancer for transposition (10,19,23,30). The Mu repressor binding sites O1 and O2 stimulate, in an orientation dependent manner, the transposition reaction a 100 fold. This enhancement is mediated by transposase A which interacts with these sites through a domain distinct from that which specifically interacts with the attachment sites (23). The exact mechanism of action of this Internal Activating Sequence (IAS) is unknown but it has been shown that it can aid in the active formation of the CDC

complex possibly by interacting with both attachment sites.

The *E.coli* host proteins HU and IHF are also involved in the Mu transposition reaction: HU in the formation of the type I complex (29) and IHF in the alleviation of supercoil dependency of the formation of this complex (28). In our group another host factor was discovered that binds to a specific site adjacent to L2 in *att*L (Fig. 1) (11). This factor was also noticed by Bétermier *et al.* who identified the protein as Fis (Factor of inversion stimulation) (3). This 12 kD heat stable protein is known to have an accessory role in several processes. For a review see Finkel and Johnson (8). Firstly, it is involved in the inversion of the G-loop of bacteriophage Mu (17) where Fis, bound to the recombinational enhancer, is thought to interact with Gin in order to align the recombination sites. Secondly, Fis stimulates the excision reaction of prophage lambda through cooperative binding with Xis at two adjacent sites in lambda *att*R (32). Also intrinsic functions of Fis in *E.coli* have been discovered. It stimulates transcription of several stable RNA operons through binding to an Upstream Activation Sequence or UAS (24,25,27). In addition Fis seems to play a role in chromosomal replication since the stability of oriC-plasmids is significantly reduced in *fis* mutant cells (7,9).

In this paper we investigate the role of Fis in the transposition reaction of Mu using an *in vivo* mini-Mu transposition assay. We show that Fis can

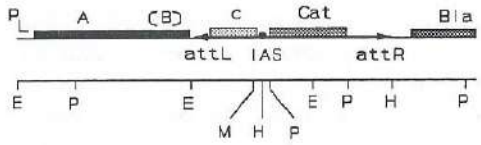

Figure 2. Schematic representation of the mini-Mu plasmids. The genes *A* and *B* are under control of the P_L promoter and *c* is expressed from Pc. Mu sequences are represented by the thick line which include the IAS. The restriction sites indicated are E=*Eco*RI, P=*Pst*I, M=*Mlu*I and H=*Hin*dIII. The plasmid confers resistance to Chloramphenicol (*cat*) and Ampicillin (*bla*).

inhibit transposition through a concerted action with Mu repressor and in a way that is dependent on sequences within the Mu enhancer.

MATERIALS AND METHODS

Bacterial strains. The strains PP135 [Δ*lac-pro*X111, *thi*209, supE, (λ)] (13) and MC1000 *fis*-767 (16) have been described before. The strain PP2542 is M72 [*lac, bio, trp* (λ*N*am7-*N*am53 *c*I857 ΔH1)] (2) with pGP655. The *fis*-767 mutation was introduced in PP2542 by P1 transduction resulting in PP2541.
Plasmids. The relevant characteristics of mini-Mu plasmids (Fig. 2) used in the transposition assays are described in tables 1 and 2. In these plasmids Mu repressor is expressed from Pc (34) as was tested by Mu titration. Plasmids

with an inactive repressor gene were constructed by introduction of a frame shift in the coding region in the following way. First a *Bgl*II site (C842 ->T) was created with site directed mutagenesis (18) followed by filling in of the site using Klenow polymerase and dNTPs. The plasmid with an inactive IAS (pGP884) was constructed by filling in of the MluI site in between O1 and O2 of pGP851. The Fis binding site in pGP851 was replaced by a random sequence in the following way. First two restriction sites were created at either site of the *fis* binding sequence using site directed mutagenesis: a *Hind*III site (C->T91) at the left and a *Cla*I site (G108->A and T112->A) at the right. Subsequently the bases between these newly created sites were replaced by a synthetic DNA fragment of the same length. The new sequence at *att*L in pGP1046 now reads from position 91 to position 112 : TTCATATGGCTGCGCACATCGA.

The repressor overproducer pGP829 contains the Mu repressor on a *Hind*III-*Sau*3A fragment (position 1000 and 60 in *att*L (11)) under control of P_L. To allow for selection of pGP829 in the *in trans* complementation assay, the β-lactamase genes of pGP852, pGP619 and pGP880 were inactivated (pGP1004, pGP1005 and pGP1007 respectively) by insertion of a kanamycin cartridge in the *Sca*I site. The Fis overproducer pGP842 (P_L-*fis*) was constructed by random cloning of a *Kpn*I-*Hind*III chromosomal DNA digest of *E.coli* and selection for complementation of the Fis phenotype of PP2541. All cloning procedures were according to Maniatis (20). The construction of plasmids pGP655 (14), pGP619 (13) and pGP660 (15) have been described before.

Transposition assay. The transposition frequencies of the mini-Mu plasmids were determined with a conjugation assay (13,14) using the F factor pGP655 as transposition target and PP135 as acceptor strain. The transposition frequency is the fraction of F factors that confer chloramphenicol resistance to the acceptor strain. The ratio of the transposition frequency in the Fis^- (PP2541) and Fis^+ (PP2542) strains (-/+) is used as an indicator of the Fis effect. The use of pGP655, lacking any known insertion sequences, resulted, under non-induced conditions, in a low background of transposition which enabled us to detect transposition levels as low as $1x10^{-8}$. The absolute level of F factor transfer is the same in all strains used.

Protein purification: Fis was purified to homogeneity from the strain M72 containing pGP842. A crude extract was prepared by gentle lysis of the cells in 0.8 M KCl in HED (25 mM HEPES pH7.6, 1 mM EDTA and 2mM DTT) followed by freeze-thawing. The proteins from the supernatant (50 min at 39.000 rpm, Beckmann 50.2Ti rotor) were precipitated with 0.35 gram/ml $(NH_4)_2SO_4$ and after centrifugation (20 min at 20.000 rpm, Beckmann 50.2Ti rotor) dissolved in 0.4 M KCl in HEDG (HED containing 20% glycerol).

This protein extract was dialysed against 0.4 M KCl in HEDG and run on a P11 column (0.4M - 1.0 M KCl in HEDG). The peak fractions were diluted to 0.3 M KCl in HEDG, heated for 10 min at 80°C and applied to a ssDNA agarose column (0.3 M - 1.0 M KCl in HEDG). Fis containing fractions were stored at -80°C.

DNaseI footprinting. The *Eco*RI-*Sal*I restriction fragment of pGP660 containing *att*L (170 base pairs) was labelled at the *Sal*I site using ^{32}P-dCTP and Klenow polymerase. About 5-10 ng of labelled fragment was incubated with 0, 5, 10, 30, 50, 70 µg/ml of repressor either with or without 2.5 µg/ml of Fis. Footprint conditions were described before (6). Densitometric scans of the protection patterns were obtained with a LKB Ultroscan XL.

RESULTS

The effect of Fis on transposition. During an isolation procedure of the Mu A protein a host protein, was co-purified which specifically bound to a site adjacent to L2 (11). This protein was also observed by Bétermier *et al.* who identified it as Fis (3). Because of the position of the Fis binding site adjacent to a transposase binding site we investigated whether Fis has a role in Mu transposition. The transposition frequencies of a series of mini-Mu constructs were determined both in Fis^+ (PP2542) and in Fis^- (PP2541) background (Table 1). These experiments resulted in two major conclusions: Firstly, an effect of Fis on transposition is only observed under special conditions (as described below). Secondly, when an effect is observed, Fis appears not to stimulate transposition but rather to reduce it.

We found that the reducing effect of Fis is only observed with mini-Mu's expressing besides the Mu genes A and/or B also the Mu repressor. For instance when we compare the frequency obtained with mini-Mu pGP851 with that of pGP880, see Table 1 Fis has only an effect (factor 20) with the mini-Mu in which the repressor is expressed. Also when the repressor is supplied *in trans* from a plasmid different from the one in which the mini-Mu is situated (Table 1, line 5) the negative effect of Fis on transposition in the presence of repressor protein is still observed. This proves that the observed reduction of transposition is due to the repressor protein and not to the process of repressor transcription within the mini-Mu.

The role of MuB in the Fis effect. The inhibition of transposition by Fis in the presence of Mu repressor is less severe when besides MuA also MuB is expressed (compare pGP852 with pGP851, Table 1). Apparently B can in some way counteract the action of Fis and repressor. Therefore one would expect that the balance between B and Fis & repressor is important for the extent of the Fis effect. In our test system B is expressed from a much stronger promoter (PL) than repressor

Table 1. The effect of Fis on transposition.

Plasmid	Characteristics *	Transposition frequency † Fis⁻	Fis⁺	Ratio -/+ ‡
pGP852	P_L:A B P_C:c	5.4×10^{-2}	2.0×10^{-2}	2.7± 1.6
pGP876	P_L:A B P_C:c⁻	4.0×10^{-2}	3.3×10^{-2}	1.2± 0.5
pGP851	P_L:A P_C:c	4.0×10^{-4}	2.0×10^{-5}	20.0± 7.4
pGP880	P_L:A P_C:c⁻	6.0×10^{-3}	4.4×10^{-3}	1.4± 0.4
pGP1007	P_L:A P_L:c	5.1×10^{-4}	5.7×10^{-5}	8.9± 3.5
pGP1004	P_L:A B P_L:c	2.4×10^{-2}	3.0×10^{-4}	80.0±42

* P_L=lambda promoter; P_C=Mu repressor promoter; c⁻=inactivated repressor; with pGP1007 and pGP1004 repressor is provided *in trans* from an additional plasmid (pGP829).
† Transposition frequency is determined as the fraction of F factors that confer chloramphenicol resistance. Experiment at least repeated 4 times with mean error of 30%.
‡ Ratio of transposition frequencies in Fis⁻ over Fis⁺ with SD indicated.

,so if the above assumption is correct one would expect that the Fis effect will increase again when besides B also repressor is overproduced. Indeed with pGP1004 (Table 1), where also repressor is expressed from PL, Fis reduces the transposition frequency more strongly (80 fold) than when the repressor is expressed from its own promoter. Evidently the amount of repressor relative to the level of B is essential for the extent of the Fis effect.

Involvement of IAS in Fis effect. Our next step was to try to identify the sites on which Fis and repressor act in the process of inhibition of Mu transposition. The IAS is a good candidate for the action of the Mu repressor. Firstly this site contains the two strong repressor binding sites O1 and O2. Moreover, it has been shown with transposition experiments *in vitro* that repressor can impede the function of the enhancer in transposition as it can compete with the A protein for the transposase binding sites (6).

When the transposition frequencies were measured with a mini-Mu in which the IAS is deleted (pGP1005) and the repressor is supplied *in trans* no effect of Fis is observed any more (Table 2). This proves that sequences in the IAS, presumably the repressor binding sites, are essential for the transposition inhibiting effect of Fis.

We have also mutated the IAS in such a way that the repressor binding sites are left intact while its enhancing activity is lost. This was achieved by inserting 4 bp into the MluI site which is in the IHF binding site which is also part of the IAS. The 4 bp insertion not only leads to the inactivation of the *ihf* site (33) but changes the helical phasing of the repressor binding sites O1 and

Table 2. The involvement of the IAS and the Fis binding site in *att*L.

Plasmid	Characteristics *	Transposition frequency † Fis⁻	Fis⁺	-/+ ‡
pGP619	P_L:A - δIAS	4.0×10^{-5}	6.5×10^{-5}	0.6±0.3
pGP1005	P_L:A P_L:c δIAS	2.6×10^{-5}	2.0×10^{-5}	1.3±0.5
pGP884	P_L:A P_C:c IAS⁻	5.5×10^{-5}	3.0×10^{-6}	18.3±9.5
pGP1046	P_L:A P_C:c IAS *fis*(Nde)	2.7×10^{-4}	2.7×10^{-5}	10.0±3.6

* P_L=lambda promoter; P_C=Mu repressor promoter; IAS⁻=4 base pairs insertion; δIAS=deleted IAS; *fis*(Nde)= mutated *fis* site in *att*L; with pGP1005 repressor in provided *in trans* from an additional plasmid.
† Transposition frequency is determined as the fraction of F factors that confer chloramphenicol resistance. Experiment repeated at least 4 times with mean error of 30%.
‡ Ratio of transposition frequencies in Fis⁻ over Fis⁺ with SD indicated.

O2 thereby impairing the enhancing activity (30). The transposition frequency in the presence of Fis is indeed strongly reduced when the mini-Mu plasmid, carrying this IAS mutation, is used (pGP884, Table 2). However, in contrast with the IAS deletion the Fis effect is not lost and remains the same as in the presence of the IAS. Apart from showing that a functional *ihf* site is not involved in the Fis effect this experiment tells us that the Fis-mediated inhibition of transposition is not merely the result of inactivating the transpositional enhancer.

Fis site near L2 not involved in Fis effect? For several reasons the *fis* site near L2 seemed the most likely site on which Fis acts in the process of Fis-mediated inhibition of transposition.

Firstly, it is the only Fis binding site which can be detected in the first 1250 bp of the Mu genome. The IAS region which is essential for the Fis effect neither contains a sequence resembling a Fis consensus, nor does an IAS containing fragment show any specific retardation during electrophoresis after incubation with purified Fis (results not shown).

Secondly the *fis* site is adjacent to a transposase binding site (L2) which is also a (weak) repressor binding site. Since the Fis-mediated transposition inhibition requires the repressor protein, Fis could possibly exert its function in this inhibiting process by facilitating the binding of repressor to L2 making the repressor a stronger competitor for the A protein.

However, the experiments directed to prove the involvement of the *fis* site turned out to be negative. Firstly, in DNaseI footprint experiments no cooperativety was found between Fis

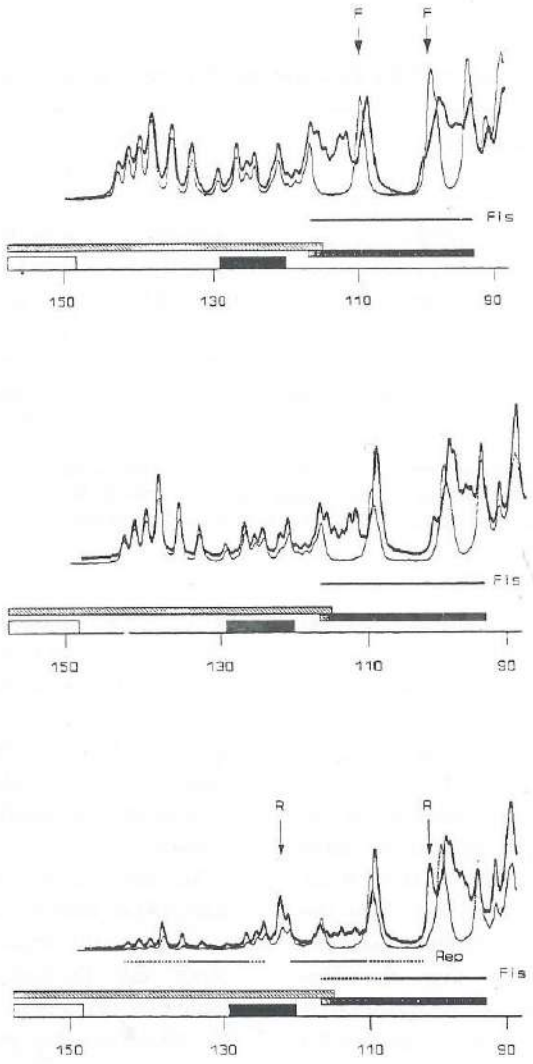

Figure 3. Densitometric scans of repressor and Fis protection patterns. In all figures the left hatched box indicates sequences known to be protected by transposase A (25) whereas the right hatched box is the Fis protected area (13,14). The two parts of the A binding consensus are YGtTTCAYt (black box) and AARYRCGAAAR (white box), here of L2 and L3 respectively. Arrows indicate strong enhanced cleavage sites by Fis (F) or repressor (R). Sequences protected are indicated just below the scans. (Amin=0.04, Amax=1.90)
A: thick line = no proteins added , thin line = +2.5 µg/ml Fis
B: thick line = 10 µg/ml Repressor, thin line = +2.5 µg/ml Fis
C: thick line = 30 µg/ml Repressor, thin line = +2.5 µg/ml Fis

and repressor for binding to L2. The only effect that can be observed is that Fis seems to influence slightly the conformation of the complex between repressor and L2. The enhanced cleavage site caused by repressor bound at L2 at position 101 nearly disappears in the presence of Fis whereas the enhanced cleavage site at position 123 is reduced (Fig. 3).

Secondly and of more importance are the results obtained with a mini-Mu in which the *fis* sequence is substituted by a 'random' DNA sequence (Materials and Methods). Since Fis has a rather degenerated consensus bindingsite, care was taken that the new sequences did not have any homology with the few conserved bases in the consensus. In a bandshift assay binding of *fis* to this sequence was strongly decreased (not shown). However, when the plasmid with the substitution was used in transposition experiments in Fis^+ and Fis^- backgrounds no differences were found in the levels of transposition when compared with those of the isogenic plasmid pGP851 which does contain a functional Fis binding site (pGP1046 in Table 2). It seems therefore that the Fis mediated inhibition of transposition is not exerted through this *fis* site.

DISCUSSION

Under normal conditions the host protein Fis seems hardly to effect Mu transposition (pGP852, Table 1). The only reason for looking at a possible effect of Fis on transposition was the occurrence of a Fis binding site adjacent to a transposase binding site at the left end of the Mu genome. We have indeed been able to detect an effect of Fis on transposition under certain conditions using mini-Mu's as transposable elements. However, the involvement of the *fis* site in *att*L could not be shown.

No stimulation of Mu transposition by Fis was observed although this might be expected but an inhibition and only under the condition that also the Mu repressor gene was expressed. The involvement of Mu repressor suggested that our observations are related to those of Bétermier *et al.* (3) who showed that a Mu*cts* lysogen is more easily induced in a Fis^- background.

How can Fis facilitate the inhibition of transposition by the repressor? The only site which we found to be involved is the Mu enhancer. Most likely the repressor binding sites in the enhancer are targets in the concerted action of Fis and repressor although this still has to be proven by site-directed mutagenesis of the O1 and/or O2 sites. In the experimental system used the repressor has no function in regulating the expression of the genes A and B, which are needed for transposition, as these are under the control of the P_L promoter. One way the repressor can inhibit transposition is competing with A for the operator sites in the enhancer. It has been shown with *in vitro* experiments that repressor protein can indeed lower

the transposition efficiency. One explanation for the affect of Fis is therefore that it favours the binding of repressor to the enhancer and prevents the binding of A to this site. However, this could not be the only explanation. When the enhancer is inactivated by a 4 bp insertion the Fis repressor effect is still seen suggesting that an inactive enhancer in the presence of repressor can further inhibit transposition to a level which is lower that in the absence of the enhancer. It has been postulated but not yet proven that the enhancer and transposase can form an unstable and temporary complex with one or both the *att* sites and that this complex facilitates the formation of the complex between *att*L and *att*R. Maybe an inactive complex is formed with the *att*L sites, enhancer and repressor protein similarly as normally is formed with transposase, enhancer and the *att* sites. Such an inactive complex could impede the interaction between *att*L and *att*R to a level which is even lower than without enhancer. However, it does not seem that Fis bound at the *fis* site in *att*L plays a role in such a presumed interaction of enhancer, the *att* sites and repressor as this *fis* site can be substituted by a random sequence without losing the Fis effect. On the other hand the *fis* consensus is strongly degenerated and we found in bandshift experiments that Fis can bind to random sequences at concentrations that are only slightly higher than necessary for binding to its consensus sequence. It has been shown also that for certain *ihf* sites (H1 in *att*L) IHF can be replaced by HU, which in contrast to IHF does not bind to a specific sequence. These considerations make it difficult to conclude with absolute certainty that Fis in a special complex and context can not bind even if its normal favourite binding site has been changed.

On the other hand it seems also possible that Fis merely influences the level of active repressor protein. It is unlikely, however, that Fis stimulates the repressor synthesis at the level of transcription. Firstly no Fis binding site is present in the promoter region of the *mu*c gene. Moreover the Fis effect is also observed when the repressor is provided *in trans* from a foreign (P_L) promoter. One possibility is therefore that Fis either directly or indirectly affects the stability of the repressor messenger or the Mu repressor protein.

What could be the function of Fis in the regulation of Mu transposition? One possibility is that Fis is a measure for the physiological condition of the cell. It has been shown that Fis is under stringent control (25,27) and nearly absent in the stationary phase of the growth cycle. If Fis affects the stability of repression as we postulated above then the absence of Fis in the stationary phase could lead to a faster break-down of Mu repressor and therefore to an enhanced chance for Mu-transposition or rearrangements, a phenomenon which has been observed in Mu-lac fusions in colonies on agar plates (26). When the Fis effect we have measured in our experimental system is due to competition between A and repressor for the IAS or an IAS-*att* complex then the presence of the B protein apparently favours a productive interaction with the transposase, because the Fis repressor effect diminishes when also B is expressed. More recently it has been shown that the formation of a transposition complex between the ends of Mu (in which the enhancer plays a role) of Mu is stabilized by an interaction of protein A with B (31). In that interaction the DNA binding property of the B protein is not involved. Possibly the complex between A and B makes it more difficult for the repressor to compete for the A binding sites and concomitantly the effect of Fis on Mu transposition is only small.

Literature cited

(1) Adzuma, K., and Mizuuchi, K. (1988) *Cell 53*:257-266.
(2) Bernard, H., Remaut, E., Herschfield, M.V., Das, H.K., Helinski, D.R., Yanofski, C. and Franklin, N. (1979). *Gene 5*:59-76.
(3) Bétermier, M., Lefrère, V., Koch, C., Alazard, R. and Chandler, M. (1989) *Mol. Microbiol. 3*:459-468.
(4) Craigie, R. and Mizuuchi, K. (1985) *Cell 41*:867-876.
(5) Craigie, R. and Mizuuchi, K. (1987) *Cell 51*:493-501.
(6) Craigie, R., Mizuuchi, M. and Mizuuchi, K. (1984) *Cell 39*:387-394.
(7) Filutowisz ,M., Ross, W., Wild, J., and Gourse, R.L. (1992) *J. Bacteriol. 174*:398-407.
(8) Finkel and Johnson (1992) *Mol. Microbiol. 6*:3257-3265.
(9) Gille, H., Egan, J.B., Roth, A. and Messer,W. (1991) *Nucleic Acids Res.* **19:** 4167-4172.
(10) Goosen, N. and van de Putte, P. (1986) in *Phage Mu* (eds. Symonds, N., Toussaint, A., van de Putte, P. and Howe, M.M.) p.41-52, Cold Spring Harbor Laboratory, Cold Spring Harbor (NY) USA.
(11) Groenen, M.A.M. (1987) *The ends of the genome of bacteriophage Mu.* (Ph.D. thesis), Leiden University, The Netherlands.
(12) Groenen, M.A.M. and van de Putte, P. (1986) *J. Mol. Biol. 189*:597-602.
(13) Groenen, M.A.M., Timmers, E. and van de Putte, P. (1985) *Proc. Natl. Acad. Sci. USA 82*:2087-2091.
(14) Groenen, M.A.M., Kokke, M. and van de Putte, P. (1986) *EMBO J. 5*:3687-3690.
(15) Groenen, M.A.M., Vollering, M., Krijgsman, P., van Drunen, C.M. and van de Putte, P. (1987) *Nucl. Acids Res. 21*:8831-8844.
(16) Johnson, R.C., Ball, C.A., Pfeffer, D., and Simon, M.I. (1988) *Proc. Natl. Acad. Sci. USA 85*:3484-3488.
(17) Koch, C., and Kahmann, R. (1986) *J. Biol. Chem. 261*:15673-15678.
(18) Kunkel, T.A. (1985) *Proc. Natl. Acad. Sci. USA 82*:488-492.
(19) Leung, P.C., Teplow, D.B. and Harshey, R.M. (1989) *Nature (London) 338*:656-658.
(20) Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning. A Laboratory Manual.* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
(21) Maxwell, A., Craigie, R. and Mizuuchi, K. (1987) *Proc. Natl. Acad. Sci. USA 84*:699-703.
(22) Mizuuchi, K. and Craigie, R. (1986) *Annu. Rev. Genet. 20*:385-429.
(23) Mizuuchi, M. and Mizuuchi, K. (1989) *Cell 58*:399-408.

(24) Nilsson, L., Vanet, A., Vijgenboom, E. and Bosch, L. (1990) *EMBO J. 9*:727-734.
(25) Nilsson, L., Verbeek, H., Vijgenboom, E., van Drunen, C.M., and Bosch, L. (1991). *J. Bacteriol. 174*:921-929.
(26) Shapiro, J.A. (1984) *J. Gen. Microbiol.* **130:** 1169-1181.
(27) Ross, W., Thompson, J.F., Newland, J.T. and Gourse, R.L. (1990). *EMBO J. 9*:9733-3742.
(28) Surette, M.G. and Chaconas, G. (1989) *J. Biol. Chem. 264*:3028-3034.
(29) Surette, M.G., Buch, S.J. and Chaconas, G. (1987) *Cell 49*:253-262.
(30) Surette, M.G., Lavoie, B.D. and Chaconas, G. (1989) *EMBO J. 8*:3483-3489.
(31) Surette, M.G., Harkness, T. and Chaconas, G. (1991) *J. Biol. Chem. 266*:3118-3124.
(32) Thompson, J.F., Moitoso de Vegas, L., Koch, C., Kahmann, R. and Landy, A. (1987) *Cell 50*:901-908.
(33) Van Rijn, P.A., Goosen, N. and van de Putte, P. (1988) *Nucl. Acids Res. 16*:4595-4605.
(34) Van Rijn, P.A., Goosen, N., Turk, S.C.H.J. and van de Putte, P. (1989) *Nucl. Acids Res. 17*:10203-10212.

Sequences in the attachment sites of bacteriophage Mu that are essential for the stimulation of transposition by the internal activating sequence

C.M. van Drunen, E.J. Mientjes, C. van Zuylen,
N. Goosen and P. van de Putte

In this chapter we determine which of the A binding sites within the attachment sites (L1/L2/L3 in *att*L and R1/R2/R3 in *att*R) are required for the stimulatory activity of the enhancer.

First a series of enhancer containing mini-Mu's with progressive internal deletions of *att*L were tested. In the absence of L3 the IAS is still able to stimulate transposition to wildtype levels while in the absence of both L3 and L2 it is not. Transposition level of a construct without L3 and L2 drops to the level of a construct with full length *att*L without IAS which indicates that the binding site L2 is required for the stimulatory activity of the enhancer. Deleting the IAS in a construct without L3 and L2 does not further reduce the efficiency of transposition showing a restrictive role for L2 in mediating the stimulatory activity of the IAS. Point mutations within the L2 binding sites that convert a Mu L2 site into the L2 site of the related phage D108 also complete abolishes the stimulatory activity of the IAS. This not only shows that L2 alone mediates the stimulatory activity of the IAS but also that in spite of the high degree of sequence homology between the attachment sites of both phages the divergent enhancer sequences impose specific sequences requirements for their respective attachment sites.

In *att*R a similar picture emerges about the role of the particular A binding sites. Deleting R3 in the presence of the IAS has no effect of the efficiency of the transposition reaction while deleting R3 and R2 results in a severe drop which is not observed in the absence of the IAS. However there is one important difference between the results of deleting L2 or R2 In contrast to L2 deleting R2 leads to a drop in the level of transposition that extends far below the level of a construct where only the IAS is deleted. In other words in the absence of R2 the IAS inhibits transposition. We believe that normally in the absence of the enhancer transposition occurs through random collision of L1 and R1 which now is hampered through interaction of *att*L with the IAS. As this phenomenon is not observed when L2 is deleted we speculate that assemble of the IAS with attachment sites proceeds in an ordered fashion with the primary interaction of the IAS with *att*L followed by the interaction with *att*R.

INTRODUCTION

Transposition, site specific recombination and initiation of replication are processes that require a high degree of precision as they affect the DNA integrity. The general mechanism of these processes involves the formation of a higher order nucleo-protein complex with multiple interactions between DNA and proteins. The formation of these complexes might on one hand increase the efficiency of the reaction but on the other hand also allow fine-tuning and tight regulation via the different components of the complex. Transposition of bacteriophage Mu is both efficient and with respect to the Mu attachment sites precise. Also in Mu transposition such a nucleo-protein complex is formed (**19,28**). The factors involved in the formation of this complex will be discussed below. Transposase A binds specifically to each end of the Mu DNA (**11,14**) and through the interaction of A with these attachment sites complex I or cleaved donor complex (CDC) is formed in which the nicked ends of the Mu genome are held together (**28,32**). The formation of this complex is furthermore dependent on the *Escherichia coli* protein HU and on supercoiling of the Mu substrate (**8,26**). The Mu B protein is a general DNA binding protein that stimulates transposition (**1,2,22**) by stabilizing both the interactions between the A molecules within the CDC and the interaction of the CDC with the target DNA. This last interaction leads to the formation of complex II or strand transfer complex (STC) where Mu DNA is covalently joined to the target site (**5,28**).

The IAS is an enhancer for transposition that is located between positions 850 and 997 at the left end of the Mu genome (**21,23,29**). This site is part of the Mu regulatory region where two diverging promoters (Pc and Pe) are involved in the choice between lysogeny and lytic development (**11**). Transposase A interacts with sequences within the IAS through a protein domain that is distinct from that which interacts with the transposase binding sites in the attachment sites (**21,23**). Homology between the domain of the repressor protein that mediates binding to the operator and the domain of A that is responsible for binding to the IAS (**15,21**) suggest that the repressor binding sites O1 and O2 are the targets for A binding in the IAS. The relative orientation of O1 and O2 as well as the correct orientation of the IAS with respect to the attachment sites, are essential for the function of the IAS. The IAS does not function when it is on the mini-Mu plasmid but outside the Mu ends (**23**) and only to a limited extent when it is inverted (**29**). The IAS however is able to stimulate transposition *in trans* of a mini-Mu plasmid that does not contain an IAS itself, but only when it is provided on a small synthetic oligo (**27**). The mechanism by which the IAS stimulates transposition has not been clarified. However as A is potentially able to interact with an attachment

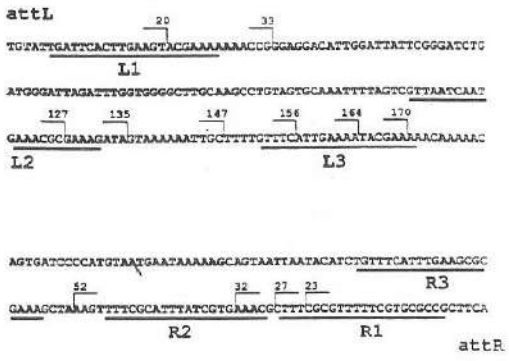

Figure 1. DNA sequence of *att*L and *att*R of Mu. Indicated are the number of bases remaining in the mini-Mu deletion derivatives.

site and IAS simultaneously, the stimulatory activity of the IAS could well be a consequence of the formation of a three-site-complex between both attachment sites and the IAS. Such a complex could facilitate the proper alignment between the attachment sites and thus lead to an enhanced formation of the cleaved donor complex.

Each Mu end contains three transposase A binding sites, L1, L2 and L3 in *att*L and R1, R2 and R3 in *att*R (Fig.1). Investigation of the CDC however revealed that in this complex only three of these sites are bound by A protein: the outermost sites L1 and R1 and the R2 site (**25**). The structural unit of the A protein within the CDC appears to be a tetramer (7) of which one subunit does not seem to be bound to the DNA.

To investigate the role of the different A binding sites in the formation of the CDC we measured the transposition frequencies of mini-Mu's that carry progressive deletions in the attachment sites. The same deletions constructs were tested with and without the IAS. Our results indicate that for transposition in the absence of the IAS only the outermost L1 and R1 sites are required, whereas in the presence of the IAS also L2 and R2 are important. This suggests that the stimulating activity of the IAS is mediated through interaction with the L2 and R2 sites. In our experimental system the L3 and R3 sites do not contribute to the efficiency of transposition.

MATERIALS AND METHODS

Bacterial strains. The strains PP135 (*thi pro*) (**14**) and MC1000 *fis*-767 (**16**) have been described before. The strain PP2542 is M72 (λ*N*am7-*N*am53 *c*I857 ΔH1) (3) with pGP655 (**13**). The *fis*-767 mutation was introduced in PP2542 by P1 transduction resulting in PP2541.

Plasmids. The most important mini-Mu constructs used in this study are schematically represented in Fig 2. The mini-Mu plasmid pGP618 (Fig. 2A) carries the *cat* gene flanked by 850 bp of *att*L and 792 bp of *att*R (**14**). In addition this plasmid has the A and B genes under control of the lambda P_L promoter. The deletion derivatives (Δ1, Δ16, Δ11, Δ26, Δ24, Δ28, Δ14, Δ20 and Δ12 in *att*L and Δ53, Δ64, Δ66 and Δ43 in *att*R) have been described before (**14**). The end-points of the deletions

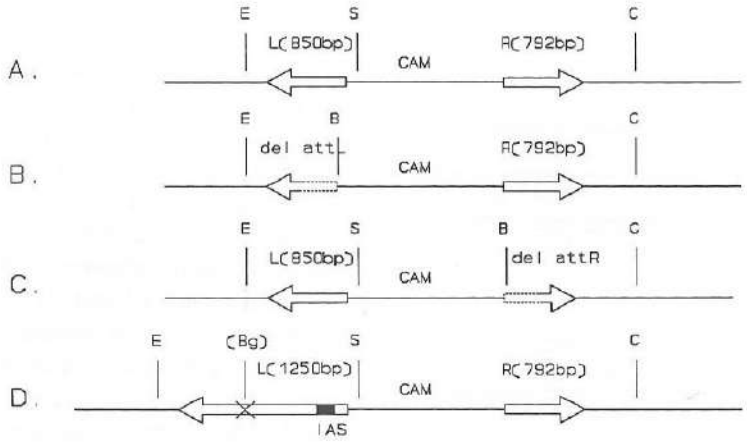

Figure 2. Schematic representation of the mini-Mu constructs used in this study. Only relevent restriction sites are indicated:B=BamHI, Bg=BglII, C=ClaI, E=EcoRI and S=SalI. A.pGP618 containing 850 bp of *att*L and 792 bp of *att*R flanking the chloramphenicol resistance gene (CAM). B. pGP618 derivatives containing deletions in *att*L. C. pGP618 derivatives containing deletions in *att*R. D. pGP866 contains 1250 bp of *att*L and 792 bp of *att*R with IAS sequence indicated. The BglII site was used for the introduction of the different *att*L deletions depicted in B. pGP875 is the same construct with a filled in BglII site leading to the inactivation (indicated by the cross of the Mu repressor gene.

points of the deletions are flanked by a *Bam*HI linker (Fig. 2B and C). For the introduction of the IAS in the mini-Mu of pGP618, the 850 bp of *att*L were replaced by an *Eco*RI-*Sal*I fragment containing 1250 bp of *att*L (including the IAS). Next by site-directed mutagenesis (C842->T) a *Bgl*II site was introduced at 840 bp from the left end resulting in pGP866 (Fig. 2C). Finally to inactivate the repressor gene located on the 1250 bp fragment, the *Bgl*II site was filled in using Klenov DNA polymerase (resulting in pGP875).

For the introduction of the different *att*L deletions in the mini-Mu containing the IAS the *Eco*RI-*Bgl*II fragment of pGP866 (containing the 840 bp of the left end) was replaced by the *Eco*RI-*Bam*HI fragments of the different truncated *att*L sequences (deletion derivatives of pGP618). This resulted in pGP1010, pGP1008, pGP1012, pGP1011, pGP1018, pGP1016 and pGP1017 (Table 2). For the introduction of the *att*R deletions in the mini-Mu containing the IAS, the *Sal*I-*Cla*I fragment of pGP875 was substituted for the *Sal*I-*Cla*I fragments of the deletion derivatives of pGP618, containing truncated *att*R sequences. This resulted in pGP896, pGP887 and pGP888 (Table 4). pGP893 is derived from pGP866 by deletion of the total *att*R region.

Substitution of the non-conserved bases of L2 in the left part of the A binding region of Mu (in pGP875) for the corresponding bases of the phage

Table 1. Transposition of mini-Mu's with deletions in *att*L without IAS.

Plasmid	Base pairs at *att*L	Transposition frequency † A Fis$^+$	B Fis$^-$
pGP618Δ1	170 bp	1.7×10^{-3}	1.8×10^{-3}
pGP618Δ16	164 bp	1.4×10^{-3}	2.3×10^{-3}
pGP618Δ11	156 bp	5.8×10^{-3}	0.9×10^{-3}
pGP618Δ26	147 bp	3.2×10^{-3}	1.0×10^{-3}
pGP618Δ24	135 bp	6.8×10^{-3}	8.2×10^{-3}
pGP618Δ28	127 bp	6.4×10^{-3}	9.7×10^{-3}
pGP618Δ14	33 bp	1.2×10^{-3}	1.7×10^{-3}
pGP618Δ20	20 bp	1.2×10^{-4}	6.0×10^{-5}

† Transposition frequency is determined as the fraction of F factors that confer chloramphenicol resistance.

D108 was through site directed mutagenesis (the bases 105-TTAGTCG TTAATCAAT-120 where replaced by 105-TTAGCCGTTACATCTT-120 corresponding to the D108 sequence).

Transposition assay. The transposition frequencies of the mini-Mu plasmids were determined with a conjugation assay as described by Groenen *et al* **(14)** using PP135 as acceptor strain. The donor strain was grown with gentle shaking at the permissive temperature of 30°C until OD_{680nm}=0.2 after which transposition was induced at 38°C for 60 min. Conjugation was allowed to proceed for 60min by mixing the donor strain (0.5ml) with exponentially growing acceptor strain (5ml of OD_{680nm}=0.2) after which the cells were plated on selective minimal medium plates. The transfer of F*tet* was monitored by selection for tetracyclin resistant transconjugants. The transposition frequency is determined as the fraction of F factors that confer chloramphenicol resistance to the acceptor strain PP135. Each experiment was repeated at least four times.

RESULTS

Analysis of sequences in *att*L involved in transposition. Each of the attachment site of bacteriophage Mu contains three A binding sites, L1-L3 in *att*L and R1-R3 in *att*R. Different deletion mutants have been isolated in which one or several of these sites have been removed (Fig. 1). To investigate the role of the different A binding sites in Mu transposition we tested the effect of the deletions on the transposition frequency of a mini-Mu using the mating-out assay as described in Materials and

Table 2. Transposition of mini-Mu's with deletions in *att*L with the IAS.

Plasmid	Base pairs at *att*L	Transposition frequency †	
		A Fis$^+$	B Fis$^-$
pGP875	170 bp	2.3×10^{-2}	2.6×10^{-2}
pGP1010	164 bp	3.3×10^{-2}	4.0×10^{-2}
pGP1008	156 bp	2.7×10^{-2}	2.6×10^{-2}
pGP1012	147 bp	2.2×10^{-2}	1.2×10^{-2}
pGP1011	135 bp	0.8×10^{-2}	2.2×10^{-2}
pGP1018	127 bp	1.4×10^{-3}	1.8×10^{-3}
pGP1016	33 bp	1.3×10^{-3}	2.7×10^{-3}
pGP1017	20 bp	1.3×10^{-4}	1.5×10^{-4}

† Transposition frequency is determined as the fraction of F factors that confer chloramphenicol resistance.

Methods. First the transposition frequencies of constructs without the IAS were measured (Table 1 column A). Deletion of either L3 alone (147bp) or L3 and L2 (33bp) does not result in a reduction of transposition. Only when part of the L1 site (20bp) is removed transposition drops by a factor of 10 compared to that of mini-Mu's with a full length *att*L. So in this mini-mu transposition assay in *att*L only L1 contributes to the efficiency of the transposition reaction and neither L2 nor L3 seems to be important for transposition in constructs where the IAS is not present. These results differ from results obtained earlier by Groenen *et al* (12,14) who showed that deletion of the DNA region containing L3 and L2 in mini-Mu's without the IAS did result in a reduction of the transposition frequency by about a factor of 10. This discrepancy is due to the difference in transposition target used in both studies. As will be described in chapter 4 the F*pro-lac* target used in the experiments of Groenen *et al* seems to carry a DNA sequence that stimulates Mu transposition towards this target in a way that is dependent on sequences located in the region containing L2 and L3. From the results with the target used in this study (pGP655) however it is now clear that at least in the absence of the IAS, L2 and L3 do not contribute to the efficiency of the transposition reaction.

The effect of the same deletions in *att*L were measured in similar mini-Mu constructs but now containing the IAS (Table 2). Transposition frequencies of these constructs are shown in column 2A. The transposition frequency of the construct with all three A binding sites (170 bp) is about tenfold higher than

Table 3. Transposition of mini-Mu's with deletions in *att*R without IAS.

Plasmid	Base pairs at *att*R	Transposition frequency †	
		A Fis$^+$	B Fis$^-$
pGP618	792 bp	1.7×10^{-3}	1.8×10^{-3}
pGP618Δ53	52 bp	2.4×10^{-3}	n.d.
pGP618Δ64	32 bp	0.6×10^{-3}	0.5×10^{-3}
pGP618Δ66	27 bp	0.7×10^{-3}	1.2×10^{-3}
pGP618Δ43	23 bp	3.9×10^{-5}	9.0×10^{-5}
pGP640	-	1.1×10^{-5}	4.5×10^{-5}

† Transposition frequency is determined as the fraction of F factors that confer chloramphenicol resistance.

of the corresponding mini-Mu without the IAS, showing that in our assay the IAS stimulates transposition by a factor of ten. Again deletion of L3 (147 bp) does not result in reduction of the transposition frequency. Apparently also in the presence of the IAS this site seems to have no important role in transposition. After deletion of part (leaving 127bp of *att*L) or all of L2 (leaving 33 bp of *att*L) however transposition drops approximally tenfold to a level comparable to that of mini-Mu's without the IAS. This strongly suggests that the L2 site is essential for the enhancing activity of the IAS. Deletion of part of L1 (20 bp) does finally lead to a further decrease in transposition as was observed in constructs without the internal activating sequence.

Besides the three A binding sites also a Fis binding site is present in *att*L (4,31). Since this binding site is located adjacent to L2, some of the deletions not only remove the L2 and L3 binding sites but also this Fis binding site. To exclude that the reduction of transposition after deletion of L2 is due to the absence of Fis binding rather than to the deletion of L2, we measured the transposition frequencies of the same mini-Mu constructs in a Fis$^-$ background (Tables 1B and 2B). These results show that both in the absence and in the presence of the IAS the frequencies are similar to those in a Fis$^+$ background. Therefore Fis binding to *att*L seems not important for the level of transposition or the stimulating activity of the IAS.

Analysis of sequences in *att*R involved in transposition. In a similar way the function of the three A binding sites at the right end of the Mu genome was studied by measuring transposition levels of mini-Mu's with successive

Table 4. Transposition of mini-Mu's with deletions in *att*R with the IAS.

Plasmid	Base pairs at *att*R	Transposition frequency †	
		A Fis$^+$	B Fis$^-$
pGP875	792 bp	1.6×10^{-2}	2.6×10^{-2}
pGP896	52 bp	4.9×10^{-2}	n.d.
pGP887	27 bp	0.1×10^{-5}	1.1×10^{-5}
pGP888	23 bp	0.8×10^{-5}	0.5×10^{-5}
pGP893	-	0.6×10^{-5}	0.6×10^{-5}

† Transposition frequency is determined as the fraction of F factors that confer chloramphenicol resistance.

levels of mini-Mu's with successive deletions in *att*R. Table 3 lists the transposition frequencies of these constructs lacking the IAS. Deletion of R3 alone (leaving 52 bp of *att*R) or R2 and R3 (leaving 27 bp of *att*R) has hardly any effect on the transposition levels in our test system and these results are not influenced by the presence or absence of Fis (Table 3A and 3B). Deletion of part of R1 however leads to a tenfold reduction of transposition that is the same for both *fis* backgrounds. Apparently R2 and R3 like L2 and L3 also are dispensable for transposition in our test system when transposition takes place in the absence of the IAS.

In the presence of the IAS quite different results were obtained (Table 4). Deletion of R3 alone (52bp) again has no effect on the transposition frequency. An additional deletion of part or all of R2 however leads to a reduction of transposition by a factor of 1000. This reduction is much more drastic than the reduction of transposition observed in the corresponding *att*L experiment where L3 and L2 were deleted (Table 2, factor of 10). Moreover the transposition level of constructs with only R1 in the presence of the IAS is hunderd times lower than transposition of mini-Mu constructs without the IAS. These results suggest that when R3 and R2 are deleted the IAS is inhibiting transposition. This effect is also observed in the absence of Fis. Summarizing it appears that R3 is not important for transposition, neither in the absence nor in the presence of the IAS and further that upon deletion of R2 the IAS seems to change from an activator to an inhibitor.

A role of the non-conserved bases in L2 in the activity of the IAS. The binding sequence of L2 is markedly different from the other A binding sites in Mu as this site only has the right part (AARYRCGAAAR) of the A consensus

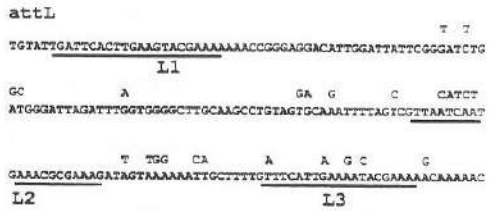

Figure 3. Sequence comparison between the *att*L sites of the phages Mu and D108. The sequence of Mu is shown with bases that differ in D108 indicated above.

(9,12). Footprint assays have shown that L2 has relative low affinity for A, probably as a consequence of this deviation from the consensus sequence. Mu and the related phage D108 have highly homologous attachment sites and also in D108 the L2 region contains only the right parts of the A binding consensus. Interestingly the sequence of the non-conserved part of L2 is quite different in spite of the high degree of similarity between the attachment sites of both phages (Fig.2). Furthermore almost the complete transposase proteins of D108 and Mu, including the *att* binding domain, are highly homologous (24,30). Therefore D108 transposase binds as efficiently to the Mu ends as the Mu A protein (7). Only the N-terminal part of both transposases which comprises the domain that interacts with the enhancers of the respective phages is different. This suggests that the enhancers (which are also non-homologous in both phages) are phage-specific, which could explain why the respective transposases cannot complement each other in mini-Mu and mini-D108 transposition (30). Since L2 appears to be important for the activity of the IAS, we wondered whether also the differences in the sequences of L2 could be related to the difference in IAS of both phages.

Using site specific mutagenesis we substituted the Mu L2 region for that of D108 (Materials and Methods). Since it has been shown that the Mu A protein binds as efficiently to the L2 site of Mu as to the comparable site in D108 (7), A binding is expected to be unaltered in our mutant. The transposition frequency (0.9×10^{-3}) of the mini-Mu with the D108 L2 region in the presence of the IAS however is 30 fold lower as compared to the frequency of the same construct with the L2 region of Mu (1.6×10^{-2}). This level is nearly the same as that of a mini-Mu without a functional enhancer (1.7×10^{-3}). Therefore it seems possible that in the construct with the D108 L2 site the IAS is no longer active and that the whole of L2, including the non-conserved part, is important for the stimulatory activity of this enhancer.

DISCUSSION

For assembly of the Mu transposition complex the Mu A protein is believed to initially bind to all six binding sites in the two attachment sites. Next the two Mu ends are brought together in a

complex where these ends are subsequently cut to form the CDC. In this complex only three of these sites (L1, R1 and R2) are occupied by A (20). Formation of the CDC requires in addition to A also HU, the IAS and a supercoiled substrate. The role of the IAS is thought to be similar to that of the enhancer element in the site-specific recombination of inversion systems. There the enhancer senses the relative orientation of the two recombination sites and through the action of two proteins (Fis and the DNA-invertase) a synaptic complex is formed between the two recombination sites and the enhancer (17). Although such a three-site complex has never been observed in Mu transposition it is conceivable that through the action of A (and HU) the IAS interacts with both attachment sites, thereby correctly aligning these sites.

Our results suggest that such an interaction of the attachment sites with the IAS is mediated through A molecules bound at L2 and R2. When these sites are removed the transposition frequency drops, but only with mini-Mu's that contain an IAS. Deletion of L2 has the same effect on the transposition frequency of a mini-Mu as deletion of the IAS. This strongly suggests that the only role of L2 is to mediate an interaction of *att*L with the IAS and that therefore the L2 site like the IAS only is involved in formation of the CDC and not in subsequent steps. Indeed in an isolated CDC complex the L2 site is not occupied by A protein, yet this complex is still functional in the strand transfer reaction (20). The L2 site was shown to be a relative weak A binding site (7). In DnaseI footprint experiments with the A protein the L2 site lacks a site of enhanced DnaseI cleavage which is present in all other A binding sites (32). This enhanced cleavage occurs in the right part of the A binding region that is conserved in all six A binding sites so including L2 suggesting that A binding to L2 leads to a somewhat different structure than A binding to the other sites. In this chapter we have shown that this non-conserved part of the L2 site could be important for in the putative interaction of L2 with the enhancer. Substitution of the non-conserved sequence in L2 for the same region from the related phage D108 seems to abolish the function of the IAS, as the resulting mini-Mu's show the same transposition frequency as mini-Mu's without the IAS. Since it has been shown that the Mu-A protein binds equally well to the L2 site of Mu as to the corresponding L2 site of D108 this defect is probably to be a qualitative rather than a quantitative effect on A binding to L2. The non-conserved part of the L2 could be essential for a correct positioning of an A molecule to L2 so that it can interact with the IAS. Since in D108 both the enhancer sequence and the IAS binding domain are different from those of Mu it is conceivable that for D108 another type of specific positioning is required. That would explain why the L2 sites of Mu and D108 are not interexchangeable in transposition with the Mu IAS and the Mu A protein.

Deletion of R2 results in a reduced transposition frequency, but again only in mini-Mu's that contain an IAS suggesting an interaction between those sequences too. In the presence of the IAS deletion of R2 however has a more drastic effect on transposition than deletion of L2. Here the transposition frequency drops to a level that even is 100 times lower than that of mini-Mu's without the IAS. So it seems that upon deletion of R2 the IAS is inhibiting transposition. How can we envisage this postulated IAS mediated inhibition? If the role of the IAS is to bring the Mu ends together in an ordered fashion, for transposition without the IAS the attachment sites will have to join in a non-ordered fashion by random collision. When the IAS is present, but can only interact with *att*L (due to the deletion of R2 in *att*R) the interaction between the IAS and *att*L might form a sterical hindrance for such a collision process. When *att*R is intact and interaction with the left end is prevented by deletion of L2 the inhibition by the IAS is not observed suggesting that the formation of the putative three-site complex is ordered, i.e. first interaction of the IAS with *att*L followed by interaction with *att*R.

The inhibitory action of the IAS that we observe under conditions where the putative interaction between *att*R and the IAS is prevented due to deletion of R2 could be related to previous observation made by R. Craigie & K. Mizuuchi (8). These workers showed that Mu DNA with precut ends (constructed by means of artificially created restriction sites) could be efficiently coupled to the target. This reaction however did only proceed with two right attachment sites. When a mini-Mu with both right and left (including the IAS) attachment sites was used only products resulting from the joining of two right ends between two distinct plasmid molecules were observed. This phenomenon could be a consequence of a similar inhibitory complex between the IAS and *att*L as proposed above. Apparently under these experimental conditions *att*L is blocked from participation in the transposition reaction.

In our studies the L3 and R3 binding sites of Mu don't seem to have a direct role in the transposition process. The only function might be to prevent Mu to insert into its own genome since target DNA that contains L3 or R3 are relative immune to Mu transposition (10). Finally our results show that for transposition of mini-Mu's that lack the IAS only the L1 and R1 sites are required. This may seem somewhat surprising, since isolated CDC complexes have been shown to contain a tetramer of A protein which is not only bound to L1 and R1 but also to R2. These CDC complexes however have been formed in the presence of the IAS and since the A protein is a monomer in solution (32) it is expected that the tetramerization is a consequence of interaction between *att*L and *att*R and possibly also the IAS. It has been suggested (20) that at least one of the A molecules in the

tetramer (the one that does not seem to interact with one of the attachment sites) was originally enhancer bound and that it was transferred to the tetramer during formation of a three-site complex between the IAS and the Mu ends. It is therefore questionable whether the structural unit of A protein in the CDC that is formed in the absence of the IAS is also a tetramer. Possibly without the IAS only a dimeric interaction between A molecules bound at L1 and R1 can take place which might also result in formation of an active CDC albeit with a lower efficiency.

Literature cited

(1) Adzuma, K., and Mizuuchi, K. (1988) *Cell 53*:257-266.
(2) Adzuma, K., and Mizuuchi, K. (1989) *Cell 57*:41-47.
(3) Bernard, H., Remaut, E., Herschfield, M.V., Das, H.K., Helinski, D.R., Yanofski, C., and Franklin, N. (1979) *Gene* 5:59-76.
(4) Betermier, M., Lefevre, V., Koch, C., Alazard, R. and Chandler, M. (1989) *Mol. Microbiol. 3*:459-468.
(5) Craigie, R., and Mizuuchi, K. (1985) *Cell 41*:867-876.
(6) Craigie, R., and Mizuuchi, K. (1987) *Cell 51*:493-501.
(7) Craigie, R., Mizuuchi, M. and Mizuuchi, K. (1984) *Cell 39*:387-394.
(8) Craigie, R., Arndt-Jovin, D.J., and Mizuuchi, K. (1985) *Proc. Natl. Acad. Sci. (USA) 82*:7570-7574.
(9) Craigie, R., Mizuuchi, M., and Mizuuchi, K. (1984) *Cell 39*:387-394.
(10) Darzins, A., Kent, N., Buckwalter, M. and Casadaban, M. (1988) *Proc. Natl. Acad. Sci. (USA) 85*:6826-6830.
(11) Goosen, N., and van de Putte, P. (1987) *Phage Mu* (ed. N. Symonds et al), p.41-52. Cold Spring Harbor Laboratory, Cold Spring Harbor.
(12) Groenen, M.A.M., and van de Putte, P. (1986) *J. Mol. Biol. 189*:597-602.
(13) Groenen, M.A.M., Kokke, M., and van de Putte, P. (1986) *EMBO J. 5*:3687-3690.
(14) Groenen, M.A.M., Timmers, E., and van de Putte, P. (1985) *Proc. Natl. Acad. Sci. (USA) 82*:2087-2091.
(15) Harshey, R.M., Getzoff, E.D., Baldwin, D.L., Miller, J.L., and Chaconas, G. (1985) *Proc. Natl. Acad. Sci. (USA) 82*:159-174.
(16) Johnson, R.C., Ball, C.A., Pfeffer, D., and Simon, M.I. (1988) *Proc. Natl. Acad. Sci. (USA) 85*:3484-3488.
(17) Kanaar, R., Klippel, A., Shekhtman, E., Dungan, J.M., Kahmann, R., and Cozzarelli, N.R. (1990). *Cell 62*:353-366.
(18) Kanaar, R., van de Putte, P., and Cozzarelli, N.R. (1989) *Cell 58*:147-159.
(19) Lavoie, B.D., and Chaconas, G. (1990) *J. Biol. Chem. 265*:1623-1627.
(20) Lavoie, B.D., Chan, B.S., Allison, R.G. and Chaconas, G. (1991) *EMBO J. 10*:3051-3059.
(21) Leung, P.C., Teplow, D.B., Harshey, R.M. (1989) *Nature (London) 338*:656-658.
(22) Maxwell, A., Craigie, R., and Mizuuchi, K. (1987) *Proc. Natl. Acad. Sci. (USA) 84*:699-703.
(23) Mizuuchi, M., and Mizuuchi, K. (1989) *Cell 58*:399-408.

(24) Mizuuchi, M., Weisberg, R.A., and Mizuuchi, K. (1986) *Nucleic Acids Res. 14*:3813-3825.
(25) Mizuuchi, M., Baker, T.A. and Mizuuchi, K. (1991) *Proc. Natl. Acad. Sci. (USA) 88*:9031-9035.
(26) Surette, M.G., and Chaconas, G. (1989) *J. Biol. Chem. 264*:3028-3034.
(27) Surette, M.G., and Chaconas, G. (1992) *Cell 68*:1101-1108.
(28) Surette, M.G., Buch, S.J., and Chaconas, G. (1987) *Cell 49*:253-262.
(29) Surette, M.G., Lavoie, B.D., and Chaconas G. (1989) *EMBO J. 8*:3483-3489.
(30) Toussaint, A., Faelen, M., Desmet, L. and Allet, B. (1983) *Mol. Gen. Genet. 190*:70-79.
(31) Van Drunen, C.M., Van Zuylen, C., Mientjes, E.J., Goosen, N., and Van de Putte, P. (1993) *Mol. Microbiol. 10*:293-298.
(32) Zou, A., Leung, P.C., and Harshey, R., (1991) *J. Biol. Chem. 266*:20476-20482

Enhanced mini-Mu transposition towards F*pro-lac* depends on sequences within the attachment sites

C.M. van Drunen, N. Goosen and P. van de Putte

In this chapter we show that the efficiency of mini-Mu transposition is enhanced when Fpro-lac is used as target as compared to Ftet. The insertion sequences (IS3 and γδ) which are present on Fpro-lac and which are absent on Ftet might be responsible for this enhancement. We also show that the elevated transposition is dependent on the Mu A binding sites L2, L3 and R2 in the attachment sites of Mu. These sequences could possibly mediate an interaction between the mini-Mu plasmid and sequences present on Fpro-lac.

INTRODUCTION

The development of an *in vivo* transposition system with small derivatives of bacteriophage Mu (mini-Mu's) allows an easy identification of Mu sequences that are involved in transposition. The mini-Mu's are used in a mating-out assay that is based on conjugation of a selectable F-factor that is the target molecule for mini-Mu transposition (5,8,9). The frequency of transposition is the fraction of F-factors into which a mini-Mu is transposed. Although the selection of the integration site for Mu transposition is considered to be at random, some target DNA molecules are less efficiently used than others (6). On the other hand also hot-spots for bacteriophage Mu integration have been reported (4).

A plasmid already containing a Mu insertion is no longer a good target for subsequent Mu insertions. This so called transposition immunity has two different aspects: one at the DNA and one at the protein level. Immunity is conferred to the target molecule by sequences within either of the Mu attachment sites. These sequences are overlapping with the innermost A binding site L3 in *att*L and the R2 and R3 sites in *att*R (Fig.1) (6). Besides these particular DNA sequences also the Mu B protein is involved in transposition immunity (1,2). MuB is a general DNA binding protein that binds to the target DNA. The interaction between A bound to the attachment sites and B bound to the target is considered to facilitate the

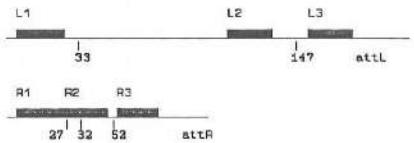

Figure 1. The Mu attachment sites. Indicated are the number of remaining basepairs of each of the attachment sites in the mini-Mu deletion mutants.

transposition reaction (11). However the presence of the immunity sequences and MuA on the target DNA triggers the intrinsic ATPase activity of B which results in dissociation of this protein from the DNA (2). As a consequence this DNA molecule is no longer an efficient target for transposition. Interestingly also the hot-spots of Mu transposition that have been found resemble MuA binding sites (4,6). The nature of the functional difference between a hot-spot and an immunity sequence is yet unresolved.

In the previous chapter we studied the involvement of the Mu A binding sites in the stimulatory activity of the enhancer. The effect of progressive deletions in either of the attachment sites on the efficiency of mini-Mu transposition towards F*tet* was determined in order to investigate the role of the particular A binding sites. In the absence of the enhancer we showed that only L1 and R1 are essential for transposition and that L2, L3, R2 and R3 can be removed without influencing the absolute level of transposition. These results are in disagreement with previous findings of Groenen *et al* who showed that the same constructs when using F*pro-lac* as a target revealed a 10-fold drop in transposition efficiency upon deletion of L3 and R2 (8). Furthermore a point mutation in L2 that prevents A binding results in a similar drop in transposition efficiency (9) whereas in our test system with F*tet* this site can completely be deleted without affecting the transposition level.

In this chapter we investigate this discrepancy between the results of M.A.M. Groenen and our results from chapter 3. We determine transposition levels of several mini-Mu constructs using these different F-factors as targets, the larger F*pro-lac* (90 kb) containing some IS elements and the smaller POX38 derivative F*tet* (58 kb) lacking all known IS elements (9,10).

MATERIALS AND METHODS

Bacterial strains. The strains PP135 [Δ*lac-pro*X111, *thi*209, supE, (λ)] (8) and M72 [*lac, bio, trp* (λ*N*am7-*N*am53 *c*I857 ΔH1)] (3) have been described before. PP2542 is M72 with F*tet* and PP2747 is M72 with F*pro-lac*.

Plasmids. The plasmids pGP634 (containing *att*L, *att*R and the Mu *A* and *B* genes) (8), F*tet* (pGP655, a POX38 derivative) (9), pGP614 (like pGP634 but lacking the Mu *A* and *B* genes) (8) and the deletion mutants of the attachment sites (Δ53, Δ64, Δ66, Δ26, Δ14 and pGP618Δ12) (Fig.1) (8) have been described before.

Transposition assay. The transposition frequencies of the mini-Mu plasmids were determined with a conjugation assay as described by M.A.M Groenen, with PP135 as acceptor strain (8,9). The transfer of F*pro-lac* was monitored by selection for Pro^+ and that of F*tet* by selection for tetracycline resistant transconjugants. Since the mini-Mu is carrying a chloramphenicol acetyl transferase gene between its ends, the transposition frequency is the fraction of F-factors that confer chloramphenicol resistance to the acceptor strain.

RESULTS AND DISCUSSION

In order to compare F*pro-lac* and F*tet* as target molecules in the transposition assay we first determined the background level of cointegrate formation between the mini-Mu plasmids and these F-factors. The background level with F*pro-lac* both under uninduced conditions (pGP634) or in the absence of the *A* and *B* genes (pGP614) is about 10^{-4} (Table 1). This level is very high when compared to the background level of approximately 10^{-8} obtained with F*tet* as target. An important difference between both F-factors is that F*pro-lac* contains several IS elements (IS2, IS3a+b and γδ (7) whereas F*tet* completely lacks any known IS elements. Although in this case we have not

Table 1. Background transposition levels with F*pro-lac* and F*tet*.

	Mu genes	Induction*	F*pro-lac*	F*tet*
pGP634	A B	–	1.0×10^{-4}	3.3×10^{-8}
	A B	+	3.6×10^{-2}	1.8×10^{-3}
pGP614	-	+	2.0×10^{-4}	nd†

* +, incubation at 38°C; -,incubation at 30°C.
† nd, not determined

analyzed the transposition products with F*pro-lac* we assume that they arise from IS-mediated transposition towards the mini-Mu plasmid which would also result in cointegrate formation.

The transposition efficiencies towards both F-factors strongly increase when the *A* and *B* genes are induced (Table 1). Also in this experiment there is a difference in the level of transposition which is dependent whether F*pro-lac* (3.6×10^{-2}) or F*tet* (1.8×10^{-3}) is used as target. However, here the difference can not be due to IS transposition, as one would expect only a small contribution (10^{-4}) to the total number of transposition events, unless also IS transposition is stimulated as a consequence of Mu A and B expression. Two observations however argue against such A and B stimulated IS transposition. Firstly, when A and B are provided but Mu transposition is prevented by deleting one of the attachment sites the transposition level is 3.0×10^{-4} and not 3.6×10^{-2}. Secondly, restriction analysis of transposition products did not reveal any IS element insertion into the mini-Mu plasmid and showed them all to be a consequence of mini-Mu transposition towards F*pro-lac* (results not shown). The difference between both F-factors could rather point to hot-spot(s) for Mu transposition located on F*pro-lac*. Such hot-spots could for instance be IS3 and γδ located on F*pro-lac* since the inverted repeat sequences of these IS elements share a high degree of similarity with the Mu A binding site consensus. The less than 2-fold difference in size between F*pro-lac* (90 kb) and F*tet* (58 kb) or their for transposition available silent regions is unlikely to cause the difference in the transposition of a factor of 20. Further experiments using deletion mutants of the mini-Mu plasmid also argue against this last explanation.

Table 2 shows the transposition frequency of mini-Mu's with progressive deletions (deletion endpoints are indicated in Fig. 1) in either of the attachment sites using the two different F-factors as targets. In this experiment we observed that the difference between the F-factors is dependent on sequences that are present in the attachment sites of Mu. The transposition frequency of

Table 2. The difference in transposition efficiency towards F*pro-lac* and F*tet* is dependent on sequences within both *att* sites.

Plasmid	Deleted A binding sites*		F*pro-lac*	F*tet*
pGP634	None		3.6×10^{-2}	1.8×10^{-3}
Deletions in *att*R				
Δ53	R3	(52 bp)	4.1×10^{-2}	2.4×10^{-3}
Δ64	R2/R3	(32 bp)	1.5×10^{-3}	0.6×10^{-3}
Δ66	R2/R3	(27 bp)	0.3×10^{-3}	0.7×10^{-3}
Deletions in *att*L				
Δ26	L3	(147 bp)	5.0×10^{-3}	3.2×10^{-3}
Δ14	L2/L3	(33 bp)	3.0×10^{-3}	1.2×10^{-3}
Δ12	L1/L2/L3	(0 bp)	3.0×10^{-4}	nd†

* numbers between brackets indicate length of attachment site (wt: 850 bp *att*L and 792 bp *att*R)
† nd, not determined

the mini-Mu's with wild type attachment sites or with deleted R3 is 20 fold higher with F*pro-lac* than with F*tet* as target. When however L3 in *att*L or R2 and R3 in *att*R have been deleted the transposition frequencies with both F-factors are the same (Table 2). So it seems that the F*pro-lac* plasmid contains one or more hot-spots for Mu transposition and that these are dependent on the L3 and R2 binding sites on the mini-Mu.

Sequences that resemble a transposase binding site can stimulate transposition towards a target molecule (4). The hot-spots on the F*pro-lac* plasmid might therefore well be the inverted repeat sequences of IS3 or γδ which resemble the Mu A binding consensus. In an attempt to identify these hot-spots we did a restriction enzyme analysis of the mini-Mu transposition products with the F*pro-lac* target (results not shown). This analysis however did not reveal any site-specific integration of the mini-Mu. In the case of the Mu related transposon Tn3 similar results were obtained (12). This transposon was shown to insert with a higher frequency into a target that carries sequences that resemble a Tn3 transposase binding site. But also in this case no insertion at a specific site could be detected. Apparently the presence of a hot-spot causes the whole of the DNA molecule to become a more efficient target. Interestingly the hot-spot(s) on the F*pro-lac* in the case of Mu transposition seem only effective when the R2 and L3 binding sites are present.

Groenen *et al* showed that a point-mutation that renders L2 unable to bind Mu A reduces transposition towards the F*pro-lac* plasmid 10-fold whereas in our experiments deletion of L2 (and L3) when using F*tet* as target does not reveal such a reduction of the transposition efficiency. This would indicate that in the observed discrepancy not only L3 and R2 but also L2 is involved. These regions coincide with the regions in the Mu attachment sites that are required for the stimulatory activity of the enhancer as the results in the previous chapter have demonstrated.

The mini-Mu plasmids described in this report do not contain the transpositional enhancer, since they only have 850 bp of the left end. Still, the reduced transposition in the absence of active L2, L3 or R2 sites that we observe when F*pro-lac* is used as a target might suggest that with this particular F-factor the mini-Mu's behave like they contain an enhancer. Possibly an enhancer-like sequence is provided by F*pro-lac*. The inverted repeats of IS3 or γδ on this F-factor could be candidates for such a sequence since they resemble the A bindingsite consensus. The enhancer has *in vitro* been shown to be able to act *in trans* (13). However it can only do so when present at high molar excess relative to the mini-Mu and most efficiently when located on a small linear DNA molecule. These requirements might be different *in vivo* however. On the other hand the interaction between the mini-Mu and a putative enhancer-like sequence on F*pro-lac* might not need to resemble the interaction of the Mu attachment sites with the Mu enhancer. Any interaction between Mu and F*pro-lac* could enhance transposition merely by bringing the target in close proximity of the Mu ends.

Literature cited

(1) Adzuma, K., and Mizuuchi, K. (1988) *Cell 53*:257-266.
(2) Adzuma, K., and Mizuuchi, K. (1989) *Cell 57*:41-47.
(3) Bernard, H., Remaut, E., Herschfield, M.V., Das, H.K., Helinski, D.R., Yanofski, C., and Franklin, N. (1979) *Gene 5*:59-76.
(4) Castilho, B.A., and Casadaban, M.J. (1991) *J. Bacteriol. 173*:1339-1343.
(5) Chaconas, G., Harshey, R.M., Sarvetnick, N., and Bukhari, A. (1981) *J. Mol. Biol. 150*:341-359.
(6) Darzins, A., Kent, N., Buckwater, M., and Casadaban, M. (1988) *Proc. Natl. Acad. Sci. (USA) 85*:6826-6830.
(7) Davidson, N., Deonier, R.C., Hu, S., and Ohtsubo, E. (1975) In *Microbiology 1974* (ed. D. Schlessinger), p.56-65. American Society for Microbiology, Washington D.C.
(8) Groenen, M.A.M., Timmers, E., and Van de Putte, P. (1985) *Proc. Natl. Acad. Sci (USA) 82*:2087-2091.
(9) Groenen, M.A.M., Kokke, M., and Van de Putte, P. (1986) *EMBO J. 5*:3687-3690.
(10) Guyer, M.S., Reed, R.R., Steitz, J.A., and Low, K.B. (1979) *Cold Spring Harbor Symp. Quant. Biol. 43*:135-140.
(11) Maxwell, A., Craigie, R., and Mizuuchi, K. (1987) *Proc. Natl. Acad. Sci. (USA) 84*:699-703.
(12) Nissley, D.V., Lindh, F., Fennewald, M.A. (1991) *J. Mol. Biol. 218*:335-347.
(13) Surette, M.G., and Chaconas, G. (1992) *Cell 68*:1101-1108.

Factor of Inversion Stimulation from *Escherichia coli* and related bacteria

Here we describe a rapid purification scheme for the *E.coli* protein Fis. This 2-step procedure results in a protein fraction that on a silver stained SDS-PAAGE gel is free from any contaminating proteins. Against the purified Fis fraction antibodies where raised. The antibodies and the cloned *fis* gene where used to investigate the distribution of *fis*-like genes and proteins in different organisms. The presence of Fis is probably restricted to the enteric group of facultative anaerobic chemoheterotroph bacteria suggesting that *fis* is a relatively recent gene.

INTRODUCTION

Several DNA binding proteins have been isolated from various prokaryotic organisms that might be considered the equivalents of eukaryotic histone proteins **(10)**. The search for these proteins was prompted because the DNA of bacteria, like the DNA of higher organisms, has to be contained in the relative small volume of the cell. This requires the bacterial DNA to be condensed about a 1000-fold which could be mediated by wrapping of the DNA around "histone-like" proteins. The general properties of histone proteins (small, basic and abundant) aided the search for and the purification of these proteins.

In *Escherichia coli* several of these histone-like proteins (H-NS, HU and H) were identified and cloned. Of these proteins HU is most similar to the histone protein since it can wrap DNA and alter the helical pitch of DNA *in vitro* **(4)**. Functionally however the action of HU is more specific as it is involved in a variety of processes (inversion **(22)**, transposition **(6,37)**, replication **(8)** and transcription **(12)**) where it aids in the formation of a higher order nucleoprotein complex by its ability to alter the global conformation of the DNA. Like HU also H-NS plays a role in transcription as a negative regulator of several different operons. For all these operons regulatory mutants have been isolated (*drdX* **(13)**, *bglY* **(7,29)**, *pilG* **(22,36)** and *osmZ* **(20)**) which all mapped in the same gene, namely the gene encoding H-NS **(13)**. The mechanism by which H-NS influences transcription is unknown but it could act like eukaryotic histones which through binding to a promoter region also can inhibit transcription. Unlike these histones however H-NS does not wrap DNA *in vitro*, although condensation of DNA has been observed **(35)**. The third histone-like protein, H is not well characterized. The only effect observed with this protein is inhibition of *in vitro* replication through a yet unknown mechanism **(12)**.

The histone-like proteins from *E.coli* have homologs in bacterial species. Genes with homology to H-NS are observed in all gram-negative bacteria as was evident from Southern analysis. The H-NS type genes from *Proteus vulgaris* **(28)**, *Serratia marcensus* **(28)** and *Salmonella thyphimurium* **(21)** have been cloned and have about an 80% homology with the *E.coli* gene **(28)**. HU like genes have also been found in a variety of organisms. These include *Salmonella typhimurium* (HU) **(19)**, *Pseudomonas aeruginosa* (HPa) **(18)**, *Bacillus subtilus* (HBa) **(38)**, *Rhizobium meliloti* (HRm) **(27)**, *Anabena* (HU) **(17)**, *Synechocystus* (HS) **(1)** and *Thermoplasma* (HTa) **(34)**. Although all these organisms have an HU like gene, not all organisms have two different HU genes like *E.coli* **(31)**. It is striking that HU is so widespread. It has been found in close relatives of *E.coli* like *Salmonella* (Enterobacter) and in more distant organisms like *Anabena* (Cyanobacteria) and Thermoplasma (Archeabacteria). This is indicative of a basic function for HU or HU like proteins although *E.coli* strains that lack HU are viable **(9)**. The distribution of H-like proteins is not well studied. Of interest however is the immunological crossreactivity of H with antibodies directed against the eukaryotic H2A histone **(reviewed in 10)**.

In addition to the proteins mentioned above, two other small and basic proteins, IHF (integration host factor) and Fis (factor of inversion stimulation) have been identified in *E.coli*. The primary sequence of IHF, which like HU is a heterodimer, is very similar to HU and its protein structure is believed to be the same **(37)**. In contrast to HU however both IHF and Fis bind to specific DNA sequences and are therefore not considered to function as histones in *E.coli*. IHF and Fis are involved in a variety of different processes (i.e. transcription **(14,32)** and recombination **(3,22)**). The major role of these proteins is thought to be bending of DNA thereby bringing DNA sequences in a more favourable conformation for the reaction to proceed. However, in some processes they can probably also act through protein-protein interaction. Fis is a relatively new member of this family. Although no primary sequence homology with any of the here described proteins is obvious there clearly is some functional homology with IHF.

In this chapter we have investigated the distribution of Fis throughout several genera of bacteria. By using Southern and Western blot analysis we found that Fis is restricted to the enteric group of bacteria, the same group that is the natural host for phage Mu **(reviewed in 26)**.

MATERIALS AND METHODS

Plasmids and DNA recombinant methods. The *fis* gene was obtained by random cloning of a *Kpn*I-*Hind*III digest of chromosomal *E.coli* DNA in pUC18 (Fig.1, pGP841) and subsequent selection for complementation of the Fis⁻ phenotype of MC1000 *fis*-767. The construction of pGP660 with the left 170 base pairs of Mu has been described before **(16)**. Southern blot analysis was essentially according to Maniatis **(30)** with hybridization overnight in 500 mM Sodiumphosphate pH7.6 and 7% SDS at 65°C. Chromosomal DNA (10 µgram) was cut with *Mlu*I and probed with the *Mlu*I-*Hind*III fragment of pGP841.

Strains. MC1000 *fis*-767 **(23)** was kindly provided by Johnson. The strains KA924 (*Citrobacter freundii*), KA927 (*Klebsiella aerogenes*), KA930 (*Enterobacter cloacae*), KA931 (*Shigella sonnei*), KA937 (*E.coli* C), KA943 (*E.coli* B), KA1060 (*E.coli* K12), KA1278 (*Bacillus subtilis*), KA1298 (*Pseudomonas aeruginosa*), KA1299 (*Pseudomonas putida*), KA1426 (*Acinetobacter calcoaceticus*), KA1515 (*Acinetobacter lwoffi*), KA1523 (*Pseudomonas acidovorans*), KA1559

(*Gluconobacter oxidans*) and KA1568 (*Pseudomonas cepacia*) are all from our collection. Yeast DNA (*Saccharomyces cerevisiae*) was a kind gift of C. Terleth and Human DNA (primary fibroblast) was kindly provided by C. Backendorf. The strain PP2540 is M72 (λ*N*am7-*N*am53 cI857 ΔH1) *fis*-767 (9).

Protein purification. Fis was purified to homogeneity from *E.coli* strain KA1060. Cells were grown in LBroth at 37°C in a 12 litre New Brunswick fermentor and were collected (35 grams wet weight, OD_{700nm}=1.0) by concentrating the culture over a Millipore filter followed by low speed centrifugation. A crude extract was prepared by gentle lysis of the cells in 0.8 M HED (800 mM KCl, 25 mM HEPES pH 7.6, 1 mM EDTA and 2 mM DTT) followed by freeze-thawing. The proteins from the supernatant obtained after centrifugation (50 min at 39.000 rpm, Beckmann 50.2Ti rotor) were precipitated with 0.35 gram/ml $(NH_4)_2SO_4$ and after a further centrifugation (20 min at 20.000 rpm, Beckmann 50.2Ti rotor) dissolved in 0.4 M HEDG (HED containing 20% glycerol). This protein extract was dialysed against 0.4 M HEDG and run on a P11 column (0.4 M - 1.0 M HEDG). The peak fractions eluting at 0.6 M HEDG were diluted to 0.3 M HEDG, heated for 10 min at 80°C and applied to a ssDNA agarose column (0.3 M - 1.0 M HEDG). Fis containing fractions (0.45 M HEDG) were stored at -80°C. During the purification Fis activity was monitored by DNaseI footprinting on a 170 bp

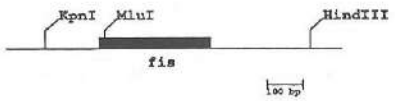

Figure 1. Physical map of *E.coli fis* region. Indicated are the position of the relevant restriction sites and the Fis coding sequence (thick line).

*att*L fragment and by an *in vitro* inversion assay (33).

DNaseI footprinting. The *Eco*RI-*Sal*I restriction fragment of pGP660 containing *att*L (170 base pairs) was labelled at the *Sal*I site using ^{32}P-dCTP and Klenow polymerase. About 5-10 ng of labelled fragment was incubated (10 min at 25 °C) with 50 ng of Fis in 20 µl of binding buffer (25 mM HEPES pH7.6, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM DTT and 150mM KCl). Subsequently DNaseI at a final concentration of 0.1 ng/ml was added and the mixture was incubated for an additional 5 min. The reactions were stopped by the addition of 45 µl of ice cold stopmix (600 mM sodiumacetate, 80 mM EDTA and 50 µg/ml Calf thymus DNA). After phenol extraction and precipitation the samples were run on a 10% sequencing gel.

Immunological methods. Against Fis directed antibodies were raised in a New Sealand rabbit by subcutal injections (25 µg of Fis in 2 ml of Freund's adjuvant, Gibco) at two weeks intervals. The antibodies were collected after 6 weeks and were screened in both ELISA and Western blot assays for specificity and titre. Western blotting was essentially according to Maniatis **(30)** with the following modifications. Electroforesic transfer was overnight (40 volts and 200 mA at 4°C) in 192 mM Glycine, 20 % (v/v) Methanol and 25 mM Ethanolamin (pH 9.0) to facilitate binding of highly basic proteins to the nitrocellulose filter. Before screening with the primary antibody the filter was floated in PBS (8 g/l NaCl, 2 g/l KCl, 14 g/l Na_2HPO_4 and 2 g/l KH_2PO_4) for 4 h to remove the SDS.

RESULTS and DISCUSSION

Purification of Fis. During the purification of transposase A of Mu M.A.M. Groenen observed that a protein copurified with the A protein **(15)** which was later identified as Fis **(2)**. This property was used to develop a convenient purification scheme for Fis by the combination of the A purification procedure **(5)** with specific steps of previously published Fis purification procedures **(22,24,25)**. The result is a rapid two-column-method that allows Fis to be purified to homogeneity which is described in the M&M section.

Raising of Fis antibodies. Antibodies were raised against purified Fis with a resulting titre of about 1:2000 as determined by an ELISA. The specificity was tested in a Western blot that allows detection of possible cross-reactivity with other proteins. In our procedure no cross-reactivity was observed neither with an extract prepared from a Fis$^-$ strain nor with purified IHF and HU which due to their properties (basic and heat stable) might be expected to be the principle contaminants of the Fis preparation (Fig.2).

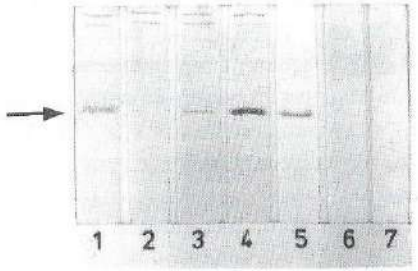

Figure 2. Immunological detection of Fis. Lane 1, Fis$^+$ extract (M72); lane 2, Fis$^-$ extract (PP2540); lane 3, extract of M72 with pGP842 not induced; lane 4, extract of M72 with pGP842 induced; lane 5, Fis; lane 6, IHF; lane 7, HU. For extracts 15 µgram and for purified samples 100 ngram of protein was applied to a 15% poly-acrylamide SDS-Tricine gel. The arrow points to the Fis protein.

Distribution of Fis. The cloned *fis* gene was used to determine whether homology to the *fis* gene can be found in other organisms which could be indicative for the presence of a similar gene in these organisms. Besides screening different bacterial DNAs also yeast and human chromosomal DNAs were probed (Fig.3). Strong positive

signals pointing to the presence of a fis-like gene were only detected in the enteric group of gram-negative bacteria. The closest relatives of *E.coli* K12 namely *E.coli* B, *E.coli* C and *Shigella* share a band of equal molecular size. This shows that possibly even the flanking sequences of *fis* with their restriction sites are conserved, indicative of their evolutionary relationship. Positive signals, but with a different molecular weight, were also obtained with *Salmonella*, *Citrobacter*, *Enterobacter* and *Klebsiella* all belonging to the enteric group of facultative anaerobe gram-negative bacteria. No signal under these stringent conditions was obtained with the other species from more distant genera.

The Southern blot analysis was only aimed at the identification of genes that share a high homology with the *fis* gene at the DNA level. In order to detect proteins that on an aminoacid level are similar to Fis we also screened a protein blot with polyclonal antibodies directed against Fis. In this Western blot we could detect Fis homologs of equal molecular weight in the same organisms that were also positive in the Southern blot assay (Fig.4). This confirms the presence of Fis in these organisms. Some cross-reactivity was observed with an unidentified protein of about 50-60 kDa in *Klebsiella*, both *Acinetobacter* species and all *Pseudomonas* species tested. Although we did not attempt to identify this protein we speculate that at least in the case of

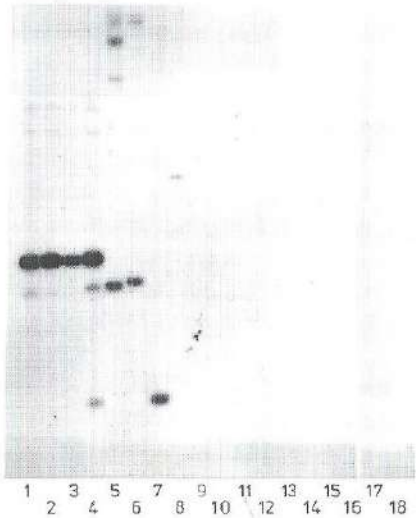

Figure 3. Southern blot analysis of *fis* in different organisms. Lane 1, *E.coli* B; lane 2, *E.coli* C; lane 3, *E.coli* K12; lane 4, *S.sonnei*; lane 5, *S.typhimurium*; lane 6, *C.freundii*; lane 7, *E.cloacae*; lane 8, *K.aerogenes*; lane 9, *B.subtilis*; lane 10, *A.calcoaceticus*; lane 11, *A.lwoffi*; lane 12, *G.oxydans*; lane 13, *P.acidovorans*; lane 14, *P.aeruginosa*; lane 15, *P.cepacia*; lane 16, *P. putida*; lane 17, *S.cerevisiae* ; lane 18, *H.sapiens*. Chromosomal DNA (10 µgram) digested with *MluI* and samples were run on a 0.8% agarose gel in Tris-borate buffer.

Klebsiella this protein could be NtrC. This protein has considerable homology with Fis in the DNA binding domain which could be a target for antibodies directed against this epitope (23). Interestingly this protein is like Fis also involved in transcription. In several bacterial species NtrC positively regulates transcription of a class of operons involved in nitrogen fixation (11) whereas Fis stimulates the expression of the stable RNA operons (32).

In conclusion, we have found Fis or Fis-related proteins only within the enteric group of bacteria making it from evolutionary point of view a recent gene. These bacteria share also the ability to propagate Mu and show in addition a growth profile with a relative short lag phase which could be related to the stimulation of transcription of stable RNA operons by Fis (32).

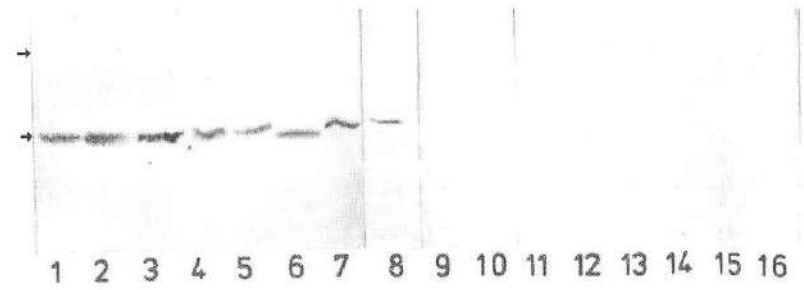

Figure 4. Immunological detection of Fis from several bacterial species.
Lane 1, *E.coli* B; lane 2, *E.coli* C; lane 3, *E.coli* K12; lane 4, *S.sonnei*; lane 5, *S.typhimurium*; lane 6, *C.freundii*; lane 7, *E.cloacae*; lane 8, *K.aerogenes*; lane 9, *B.subtilis*; lane 10, *A.calcoaceticus*; lane 11, *A.lwoffi*; lane 12, *G.oxydans*; lane 13, *P.acidovorans*; lane 14, *P.aeruginosa*; lane 15, *P.cepacia*; lane 16, *P.putida*. The lanes contain 15 µgram of crude extract and the samples were run on a 15% polyacrylamide SDS-Tricine gel. Lower arrow points to Fis; Upper arrow points to a 55 kDa protein.

Literature cited

(1) Aitken, A., and Rouvière-Yaniv, J. (1979) *Biochem. Biophys. Res. Commun. 91*:461-467.
(2) Bétermier, M., Lefèvre V., Koch C., Alazard, R., and Chandler, M. (1989) *Mol. Microbiol. 3*:459-468.
(3) Better, M., Wickner, S., Auerbach, J., and Echols, H. (1983) *Cell 52*:161-168.
(4) Broyles, S.S., and Pettijohn, D.E. (1986) *J. Mol. Biol. 187*:47-60.
(5) Craigie, R., and Mizuuchi, K. (1986) *J. Biol Chem. 260*:1832-1835.
(6) Craigie, R., Arndt-Jovin, D., and Mizuuchi, K. (1985) *Proc. Natl. Acad. Sci. (USA) 82*:7570-7574.
(7) Defez, R., and Defelice, M. (1981) *Genetics 97*:11-25.
(8) Dixon, N., and Kornberg, A. (1984) *Proc. Natl. Acad. Sci. (USA) 81*:424-428.
(9) Dri, A.M., Rouvière-Yaniv, J., and Moreau, P.L. (1991) *J. Bacteriol. 173*:2852-2863.
(10) Drlica, K., and Rouvière-Yaniv, J. (1987) *Microbiol. Rev. 51*:301-305.
(11) Drummond, M., Whitty, P., and Wootton, J. (1986) *EMBO J. 5*:441-447.
(12) Flashner, Y., and Gralla, J. (1988) *Cell 54*:713-721.

(13) Göransson, M., Sonden, B., Nilsson, P., Dagberg, B., Forsman, K., Emanuelsson, K., and Uhlin, B.E. (1990) *Nature (London) 344*:682-685.
(14) Goosen, N., van Heuvel, M., Moolenaar, G.F., and Van de Putte, P. (1984) *Gene 32*:419-426.
(15) Groenen, M.A.M. (1986) Ph.D. thesis, Leiden University, The Netherlands.
(16) Groenen, M.A.M., Vollering, M., Krijgsman, P., Van Drunen, C.M., and Van de Putte, P. (1987) *Nucleic Acids Res. 21*:8831-8844.
(17) Haselkorn, R., and Rouvière-Yaniv, J. (1976) *Proc. Natl. Acad. Sci. (USA) 73*:1917-1920.
(18) Hawkins, A.R., and Wooton, J.C. (1981) *FEBS Lett. 96*:395-398.
(19) Higgins, N.P., and Hillyard, D. (1988) *J. Bacteriol. 170*:5751-5758.
(20) Higgins, C.F., Dorman, C.J., Stirling, D.A., Waddell, L., Booth, I.R., May, G., and Bremer, E. (1988) *Cell 53*:569-584.
(21) Hulton, C.S.J., Seirafir, A., Hinton, J.C.D., Sidebotham, J.M., Waddell, L., Pavitt, G.D., Owen-Hughes, T., Spassky, A., Buc, H., and Higgins, C.F. *Cell 63*:631-642.
(22) Johnson, R.C., Bruist, M., and Simon, M. (1986) *Cell 46*:531-539.
(23) Johnson, R.C., Ball, C.A., Pfeffer, D., and Simon, M.I. (1988) *Proc. Natl. Acad. Sci. (USA) 85*:3484-3488.
(24) Kanaar, R., Van de Putte, P., and Cozzarelli, N.R. (1986) *Biochim. Biophys. Acta 866*:170-177.
(25) Koch, C., and Kahmann, R. (1986) *J. Biol. Chem. 261*:15673-15678.
(26) Koch, C., Mertens, G., Rudt, F., Kahmann, R., Kanaar, R., Plasterk, R.H.A., Van de Putte, P., Sandulache, R., and Kamp, D. (1986) in *Phage Mu* (eds. Symonds *et al.*), p.75-91. Cold Spring Harbor Laboratory, Cold Spring Harbor.
(27) Laine, B., Bèlaïche, D., Khanaka, H., and Sautière, P. (1983) *Eur. J. Biochem. 131*:325-331.
(28) La Teana, A., Falconi, M., Scarlato, V., Lammi, M., and Pon, C.L. (1989) *FEBS lett. 244*:34-38.
(29) Lejeune, P., and Danchin, A. (1990) *Proc. Natl. Acad. Sci. (USA) 87*:360-363.
(30) Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) *Molecu-lar cloning. A Laboratory Manual.* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
(31) Mende, L., Timm, B., and Subramanian, A.R. (1978) *FEBS Lett. 96*:395-398.
(32) Nilsson, L., Vanet, A., Vijgenboom, E., and Bosch, L. (1990) *EMBO J. 9*:727-734.
(33) Plasterk, R.H.A., Kanaar, R., and Van de Putte, P. (1984) *Proc. Natl. Acad. Sci. (USA) 81*:2689-2692.
(34) Searcy, D.G. (1975) *Biochim. Biophys. Acta 395*:535-547.
(35) Spassky, A., Rimsky, S., Garreau, H., and Buc, H. (1984) *Nucleic Acids Res. 12*:5321-5340.
(36) Spears, P.A., Schauer, D., and Orndorff, P.E. (1986) *J. Bacteriol. 168*:179-185.
(37) Surette, M., Buch, S., and Chaconas, G. (1987) *Cell 49*:253-262.
(38) Wilson, K.S., Vorgias, C.E., Tanaka, I., White, S.W., and Kimura, M. (1990) *Protein engineering 4*:11-22.

GENERAL OVERVIEW AND SUMMARY

The results of this thesis zoom in on the role of the Mu enhancer (IAS) in regulation of transposition and it is shown that the IAS not only mediates positive but also negative regulation. The stimulatory activity of the IAS seems to reside in the ability of the IAS, via transposase A, to interact with both attachment sites of Mu. This presumably facilitates the correct interaction between *att*L and *att*R and the subsequent formation of the cleaved donor complex (CDC). The inhibitory activity of the enhancer is mediated through the Mu repressor (c) which, in concert with the *E.coli* protein Fis, is able to inhibit transposition.

In both the Mu attachment sites there are three A binding sites designated L1-L3 in *att*L and R1-R3 in *att*R. Chapter 3 identifies the sequences in both attachment sites that are required for the stimulatory activity of the IAS. We suggest that the interaction of the attachment sites with the IAS is mediated by transposase A through the L2/R2 binding sites in these attachment sites. Transposase A has two distinct DNA binding domains one of which is able to bind to the attachment sites whereas the other is able to bind to the IAS. The A molecules bound at L2 and R2 are therefore potentially also able to bind to the IAS, thus mediating the interaction between the attachment sites and the IAS. The target of A in the IAS overlaps with repressor binding sites O1 and O2. Noteworthy in this respect is the DNA binding helix-turn-helix motive which is conserved between repressor and A, suggesting that A indeed recognizes O1 and O2 within the enhancer. The function of A bound at L1 is most likely to interact with its opposite partner at R1, in order to correctly align the attachment sites for the strand transfer reaction at the target site.

We propose a chain of events that leads to the formation of the CDC and the role of the enhancer in this. A central point in this model is the formation of a three-site-complex (or "transition complex") between the attachment sites and the IAS. Under conditions where the interaction between the IAS and *att*R is presumably disturbed (deletion of R2), the IAS inhibits rather than stimulates transposition. This could mean that the formation of the "transition complex" proceeds in two steps. The first step would be the interaction of *att*L with the IAS, which will be facilitated due to the close proximity of these sites. The second step would be the interaction of *att*R with this "docking complex", followed by the formation of the CDC after the IAS has left the "transition complex". In the absence of the enhancer the attachment sites will have to find each other through random collision, rather than in an ordered fashion. In the presence of the IAS but when R2 is deleted, random collision of the ends could well be hampered when *att*L, via its interaction with the IAS, is shielded from interacting with *att*R.

Also in negative regulation of transposition the interaction between *att*L and the IAS could play a role. In

chapter 2 we investigated a potential inhibitory complex between *att*L and the IAS, which we hypothesized to be formed through interaction of Fis bound to *att*L and repressor bound to the IAS. Such an inhibitory complex could impede the formation of the stimulatory complex through direct competition. We could establish the role of the enhancer. Further we showed that the role of the repressor was not confined to a mere inactivation of the IAS or to competition for A binding at the attachment sites. However, we were not able to support the idea that the action of Fis is mediated through the strong Fis binding site in *att*L.

The complexes we have described, one involved in stimulation and one involved in inhibition of transposition seem different by nature. However, this does not imply that these complexes are organized in two distinct ways. There are several arguments that the complexes are in fact quite similar. The transposase A in one complex and repressor in the other have similar binding characteristics (both have affinity for each others binding sites). We speculate that the principle difference between the potential stimulating and inhibiting complex originates from the proteins that mediate the interaction between *att*L and the IAS (transposase A in the case of the stimulating complex; and Fis/repressor in the case of the inhibiting complex). Repressor and transposase A share a similar DNA binding domain with which these proteins bind to the IAS, so the target of both complexes in the IAS is expected to be the same. Furthermore the potential target of interaction within *att*L is for both processes the region of L2. Although repressor, unlike A, does not contain a second DNA binding domain, this function could possibly be provided by interaction of c with Fis in a yet not clearly established way. Taking this into account both complexes can be considered to be more similar than the action of these complexes would imply, as in both processes there is a role for the interaction of the IAS and *att*L.

Is the interaction between *att*L and the IAS in both the inhibitory and stimulatory complex merely a reflection of the spatial arrangement of these sites which in the Mu genome are in close proximity? We believe that this is not the case and would like to postulate that this interaction has arisen from some early phase in Mu evolution where the present-day *att*L and IAS formed the attachment sites of an ancestral Mu with the present-day repressor as its transposase. The following observations concerning the organization of the left 1300 base pairs of Mu form the basis of this idea: a reading frame that encodes a protein with binding sites for this protein at either end of the gene, the overall size of this potential transposon genome and the organization of the repressor promoter within the proposed attachment sites. An example of a present-day transposon featuring the same aspects as summarized above is IS10R. This insertion sequence is

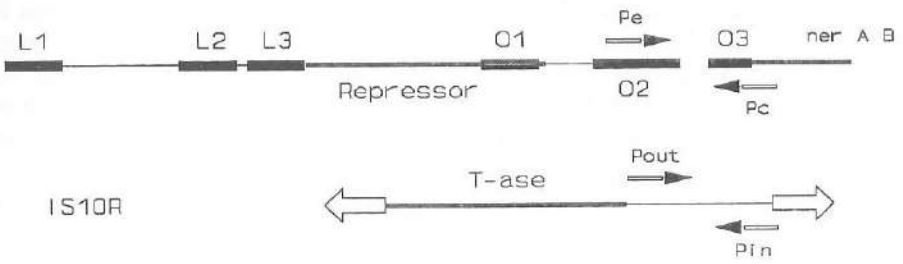

Figure 1. Comparison of Mu attL (top) with IS10 (bottem)

both an autonome transposon and a functional part of Tn10, a composite transposon comprising of two IS10 sequences. In figure 1 a schematic representation of IS10R is provided, which clearly illustrates the similarities between the organization of the left attachment site of Mu and this transposon. In IS10R the open reading frame of the transposase is flanked by two inverted repeats, which contain binding sites for the transposase. The promoter (P_{in}) that transcribes the transposase is situated within the right inverted repeat, with tight regulation of transposition by a binding site of the transposase that overlaps its own promoter. An additional level of regulation is mediated by P_{out}, an outward directed promoter, with a transcript that partly overlaps with that of P_{in}. The configuration of these converging promoters leads to transcription interference and subsequent repression of the transposase expression. These features, as stated above, can also be observed within the left 1300 bases of Mu, which could suggest that this part of Mu, in some early stage of Mu evolution, was an autonome transposon. In this ancestral Mu, the transposase (now repressor) would be located within the attachment sites (IR-left and IR-right) that would each contain three binding sites for the transposase. The binding sites in IR-right (now IAS) would regulate the level of transposase via P_{in} (now Pc) aided by transcription interference by P_{out} (now Pe). In this ancestral transposon both *att* sites would have been brought together by its transposase in order to mediate transposition. The interaction between the IAS and *att*L in the present-day Mu, which seems to be important for correct regulation of Mu transposition, could be a reflection of the interaction these sites once had in the ancestral Mu. So, the differences between positive and negative regulation of Mu transposition, seem to disappear against the age of time.

SAMENVATTING

Het thema van mijn proefschrift is het transpositiemechanisme van bacteriofaag Mu en meer in het bijzonder haar regulatie. Deze regulatie door bij transpositie betrokken eiwitten kent twee aspecten. Ten eerste op het niveau van transcriptie/ translatie van deze eiwitten en ten tweede op het mechanistisch niveau waarbij door interactie tussen de bij transpositie betrokken eiwitten de afzonderlijke functies van deze eiwitten beinvloed worden. In het onderzoek op het gebied van transpositieregulatie stond men tot nu toe hoofdzakelijk stil bij het eerste aspect, terwijl in dit proefschrift juist het mechanistische aspect centraal staat.

Het vroege promotergebied van bacteriofaag Mu heeft twee divergente promoters; Pe welke *ner* met de transposases *A* en *B* afschrijft en Pc welke de repressor *c* afschrijft. Binding van Ner aan een specifieke plaats in het promotergebied verlaagt de expressie van Pc, terwijl binding van c in het promotergebied juist de expressie van Pe verlaagt. De uitkomst van de competitie tussen Ner en c is dus van invloed op de ontwikkeling van de Mu profaag. Opvallend genoeg heeft het vroege promotergebied van Mu naast zijn rol in transcriptieregulatie ook een rol in het transpositiemechanisme zelf. De repressorbindingsplaatsen O1 en O2 kunnen naast c ook het transposase binden en fungeren dan als een transpositie enhancer. Mijn onderzoek laat zien dat deze "Internal Activating Sequence" (IAS) niet alleen in staat is de efficientie van de transpositiereactie te verhogen, maar tevens -onder bepaalde condities - om deze te verlagen.

Het transpositiemechanisme is erop gericht beide faaguiteinden (*att*L en *att*R) via het transposase op een geordende wijze bij elkaar te brengen. De vorming van dit "Cleaved Donor Complex" (CDC) wordt geacht door de IAS gestimuleerd te worden. In de hoofdstukken 3 en 4 wordt de rol van de diverse transposasebindingsplaatsen in *att*L en *att*R onderzocht in relatie tot de stimulerende werking van de IAS. Hiertoe heb ik systematisch de diverse bindingsplaatsen uit beide faaguiteinden gedeleteerd en gekeken of de IAS de transpositiereactie van deze deletiemutanten nog kan stimuleren. Wanneer L3 of R3 ontbreken, stimuleert de IAS de transpositiereactie nog volledig. Deze bindingsplaatsen zijn blijkbaar niet betrokken bij de stimulerende activiteit van de IAS. Deletie van L2 of R2 heeft echter wel een duidelijk effect. In beide gevallen is de IAS niet meer in staat de efficientie van de transpositiereactie te verhogen. In het geval dat R2 gedeleteerd wordt, remt de IAS zelfs de reactie. De transpositiefrequentie komt nu zelfs onder het niveau van een construct zonder IAS te liggen. In de afwezigheid van de IAS hebben deze deleties echter geen effect, zodat de functie van L2 en R2 blijkbaar beperkt is, in relatie tot de stimulerende werking van de IAS. Plaatsgerichte mutagenese van L2, waarbij deze vervangen wordt door de overeenkomstige bindingsplaats uit de met Mu verwante faag D108 ,leidt eveneens tot het verliezen van de enhanceractiviteit van de IAS. Waarschijnlijk hebben beide fagen enhancers welke op een specifieke manier gekoppeld zijn met

de bindingsplaatsen voor hun eigen transposase in hun respectievelijke uiteinden.

Een verdere reductie in de transpositiefrequentie treedt op wanneer L1 of R1 gedeleteerd worden. Daar dit ook het geval is in de afwezigheid van de IAS, moet dit los gezien worden van de stimulerende activiteit van de IAS en aan het mechanisme van transpositie toe geschreven worden. Ik suggereer in dit hoofdstuk dat L2 en R2 betrokken zijn bij de vorming van een "three-site complex" tussen *att*L *att*R en de IAS. Dit complex zou dan een intermediair zijn tijdens de vorming van het CDC waarbij de IAS *att*L en *att*R op de juiste wijze bijeen brengt. Daar bovendien deletie van R2 in de aanwezigheid van de IAS een extreme reductie van de transpositie tot gevolg heeft, verloopt de vorming van dit "three-site-complex" mogelijk geordend. In eerste instantie vormen *att*L en de IAS een binair complex waarna vervolgens *attR* de IAS uit dit binaire complex verdringt en samen met *att*L het CDC vormt.

Hoofdstuk 4 laat zien dat de rol welke aan de diverse transposasebindingsplaatsen kan worden toegeschreven, afhangt van het soort DNA molecule dat als target voor mini-Mu transpositie wordt gebruikt. Wanneer F'*pro-lac* de target is, blijkt deletie van de transposase bindingsplaatsen L2 en R2 in afwezigheid van de IAS een verlaging van de transpositiefrequentie te geven, terwijl wanneer een pOX38 afgeleide de target is, dit alleen in aanwezigheid van de IAS het geval is. Op grond van deze resultaten geef ik aan dat het verschil tussen beide targetmoleculen gezocht kan worden in het voorkomen van Mu-gerelateerde transposons op F'*pro-lac*. Deze kunnen mogelijk als hotspot voor Mu integratie of als een *in trans* werkende Mu transpositieënhancer werken.

Hoofdstuk 2 onderzoekt of het *Escherichia coli* eiwit Fis (Factor of inversion stimulation), naast zijn reeds bekende rol bij de inversie van het bacteriofaag Mu G-gebied, betrokken zou kunnen zijn bij de regulatie van Mu transpositie. Dit is ingegeven door het voorkomen van een sterke Fis bindingsplaats in het linker uiteinde van Mu, vlak naast de voor de werking van de IAS zo belangrijke transposasebindingsplaats L2. Ik toon aan dat Fis, samen met de Mu repressor (c), zelfs na inductie van transpositie nog instaat is te competeren met het transposase. Voor deze remming moet echter wel de IAS aanwezig zijn: zonder de IAS kunnen Fis en c de transpositie niet remmen. De repressor bindingsplaatsen O1 en O2 in de IAS binden naast repressor ook transposase A. Deze binding is noodzakelijk voor de stimulerende werking van de IAS tijdens transpositie, wat zou suggereren dat inactivatie van de IAS, door Fis gestimuleerde binding van repressor, een mogelijk mechanisme van inhibitie is. Dit blijkt niet het geval. Ook transpositie van een construct met een voor transpositie inactieve IAS, waarbij O1 en O2 intact blijven, wordt door Fis en c geremd. Een remmend complex tussen de IAS en *att*L, gemedieerd via de repressorbindingsplaatsen van de IAS en de Fis bindingsplaats in *att*L, lijkt een

interessant alternatief, maar deze rol van de Fis bindingsplaats kon niet worden aangetoond. Een construct waar deze bindingsplaats gemuteerd is zodat Fis *in vitro* niet meer kan binden, vertoont nog steeds transpositie inhibitie via coöperatie tussen Fis en c. Daar de werking van Fis blijkbaar onafhankelijk van zijn bindingsplaats is, lijkt de vorming van een remmend complex onwaarschijnlijk. Ik kan echter niet uitsluiten dat *in vivo* bij lage Fis concentraties de cooperatie nog wel via de Fis bindingsplaats verloopt.

In hoofdstuk 5 staat het eiwit Fis zelf centraal. In dit hoofdstuk heb ik gekeken bij welke bacteriesoorten dit eiwit of een homoloog van dit eiwit voorkomt. Na analyse op zowel DNA- als eiwitniveau kan ik alleen bij de leden van de Enterobacter groep een Fis-achtig eiwit aantonen. Dit voorkomen correleert opvallend genoeg met het gastheerbereik van bacteriofaag Mu wat de rol welke Fis in bacteriofaag Mu ontwikkeling speelt onderstreept.

Hoofdstuk 6 tenslotte probeert beide hoofdthemas van mijn proefschrift, de stimulerende en remmende werking van de IAS te verenigen. Vanuit een evolutionair gezichtspunt kunnen de huidige interacties tussen *att*L en de IAS gezien worden als een rudimentaire interactie tussen beide sequenties toen zij mogelijk zelf de uiteinden van een primitief transposon vormden. Dit idee wordt mede ingegeven door de structuur van het linker uiteinde, dat grote overeenkomsten heeft met heden ten dage voorkomende IS elementen. Het oer-transposase zou zich tot repressor gespecialiseerd hebben, terwijl het de oorspronkelijke interactie plaatsen behouden heeft. Een tweede copie van het oer-transposase is verder ontwikkeld tot het transposase zoals we dat nu kennen, waarbij de affiniteit van A voor de repressorbindingsplaatsen in de IAS mogelijk zijn afkomst verraadt. Mocht dit idee juist zijn dan kan zowel de stimulerende werking van de IAS via A, als de remmende werking via c gezien worden als exponenten van een universeel mechanisme, dat zijn wortels vindt in de vroege evolutie van faag Mu.

Abbreviations

A	Adenosine
am	Amber mutation
attL	Left attachment site
attR	Right attachment site
bio	Biotin
bla	ß-lactamase
C	Cytidine
cat	Chloramphenicoltransferase
CDC	Cleaved donor complex
DTT	Dithiothreitol
EDTA	(Ethylene-dinitrilo)tetraacetic acid
Fis	Factor of inversion stimulation
HEPES	(Hydroxyethylpiperazine)ethanesulphonic acid
G	Guanosine
IAS	Internal activating sequence
IHF	Integration host factor
lac	Lactose
Mu	Mutator
N	The bases G, A, T or C
PBS	Phosphate buffered saline
pro	Proline
R	The bases A or G
T	Thymidine
Y	The bases C or T
SDS	Sodium dodecylsulphate
STC	Strand transfer complex
sup	Suppressor
thi	Thiamin
Tris	Tris(hydroxymethyl)aminomethane
trp	Tryptophan

Curriculum vitae

De auteur is geboren op 27 juni 1961 te Rotterdam. Het eindexamen V.W.O werd in 1981 afgelegd aan de scholengemeenschap "Maarten Luther" te Rotterdam waarna een aanvang werd gemaakt met de studie biologie aan de Rijksuniversiteit Leiden. Het kandidaats examen biochemie werd behaald in 1984. In 1987 werd het doctoraal examen biologie afgelegd met als hoofdvak moleculaire genetica (vakgroep Biochemie der faculteit scheikunde, dr. M.A.M Groenen en prof. dr. ir. P. van de Putte) en als bijvakken medische biochemie (vakgroep medische biochemie der medische faculteit, dr. J. van de Lubbe en prof. dr. A. van der Eb) en celbiologie (vakgroep celbiologie en gist genetica der faculteit biologie, dr. W. Spek en prof. dr. T. Konijn). Van december 1986 tot december 1990 was hij, in dienst van de Rijksuniversiteit Leiden, werkzaam als assistent in opleiding bij de afdeling Moleculaire Genetica (prof. dr. ir. P. van de Putte). Vervolgens werd hij ,in dienst van de Rijksuniversiteit Groningen, aangesteld als onderzoeker bij de afdeling celbiologie (vakgroep biochemie der faculteit scheikunde, prof. dr. P.J.M. van Haastert). Vanaf mei 1994 is hij als onderzoeker, in dienst van N.W.O, verbonden aan van het E.C. Slater instituut (vakgroep biochemie der Universiteit van Amsterdam, prof. dr. R. van Driel).

List of publications

J.L.M. van der Lubbe, C.M. van Drunen, J.J.L. Hertoghs, J.J. Cornelis, J. Rommelaere and A.J. van der Eb. (1985). Enhanced induction of SV40 replication from transformed mammalian cells by fusion with UV-irradiated untransformed cells. *Mutation Research 151*:1-8.

J.L.M. van der Lubbe, P.J. Abrahams, C.M. van Drunen and A.J. van der Eb. (1986). Enhanced induction of SV40 replication from transformed rat cells with UV-irradiated normal and repair-deficient human fibroblasts. *Mutation Research 165*:47-56.

M.A.M. Groenen, M. Vollering, P. Krijgsman, C.M. van Drunen and P. van de Putte. (1987).Interactions of the transposase with the ends of Mu: Formation of specific nucleoprotein structures and non-cooperative binding of the transposase to its bindings sites. *Nucleic Acid Research 15*:8831-8844.

W. Spek, C.M. van Drunen, R. van Eijk and P. Schaap. (1988). Opposite effects of adenosine on two types of cAMP-induced gene expression in *Dictyostelium* indicates the involvement of at least two different intracellular pathways for the transduction of cAMP signals. *FEBS Letters 228*:231-234.

L. Nilsson, H. Verbeek, E. Vijgenboom, C.M. van Drunen, A. Vanet and L. Bosch. (1992). Fis-dependent *trans* activation of stable RNA operons of *Escherichia coli* under various growth conditions. *Journal of Bacteriology 174*:921-929.

C.M. van Drunen, C. van Zuylen, E.J. Mientjes, N. Goosen and P. van de Putte. 1993). Inhibition of bacteriophage Mu transposition by Mu repressor and Fis. *Molecular Microbiology 10*:293-298.

C.M. van Drunen, E.J. Mientjes, C. van Zuylen, N. Goosen and P. van de Putte. (1994). Transposase A binding sites in the attachment sites of bacteriophage Mu that are essential for the activity of the enhancer and A binding sites that promote transposition towards Fpro-lac. *Nucleic Acid Research* 22: 773-779.

Nawoord

Bijna is het dan zo ver. De doctorstitel ligt in het verschiet en het moment is daar om terug te kijken op de gebeurtenissen die tot dit heugtelijk feit hebben geleid. Een ding is duidelijk: velen hebben een bijdrage geleverd die niet onvermeld mag blijven. Waar kan ik anders beginnen dan bij het begin.

Eerst, en boven al, zijn daar mijn ouders die vanaf het prille begin alles gedaan hebben om mijn ontwikkeling zo goed en plezierig mogelijk te laten verlopen. Er is geen taal rijk genoeg om mijn liefde en waardering onder woorden te brengen.

De tijd vliegt en inmiddels sta ik ingeschreven bij biologie van de Rijksuniversiteit Leiden: ik studeer. De weg naar mijn promotie zou onmogelijk zijn geweest zonder de morele steun van Oma, Mimi en in een vroege fase Opa.

Aan goede studiegenoten/vrienden geen gebrek. Een select gezelschap van Rob, Marieke, Valentina en Frans, reisde dag-in-dag-uit tussen Rotterdam en Leiden heen en weer. Via hen en de cursus planten/dieren-determinatie-vanuit-rijdende-trein leerde een stadsjongen ook nog eens vele planten, vogels en insekten kennen. Hans en Bert vormden het Leidse kader van mijn vriendenclub terwijl Ton en Jannie de mobiele brigade vormden. Na het verplichte programma volgde de stages en de kennismaking met de wetenschap. Mijn begeleiders hebben elk op meer dan voortreffelijke wijze een gedeelte van mijn wetenschappelijke vorming voor hun rekening genomen. Jos, Martien en Wouter mogen niet onvermeld blijven.

Na het afstuderen was het tijd het geleerde in de praktijk te brengen: met andere woorden ik werd AIO. Na de eerste week lag het concept van mijn proefschrift klaar: het 68 kDa eiwit zuiveren, het gen kloneren, even de functie bij Mu transpositie bepalen en klaar is de spreekwoordelijke Kees. Dat liep anders zoals de meeste van jullie wel zullen weten. De resultaten van de proeven waren er niet gekomen zonder de inzet en toewijding van hen, die sommige waarschijnlijk "mijn studenten" zouden noemen, maar ik liever als mijn collega's en vrienden zou beschrijven. Waar vindt je mensen zoals Karin, Carol en Edwin die midden in de nacht nog even wat doen, een interactie model helpen ontwerpen en 100 constructen maken om een en ander te testen. Furthermore I would like to acknowledge the colaboration with Dr. Lars Nilson.

Kamergenoten, labgenoten, Jan de SRV man en Nicolien tesamen met de bijzonder bijzondere personen van Liesbeth en Ingrid hebben mijn verblijf in Leiden tot een groot feest gemaakt.

Tenslotte mag ik mij verheugen op het vertrouwen dat Peter en Roel in mij gesteld hebben. Het leren kennen van Groningen en Amsterdam betekende meer voor mij dan menigeen kan bevroeden en ik op papier kan vastleggen. Ineke, de wijze levenslessen van Robert, mijn klasgenoot Mari en mijn fietsgenoot Romi zal ik met node missen.

Stellingen

-1-

Het uitgangspunt dat een wetenschappelijk resultaat reproduceerbaar moet zijn, gaat voorbij aan het potentiële belang van chaotische systemen

-2-

Het artikel van Mirkovitch *et al* wordt ten onrechte geciteerd als bewijs dat de boundary elementen SCS en SCS' niet aan de kern matrix binden.

J. Mirkovitch, M.-E. Mirault en U.K. Laemmli (1984) Cell 39:223-232
E.R. Jupe, R.R. Sinden en I.L. Cartwright (1995) Biochemistry 34:2628-2633

-3-

Bij het bestuderen van de invloed van matrix attachments regio's op de expressie van transgenen realiseren weinigen dat sommige van de reporter constructen zelf al sequenties bevatten die aan de kern matrix kunnen binden

E.M. Thompson, E. Christians, M.-G. Stinnakre en J.-P. Renard (1994) Mol. Cell. Biol. 14:4694-4703
L. Poljak, C. Seum en U.K. Laemmli (1994) Nucleic Acids Res. 22:4386-4394
Y. Pommier, P.N. Cokcerill, K.W. Kohn en W.T. Garrard (1990) J. of Virology 64:419-423

-4-

Wanneer men de rol van methylering in eukaryote cellen bestudeert door het binnen brengen van constructen mag men de invloed van het methyleringspatroon dat mogelijk in *Escherichia coli* is ontstaan, niet veronachtzamen

-5-

De *science citation index* geeft een nuttige indicatie van de kwaliteit van het gepresenteerde onderzoek, maar vormt niet altijd een objectieve maatstaf

-6-

Technieken beschreven in de methodensectie van een artikel dienen geen samenvatting te zijn van het werk van anderen

H. Verbeek, L. Nilson, G. Baliko en L. Bosch (1990) Biochim. Biophys. Acta 1050: 302-306

-7-

Op een oneindig groot grasveld grazen de koeien bij benadering in een zelfde richting

-8-

Het is niet altijd mogelijk gedachten en gevoelens volledig en juist over te brengen

-9-

Het verdient aanbeveling bij de afvaardiging naar een sportbijeenkomst niet alleen voor deelnemende sporters maar ook voor het aantal begeleidende bondsbestuursleden een limiet in te stellen

-10-

Ondanks het huidige falen van de moleculair biologen om het guanylylcyclase gen van *Dictyostelium discoideum* te kloneren, mag men vooralsnog niet concluderen dat dit organisme cGMP kan produceren met een enzyme waarvoor geen coderende sequentie bestaat

Stellingen behorende bij het proefschrift getiteld
Regulation of phage Mu transposition van C.M. van Drunen