MOBILISATION OF THE NON-CONJUGATIVE PLASMID RSF1010

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A Thesis presented for the Degree of
Doctor of Philosophy

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September 1983.

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ABSTRACT

A survey of the mobilisation of a range of independent, naturally occurring, non-conjugative plasmids by conjugative plasmids was carried out. This illustrated the specificity of interaction between non-conjugative and conjugative plasmids and showed that mobilisation is a general property of non-conjugative plasmids.

RSF1010, a $Sul^{R}Sm^{R}$, non-conjugative IncQ plasmid was mobilised efficiently by representative plasmids of IncM, IncX and IncIα groups but especially by IncP plasmids. As RSF1010 has been developed as a broad-host-range cloning vector then its mobilisation is of considerable importance and so this was investigated in more detail. A 1.7Kb region of RSF1010, cloned in a pBR322-derived vector, was shown to contain both the origin of transfer (*oriT*) and the genes responsible for mobilisation. The nucleotide sequence of this region was determined.

oriT was located on an 80bp segment of DNA by subcloning and *in vitro* deletion. Symmetrical sequences, possibly involved in transfer, were identified.

Complementation analysis identified two genes required for mobilisation of RSF1010. One of these, *cisB*, was identified from the nucleotide sequence and would encode a protein of 15Kd which was in excellent agreement with a protein synthesised from mini-cells. A second gene, *cisA*, identified from the sequence, overlapped *cisB* and encoded a protein of >65Kd. The available data suggests that this protein is involved in replication in *E. coli* and *Pseudomonas*. A second mobilisation gene (*cisC*) was tentatively identified by the mapping of an insertion mutant. *cisC* overlaps *cisA* but is transcribed from the opposite strand and would encode a protein of 22Kd.

DECLARATION

I hereby declare that I alone have composed this thesis, and that, except where stated, the work presented within is my own.

September 1983.

For Vicky

ACKNOWLEDGEMENTS

I am indebted to many members of this Department for their willing help and advice. In particular, I would like to thank Wendy Smith and Liz Marson for their help in the lab; George Coupland, Tony Brown and John Maule for their friendship, advice and helpful discussion; Graham Hatfull for his friendship and invaluable collaboration in sequencing and Gordon Langsley for his companionship while writing this thesis.

I am deeply indebted to Richard Hayward and especially Noreen Murray for their time, patience and helpful advice in the absence of Neil Willetts and also to David Finnegan for his reassurance and friendly advice.

I am very grateful to Neil Willetts for his excellent supervision and guidance, and for devising such an interesting project.

My final acknowledgement is to Vicky, without whom this thesis would not have been possible.

ABBREVIATIONS

Ap	ampicillin
bp	base pair
Cm	chloramphenicol
kb	kilobase
Km	kanamycin
Nal	nalidixic acid
Sm	streptomycin
SpC	spectinomycin
Sul	sulphonamide
Tc	tetracycline
Tp	trimethoprim
Tra	transfer
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
MRNA	messenger RNA

Chapter 1

INTRODUCTION

(a) Transferable antibiotic resistance

Plasmids are naturally occurring extrachromosomal elements which, in addition to specifying functions for their own maintenance, often encode other characteristics. One of these is the process of conjugation which is an important route for genetic exchange amongst bacteria and requires cell-to-cell contact. The discovery of gene transfer amongst bacteria allowed the identification of the sex factor F as the 'causative agent'. As a consequence the F conjugation system is the most comprehensively studied. Other plasmids have since been isolated from many bacterial species and are often associated with the transfer of antibiotic resistance.

Transferable antibiotic resistance was first identified when antibiotic resistant Shigellae, isolated from human cases of dysentry in Japan, were shown to be capable of passing on this resistance to Eschericia coli. Bacterial resistance to antibiotics arose with an increasing frequency and multiple resistance was often correlated with the sequential use of different antibiotics during a treatment (reviewed by Watanabe, 1963).

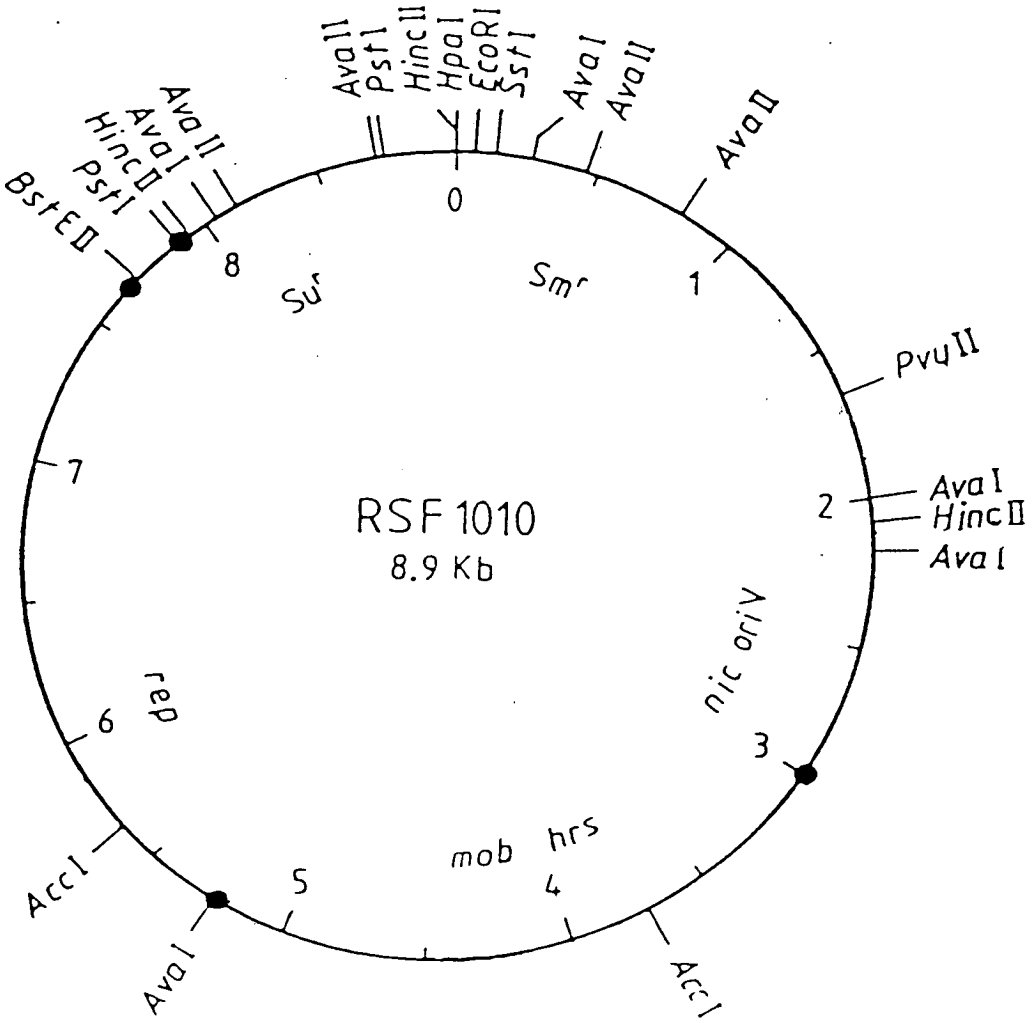
Anderson (1968) documented a similar example of an increase in multiple antibiotic resistance occurring in Salmonella typhimurium phage type 29 infections in animals and humans. In 1963 streptomycin and sulphonamide resistant S. typhimurium were isolated and by 1965 this strain had acquired resistance to kanamycin, tetracycline, ampicillin and furazolidone. These resistances, apart from furazolidone which is chromosomally linked, were shown to be mobilised by a conjugative plasmid, Δ, isolated from the same strain; they could not transfer themselves. It was the ability of these resistances to be mobilised which was instigated in the spread of the resistances from harmless commensal organisms to pathogenic ones and led to the processes of mobilisation being characterised.

Genetic evidence suggested that the resistances to sulphonamides and streptomycin were linked, while those of kanamycin and ampicillin were independent of each other, although all were capable of being mobilised by the Δ factor. Resistance to tetracycline was initially

Fig. 1.1 Physical and genetic map of RSF1010

Abbreviations: Su^r, Sm^r resistance to sulphonamide and streptomycin, respectively; *mob*, *nic*, *oriV*, *rep*, *hrs*, determinants for plasmid mobilisation, relaxation nick site, origin of vegetative replication, positive replication factor and ability to survive in *Pseudomonas*, respectively; filled circles are RNA polymerase binding sites. (Bagdasarian *et al.*, 1982; M. Bagdasarian, pers. comm.)

FIG.1.1


RSF 1010
8.9 Kb
0
1
2
3
4
5
6
7
8
Su
Sm
rep
nic oriV
mob
hrs
AvaII
PstI
HincII
HpaI
EcoRI
SstI
AvaI
AvaII
AvaII
PvuII
AvaI
HincII
AvaI
AccI
AvaI
AccI
BstEII
AvaII
AvaI
HincII
PstI

transferred at a very low frequency. However, the tetracycline resistant exconjugants could now retransfer tetracycline resistance at very high frequencies. Milliken and Clowes (1973) demonstrated that this was due to covalent linkage of the tetracycline determinant and Δ. They also showed that the resistance to sulphonamide and streptomycin was determined by a 8.9Kb plasmid, later shown to be RSF1010 (Fig. 1.1).

The importance of transfer in the spread of antibiotic resistance became increasingly apparent with the isolation and characterisation of more antibiotic resistant plasmids (Fredericq et al., 1971; Van Embden and Cohen, 1973; Williams Smith and Heller, 1973; Guerry et al., 1974; Barth and Grinter, 1974). Plasmids were classified into two basic groups; those capable of conjugation and those capable of being mobilised by the conjugative plasmids but not capable of self-transmission.

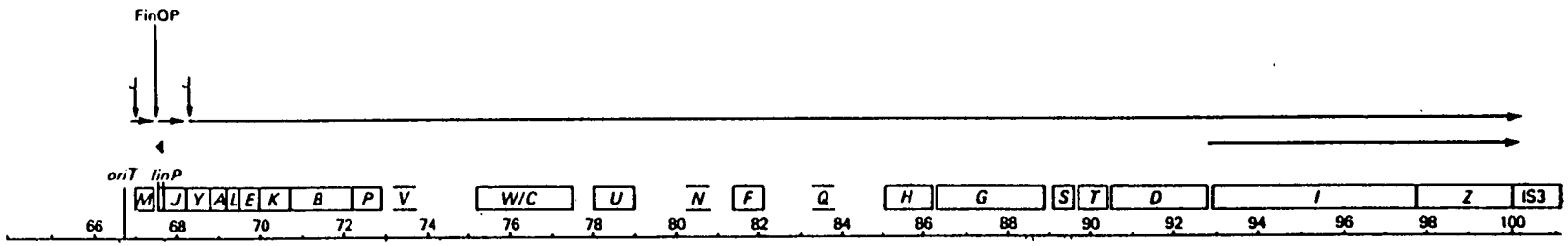
Conjugative plasmids are usually large (>50Kb) and have a low copy number, R6K being the only documented exception (Kolter and Helinski, 1978). In contrast, non-conjugative plasmids are relatively small (<10Kb) and multicopy. One of the reasons for the difference in size of the two plasmid types is presumably a reflection of their roles in conjugation. Conjugative plasmids carry all the necessary information for mating pair formation and transfer, which often accounts for large proportions of their genome e.g. F, (Willetts and Skurray, 1980), R46 (Brown and Willetts, 1981), RP4 (Barth et al., 1978b). The non-conjugative plasmids so far characterised carry only information necessary for their conjugal metabolism, but even so as much as a third of their genome may be devoted to this e.g. ColE1 (Dougan et al., 1978), CloDF13 (Van de Pol et al., 1978), RSF1010 (this thesis), which reflects the importance and complexity of the transfer mechanism. Despite depending on conjugative plasmids for their transfer, non-conjugative plasmids are often efficiently mobilised. F can mobilise ColE1 and CloDF13 at frequencies equivalent to its own transfer (Clowes, 1963; Van de Pol et al., 1978).

Similarly, the non-conjugative plasmid RSF1010 is efficiently mobilised by representatives of the broad-host-range conjugative IncP plasmids (Willetts and Crowther, 1981). RSF1010 also has the ability to replicate in a wide-range of hosts (Table 1.1) and it would seem more than a coincidence that IncP plasmids mobilise it most efficiently.

Table 1.1 The Host-range of IncQ Plasmids

Bacterial species	Reference
E. coli	Barth and Grinter (1974)
Pseudomonas aeruginosa	Nagahari and Sakaguchi (1978)
Ps. putida	Nagahari and Sakaguchi (1978)
Azotobacter vinelandii	David *et al.* (1981)
Methylotrophus methylotrophus	Windass *et al.* (1980)
Rhizobium meliloti	Simon *et al.* (1983)
R. leguminosarum	Simon *et al.* (1983)
R. trifolii	Simon *et al.* (1983)
Agrobacterium tumefaciens	Simon *et al.* (1983)
Acetobacter xylinum	S. Valla cited in Bagdasarian *et al.*(1981)
Alcaligenes eutrophus	Barth *et al.* (1981)
Acinetobacter calcoaceticus	T. Schmidthauser cited in Barth *et al.*(1981)
Rhodopseudomonas spheroides	W. Tucker cited in Bagdasarian *et al.* (1981)
Salmonella sp.	Barth and Grinter (1974),Smith *et al.* (1974)
Proteus mirabilis	Barth and Grinter (1974)
P. morgani	Barth and Grinter (1974)
Providencia sp.	Barth and Grinter (1974)
Serratia marcesens	Barth *et al.* (1981)
Klebsiella aerogenes	Barth *et al.* (1981)

Fig. 1.2



A physical and genetic map of the transfer region of plasmid F. The numbers show kilobase co-ordinates and the horizontal lines above the genes represent transcripts. The direction of DNA transfer from oriT is such that the transfer region is transferred last. finP may be transcribed from the DNA strand opposite the long leader sequence of the traJ mRNA (N. Willetts and R. Thompson, unpublished data). Transcription from the promoters for traM and for the traY-Z operon is dependent on the product of traJ which is in turn negatively regulated by the FinOP repressor (Finnegan and Willetts, 1973; Willetts, 1977; Gaffney et al., 1983). Roles attributed to the genes are regulation - finP and traJ; pilus formation - traA, L, E, K, B, V, W/C, U, F, Q, H, and G; stabilisation of mating pairs - traN and G; conjugative DNA metabolism - traM, Y, D, I, and Z; surface exclusion - traS and T. (Taken from Willetts and Wilkins, 1983).

It suggests that there is a specific relationship between conjugative and non-conjugative plasmids. As RSF1010 can exist in, and be mobilised into, many hosts it has been developed as a broad-host-range cloning vector. The ability to be mobilised overcomes the problems of transformation into these varied and poorly understood hosts, and is therefore a system warranting detailed investigation. The work presented in this thesis is a molecular and genetic analysis of the mobilisation system of RSF1010 and its relationship with the conjugative IncP plasmid RP1.

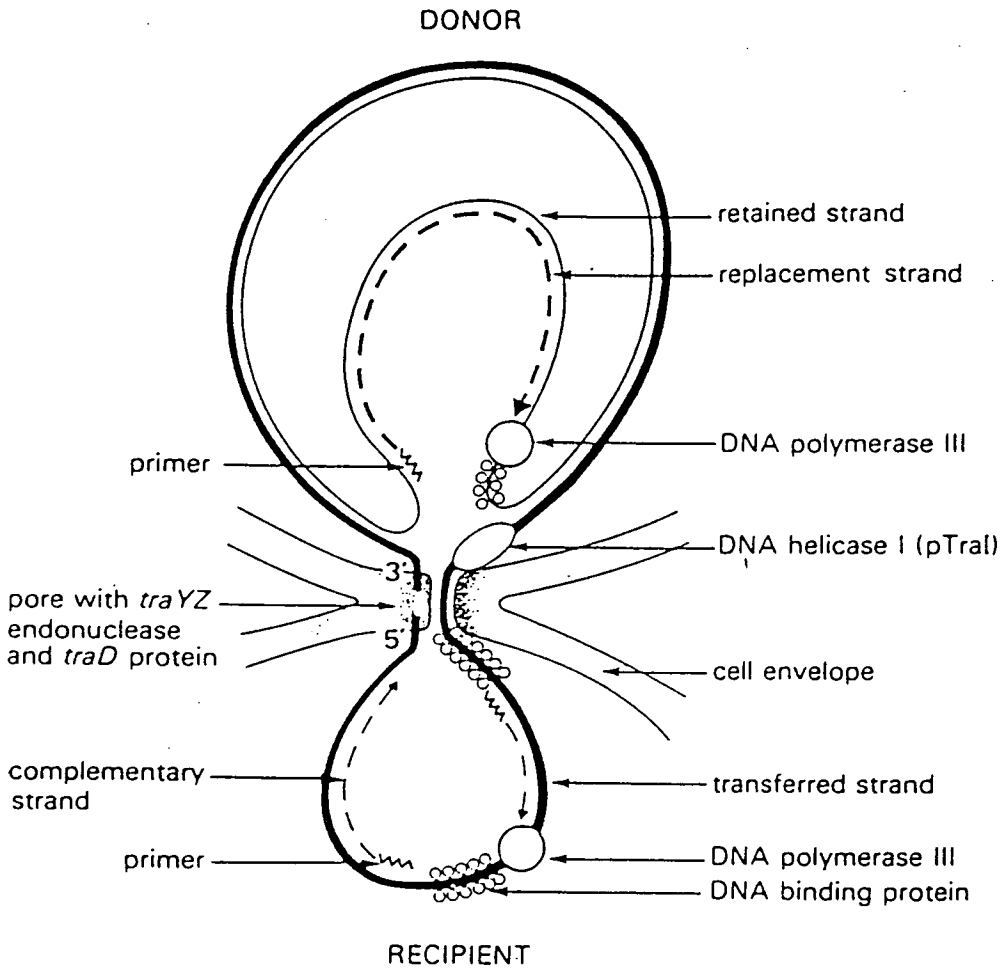
(b) Conjugation Systems

Knowledge of the mechanism of transfer is mainly restricted to F (reviewed by Willetts and Skurray, 1980) and so although other transfer systems have been shown to be genetically distinct (Alfaro and Willetts, 1972; Willetts, 1977a), they are assumed to have the same basic components; mating pair formation, conjugative DNA metabolism and surface exclusion. These will be outlined below with special reference to F.

Twenty-two genes of F, covering a third of the molecule (30Kb), have been identified thus far as being involved in conjugation (Fig. 1.2). Twenty of these genes are transcribed as a single major operon under the positive control of the traJ product (Finnegan and Willetts, 1973; Willetts, 1977b). The remainder of the transfer genes can be conveniently divided into functional groups concerned with mating pair formation, conjugative DNA metabolism and surface exclusion.

Mating pair formation is the first step in conjugation and is the process which brings about cell contact. At least 13 gene products traALEKBVWCUFQHG are involved in this process by directing synthesis of the sex pilus. Sex pili are extracellular filamentous organelles which are responsible for recipient cell recognition. All conjugative plasmids so far examined, in gram-negative bacteria, produce pili (Bradley, 1980a) but these pili are very different showing the diversity of transfer systems. As a consequence pili provide an important means of classifying conjugative plasmids since they differ in their morphology, serology, sensitivity to male-specific phage and in their ability to allow conjugation on solid or in liquid media (Bradley, 1980b; Bradley et al., 1980). traG is also required together with traN for stabilisation

Fig. 1.3



A model for the conjugative transfer of F. a specific strand of the plasmid (thick line) is nicked at oriT by the traYZ endonuclease and transferred in the 5' to 3' direction through a pore formed between the juxtaposed donor and recipient cell envelopes. The termini of this strand are attached to the cell membrane by a complex that includes the endonuclease. DNA helicase I (traI product) migrates on the strand undergoing transfer to promote unwinding of the DNA. The plasmid strand retained in the donor cell is shown by a thin line. DNA transfer is associated with synthesis of a replacement strand in the donor and of a complementary strand in the recipient cell (broken lines); both processes require de novo primer synthesis and the activity of DNA polymerase III holoenzyme. The model assumes that a single-strand binding protein coats DNA and is transferred between the conjugating cells. (Taken from Willetts and Wilkins, 1983.)

of the mating pairs once recipient contact has been made (Achtman and Skurray, 1977; Manning et al., 1981).

When mating pairs have formed DNA transfer can begin. In F this involves the transfer of a single pre-existing strand from the donor to the recipient, where the transferred strand is recircularised and its complement synthesised, whilst the strand retained in the donor serves as a template for synthesis of DNA to replace that transferred (Rupp and Ihler, 1968; Ohki and Tomizawa, 1968; Vapneck and Rupp, 1970). This overall process is termed conjugative DNA metabolism (reviewed by Willetts and Wilkins, 1983).

The traMYDIZ gene products are known to be involved in this process (Kingsman and Willetts, 1978; Everett and Willetts, 1980). traD mutants were shown to have a reduced level of donor conjugal DNA synthesis (D.C.D.S.) (Kingsman and Willetts, 1978) and, consistent with its location in both inner and outer membranes (Achtman et al., 1979), it was suggested that the primary role of the traD product was in transfer of the single-strand, disruption of the transfer had a secondary effect on the rate of DNA synthesis.

Genetic experiments demonstrated that the transfer of F was initiated from a specific cis-acting site, oriT (Willetts, 1972; Guyer and Clark, 1976). More recently the region containing the oriT has been accurately mapped (Everett and Willetts, 1980) and sequenced (Thompson et al., 1983). Characterisation of an in vivo system for nicking at oriT has allowed the accurate location of the nick site and has demonstrated that the endonuclease activity is attributable to the traY and Z gene products (Everett and Willetts, 1980; 1982).

The traM product, although not required for pilus synthesis, formation or stabilisation of mating pairs, or nicking at oriT, is essential for DNA transfer and conjugative DNA synthesis (Kingsman and Willetts, 1978). A role of triggering transfer has therefore been suggested for it. The traI product has recently been demonstrated to be DNA helicase I (M. Abdel-Monem, cited in Willetts and Wilkins, 1983) and its role has therefore been postulated to be that of unwinding the DNA strands of F for transfer (Fig. 1.3).

Both donor and recipient have roles in conjugal DNA metabolism. In the recipient the synthesis of the complementary strand of F is thought to be discontinuous as the 5' terminus is transferred first

(Rupp and Ihler, 1968; Ohki and Tomizawa, 1968). Consistent with this, an RNA primer is required for synthesis of the complementary strand by DNA polymerase III (Wilkins and Hollom, 1974). This RNA primer may be synthesised by RNA polymerase or, in the case of some conjugative plasmids other than F, the plasmid may encode a primase e.g. RP1 (Lanka and Barth, 1981), ColI (Wilkins et al., 1981). Recircularisation in the recipient is recA-independent and does not require expression of any plasmid gene in the recipient (Hiraga and Saitoh, 1975). As the 3' end of the nick site could not act as a primer for DCDS in vivo (Kingsman and Willetts, 1978) and the 5' end is modified (Everett and Willetts, 1980) it was proposed that both termini are protein bound and that this aided recircularisation in the recipient. DCDS, although not essential for transfer, requires an RNA primer and DNA polymerase III (Kingsman and Willetts, 1978). This primer is not synthesised by the primosome as it occurs independently of the dnaB protein (Bresler et al., 1968) and so the likely candidate is either RNA polymerase (Kingsman and Willetts, 1978) or a plasmid primase (Chatfield et al., 1982).

The third component believed to be common amongst transfer systems is that of surface exclusion. Two gene products traS and T are involved in reducing the recipient ability of an F^+ cell. These products are not required for transfer per se (Achtman et al., 1980). Surface exclusion has been shown to be a property of other conjugative plasmids (Barth, 1979; A.M.C. Brown, 1981).

Although all conjugative plasmids probably have the same basic components in their transfer system (discussed above) direct comparisons with F should be carefully drawn. The conjugative plasmids RP1, R91-5, R751 and Ti have their transfer genes in distinct regions (Barth et al., 1978b; Moore and Krishnapillai, 1982; Meyer and Shapiro, 1980; Holsters et al., 1980) and certain conjugative plasmids, those coding for rigid pili, only transfer efficiently on solid media (Bradley et al., 1980) implying that they have different transfer mechanisms.

(c) Mobilisation Systems

The mobilisation of ColE1 by F was the first mobilisation system identified (Clowes, 1963) and as a consequence it has been extensively characterised. ColE1 has since been shown to be mobilised by a wide

variety of conjugative plasmids (Reeves and Willetts, 1974; Warren et al., 1979; Willetts, 1981; Chapter 3) but as F is the most characterised of these it is ColE1's relationship with F that has been most studied. The cloacinogenic non-conjugative plasmid CloDF13 is also mobilised efficiently by F and R64-11 (IncIα) and its mobilisation system has also been characterised (Van de Pol et al., 1978; Van de Pol, 1980). RSF1010 mobilisation by IncP plasmids therefore represents an entirely different mobilisation system to be investigated. As many analogies are drawn between the mobilisation of RSF1010 by RP1 and that of ColE1 by F throughout this thesis, the mobilisation system of ColE1 will be described below.

Clewell and Helinski (1969) demonstrated that ColE1 DNA could be induced, by ionic detergents or a protease, to convert from a covalently closed circular form to an open circular form by introduction of a nick in the DNA. This involved a protein-DNA complex and the DNA was said to be relaxed when in the open circular form. Blair et al., (1971) showed that this nick was specific to the more dense strand (in poly (U,G) gradients) of ColE1. As the replication origin (oriV) of ColE1 mapped very close to the nick site it was assumed to be involved in replication (Lovett et al., 1974a;b). This was later disproved since the nick site could be physically separated from oriV (Bastia, 1978; Tomizawa et al., 1977). Further experiments showed that relaxation involved the association of proteins with the DNA and that the nick occurred at a specific site (Lovett et al., 1974b; Blair and Helinski, 1975). There were three proteins of molecular weights 11Kd, 16Kd and 60Kd associated with the DNA and it was shown that on relaxation the largest protein became covalently attached to the 5' end of the nick (Lovett and Helinski, 1975; Guiney and Helinski, 1975). The nucleotide sequence surrounding the relaxation nick site was determined and the actual nick site was shown to be over 250bp away from the origin of replication (Bastia, 1978). A role in the transfer of ColE1 was suggested for the nick site and this was confirmed by genetic experiments.

Hershfield et al. (1976) isolated a deletion mutant of ColE1 that was unable to transfer and could not be relaxed. Transposons were also used to generate insertion mutants of ColE1 that were of a similar phenotype and a correlation was noted between the ability to form

relaxation complexes and the ability to transfer (Inselberg, 1977a; b; Dougan and Sherratt, 1977; Warren and Sherratt, 1977). It was shown that these mutants could be complemented for transfer by wild-type ColE1. This demonstrated that a *trans*-acting product was supplied by the non-conjugative plasmid. The fact that a second small non-conjugative plasmid ColK, but not ColE2, could complement mobilisation deficient (*mob*$^-$) mutants of ColE1 implied that there was some specificity of interaction between the protein and the DNA. It was suggested that this site of interaction was at the origin of transfer, *oriT*, which might be the relaxation nick site (Inselberg, 1977b; Warren and Sherratt, 1977).

This was confirmed by Warren *et al*. (1978) who proposed that as the *oriT* site, by definition, is *cis*-acting, then when cloned into another non-mobilisable, non-relaxable plasmid, it should confer on this replicon the ability to be relaxed and mobilised, if the *mob* proteins are supplied in *trans*. This was shown to be true and a model was proposed for ColE1 mobilisation. This involved the covalent linkage of a relaxation protein to the 5' side of the nick site. The DNA was then transferred as a single-strand with the 5' terminus leading. Recircularisation occurred by recognising either the 3' end of the molecule or a second resynthesised *oriT* site. This model however is based on the assumption that ColE1 transfers a single strand with the 5' terminus leading as has been shown for F. This model has still not been proven and analogies with F must be carefully drawn as although it is transferred from a unique site, *oriT*, no relaxation complex has been associated with F *oriT* (Johnson *et al*., 1981). The assumption that *oriT* is the relaxation nick is substantiated by further examples of the *oriT* site and the relaxation site being mapped to the same region (Guiney and Helinski, 1979; Nordheim *et al*., 1980)

The insertion (Inselberg, 1977a; Dougan and Sherratt, 1977) and deletion mutants of ColE1 (Inselberg and Ware, 1977) showed that the mobilisation region was contained within a 2Kb segment of the ColE1 genome adjacent to *oriT* (Dougan *et al*., 1978; Warren *et al*., 1978; Fig. 1.4a). Analysis of proteins synthesised in mini-cell derivatives containing these various mutant plasmids has failed to conclusively identify any proteins which may be synthesised from the mobilisation region. Proteins of 16Kd and 11Kd were tentatively identified as being those involved in the relaxation complex (Inselberg and Applebaum

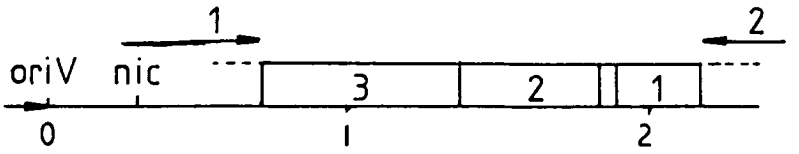
Fig. 1.4 Comparative genetic maps of the mobilisation regions of ColE1 and CloDF13

(a) Map of the mobilisation region of ColE1. The map is based on that of Dougan et al. (1978) and a nucleotide sequence of part of this region Oka et al. (1981). Kilobase co-ordinates extend from the origin of replication, and the arrow indicates the direction of replication fork movement. nic is the putative origin of transfer. The horizontal line indicates the extent of the mobility region mapped using transposon inserts (Dougan et al., 1978) and the vertical lines show the approximate limits of segments defining three complementation groups mob-1, 2 and 3. (Inselberg and Ware, 1979). Direction of transcription across the mobilisation region is conflicting; Ebina et al. (1979) suggest transcription is left to right (arrowed, 1) while complementation and mapping data suggests that at least mob-1 and 2 are transcribed from right to left (arrow, 2) (Inselberg and Applebaum, 1978; Inselberg and Ware, 1979) (Taken from Willetts and Wilkins, 1983).

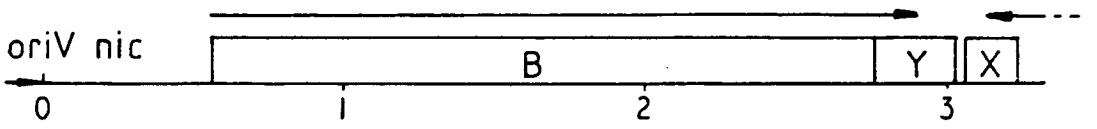
(b) Map of the mobilisation region of CloDF13. (Van de Pol, 1978; Veltkamp and Stuitje, 1981). The Kilobase co-ordinates extend from the origin of replication (oriV) and the arrow indicates the direction of replication fork movement. There are three mobilisation genes B, Y and X and their direction of transcription is indicated. nic is the putative origin of transfer.

FIG.1.4

a)



b)



1978; Dougan and Sherratt, 1977), but no other workers have identified these proteins and all fail to agree on any mobilisation protein (Meagher et al., 1977; Ebina et al., 1979; summarised by Collins, 1979).

Inselberg and Ware (1979) carried out a complementation analysis using their insertion and deletion mutants (Inselberg and Ware, 1977) and a set of point mutants. They tentatively identified three complementation groups, which could be roughly mapped (Fig. 1.4a). Two of the groups would be sufficient in size to code for the two small relaxation proteins (16Kd and 11Kd), however, there was not enough DNA to code for the 60Kd protein unless it overlapped the other two complementation groups. Two of the insertion mutants were known to affect the product of a 15Kd protein (Inselberg and Applebaum, 1978). However, these were in different complementation groups and so it was suggested that these had a polar effect on transcription. The direction of transcription, towards the oriT site, although in agreement with the earlier work of Inselberg and Applebaum (1978), was in contrast to that of Ebina et al. (1979). One way of reconciling this data would be to suggest that there are overlapping genes transcribed in both directions. However, more evidence is needed to clarify these conflicting data and to demonstrate that proteins involved in mobilisation are not supplied by the host.

The plasmid CloDF13 is similar in many ways to ColE1 (see Warren and Clark, 1980; Veltkamp and Stuitje, 1981). It too has a cis-acting sequence required for mobilisation and three gene products have been shown to be involved in mobilisation (Van de Pol et al., 1978; Van de Pol, 1980; Veltkamp and Stuitje, 1981; Fig. 1.4b). Although both ColE1 and CloDF13 can be mobilised by F and R64-11, they cannot complement mob$^-$ mutants of each other (Van de Pol, 1980), again demonstrating the specificity of their mob products and oriT. Neither of the plasmids requires any of the conjugal DNA synthesis genes of F so far tested (traMIZ) (Willetts, 1980), which suggests they carry all the necessary information for their conjugal DNA metabolism. They do however require all the F genes for mating pair formation (Alfaro and Willetts, 1972; Willetts and Maule, 1979; Van de Pol et al., 1980) and in addition ColE1, but not CloDF13, requires the traD product (Willetts, 1980). This suggests a specificity of interaction between the conjugative and non-conjugative plasmid, as would the fact that

not all conjugative plasmids will mobilise a particular non-conjugative plasmid (Willetts, 1981). This is examined in more detail in Chapter 3.

(d) IncQ Plasmids

The highly efficient mobilisation of the IncQ plasmid, RSF1010 by IncP plasmids (Willetts and Crowther, 1981) represents a completely different mobilisation system to those previously studied. In addition, as the broad-host-range of IncQ plasmids (Table 1.1) has been exploited to allow the generation of broad-host-range vectors, then the mobilisation system of these plasmids warrants detailed investigation.

The mobilisation of a Sm^RSul^R (SSu) plasmid by the IncI plasmid, Δ, was first described in detail by Anderson (1968). The prototype Sul^RSm^R plasmid, NTP2, was used to classify a whole range of similarly resistant, independent, naturally occurring plasmids, within the same incompatibility group (Smith et al., 1974). Milliken and Clowes (1973) characterised NTP2 as a small 8.5Kb multicopy plasmid. Guerry et al. (1974) characterised another Sul^RSm^R plasmid isolated from E. coli by Frédéricq et al.(1971); this plasmid, which they called RSF1010, had identical properties to the Sul^RSm^R plasmid, NTP2. Barth and Grinter (1974) compared the molecular weights and homologies of plasmids isolated from Sul^RSm^R strains from a wide geographical and taxanomic distribution and showed they had considerable sequence homologies. They later compared the incompatibility properties of these Sul^RSm^R plasmids with RSF1010::TnA (Heffron et al., 1975). These were incompatible with each other but compatible with all other characterised plasmids and were assigned to a new incompatibility group, IncQ (Grinter and Barth, 1976). The IncQ plasmids therefore have a broad-host-range, the ability to be mobilised and are Sul^RSm^R. The four prototype plasmids NTP2, RSF1010, R1162 (Bryan et al., 1972; Meyer et al., 1982a), R300B (Barth and Grinter, 1974) are very similar if not identical (Barth and Grinter, 1974; Grinter and Barth, 1976; Barth, 1979; Meyer et al., 1982a).

It was shown by transpositional inactivation that the Sul^R and Sm^R genes were transcribed as a single operon from a promoter proximal to the Sul^R gene (Heffron et al., 1975; Rubens et al., 1976; Fig. 1.1). The replication origin of RSF1010 was mapped by de Graaf et al. (1978), 2.6Kb from the EcoRI site (Fig. 1.1). Replication was shown to proceed either uni- or bi-directionally from this site at equal frequencies.

The terminus of bi-directional replication was not a genetically defined site. IncQ plasmids are multicopy but estimates of the actual copy number vary between 15 and 60 (Bagdasarian and Timmis, 1981; Meyer et al., 1982a). Unlike some multicopy plasmids e.g. ColE1, IncQ plasmids are not amplified in the presence of chloramphenicol implying that their replication is not repressed by a protein (Grindley and Kelly, 1976). At least one replication function of IncQ plasmids is physically separable from oriV (Meyer et al., 1982a;b; Bagdasarian et al., 1981; 1982), which is similar to the IncP broad-host-range plasmids e.g. RK2 (Thomas et al., 1980) R751 (Meyer and Shapiro, 1980).

The broad-host-range, relatively high copy number and ability to be mobilised give IncQ plasmids unique properties, which can be readily exploited to allow the generation of broad-host-range vectors that can be mobilised between hosts without the need for a transformation system. A restriction map of RSF1010 is shown in Fig. 1.1 and it can be seen that there is a sparsity of hexanucleotide sites suitable for cloning. A wide range of cloning vectors has therefore been constructed from RSF1010 with restriction sites more suitable for cloning (Bagdasarian et al., 1979; 1981; 1982; Bagdasarian and Timmis, 1981; Barth et al., 1981). By using these broad-host-range, mobilisable, cloning vectors the genetic analysis and manipulation of soil bacteria, and the pseudomonads in particular, whose many metabolic activities are of great scientific, medical, agricultural and economic importance, may now be possible.

Chapter 2

MATERIALS AND METHODS

(a) Growth Media and Buffers

L-Broth contained, per litre: Difco Bacto Tryptone, 10g; Difco Bacto yeast extract 5g; NaCl, 5g; pH 7.2.

Nutrient Agar: Oxoid no.2 Nutrient Broth, 25g; Davis New Zealand agar, 12.5g per litre.

M9 Minimal Agar: Davis New Zealand agar, 20g; NH_4Cl, 1g; NaCl, 0.5g; Na_2HPO_4, 7g; KH_2PO_4, 3g; $MgSO_4 . 7H_2O$, 0.4g; Glucose, 2.5g; Thiamine, 2µg per litre.

BBL Agar: Baltimore Biological Laboratories trypticase, 10g; NaCl, 5g; Difco agar, 10g per litre.

BBL Top Agar: As BBL agar, but only 6.5g Difco agar per litre.

Phage buffer contained, per litre: KH_2PO_4, 3g; Na_2HPO_4, 7g; NaCl, 5g; 0.1M $MgSO_4$, 10ml; 0.01M $CaCl_2$, 10ml; 1% (w/v) gelatin, 1ml.

TE buffer: 10mM Tris-HCl (pH 8.0), 1mM EDTA.

TES buffer; TE containing 1% (w/v) NaCl.

TAE buffer: 40mM Tris, 20mM Sodium acetate, 5mM EDTA (pH 8.2).

TBE buffer: 90mM Tris, 89mM boris acid, 2.5mM EDTA.

(b) Plasmids and *Escherichia coli* K12 Strains

See Tables 2.2 and 2.3.

(c) Conjugation Techniques

Generally all matings were carried out with static overnight cultures. In the cloning experiments, when selection for *oriT* needed to be most efficient, mid-exponential cultures were used and the matings were filter matings. All transfer and mobilisation frequencies stated throughout this thesis are the average of at least two experiments. Prior to counting, 25 donor cell colonies were patched on nutrient agar, grown for 6 hours at 37°C and replicated onto selective media to confirm the presence of the plasmid marker. When transfer of two or more different plasmids from the same donor strain was selected on different media and the proportion of recipients receiving more than one plasmid needed to be determined, 50 transconjugants

Table 2.1 Final concentration of additives to minimal and nutrient media.

Antibiotics	Minimal (μg/ml)	Nutrient (μg/ml)
Ampicillin (Penbritin)	50	50
Chloramphenicol (Chloromycetin)	50	50
Kanamycin (Kantrex)	25	20
Nalidixic Acid	40	40
Spectinomycin	100	20
Streptomycin		
(low)	20	20
(high)	500	500
Sulphonamide (Sulphadimidine)	100	-
Tetracycline (Achromycin)	10	20
Trimethoprim	50	-
Amino Acids	20	-

Note: When plating out transfections of M13 recombinants on CMK603, 25μl of 2% X-gal (5-Bromo-4-Chloro-3-Indolyl-β-D-Galactopyranoside) and 25μl of 2.5% IPTG (Isopropyl-β-D-Thiogalactopyranoside) were added to 3ml of BBL top agar.

Table 2.2 Plasmids

Plasmid	Phenotype, Genotype or Description	Incompatibility Group	Reference/Source
RSF1010	Sul^R, Sm^R; Mob^+	Q	Guerry et al., 1974
pKT228	Sul^R, Sm^R; RSF1010::Tn3 Mob^-	Q	Badgasarian et al., 1982
pKT229	Sul^R, Sm^R; RSF1010::Tn3 Mob^-	Q	Bagdasarian et al., 1982
pKT260	Sul^R, Sm^R; RSF1010;;Tn3 Mob^-	Q	Bagdasarian et al., 1982
pKT19	Sul^R, Sm^R; RSF1010::Tn3 Mob^-	Q	Bagdasarian et al., 1982
RSF1010 Δ18	Sul^R, Sm^R; Mob^- Δ derivative of RSF1010	Q	Bagdasarian et al., 1982
RSF1010 Δ5	Sul^R, Sm^R; Mob^- Δ derivative of RSF1010	Q	Bagdasarian et al., 1982
RSF1010 Δ20	Sul^R, Sm^R; Mob^- Δ derivative of RSF1010	Q	Bagdasarian et al., 1982
PDS1101	Ap^R, Iel^+ ColE1::Tn1		Dougan and Sherratt, 1977
RSF2011	Ap^R, Iel^+ Cel^+ ColE1::Tn3		So et al., 1975
pML2	Km^R, Iel^+ mini-ColE1		Hershfield et al., 1974
NTP24	Sul^R, Sm^R		H. Smith
R831a	Km^R, Sm^R		Barth et al., 1978a
NTP5	Tc^R		Smith et al., 1974
NTP6	Ap^R		Smith et al., 1974
NTP1	Ap^R		Anderson and Lewis, 1965
NTP11	Km^R		Grindley and Kelley, 1976
pBR322	Ap^R, Tc^R		Bolivar et al., 1977
pED825	Ap^R; Δ derivative of pBR322		Everett and Willetts, 1982
RP1	Ap^R, Km^R, Tc^R, Tra^+	P	Sykes and Richmond, 1970

Plasmid	Phenotype, Genotype or Description	Incompatibility Group	Reference/Source
pM0481	cis-dominant Tra$^-$ mutant of R18	P	Stokes et al. (1981)
pED525	RP1 Tra$^-$	P	Watson et al., 1980
pED591	RP1 Tra$^-$	P	L. Schmidt, 1981
pED600	RP1 Tra$^-$	P	L. Schmidt, 1981
pED623	RP1 Tra$^-$	P	L. Schmidt, 1981
pED516	RP1 Tra$^-$	P	Watson et al., 1980
pED615	RP1 Tra$^-$	P	Watson et al., 1980
pED562	RP1 Tra$^-$	P	Watson et al., 1980
pED511	RP1 Tra$^-$	P	Watson et al., 1980
pUB307	Km^R, Tc^R, Tra$^+$; Ap^S Δ derivative of RP1	P	Bennett et al., 1977
R751	Tp^R, Tra$^+$	P	Jobanputra and Datta, 1974
JCFL0	F lac	FI	Achtman et al., 1971
R64-11	Tc^R, Sm^R, Tra$^+$	Iα	Meynell and Datta, 1967
pED904	Ap^R, Spc^R, Tra$^+$; Δ derivative of R46	N	Brown and Willetts, 1981
pED918	Ap^R, Spc^R, Tra$^+$; Δ derivative of R46	N	Brown and Willetts, 1981
pAr-32	Cm^R, Sul^R, Tra$^+$	U	Bradley et al., 1982
R388	Sul^R, Tp^R, Tra$^+$	W	Datta and Hedges, 1972
R6K	Ap^R, Sm^R, Tra$^+$	X	Heffron et al., 1975
Tp231	Ap^R, Tc^R, Cm^R, Tra$^+$	X	Bradley, 1980a
R446b	Tc^R, Sm^R	M	Hedges et al., 1973
R471a	Ap^R, Km^R, Cm^R	M	Hedges et al., 1975
Rts1::Tn1725	Km^R, Cm^R, Non-Permissive at 42°C	T	R. Schmitt

Table 2.3 *Escherichia coli* K12 strains

Strain	Relevant Genotype	Reference or Source
JC3272	*his lys trp str gal lac* Δx74	Achtman *et al.*, 1971
JC6310	*recA56 lys*$^+$ derivative of JC3272	Willetts 1975
ED8654	*supE supF metB hsdR*$^-$ *hsdM*$^+$	Borck *et al.*, 1976
ED24	Spc^R T6^R p1^R	N. Willetts
ED3886	*recA*$^-$ derivative of ED24	J. Maule
ED4134	*lac* ΔU124 T6^R	N. Willetts
CMK603	Restriction-minus derivative of JM101	MRC Laboratory of Molecular Biology, Cambridge
JM101	Δ(*lac pro*), *thi*, *strA*, *supE*, *endA sbcB*, F'*traD*, *proAB*, *lacI*, *lacZ*ΔM15	
DS410	Str^R, mini-cell producing strain	D. Sherratt
ED3818	Na1^R derivative of JC3272	N. Willetts
ED3826	Col^R derivative of JC3272	N. Willetts

of each type were patched and replicated.

(i) Broth matings

0.2ml of donor cell culture were added to 1.8ml of recipient in a glass test-tube (18mm x 150mm) and left to stand in a water bath at 37°C for 30 minutes. The culture was then chilled briefly on ice and diluted in phage buffer. 0.1ml volumes of appropriate dilutions were then spread onto selective plates. These selective plates had additives to select for transconjugants (Table 2.1). Donor cells were usually contraselected with streptomycin or spectinomycin. Viable counts of donor cells were obtained by plating 0.1ml of a 10^{-5} dilution of donor culture on nutrient agar. Colonies were counted after incubation for approximately 1 day (nutrient agar) and 2 days (minimal agar).

(ii) Filter matings

0.2ml of donor and 1.8ml of recipient were mixed in a chilled 5ml bijou bottle and drawn up into a 2ml syringe. The cells were then carefully filtered through a 20mm diameter membrane filter (Millipore Corporation, pore size 0.45μm), and the filter transferred to a pre-warmed nutrient plate. After incubation at 37°C for 30 minutes the filter was introduced into a 20ml chilled Universal bottle containing 2ml of fresh L-broth and the cells resuspended by vigorous agitation on a Whirlimixer for 30 seconds. 0.1ml volumes of appropriate dilutions were spread onto selective plates and incubated at 37°C. Donor colonies and transconjugants were checked as above.

(iii) Plate matings

0.1ml volumes of appropriate dilutions of donor culture and undiluted recipient culture were spread directly onto selective plates and incubated at 37°C. Particular resistances of some plasmids e.g. the Km^R plasmid pML2, could not be selected in this way and so mobilisation experiments with this plasmid were always carried out using filters.

(iv) Replica-Plate matings

This type of mating was used as a preliminary screen to distinguish between *mob*$^+$ and *mob*$^-$ mutants. Donor colonies to be

screened were patched onto nutrient plates and incubated at 37°C for 6 - 8 hours. They were then replicated onto a selective plate that had been spread with 0.1ml of the recipient. Patches could be screened + or - after overnight incubation at 37°C.

(v) Spot matings

This type of mating was also used as a screen to distinguish between mob$^+$ and mob$^-$ mutants. It was more quantitative than (iv) as approximate levels of transfer could be estimated. A transconjugant selective plate was spread with 0.1ml of the recipient. An appropriate set of dilutions of the donor was then made and 50µl spots applied to the surface of the plate. In this way three or four matings, with three dilutions per mating, could be carried out on a plate. After the spots had dried the plates were inverted and incubated overnight at 37°C.

(d) Plasmid DNA Preparations

(i) Small-scale cleared lysate preparations for screening purposes

1.5ml of stationary phase cultures were pelleted in 1.5ml 'snap-cap' polypropylene microfuge tubes using an Eppendorf microfuge (30 seconds). The cells were resuspended in 50µl lysis buffer (35mM Tris-HCl, pH 8.0; 70mM EDTA; 18%(w/v) sucrose; 1mg/1ml lysozyme (sigma)) and incubated at 37°C for 5 minutes. 50µl of Triton lysis solution were then added (0.2% Triton X-100(v/v); 50mM Tris-HCl, pH 8.0; 60mM EDTA), mixed, and the tubes left to stand at room temperature for 10 minutes. The lysates were cleared by centrifuging (Eppendorf microfuge, 5 minutes) and the viscous pellets carefully removed with pasteur pipettes. 40µl of supernatants were then mixed with 10µl of cleared lysate loading buffer (SDS 5% (w/v); Ficol 10% (w/v); Bromophenol blue 0.1%(w/v), in TAE buffer), incubated at 65°C for 10 minutes before loading onto agarose gels for electrophoresis. Approximate sizes of plasmids were subsequently estimated from the mobility of supercoiled closed circular forms on the gel, with reference to supercoiled molecules of known size.

(ii) Small-scale plasmid DNA preparations

This was based on the method of Birnboim and Doly (1979). If

DNA prepared in this way was to be used to visualise small restriction fragments after electrophoresis, it was predigested with ribonuclease A (1µg/1ml, 65°C, 30 min, Sigma).

(iii) Large-scale purification of plasmid DNA

This was based on the method of Humphreys et al. (1975). In some cases DNA was purified further by digestion with ribonuclease (as in (ii)) followed by extraction with phenol, extraction with ether and ethanol precipitation. DNA concentration and protein contamination were estimated from measurements of OD_{260} and OD_{280} of a forty-fold dilution, using a Unicam spectrophotometer. Protein contamination was considered negligible if the OD_{260}/OD_{280} ratio was ≧1.7.

(e) DNA Techniques

All DNA solutions were stored at 4°C in TE buffer.

(i) Ethanol precipitation

DNA was precipitated by the addition of 0.1 vol 3M sodium acetate (pH 5.0) and 2 vols of ethanol. This was usually carried out in a microfuge tube. The DNA was then precipitated at -70°C for 15 minutes followed by centrifugation (Quickfit microfuge, 5 minutes, 4°C). Pellets were washed with ethanol, left at -70°C for a further 5 minutes, centrifuged again for 2 minutes and dried in a vacuum dessicator, prior to resuspension in TE buffer.

(ii) Restriction endonuclease digestions

Appropriate quantities of DNA, restriction endonuclease, 10x restriction buffer, and distilled H_2O were mixed thoroughly in a microfuge tube and incubated at 37°C (65°C for TaqI) for at least one hour. Digestion was stopped by incubating at 65°C for 10 minutes (or by phenol extraction for TaqI). 10x restriction buffer was: 0.3M Tris-Acetate (pH 7.9); 0.1M magnesium acetate; 0.66M potassium acetate; 5mM dithiothreitol; 1µg/1ml nuclease-free Bovine serum albumin; except for HaeII restriction when the 10x restriction buffer was 500M Tris-HCl (pH 7.5); 50mM Magnesium Chloride; 60mM 2-mercaptoethanol. Restriction endonucleases used are listed in Table 2.4. They were obtained from Boehringer Mannheim GmbH, Bethesda Research Laboratories Inc., New England Biolabs Inc.

Table 2.4 Restriction endonucleases used and their recognition sites.

Restriction Endonuclease	Recognition Site
AccI	GT↓(AG/CT)AC
AluI	AG↓CT
AvaI	C↓PyCGPuG
BamHI	G↓GATCC
ClaI	AT↓CGAT
EcoRI	G↓AATTC
HaeII	PuGCGC↓Py
HindIII	A↓AGCTT
HinfI	G↓ANTC
MspI	C↓CGG
PstI	CTGCA↓G
SalI	G↓TCGAC
Sau3A	↓GATC
SmaI	CCC↓GGG
TaqI	T↓CGA

(iii) DNA fragment purification

Agarose gels

After the DNA had been electrophoresised, the gel was stained with ethidium bromide to localise the required band. This fragment was then cut out of the gel using a scalpel. The slice of agarose was placed in dialysis tubing containing 1ml of TE. The tubing was then immersed in a shallow layer of TAE in an electrophoresis tank and the DNA electroeluted at 200V for 30 minutes. The polarity was reversed for 2 minutes to release the DNA from the wall of the dialysis tubing. The TE was then removed from the dialysis tubing and the DNA purified as follows.

A small DEAE-cellulose (Whatman, DE52) column was constructed in a 200µl gilson tip. The column was washed twice with 1ml of TE before the DNA containing solution was passed through it. The DNA was then eluted with 0.5ml of TE containing 2M NaCl. The eluate was then extracted with phenol, extracted with ether and ethanol precipitated. Fragments prepared in this way were suitable for ligation.

Acrylamide gels

Small fragments (<1Kb) suitable for sequencing, ligating and restricting were prepared in this way. The method was identical to that of Maxam and Gilbert (1977) except that the gel elution buffer was 500mM ammonium acetate, 10mM magnesium acetate, 1mM EDTA, 0.1% sodium dodecyl sulphate (w/v) (pH 8.0). tRNA (Sigma) was added at 10µg/ml.

(iv) In vitro recombination of DNA fragments

DNA restriction digests were diluted to 10 - 40µg/ml with TE (pH 7.2). 0.1 vol of 10x T4 ligase cocktail (660mM Tris-HCl (pH 7.2); 10mM EDTA; 100mM $MgCl_2$; 100mM DTT; 10mM ATP) were added together with T4 ligase (New England Biolabs Inc.). Ligations were carried out at 16°C for 3 - 16 hours and stored, if necessary, at -8°C.

(v) End-labelling of DNA fragments with [^{32}P]

Filling recessed 3' ends of double-stranded DNA

1µCi of [α-^{32}P]-labelled nucleoside triphosphate (dCTP, Amersham Radiochemicals) in ethanolic solution was dispensed into a microfuge

tube containing 5µl sterile H_2O, and dried in a vacuum dessicator. 1µg of linearised DNA in 30µl of restriction buffer was added, together with the 3 remaining unlabelled dNTPs (50µM final conc.), and Klenow polymerase (1u/µg) (Boehringer). The mixture was incubated for 30 minutes at 37°C.

Terminally labelling the 5' ends of double-stranded DNA

Linearised DNA was initially dephosphorylated by incubation with Calf Intestinal Phosphatase (a gift from B.M. Will) for 30 minutes at 37°C in the restriction buffer. The enzyme was inactivated by the addition of EDTA (20mM final conc.) and heating to 65°C for 10 minutes. The DNA was then ethanol precipitated. Sufficient [γ-^{32}P]-ATP (3000 Ci/mMole. Amersham Radiochemicals) was added to the dessicated DNA and again vacuum dessicated. 10µl of H_2O were added to the tube and again vacuum dessicated. Finally the mix was re-suspended in 7µl H_2O and 2µl of 5x kinase buffer (100mM Tris-HCl (pH 7.5); 50mM $MgCl_2$; 5mM β-Mercaptoethanol). 2µl of T4 polynucleotide kinase (5 units/µl Collaborative Research Inc.) were added and the mixture was incubated at 37°C for 2 hours. Unincorporated label was removed by the addition of 200µl of 2.5M ammonium acetate and 800µl of cold ethanol. The DNA was then precipitated at -70°C for 15 minutes and pelleted in a microfuge (5 minutes, 4°C). The pellet was then dissolved in 100µl 0.3M sodium acetate and precipitated with 500µl ethanol at -70°C for 15 minutes. The DNA was again pelleted by centrifugation (microfuge, 4°C, 5 minutes), washed with 500µl ethanol and dried in a vacuum dessicator.

(vi) Transformation of E. coli with DNA

1ml of fresh overnight culture was diluted to 20ml in L-broth, grown to early log phase by shaking at 37°C, chilled and pelleted (5,000g, 5 minutes). The cells were resuspended in 10ml ice-cold $MgCl_2$ (100mM) and immediately re-centrifuged (5,000g, 5 minutes). After resuspension in 10ml $CaCl_2$ (75mM), they were left on ice for 20 minutes before pelleting (as before). The cells were finally re-suspended in 1ml 75mM $CaCl_2$, 15% Glycerol (v/v). The competent cells were either used immediately for transformation or stored at -70°C until needed. DNA for transformation (100 - 500ng) was diluted to

0.1ml with TE and added to 0.2ml of competent cells on ice. After 30 minutes the cells were transferred to 42°C for a 5 minute heat shock. The cells were then cooled briefly before adding 0.5ml L-broth and shaking the cells at 37°C. The expression period allowed before plating on selective media varied depending on the antibiotic.

(f) Protein Analysis: Labelled Proteins Synthesised in Minicells

Minicells prepared from plasmid-carrying DS410 were purified as described by Johnson et al. (1981). 20μCi of [^{35}S] methionine (Amersham Radiochemicals) were used to label the proteins as described by Johnson et al. (1981). Samples were subjected to electrophoresis on sodium dodecyl sulphate/polyacrylamide gels. Gels were normally run overnight in denaturing SDS buffer (0.025M Tris-HCl; 0.19M glycine; 0.1% SDS) at 13mA. The gel was then fixed (45%(v/v) methanol, 9% acetic acid (v/v)) for 10 minutes at 37°C. It was then stained for 15 minutes at 37°C with stain (0.1% coomassie brilliant blue (BDH R250)(w/v), 0.1% Cupric acetate (w/v) in fix solution). The gel was then destained with three washes of destain (7% acetic acid (v/v), 5% methanol (v/v)) for a total of 3 hours. Finally the gel was dried down onto Whatman 3MM paper using a Bio-rad gel drier (2hr) and exposed against Fuji Rx film at room temperature. The sizes of the labelled proteins were estimated by comparison with molecular weight standards (BRL Protein Marker Set 6,000 SA).

(g) Gel Electrophoresis

(i) Horizontal agarose gels

Agarose slab gels (15cm x 25cm x 0.5cm; 0.7% - 1.5% agarose (w/v) in 200ml TAE buffer) were placed between two perspex tanks (each containing 500ml TAE buffer) and absorbent wicks ('Access-matting', saturated with TAE buffer) were positioned at each end to make electrical contact between the gel and buffer tanks. 30μl DNA samples were mixed with 10μl loading buffer (0.1% (w/v) bromophenol blue, 10% (w/v) Ficol, in TAE) and loaded by Gilson automatic pipette. Each well was then filled to the top with TAE and initial electrophoresis carried out at 150V for 15 minutes. After this period the

wells were again filled with TAE, the gel covered with a thin polythene sheet ('Saran-Wrap'), and electrophoresis resumed at 45V for approximately 16 hours. After running, the gel was stained with ethidium bromide (Sigma, 5µg/ml) for 15 minutes, and destained in H_2O for 15 minutes. DNA was visualised by placing the gel on a long-wave UV transilluminator (Ultraviolet Products Inc., 365mm peak transmission). Photography was with Ilford FP4 film and an exposure time of 10 seconds through a red filter (Hoya R (25A)).

(ii) Polyacrylamide Gels (Maniatis et al., 1975)

5% or 8% (w/v) acrylamide gels in TBE buffer were prepared by mixing appropriate volumes of 37.5% (w/v) acrylamide and 2% (w/v) bis-acrylamide stock solutions with 10xTBE and H_2O. The acrylamide to bis-acrylamide ratio was 29:1 (w/v) and the gel volume either 50ml or 80ml. When run overnight the gel also contained 5% (v/v) glycerol. 0.5ml of 10% (w/v) ammonium persulphate and 60µl TEMED (N,N,N',N' - tetramethylethylenediame, Serva) were added to initiate polymerisation. The mixture was then poured between two vertical glass plates (either 20 x 20cm or 20 x 30cm) separated by 1.5mm perspex spacers at the side edges, and sealed at the bottom edge with water agar. A toothed perspex well-former was inserted at the top of the gel immediately after pouring, and 30 - 60 minutes allowed for polymerisation. The well-former and seal were then removed and the gel connected to a perspex gel apparatus containing 1 litre TBE buffer in each tank. 20µl DNA samples (previously digested with RNAseA if prepared by the method of Birnboim and Doly (1979)) were mixed with 5µl of loading buffer (50% (v/v) glycerol; 0.125% (w/v) Xylene cyanol FF; 0.125% (w/v) bromophenol blue; in TBE) and loaded with a Hamilton micro-syringe. Large gels (20 x 30cm) were run overnight at 200V, small gels (20 x 20cm) were run for 3 hours at 310V. Gels were stained for 5 minutes in ethidium bromide (5µg/ml), de-stained for 10 minutes in H_2O, and DNA bands visualised and photographed as in (i).

(iii) Sodium dodecyl sulphate/gradient polyacrylamide gels for minicell analysis

Labelled samples were subjected to electrophoresis in 10% - 20% gradient gels. Equal volumes (18ml) of 10% acrylamide (10% (w/v)

acrylamide; 0.27% (w/v) bis-acrylamide; 0.1% (w/v) SDS; 0.37M Tris-HCl pH 8.8) and 20% acrylamide (20% (w/v) acrylamide; 0.53% (w/v) bis-acrylamide; 0.1% (w/v) SDS; 0.37M Tris-HCl pH 8.8) solutions were placed into separate chambers of a 2-chamber linear gradient-maker. 50µl of 10% (w/v) ammonium persulphate and 5µl of TEMED were added to each before allowing the chambers to mix and pour between a glass sandwich (20 x 30 x 0.1cm) with teflon spacers. The gradient was allowed to polymerise after being overlaid with 1ml of H_2O. After the H_2O was removed a 3% stacking gel (3% (w/v) acrylamide; 0.8% (w/v) bis-acrylamide; 1% (w/v) SDS; 0.062M Tris-HCl pH 6.8) was poured on top using 200µl of 10% ammonium persulphate and 20µl TEMED to initiate polymerisation. A teflon comb was used to make wells. After polymerisation the gel was connected up to a perspex gel apparatus and electrophoresised as in (f).

(iv) Sequencing gels (Sanger and Coulson, 1978)

8% polyacrylamide gels were used for all sequencing by the method of Sief et al. (1980). Urea (8M) was included in the gels which had an acrylamide to bis-acrylamide ratio of 20:1. A 60ml volume of acrylamide solution in TBE was generally used and 50µl of TEMED and 1.95ml of 1.6%)w/v) ammonium persulphate added immediately before pouring to initiate polymerisation. The mixture was then poured between two glass plates (40 x 20cm) separated by 0.35mm 'plasticard' strips. Wells were formed using a toothed plasticard comb and the gel allowed to polymerise for 30 minutes before connecting to a sequencing gel apparatus with 500ml of TBE in each buffer tank. Gels were run at 1.1KV for approximately two hours. After separating the plates the gel was fixed in 10% acetic acid for 10 minutes and then washed in H_2O for 10 minutes before drying down onto Whatman 3MM paper using a Bio-rad gel drier (1 hour). The gel was then autoradiographed overnight.

6% polyacrylamide gradient gels were used for all sequencing carried out in Chapter 8. The buffer gradient gels were prepared exactly as described by Biggin et al. (1983) for (20 x 40cm) gels.

(v) Estimation of DNA fragment sizes

The migration distances of standard fragments (of known molecular weight) were plotted against the logarithm of their sizes (using semi-log

graph paper) and the sizes of unknown fragments on the same gel determined from the graph. The size standards were HindIII restriction fragments of EDλ7 (23.9, 9.6, 6.64, 4.45, 2.29, 1.95, 0.59, 0.13 Kb, Daniels et al., 1980; Willetts and McIntire, 1978) and various restriction fragments of pBR322 (Sutcliffe, 1978) or pED825 (Everett and Willetts, 1982).

(h) Transposon Mutagenisis with Tn1725 (R. Schmitt person. comm.)

Donor strain RU2901 (Rts1::Tn1725) and the recipient (JC6310) (containing the replicon to be mutagenised) were grown separately at 30°C overnight. Equal volumes were mixed and further incubated without shaking at 30°C for 90 minutes. Aliquots were plated on media selective for the transfer of Rts1::Tn1725 to JC6310 and incubated at 30°C. Transconjugants were patched onto nutrient medium and incubated at 30°C before replicating onto chloramphenicol plates and incubating at42°C for 24 hours to eliminate Rts1. Throughout the above procedure selection was always made for the antibiotic resistance carried by the target plasmid, to prevent insertion into those genes. The target plasmid was then isolated (Birnboim and Doly, 1979) and transformed into JC6310, this eliminated Cm^R isolates that had Tn1725 in their chromosome, selecting for Cm^R transformants. Plasmids were then isolated and the point of insertion of Tn1725 mapped by appropriate restriction enzyme digestion. Only one Cm^R transformant, from DNA prepared from a single patch, was kept to prevent the isolation of siblings. By the above procedure fifty independent transposon insertions could be easily isolated. A restriction map of Tn1725 is presented in Figure 4.8.

(i) Hydroxylamine Mutagenisis (Humphreys et al., 1975)

Mutagenisis was carried out as described by Humphreys et al. (1976). 300ng of mutagenised DNA were used to transform ED8654 (R751). The expression period after transformation was only 15 minutes to prevent siblings. Appropriate aliquots were then plated onto selective media for RSF1010 (Sul^R) or pED350 (Ap^R). Mob^- mutants were then screened for by the lack of ability to be mobilised in patch matings (2(c)(iv)) with JC3272 as the recipient.

(j) Nucleotide Sequencing

(i) The oriT clone pED360 was sequenced by the method of Maat and Smith (1978) which was modified by Sief et al.(1980). The sequencing was carried out as described by Sief et al. (1980) with the following exceptions. 0.1µl deoxyribonuclease (2×10^{-5} mg/ml, Sigma) was added per reaction mix to introduce nicks into the DNA and DNA polymerase 'large fragment' was always used. Each reaction mix consisted of: 8µl 5x buffer; 5µl DNA polymerase (1u/µl, Boehringer); 0.1µl Deoxyribonuclease; 18.5µl of terminally labelled ^{32}P DNA. This mixture was then aliquoted into each of the forward and backward reaction tubes (4µl per tube). These contained the nucleotide solutions (1µl) as described by Sief et al. (1980). The 'plus' reactions were not carried out. The reactions were carried out at 37°C for 20 minutes and then stopped by the addition of 5µl of formamide dye (99% formamide (deionised) containing 10mM EDTA, 0.1% Xylene Cyanol FF, 0.1% Bromophenol Blue). The samples were then boiled for 2 minutes and 3µl loaded onto a sequencing gel.

(ii) The region of RSF1010 between the AvaI sites at 2Kb and 5.2Kb (Fig. 1.1) was sequenced by the M13 sequencing method as described by Sanger et al. (1977), Sanger et al. (1980) and more recently by Deininger (1983), Biggin et al. (1983). All the cloning, sequencing and computing was carried out at the MRC Laborabory of Molecular Biology, Hills Road, Cambridge in collaboration with Dr G. Hatfull using the equipment and laboratories of Dr B. Barrell and Dr F. Sanger. I am very grateful to these people who allowed me the opportunity to sequence this region and also to Alan Bankier and Mark Biggin for advice on the sequencing and computing. The sequencing was carried out by randomly cloning sonicated fragments of RSF1010 into M13mp8 and then sequencing by the modified dideoxy method of Biggin et al. (1983) using [^{35}S]-dATP and buffer gradient gels. The data was then analysed using the computer programme of Staden (1982).

Approximately 10µg of the AvaI fragment of RSF1010, containing the mobilisation genes, was purified from an agarose gel as in (e)(iii) and cloned into M13mp8 by Dr G. Hatfull according to the procedure of Deininger (1983) with the following modifications. After ligation of the purified fragment the DNA was sonicated using a sonicator

(Heat Systems Ultrasonics W-375 sonicator) which had been filled with cold H_2O to a depth of 1" above the probe. Sonication was 4 x 40 seconds with a brief spin in a microfuge to drive the DNA back to the bottom of the tube in between each sonication. The H_2O was also replaced after 80 seconds. The ends of the sheared DNA were repaired overnight at 15°C using DNA polymerase (large fragment). The DNA was then size-fractionated in an agarose gel and samples of DNA 200 - 600 bpr long extracted and purified. Appropriate amounts of this sonicated DNA were then ligated to M13mp8 (Messing and Vieira, 1982; gift of G. Hatfull) predigested with SmaI and calf-intestinal phospatase before transforming CMK603 and screening for white plaques, i.e. recombinants.

Recombinant DNA was then prepared as described by Sanger et al. (1980) except that the cultures were only grown for 5 hours and the DNA resuspended in a final volume of 30µl of TE(pH 8.5) before freezing at -20°C.

The reaction and annealing procedure was carried out as described by Biggin et al. (1983) for sequencing with [^{35}S]α-thiodATP (Amersham International, specific activity 400 Ci/mmol). The nucleotide reaction mixes were as follows:

'T' : 0.125mMdCTP; 0.125mMdGTP; 0.00625mMdTTP; 0.25mMddTTP
'C' : 0.125mMdGTP; 0.125mMdTTP; 0.00625mMdCTP; 0.04mMddCTP
'G' : 0.125mMdTTP; 0.125mMdCTP; 0.00625mMdGTP; 0.08mMddGTP
'A' : 0.125mMdTTP; 0.125mMdCTP; 0.125mMdGTP; 0.005mMddATP

2µl of each of these reaction mixes was dispersed into capless Sarstedt tubes with 2µl of primed template. The [^{35}S]-dATP was aqueous and was dispensed with the DNA polymerase I (large fragment), 2µl per tube, after being diluted in 10mMDTT, 10mMTris (pH 8.0) to 8µl. 1.5 units of DNA polymerase and 4 µCi of ^{35}S-dATP were used per set of four reactions. These tubes were then centrifuged briefly to initiate the reactions. After 20 minutes at room temperature 2µl of chase (0.25mMdNTPs) were added and the reactions left for a further 15 minutes. Reactions were then stored at -20°C or 2µl of dye mix was added immediately before boiling. Samples were boiled for 3.25 minutes before loading onto a buffer gradient gel.

After autoradiography the sequences were fed into the computer

using a digitiser device and analysed by the automatic DB system of Staden (1982).

All sequencing work carried out in Cambridge was a joint collaboration with Dr G. Hatfull.

Chapter 3

MOBILISATION OF NON-CONJUGATIVE PLASMIDS

(a) Introduction

Mobilisation of ColE1 by the F factor of *E. coli* has long been documented (Clowes, 1963). ColE1 has since been shown to be mobilised by a wide variety of F-like plasmids (Reeves and Willetts, 1974) as well as plasmids from other incompatibility groups with genetically dissimilar transfer systems, such as R388 (IncW), R751 (IncP) and R64-11 (Inc-Iα) (Warren *et al.*, 1979). The efficiency of mobilisation of ColE1 by these plasmids varies considerably, from 100% mobilisation by F to 1% mobilisation by R388 (compared to the conjugative plasmid's transfer). This suggests that there is a specific interaction between the transfer system of the conjugative plasmid and the mobilisation system of a successfully-mobilised non-conjugative plasmid.

Four requirements must be satisfied for a non-conjugative plasmid to be mobilised by a conjugative plasmid. The first is that the non-conjugative plasmid must have an origin of transfer, which includes the nick and recircularisation site. Secondly, the *oriT* site must also contain the recognition sites for mobilisation proteins which nick, transfer and recircularise the DNA. The conjugative plasmid must supply a mating pair system so that efficient cell contact is made. The conjugative plasmid may supply, in addition to the mating pair system, a product required for transfer such as the *traD* product in ColE1 mobilisation by F. Finally, there must be an interaction between the mobilisation system and mating pair system to allow recognition of the 'mobilisation complex' and its subsequent transfer. This latter interaction is distinct from the recognition of the *oriT* and its preparation for transfer and to avoid confusion will be called the mobilisation recognition system throughout this thesis. An excellent example of the specificity of this mobilisation recognition system is demonstrated by ColE1 and ColK mobilisation by F and R64-11. ColE1 *mob*$^-$ mutants can be complemented by ColK when mobilised by R64-11. This implies that their mobilisation products must be very similar. However, unlike ColE1, ColK is not mobilised by F (Young and Poulis, 1978) and so ColK is not recognised by the F mobilisation recognition system.

There is a general sparsity of information on the ability of non-conjugative plasmids to be mobilised by conjugative plasmids and as a consequence it is difficult to observe any particular pattern involved in the mobilisation recognition system. To generate a more comprehensive base from which to investigate such a recognition system, a survey of the mobilisation ability of non-conjugative plasmids by a variety of conjugative plasmids was undertaken. This survey would also identify non-conjugative plasmids capable of very efficient mobilisation, other than ColE1, whose mobilisation would warrant further investigation.

(b) Non-Conjugative Plasmids

For the purposes of this investigation it was preferable to survey as wide a range of independently isolated non-conjugative plasmids as possible. The following plasmids were chosen:

(i) RSF1010

RSF1010 is a $Sul^{R}Sm^{R}$, broad-host-range, multicopy IncQ plasmid (see Introduction). IncQ plasmids can be mobilised by a variety of transfer factors (Anderson, 1968; Smith et al., 1974; Guerry et al., 1974; Bryan et al., 1971; Barth and Grinter, 1974), but RSF1010, in particular, has been shown to be efficiently mobilised by IncP plasmids (Willetts and Crowther, 1981). As a consequence of this latter work and the fact that RSF1010 has been developed as a broad-host-range cloning vector (Bagdasarian et al., 1979, 1981a), RSF1010 was chosen for more detailed study.

(ii) ColE1

Mobilisation of ColE1 by various transfer factors has been studied (Reeves and Willetts, 1974; Warren et al., 1979) but the data are not comprehensive. The mobilisation system of ColE1 has, to some extent, been characterised (see Introduction) and so this made it an obvious choice for inclusion. Ampicillin or kanamycin resistant derivatives of ColE1 were used to provide selection for ColE1 transconjugants.

(iii) NTP24

NTP24 is a $Sul^{R}Sm^{R}$, non-conjugative plasmid that is compatible with IncQ plasmids (H. Smith, pers. comm.). It is identical in size and has exactly the same restriction patterns for HaeII and AvaI (data not

shown) as an independently isolated $Sul^R Sm^R$, non Inc-Q plasmid, pBP1 (Van treek et al., 1981); they are probably identical (H. Smith, pers. comm.). DNA heteroduplex analysis suggests that RSF1010 and pBP1 have similar $Sul^R Sm^R$ genes but there is no detectable homology between their replication and mobilisation regions (Van Treek et al., 1981).

(iv) NTP1

NTP1, an Ap^R non-conjugative plasmid isolated from S. typhinurium type 29 (Anderson and Lewis, 1965), is mobilised by the I-like transfer factor Δ. Grindley and Kelley (1976) showed that it required DNA polymerase I for replication like the bacteriocinogenic plasmids ColE1 and CloDF13 and can replicate in the presence of chloramphenicol. Nucleotide sequence analysis has also revealed many similarities between the replication regions of these plasmids (Grindley and Nakada, 1981). The β-lactamase gene for Ap^R resides on a transposon Tn1701 (Calame et al., 1979), which is very similar to Tn3. Mini-cell analysis has shown that NTP1 encodes at least six proteins in addition to β-lactamase, but it is not known whether they are plasmid or transposon encoded (Grindley et al., 1977).

(v) NTP6

NTP6, an Ap^R plasmid isolated by Smith et al. (1974) is also mobilised by Δ. A small amount of homology does exist between NTP1 and NTP6 (Smith et al., op. cit.) but these plasmids are probably not related as they were shown to be different in size, copy number (Smith et al., op. cit.), restriction pattern (data not shown) and, as will be described, mobilisation properties. The homology between the two plasmids may be a consequence of their having similar β-lactamase genes (Smith et al., op. cit.).

(vi) NTP5

NTP5 is a Tc^R, non-conjugative plasmid isolated by Smith et al. (1974). It was shown to have no replication requirement for DNA polymerase I (Grindley and Kelley, 1976). The only other known non-conjugative plasmid with similar properties is pSC101 (Cohen and Chang, 1973), which is now recognised to be the same as the naturally occurring plasmid Sp219 (Van Embden and Cohen, 1973; Cohen and Chang, 1977). NTP5, apart from several extra fragments, was shown to have similar restriction enzyme patterns for HaeII and AvaI as Sp219 (data

not shown) implying they may be closely related. NTP5 was chosen as the representative of this particular class of plasmid. Sp219 can be mobilised by various conjugative plasmids (Van Embden and Cohen, 1973; Guerry et al., 1974; Nordheim et al., 1980).

(vii) NTP11

NTP11 is a Km^R, non-conjugative plasmid and is similar to NTP1 in that it requires DNA polymerase I for replication and is amplified in the presence of chlorampehnicol (Grindley and Kelley, 1976).

(viii) R831a

R831a is a $Sm^R Km^R$, non-conjugative plasmid isolated from Serratia marcesens (Barth et al., 1978a).

(c) Conjugative Plasmids

Plasmids are classified by their inability to coexist in the same bacterial cell (incompatibility). Amongst naturally occurring plasmids these incompatibility groups also represent different conjugation systems. Ideally in a comprehensive survey, a representative plasmid should be chosen from each conjugation system. However this was not feasible and so plasmids were chosen from different conjugation systems based on the classification of pilus type (Bradley, 1980a;b). Pili encoded by plasmids from all the incompatibility groups have been examined by electron-microscopy and classified on a morphological basis into three types: thin flexible; thick flexible and rigid. Pili synthesised by plasmids from within an incompatibility group or sub-set of a complex of groups such as the IncF complex, are not only morphologically similar but are also serologically related. The IncI complex is an exception as it has two unrelated serotypes. The ability of a plasmid to transfer in liquid or on solid media can be correlated with the type of pilus it encodes (Bradley et al., 1980).

Plasmids determining rigid pili, those of Inc groups M, N, P, U (Bradley et al., 1982) and W, have an absolute preference for mating on solid media i.e. they transfer several thousand times more efficiently on solid media than in liquid culutre. This is thought to be a consequence of the rigid pilus being very fragile and, therefore, easily broken in liquid culture thereby preventing cell-to-cell contact. Representatives of all five incompatibility groups were used in this survey.

Plasmids determining thin flexible pili such as those of the IncI complex, K and B have equally efficient transfer frequencies in liquid or on solid media. The IncIα group was chosen as representative of this particular pilus type.

The third class of plasmid, those producing thick flexible pili, can be subdivided into two types depending on preference for transfer on solid or in liquid media. The majority of the Inc groups show no preference e.g. IncF complex, J, V. Plasmids belonging to groups C, D, T and X show a preference for mating on solid medium. The transfer frequencies of these plasmids are generally several hundred-fold more efficient on a solid surface. It was suggested that the reduced efficiency in liquid culture was due to a weak linkage between pili and either the donor or recipient, which would cause breakage of cell contact in liquid culture, where the shearing forces are greater than on solid media. Flac was chosen to represent those plasmids which transfer equally well on solid or in liquid culture and IncX plasmids to represent those which transfer better on solid medium.

(d) Mobilisation Survey

Strains carrying both the non-conjugative plasmid and the conjugative plasmid were constructed and the transfer frequency of each plasmid measured. Plate matings were generally used as the plasmids coding for rigid pili only transfer efficiently on solid media. The transfer frequency of each conjugative plasmid was compared using a mid-exponential culture and a static overnight (late-exponential) culture. As the frequencies of transfer between the two culture types were similar, overnight cultures were always used as sources of donor and recipient cells. The results of the survey are presented in Table 3.1

It is immediately obvious from the table that there is no pattern of mobilisation. All the non-conjugative plasmids, except R831a, are mobilised efficiently by at least one conjugative plasmid and so the differing efficiencies of mobilisation are a consequence of the recognition of a mobilisation system by a particular conjugative plasmid. The mobilisation efficiencies can be conveniently divided into three groups: high (1), such as RSF1010 mobilisation by R751; medium (10^{-3}), such as NTP6 mobilisation by R751; low mobilisation

Table 3.1 Ratios [b] of mobilisation of non-conjugative plasmids by conjugative plasmids [a]

		Conjugative Plasmids							
Pilus Type		Rigid					Thick Flexible		Thin Flexible
Incompatibility Group		IncP	IncM	IncN	IncU	IncW	IncF	IncX	IncIα
Plasmid and selection used		R751(Tp^R)	R446b(Tc^R)	pED904(Spc^R)	pAr-32(Cm^R)	R388(Tp^R)	Flac(lac^+)	Tp231(Tc^R)	R64-11(Tc^R)
% Transfer frequency [c]		1.2	2.3	4.7	11.0	5.0	15.5	0.95	0.3
non-conjugative plasmid	antibiotic selected								
RSF1010	Sul^R	22	4	3.5×10^{-4}	ND	4×10^{-4} [e]	6.3×10^{-4}	4.23	1.6
NTP24	Sul^R	4.4	2.6×10^{-3}	4.4×10^{-2}	ND	ND	1.3×10^{-4}	ND	9×10^{-6}
NTP5	Tc^R	.24	ND [i]	$<10^{-5}$ [d]	5.2×10^{-2}	$<10^{-6}$	$<10^{-7}$	3.3×10^{-3} [g]	ND
NTP6	Ap^R	1.5×10^{-3}	4.7×10^{-3}	ND	1.9×10^{-3}	6.5×10^{-4}	4.7×10^{-7}	ND	.36
NTP1	Ap^R	$<10^{-6}$	1.1×10^{-3}	ND	6×10^{-6}	$<10^{-7}$	$<10^{-7}$	ND	1.1
ColE1 [f]	Ap^R	0.63	1	10^{-6}	0.47	2.2	0.26	4.8×10^{-4} [g]	ND
NTP11	Km^R	8×10^{-3}	0.14	$<10^{-6}$	1.7×10^{-2}	2×10^{-4}	10^{-2}	2.8×10^{-2}	2.2×10^{-2}
R831a [h]	Km^R	5×10^{-3}	6.3×10^{-3}	ND	8.3×10^{-5}	ND	1.78×10^{-4}	ND	9.5×10^{-4}

Table 3.1 (continued)

(a) Unless noted, the donor was ED8654 and the recipient JC3272. The contraselection was Sm^R at 500μg/ml.

(b) The mobilisation ratio is the frequency of mobilisation of the non-conjugative plasmid divided by the transfer frequency of the conjugative plasmid.

(c) Average number of transconjugants per hundred donor cells from all the mobilisation experiments with that particular conjugative plasmid.

(d) pED918 is the conjugative plasmid. Spc^R was used to select its transfer.

(e) The recipient was ED24 and the contraselection was Spc^R. RSF1010 mobilisation was measured using Sm^R at 20μg/ml.

(f) pDS1101 was the ColE1 derivative used except for IncN and IncX mobilisation when pML2 was used and IncW mobilisation when RSF2011 was used. pML2 transfer was selected using Km^R and these were filter matings. The recipient for RSF2011 mobilisation was ED3826 (Col^R derivative of JC3272).

(g) R6K is the conjugative plasmid. Ap^R was used to select its transfer.

(h) This plasmid, unlike all the others used, was unstable and lost at approximately 20%, after overnight incubation.

(i) ND = not done.

($<10^{-5}$), such as NTP1 mobilisation by R751. Presumably this latter level of mobilisation is due to cointegrate formation during transposition (Crisona et al., 1981), while the other two levels of mobilisation reflect the efficiency of the mobilisation recognition system. The efficient level of mobilisation does not take place via cointegrate formation as transposition and recombination occur at low frequencies while mobilisation is a high frequency occurrence. Furthermore, the mobilisation where tested, is independent of RecA (Table 3.2). RSF1010 is mobilised efficiently by IncP, X and Iα plasmids, in agreement with the data of Willetts and Crowther (1981). In addition it was shown that RSF1010 is mobilised efficiently by the IncM plasmid R446b. The mobilisation of RSF1010 was shown to be a general property of IncM plasmids by using a second IncM plasmid, R471a (Bradley, 1980a). R446b and R471a both mobilised RSF1010 efficiently in a $RecA^-$ donor strain confirming that mobilisation does not occur via homologous recombination (Table 3.2). The mobilisation of RSF1010 was not correlated with any particular pilus type, as even though it is mobilised by both IncP and IncM plasmids, these plasmids synthesise pili which are not serologically related (Bradley, 1980b). Furthermore, IncN plasmids which synthesise rigid pili do not mobilise RSF1010, whereas IncX and Iα plasmids encoding flexible pili do mobilise RSF1010, in contrast to IncF plasmids.

ColE1 derivatives were mobilised efficiently by R388 (IncW), R751 (IncP) and Flac (IncF) in agreement with previous data (Clowes, 1964; Warren et al., 1979). In addition it was also shown that the IncU (pAr-32) and IncM (R446b) plasmids mobilised ColE1 efficiently. This was not due to homologous recombination as transfer was equally efficient in a $RecA^-$ donor (Table 3.2). Warren et al. (1979) reported that apart from the main mobilisation region (A) of ColE1 required for mobilisation by Flac and R64-11, there are three additional regions (B, C and D) required for mobilisation by R751 and R388. Initially in the survey two ColE1::TnA derivatives were used, pDS1101 and RSF2011. In RSF2011, Tn3 is inserted in a region not required for mobilisation and so the plasmid is therefore mobilised equally efficiently by R64-11, R388, R751 and Flac. pDS1101, however, has Tn1 inserted in a region (C) essential for mobilisation by R751 and R388. In contrast to this, pDS1101 and RSF2011 were mobilised by

Table 3.2 Mobilisation in a RecA$^-$ donor[a]

Conjugative Plasmid	Incompatibility	Non-conjugative Plasmid	Ratio of Mobilisation[b]
R446b	M	RSF1010	2
R471a	M	RSF1010	0.23
R446b	M	pDS1101	.9
Flac	F	NTP11	0.3
pAr-32[c]	U	NTP11	0.25
pAr-32[c]	U	pDS1101	0.3
pAr-32[c]	U	NTP5	0.119

(a) Donor strain was JC6310 and recipients were ED24 unless otherwise stated. The contraselection was Spc^R.

(b) The ratio of mobilisation frequency to transfer frequency.

(c) The recipient was ED3818 and the contraselection was Nal^R.

R751 with equal efficiency. This contradicts the proposal of Warren et al. (1979) that there were extra regions required for mobilisation of ColE1, at least as far as region C is concerned.

One interesting anomaly was noted for pML2, a Km^R derivative of ColE1 (Hershfield et al., 1974), when it was used to test mobilisation efficiency by Flac in plate matings. In contrast to the data of Warren et al. (1979) there was no mobilisation of ColE1 by F (Table 3.3). However, when the mating was repeated in broth and then plated on selective plates, transfer levels in agreement with those previously found were obtained. In a control experiment, the mobilisation of pDS1101 (ColE1::Tn1) was tested and shown to be equally efficient in broth and on plates. It seems therefore that the plasmid pML2 requires an expression period after transfer before transconjugants can grow on kanamycin selective plates. For this reason all other matings involving this plasmid were carried out on filters. This phenomenon has also been observed with RP1 (where transfer measured by selection for Km^R is approximately 10% of that if measured when Tc^R is selected), but not with R831a or NTP11 (Table 3.3). This is presumably a consequence of the enzyme involved in the resistance phenotype.

Table 3.3 Effect of kanamycin selection on transfer.

Conjugative Plasmid (a)	Non-conjugative Plasmid	Selection	Surface	Mobilisation Ratio (b)
Flac	pML2	Km^R	Liquid	0.28
Flac	pML2	Km^R	Plate	$<10^{-7}$
Flac	pDS1101	Ap^R	Liquid	0.24
Flac	pDS1101	Ap^R	Plate	0.04
Flac	NTP11	Km^R	Liquid	0.05
Flac	NTP11	Km^R	Plate	0.01
Flac	R831a	Km^R	Liquid	10^{-4}
Flac	R831a	Km^R	Plate	10^{-4}

(a) The donor was ED8654. The recipient was JC3272 and Sm^R contraselection was used.

(b) The ratio of mobilisation frequency to transfer frequency.

(e) The Interaction of RP1 and RSF1010 in Mobilisation

Non-conjugative plasmids can only be mobilised in the presence of a conjugative plasmid as the conjugative plasmid is required to bring about cell contact by synthesis of the pilus. Mobilisation of ColE1 by Ftra mutants has shown that the traD product, in addition to the products involved in pilus synthesis, is required for efficient mobilisation (Alfaro and Willetts, 1972), whereas CloDF13 requires only a functional pilus to be supplied to be mobilised by F (Van de Pol et al., 1978; Willetts, 1980). Different non-conjugative plasmids may therefore require that different functions be provided by the conjugative plasmids which mobilise them. The aim of these experiments was to determine if any products of RP1, in addition to those required for pilus synthesis, were required for mobilisation of RSF1010.

RP1 is a plasmid belonging to the incompatibility group P-1. This is of particular interest because of the ability of these plasmids to transfer to, and replicate within, many different Gram-negative bacteria (Appendix B of Bukhari et al., 1977). In this regard its conjugation system is of interest since it must be able to recognise some structure or component common to cell surfaces of the wide range of bacterial genera to which the plasmid can transfer. Preliminary investigations have revealed that RP4, which is thought to be identical to RP1, RK2 and R18 (Grinsted et al., 1977; Appendix B of Bukhari et al., 1977; Burkardt et al., 1979) has three transfer regions, Tra1, Tra2, Tra3, which are physically distinct (Fig. 3.1) (Barth et al., 1978b; Barth, 1979). These regions were identified by mapping transfer deficient insertion mutants of RP1, which were characterised for loss of surface exclusion and phage sensitivity. Mutants showing reduced surface exclusion mapped in Tra2 and Tra3.

Cells harbouring RP1 synthesise P-1 pili (Bradley, 1974) which are assumed to be involved in RP1 conjugal transfer. The P-1 pili adsorb the male-specific phages PRR1, Pf3 and PR4. PRR1 and Pf3 are IncP specific, whilst PR4 is specific for pili produced by P, N and W incompatibility group plasmids (Bradley and Rutherford, 1975). Transfer deficient insertion mutants of RP1 that were still sensitive to all three phage mapped to the Tra1 region (Barth et all, 1978b). In agreement with this, Watson et al. (1980) showed that all the phage-

sensitive point mutants they tested were complemented by a clone carrying the Tra1 region. These mutants, by analogy with F, produce functional pili and are presumably mutated in transfer genes either involved in DNA metabolism or stabilisation of mating pairs. At least four complementation groups (A-D) were found in Tra1 (Barth et al., 1978b) and these corresponded to three different classes of pilus sensitivity (see Fig. 3.1 and Table 3.4). The site of the relaxation nick and origin of transfer have also been mapped to the end of Tra1 nearest to the single EcoRI site (Guiney and Helinski, 1979).

Lanka and Barth (1981) have shown that RP4 codes for a primase (pri) which maps to the opposite end of Tra1 from oriT. This primase is not required for transfer of RP4 in E. coli, however, pri mutants show a slightly reduced transfer into certain bacterial species. R300B (equivalent to RSF1010) does not produce a primase, but does show a very small reduction in mobilisation by some RP4 pri mutants into S. typhimurium and P. mirabilis. The primase coded by RP4 appears to be only required for efficient transfer into certain species which perhaps do not efficiently recognise the RP4 and R300B priming sites for complementary strand synthesis.

Various transfer-deficient point mutants of RP1, isolated in this laboratory by L. Schmidt, were tested for their ability to mobilise RSF1010 to see if any other functions of RP1 play a more important role in RSF1010 mobilisation. Mutants with defective pili would not be capable of mating pair formation and therefore, by analogy with F and ColE1, would be unable to mobilise RSF1010. For this reason mutants in the Tra1 region were of particular interest, as some of these still produced functional pili and so might be mutated in the DNA metabolism genes. One mutant, which is phage resistant and maps outside Tra1 was also used. Four of the mutants mapped to Tra1 (Watson et al., 1981; L. Schmidt, 1981) were phage sensitive and could correspond to either complementation groups A or D (according to Barth et al., 1977; Lanka and Barth, 1981) (see Fig. 3.1, Table 3.4). Two more mutants were phage resistant and would probably belong to complementation group C, while a third mutant was sensitive to PR4 but resistant to PRR1 and Pf3. This probably contains a mutation in complementation group B. The allocation of these mutants to complementation groups is based purely on assumptions from phage sensitivity

Table 3.4 Mobilisation of RSF1010 by RP1 Tra mutants[b]

Probable region of RP1 mutant	Phage sensitivity[d] PRR1	Pf3	PR4	Plasmid	RP1 transfer[a]	RSF1010 Mobilisation[a]
+ type	S	S	S	RP1	.5	.5
oriT	S	S	S	pM0481	1.3×10^{-6}	0.16
A/D	S	S	S	pED525[c]	5.8×10^{-6}	1.1×10^{-4}
A/D	S	S	S	pED591	1.2×10^{-6}	1×10^{-6}
A/D	S	S	S	pED600	6.8×10^{-6}	4.3×10^{-6}
A/D	S	S	S	pED623	1.7×10^{-6}	2.0×10^{-6}
B	R	R	S	pED516[c]	2.7×10^{-7}	4.7×10^{-6}
C	R	R	R	pED615[c]	1.3×10^{-7}	1.7×10^{-5}
C	R	R	R	pED511[c]	1.6×10^{-6}	10^{-5}
Tra2/3	R	R	R	pED562	9.1×10^{-4}	2.9×10^{-4}

(a) The average number of transconjugants per donor.

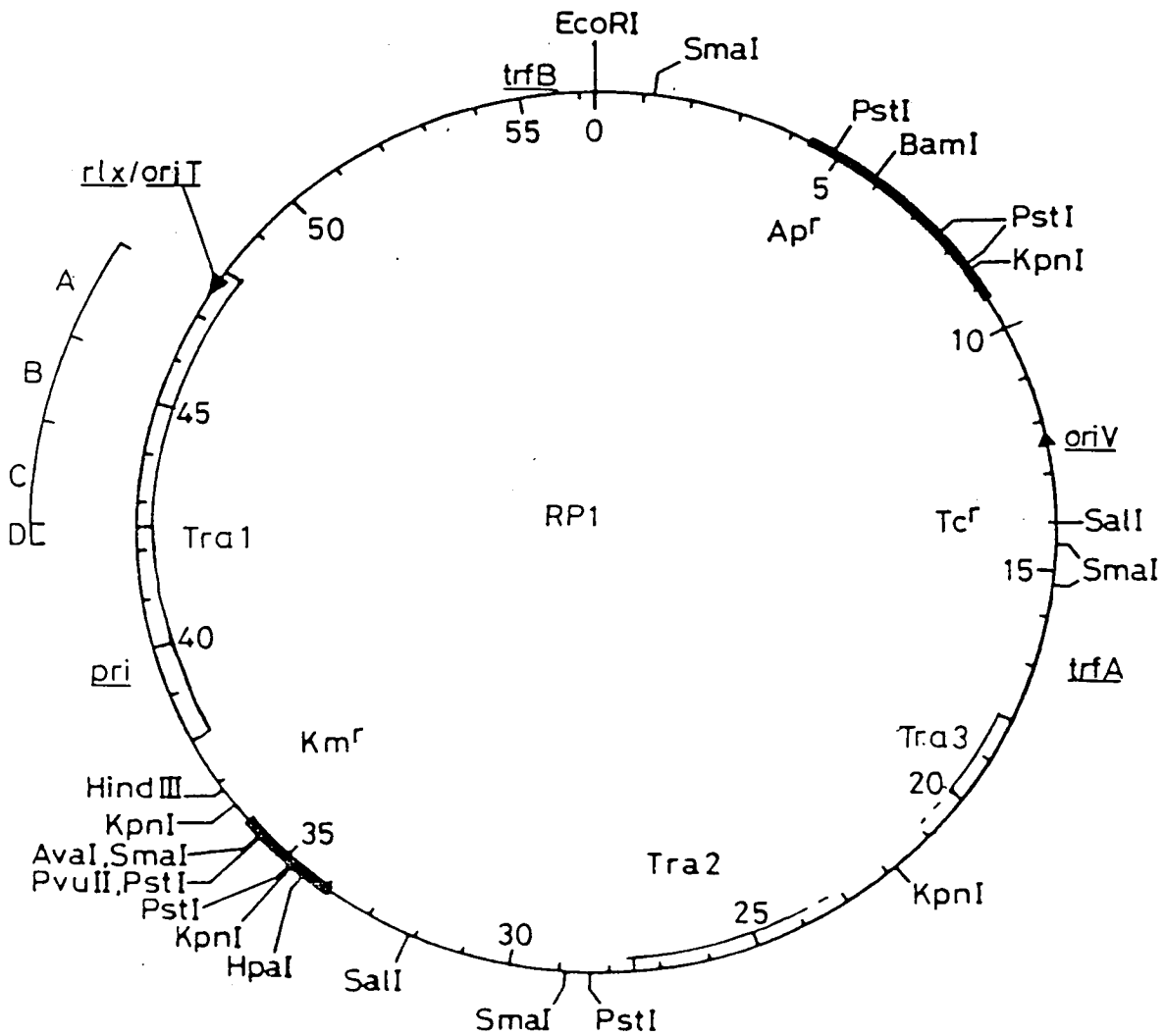
(b) The donor was ED4134 and the recipient was JC3272. The contraselection was Sm^R. All matings were plate matings carried out with overnight cultures.

(c) Data is that of N. Willetts and C. Crowther (personal communication).

(d) S = sensitive R = resistant.

Figure 3.1 A physical and genetic map of the plasmid RP1.

A composite map based on the data of Thomas (1981), Lanka and Barth (1981) and Barth et al. (1978). Key to nomenclature used: oriV, origin of vegetative plasmid replication; trf, regions specifying trans-acting replication or maintenance functions; rlx, site of relaxation complex nick site, oriT origin of conjugal transfer, thought to be the same as rlx; Tra, the regions required for conjugal transfer. A - D represent the complemention groups defined by Barth et al. (1978); pri, the RP1 primase gene as defined by Landa and Barth (1981).



corresponding to the complementation groups identified by Barth et al.(1978b). The final transfer deficient mutant used was a cis-dominant mutation which one would predict, by definition, to be in the origin of transfer of RP1 (Stokes et al., 1981). Table 3.4 lists the mobilisation frequencies of RSF1010 by these various RP1 mutants.

Mutants producing defective pili (i.e. phage resistant) were incapable of transferring themselves and of mobilising RSF1010. This is presumably because they could not form mating pairs. Surprisingly, mutants producing apparently normal pili (i.e. phage sensitive) were not capable of mobilising RSF1010. RP1 must therefore supply at least one essential function, other than in pilus formation, for RSF1010 mobilisation. One would predict, by analogy to F, that this function is probably involved in DNA metabolism or mating pair formation. The DNA metabolism genes of conjugative plasmids are thought to be plasmid specific (see Table 1 of Willetts and Skurray, 1980) and as RSF1010 is mobilised by several unrelated conjugative plasmids it seems unlikely that each conjugative plasmid supplies RSF1010 with a similar DNA metabolism component. It is also known that RSF1010 can be relaxed (i.e. nicked at oriT) in the absence of RP1 (Nordheim et al., 1980) and so this RP1 encoded function required for RSF1010 mobilisation must be involved in transfer of the DNA or stabilisation of mating pairs. Analogous mutants have been isolated in F that are phage sensitive but are not involved in DNA metabolism, these are traD (DNA transfer) and traG and traN (stable mating pair formation).

F transfer is not required for ColE1 mobilisation (Alfaro and Willetts, 1972). Similarly, RSF1010 mobilisation does not need RP1 transfer; the RP1 oriT mutant pMO481, although not transferring itself, does mobilise RSF1010 as efficiently as RP1. Again, further evidence that mobilisation of RSF1010 by RP1 is not via covalent union of the two plasmids.

(f) Discussion

It is difficult to draw any conclusions from the survey when no pattern is observed, but it does emphasise the complexity and specificity of the mobilisation recognition system. The two plasmids, RSF1010 and ColE1, which were efficiently mobilised most often must have the most adaptable mobilisation recognition systems. The

mobilisation recognition system could be the recognition of a common DNA sequence at oriT by a conjugative plasmid encoded protein which would then initiate transfer (Willetts, 1981). Alternatively, it could be the recognition of a mobilisation protein, perhaps in the relaxation complex, which has a common structure recognised by a conjugative plasmid encoded product(s). The former possibility seems less likely as the same recognition sequence would have had to evolve independently in different non-conjugative plasmids which are all recognised by the same conjugation system. Furthermore, this would have to be closely associated with the oriT as these sites can be cloned on very small fragments (Chapter 4). It is simpler to suppose that recognition of a particular component of the transfer or mobilisation system by the conjugative plasmid's mating pair system occurs.

The fact that all the non-conjugative plasmids, other than R831a, were mobilised efficiently by at least one conjugative plasmid, implies that mobilisation plays an important role in nature. Indeed ColE1, CloDF13 and RSF1010 (Dougan et al., 1978; Van de Pol, 1978; this thesis) have approximately a third of their genome involved in mobilisation and the naturally occurring plasmid P15A (Chang and Cohen, 1978), although having a defective mobilisation system, still retains an oriT. It may be that R831a is efficiently mobilised by a conjugative plasmid that was not used or, that like P15A, a second plasmid is required to supply mobilisation functions which will efficiently recognise its oriT i.e. R831a could be $oriT^{+}Mob^{-}$.

Plasmids often only encode genes that give it a transient advantage to their host, such as antibiotic resistance, and one could imagine that these functions would quickly be lost, with the plasmid, in a non-selective environment. However, if the plasmid was capable of mobilisation or transfer it would be able to move between hosts and environments and thereby continue to be transiently selected. Transfer is essentially a replication process which allows plasmid genes to replicate faster than chromosomal genes by virtue of the fact that they can be inherited 'vertically', as a cell devides, as well as 'horizontally' through a population by transfer. The ability of a non-conjugative plasmid to utilise various transfer systems could allow it to spread more rapidly to a wider host range. Very interestingly, RSF1010 is most efficiently mobilised by another broad-host-range

plasmid, RP1 (IncP). One could imagine that the wide distribution of IncQ plasmids throughout the bacterial population (Barth and Grinter, 1974; Smith et al., 1974) is a consequence of its mobilisation by IncP plasmids to these various hosts. The fact that some non-conjugative plasmids do have their own mobilisation genes implies that it is an advantage to be able to recognise their own oriT, rather than rely on a second plasmid to supply this function. Presumably, this would enable the non-conjugative plasmid to be able to utilise a variety of transfer systems.

If it is an advantage for non-conjugative plasmids to be mobilised by conjugative plasmids, then one would expect to find both types of plasmid within the same cell when plasmids are isolated from natural isolates. This has been well documented by Anderson (1968) for the Δ factor and A and SSu determinants. Evidence for this parasitic-type of relationship is accumulating for other plasmids which have very important medical implications. The heat-stable and heat-labile enterotoxins, that cause diarrhoea, are known to be plasmid coded. One particular isolate analysed by Yamamoto and Kokota (1983) has been shown to contain two large non-conjugative plasmids which encode the colonisation factor antigen I, responsible for adhesion of bacterial cells to the intestinal epithelial cells, and both the heat-stable and -labile enterotoxins. In the same isolate a large conjugative plasmid was found which could efficiently mobilise the virulence plasmids. The role of mobilisation in this example could be very important in converting a harmless bacterium into a pathogenic one. Many examples of the mobilisation of virulence plasmids exist and one alarming aspect is that with continual antibiotic treatment the generation of antibiotic resistant virulence factors, or the association of transferable drug resistance plasmids with the virulence factors may occur (Murray et al., 1983).

Penicillin resistance in both Haemophilus and Neisseria has been shown to be due to an almost identical non-conjugative Ap^R plasmid. This plasmid can be mobilised between strains of Neisseria and into E. coli by a cryptic conjugative plasmid found in certain strains of Neisseria (Deneer et al., 1982; Ensenstein et al., 1977). Guiney and Itoh (1982) have also shown that this Ap^R plasmid can be mobilised by IncP plasmids. As the Ap^R on this plasmid is due to part of the

transposon TnA, commonly found on plasmids of enteric gram-negative bacteria, it would seem likely that the dissemination of the β-lactamase gene to pathogenic gram-negative bacteria like Haemophilus and Neisseria has been caused by mobilisation. The medical and environmental implications of mobilisation should therefore never be underestimated.

Chapter 4

PHYSICAL CHARACTERISATION OF THE oriT REGION OF RSF1010 AND RP1

(a) Introduction

Plasmid DNA transfer is initiated at a specific site on the plasmid genome, oriT. This cis-acting site was first assigned in F and was positioned at one end of the transfer region (Willetts, 1972). The strand transferred in F has been identified and transfer is oriented such that the Tra region is transferred last (Ohki and Tomizawa, 1968; Rupp and Ihler, 1968; Vapneck and Rupp, 1970). RP1 also has an oriT located at one end of the transfer region (Tra1) (Guiney and Helinski, 1979; Guiney and Yakobson, 1983) and transfer is again oriented such that the transfer region (or at least Tra1) is transferred last (Aldoori et al., 1982). RP1 can be isolated as a DNA-protein relaxation complex, the nick site of which maps within the same region as the origin of transfer (Guiney and Helinski, 1979). F, however, has no relaxation complex (Johnson et al., 1981) but in vivo a system has been characterised which demonstrates nicking of F within its oriT region by the traYZ gene products (Everett and Willetts, 1980; 1982).

The relaxation complex is implicated in conjugation because the in vitro single-stranded cleavage reaction is thought to mimic a similar in vivo process in transfer. Evidence for involvement of the relaxation complex in mobilisation of ColE1 is particularly strong, as both processes have been well characterised. The relaxation nick site of ColE1 has been located and sequenced (Bastia, 1978) and shown to be within the same restriction fragment as the cis-acting site, oriT. Deletion of this restriction fragment from ColE1 abolished both relaxation and mobilisation, while its inclusion in a non-mobilisable, non-relaxable plasmid allows this plasmid to be relaxed and mobilised (Warren et al., 1978). Insertion mutants of ColE1 that are incapable of being relaxed cannot transfer (Inselberg, 1977b; Dougan and Sheratt, 1977) again implying that relaxation mutants are mobilisation mutants. Other indirect evidence for the relaxation nick site of ColE1 being oriT relies on the physical similarity of the nick to the F oriT nick (Blair et al., 1974; Lovett and Helinski, 1975; Everett and Willetts, 1980; Chapter 1).

Fig. 4.1 HaeII restriction analysis of the four smallest HaeII clones in 1.1% agarose.

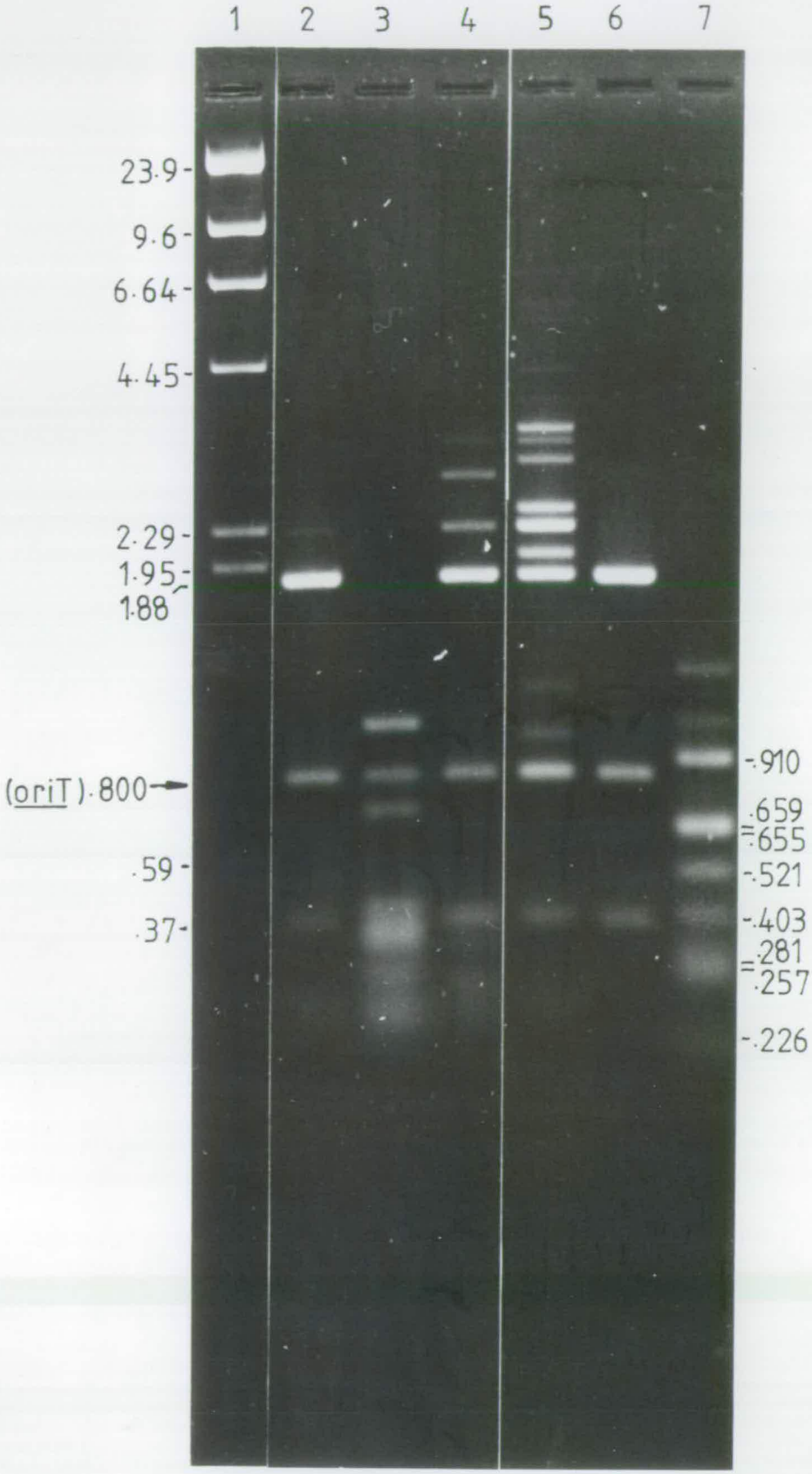
Track	DNA	Restriction Endonuclease
1	EDλ7	HindIII
2	pED354	HaeII
3	RSF1010	HaeII
4	pED350	HaeII
5	pED362	HaeII
6	pED361	HaeII
7	pBR322	AluI

Notes:

(i) Sizes of fragments are marked in Kb. The sizes of the AluI fragments of pBR322 are derived from nucleotide sequence data (Sutcliffe, 1978) as are the HaeII fragments of pED825. The 800bp HaeII fragment size was estimated from this and other gels (not shown).

(ii) The 800bp HaeII fragment common to all four clones and RSF1010 is clearly visible in tracks 2, 3, 4, 5 and 6.

FIG.4.1


1
2
3
4
5
6
7
23·9
9·6
6·64
4·45
2·29
1·95
1·88
(oriT)·800
·59
·37
·910
·659
=·655
·521
·403
·281
=·257
·226

It would appear, therefore, that the relaxation nick site of ColE1 is the same as its oriT and that a site specific nick is introduced by an endonuclease equivalent to the traYZ endonuclease of F, probably the relaxation proteins, and this is a prelude to transfer. Therefore, in addition to the nick and recircularisation site oriT must contain recognition sequences for the relaxation proteins which nick it. Clones containing such cis-acting sequences can be directly selected by their ability to be mobilised. This chapter describes the isolation of the oriT of RSF1010 and RP1 as a prelude to their further characterisation and sequencing.

(b) Cloning of oriT of RSF1010

(i) oriT and nic are on the same HaeII fragment

Functional oriT sites have been cloned on relatively short DNA fragments e.g. F (Everett and Willetts, 1982), RK2 (Guiney and Yakobson, 1983), ColE1 (Warren et al., 1978). To enable the isolation of RSF1010 oriT on a similarly small restriction fragment, an enzyme was chosen that was known to have many recognition sites within RSF1010. The restriction enzyme HaeII was appropriate because, apart from three restriction fragments of 1.2Kb, 0.8Kb and 0.6Kb in size, all other fragments are less than 0.4Kb (Fig. 4.1). To clone the HaeII fragments the vector pED825 (Fig. 4.2) was used (Everett and Willetts, 1982). This has two HaeII sites, one of which must be reconstituted to enable the plasmid to replicate, the other is available for insertion of HaeII fragments. pED825 is particularly useful for cloning oriT sequences, as the oriT of pBR322 (Twigg and Sherratt, 1980) has been deleted (Everett and Willetts, 1982). pED825 is therefore non-mobilisable (Table 4.1).

RSF1010 and pED825 were digested to completion with HaeII, mixed and ligated. The ligation mixture was used to transform the highly transformable strain ED8654 containing R751 and RSF1010. R751 supplies the IncP transfer system necessary for efficient mobilisation of IncQ plasmids (Chapter 3; Willetts and Crowther, 1981), while RSF1010 supplies any mobilisation genes whose products are necessary for recognition and nicking of the cloned RSF1010 oriT. Ap^R transformants were selected overnight in liquid culture, diluted into similar fresh selective media and grown to mid-exponential phase.

Fig. 4.2 Map of the 2246bp cloning vector pED825.

This is composed of the two HaeII fragments located from positions 2349-2719 and 2719-235 on the pBR322 map (4362bp; Sutcliffe, 1978). Co-ordinates are those of the base to the right of the dyad axis of the restriction endonuclease cleavage site. In pED825 these co-ordinates are as follows, reading clockwise: EcoRI (1), ClaI and TaqI (26), HindIII (32), HaeII (235) HinfI (259.5 and 334.5), TaqI (458), HaeII (603), HinfI (730.5 and 1246.5), PstI (1493), PvuI (1620), HindII (1791), TaqI (1901). The HaeII site at nucleotide 603 must be reconstituted in order for the plasmid to replicate, and TaqI, MspI, or HpaII fragments can be cloned into the unique ClaI site. (Everett and Willetts, 1982.)

FIG.4.2

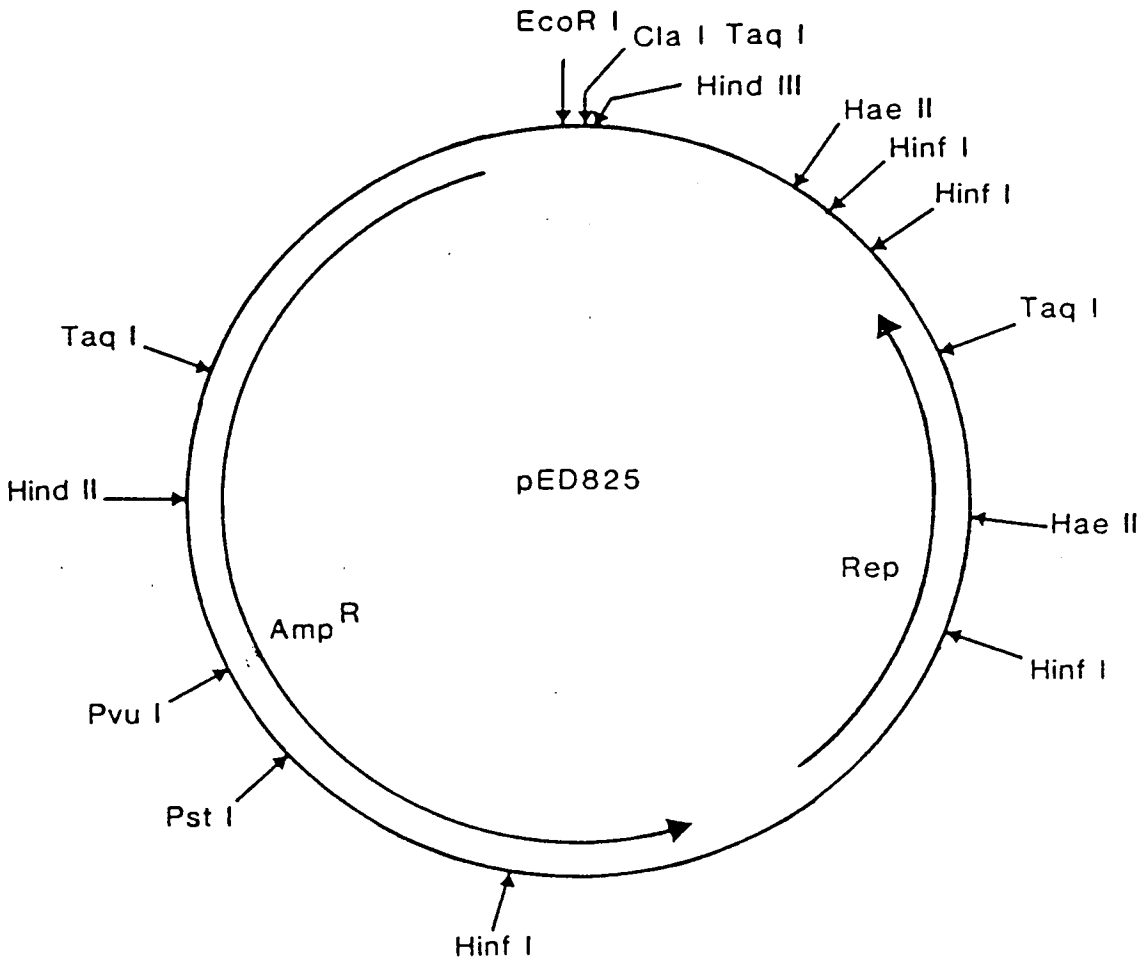

EcoR I
Cla I Taq I
Hind III
Hae II
Hinf I
Hinf I
Taq I
Taq I
Hind II
pED825
Hae II
Rep
Amp R
Hinf I
Pvu I
Pst I
Hinf I

Table 4.1 Mobilisation ratios of *oriT* clones

Plasmid	*oriT* cloned	Mobilisation Ratio
pED825	none	$<10^{-6}$
pED361	RSF1010	1.3
pED360	RSF1010	0.1
pED366	RSF1010	0.1
pED367	RSF1010	0.08
pED374	RP1	9.45
pED375	RP1	0.18
pED361 (a)	RSF1010	$<10^{-6}$

(a) The donor was JC6310 (pUB307)

All matings were plate matings. The donor was JC6310 (pUB307, RSF1010) and the recipient ED24. The contraselection was Spc^R. Mobilisation ratio is the ratio of the mobilisation frequency of the *oriT* clone to the transfer frequency of pUB307.

Ap^R transconjugants were then selected using this culture as a donor in a 30min filter mating with JC3272. Ap^R transconjugants could only arise from the mobilisation of pED825 if it contained a functional *oriT* sequence. Twenty-four such transconjugant colonies were picked and their DNA estimated by the small-scale cleared lysate method (2(d)(i)). The four smallest plasmids were chosen and their purified DNA was digested with *Hae*II and the resulting fragments separated by agarose gel electrophoresis (Fig. 4.1). The smallest clone pED361 contains a single 800bp *Hae*II fragment in addition to the two *Hae*II fragments of pED825. All the other clones contain several *Hae*II fragments but they all have the 800bp *Hae*II fragment in common. These data would imply that the *oriT* site of RSF1010 is contained within this 800bp *Hae*II fragment.

To confirm this, the frequency of mobilisation of pED361 was measured from the *recA*$^-$ strain JC6310 (pUB307, RSF1010) in plate matings with ED24 (Table 4.1). As pED825 is mobilised at frequencies of less than 10^{-6} then the high frequency mobilisation of pED361 confirms that the 800bp *Hae*II fragment contains *oriT*. pUB307 is an Ap^S derivative of RP1 (Bennett *et al.*, 1977) and is preferred to R751 as it has a higher transfer frequency. The mobilisation of pED361 from a *recA*$^-$ donor rules out the possibility of mobilisation occurring by homologous recombination.

The mapping of the *oriT* to this *Hae*II fragment is in agreement with the data of Nordheim *et al.* (1980), who showed that the relaxation nick site of RSF1010, theoretically its *oriT* site, resides on a single 800bp *Hae*II fragment. This is another example of the relaxation site and *oriT* site residing on the same small restriction fragment. pED361 could not be mobilised by pUB307 alone (Table 4.1). This shows that RSF1010 must supply some mobilisation function or functions in *trans* which act at its *oriT*. These are not present on the 800bp *Hae*II fragment contained within pED361.

(ii) Subcloning of *oriT* from pED361

In order to define more precisely the minimum region required for *oriT*, it was necessary to sub-clone *oriT* on smaller restriction fragments. There are at least five *Taq*I cleavage sites within the 800bp *Hae*II fragment of pED361 (confirmed in Chapter 7) and so this

Fig. 4.3 TaqI restriction analysis of pED360 in an 8% polyacrylamide gel.

Track	DNA	Restriction Endonuclease
1	pED360	TaqI
2	pED825	TaqI
3	pED825	MspI

Notes:

(i) Sizes of the TaqI and MspI digests of pED825 are derived from the nucleotide sequence data of Sutcliffe (1978) and are given in base pairs. The novel TaqI fragments of pED360 are estimated from this gel.

(ii) Above the 150bp TaqI fragment is a partial fragment of 180bp.

==========

Fig. 4.5 TaqI restriction analysis of pED366 in an 8% poly acrylamide gel.

Track	DNA	Restriction Endonuclease
1	pED825	MspI
2	pED360	TaqI
3	pED366	TaqI
4	pBR322	TaqI

Notes:

(i) Sizes of TaqI and MspI fragments of pBR322 and pED825 are derived from the nucleotide sequence (Sutcliffe, 1978) and are given in base pairs. The 150bp TaqI fragment common to pED360 and pED366 has been measured previously (Fig. 4.3).

FIG.4.3

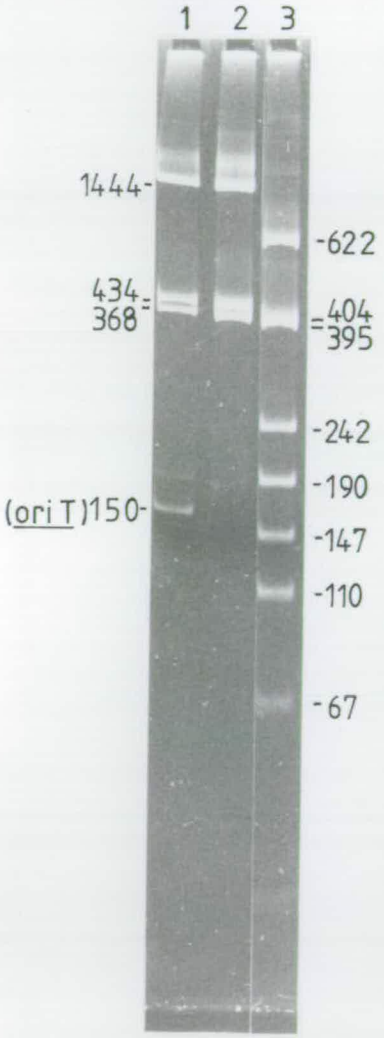
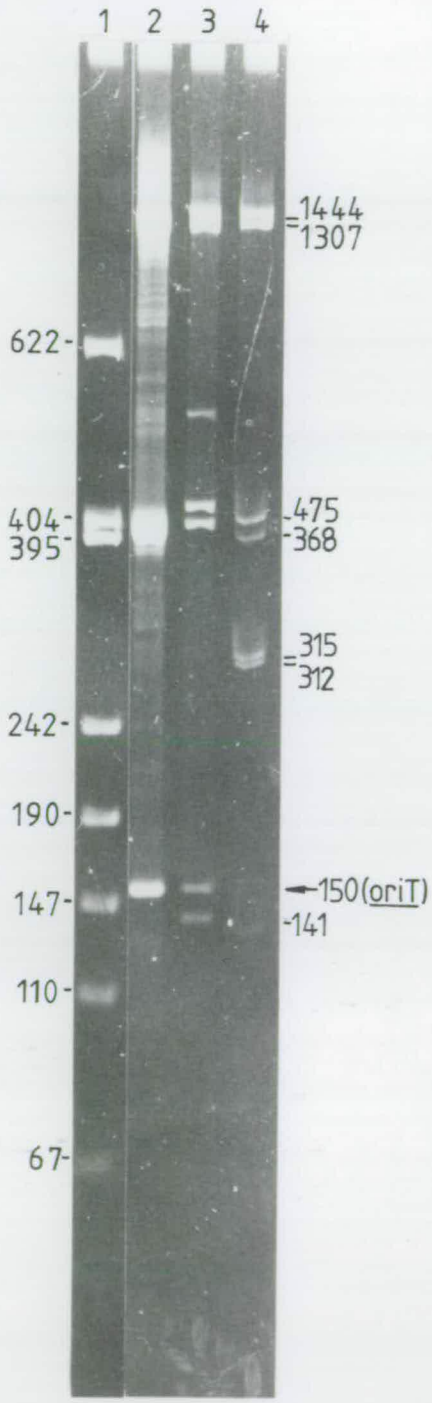

1 2 3
1444-
434
368
=
(oriT)150-
-622
=404
395
-242
-190
-147
-110
-67

FIG.4.5


1 2 3 4
=1444
1307
622-
404-
395-
-475
-368
=315
312
242-
190-
147-
110-
67-
150(oriT)
-141

enzyme was used to sub-clone TaqI fragments into the ClaI site of pED825 (Fig. 4.2).

pED361 was digested to completion with TaqI and then mixed with ClaI digested pED825. This mixture was then ligated and used to transform ED8654 (R751, RSF1010). Ap^R recombinants were selected and screened as described in 4(b)(i). The smallest clone, pED360, apparently contained one single TaqI fragment of 150bp (Fig. 4.3). The efficient mobilisation of pED360 from JC6310 (pUB307, RSF1010) (Table 4.1), confirmed the presence of the oriT site. However, the frequency of mobilisation is 10 to 20-fold less than that of pED361. Nevertheless, pED360 is efficiently mobilised and therefore contains a functional oriT site; it may be that part of a recognition sequence of oriT has been deleted and so, for example, the putative nicking protein recognises the site at a lower efficiency, which is reflected in the reduced mobilisation. Alternatively, it may be that an adjacent vector sequence is interfering in some way with recognition or initiation at oriT. These points will be discussed further in Chapter 7.

(iii) Re-cloning the single TaqI fragment

Digestion of pED360 with TaqI generates four restriction fragments. Three of these (1.44Kb, 0.8Kb and 0.36Kb) are derived from the vector, while the fourth is the 150bp TaqI fragment from RSF1010 (Fig. 4.3). However, it was noticed in several gels (including Fig. 4.3) that there was a faint band of 180bp in size above the oriT TaqI fragment, This band could be contaminating DNA or a partial digest generated from the 150bp TaqI fragment and a smaller TaqI fragment of about 30bp in size, which would not be seen on the polyacrylamide gels used. The presence of two extra TaqI fragments of 12bp and 18bp was confirmed by sequencing (Chapter 5). It was thought initially that as the clone had been selected by its ability to be mobilised then these fragments were involved with oriT and were presumably contiguous with the 150bp TaqI fragment in RSF1010. However, data presented in this chapter and in Chapter 7 demonstrates that the cloning of these two extra fragments was fortuitous.

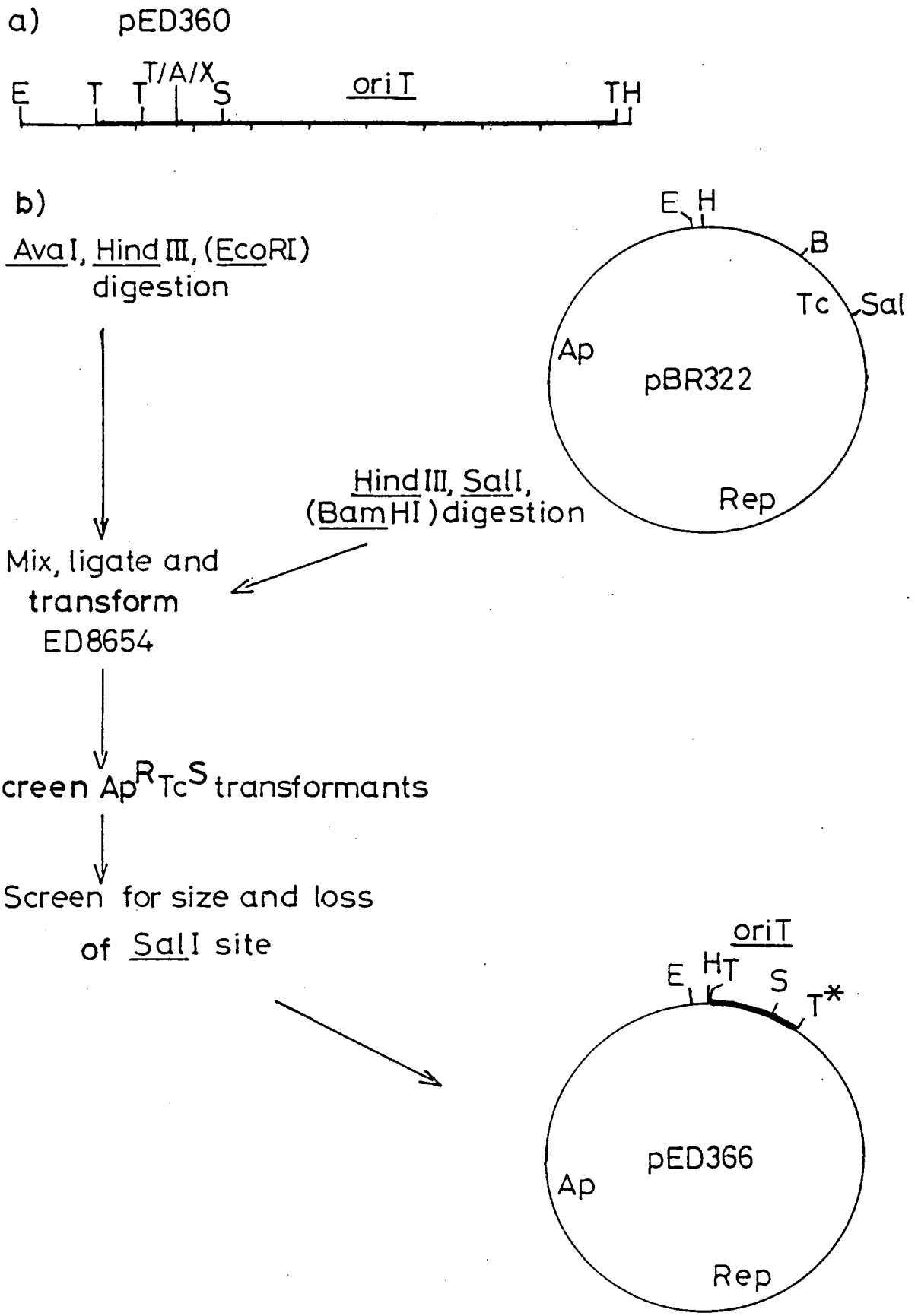
It was shown from the sequence of pED360 (Chapter 5) that the two small TaqI fragments were adjacent and were cloned so that they were closest to the EcoRI site of pED825 (Fig. 4.4). Very fortunately

Fig. 4.4

(a) A detailed map of the oriT TaqI clone pED36o between the EcoRI and HindIII sites of the vector (Fig. 4.2). The location of the two small TaqI fragments is shown and the multiple AvaI, TaqI, XhoI recognition site. The position of the Sau3A restriction site within the clone is also shown. The cloned fragment is indicated by thicker lines and the graduations represent 20bp.

(b) The cloning strategy for replacement of the HindIII-SalI fragment of pBR322 with the AvaI-HindIII fragment of pED360. The maps of pBR322 and pED366 are not drawn to scale. The * in pED366 represents the AvaI/SalI junction of the recombinant neither of these two sites is reconstituted, but the TaqI site is still present. Note that the oriT-containing TaqI fragment is now in the opposite orientation with respect to Rep, compared to pED36o.
Key to Restriction Enzyme sites: E, EcoRI; S, Sau3A; T, TaqI; A, AvaI; X, XhoI; H, HindIII; B, BamHI.

FIG.4.4


a)
pED360
E
T
T
T/A/X
S
oriT
TH
b)
AvaI, HindIII, (EcoRI) digestion
E
H
B
Tc
Sal
Ap
pBR322
Rep
HindIII, SalI, (BamHI) digestion
Mix, ligate and transform ED8654
Screen Ap^R Tc^S transformants
Screen for size and loss of SalI site
oriT
E
H
T
S
T*
pED366
Ap
Rep

the TaqI site reconstituted between the 150bp fragment and the 12bp TaqI fragment was also an AvaI site. AvaI recognises and cleaves the partially degenerate hexanucleotide sequence C↓PyCGPuG. At the junction, the degenerate nucleotides are T and A respectively. Thus the central four bases are TCGA which is the recognition and cleavage site of TaqI. The AvaI site in this particular sequence is also the cleavage site for XhoI. This provided the first evidence that these fragments were not contiguous in RSF1010, as it is known that there are no XhoI sites in the plasmid (Barth, 1979). The presence of the AvaI site was confirmed in pED360 but was shown to be absent in pED361 (data not shown). The AvaI restriction map of RSF1010 is known (Fig. 1.1) and, as confirmed by sequencing (Chapter 7), there are no AvaI sites within 900bp of oriT. These data therefore proved that these three TaqI fragments are not contiguous in RSF1010. However, their restriction sites allowed the separation of the large TaqI fragment from the two small fragments.

The 5' extensions generated by digestion with XhoI (and in pED360, AvaI) are complementary to those produced by SalI, allowing XhoI fragments to be cloned into SalI sites. To sub-clone the large TaqI fragment, the HindIII-SalI fragment of pBR322 was replaced with the AvaI-HindIII fragment of pED360 (Fig. 4.4). To avoid recircularisation of the parental molecules and the cloning of the wrong SalI-HindIII fragment, pBR322 was digested with BamHI while pED360 was digested with EcoRI. The ligated DNA was used to transform ED8654 and Ap^R transformants which were Tc^S, purified. As pED360 would also be $Ap^R Tc^S$, the plasmid sizes were screened and two of the expected size were chosen. Analysis of the SalI digestion products of these two plasmids confirmed that as expected they had lost the SalI site of pBR322 (ligating an AvaI fragment into a SalI site does not reconstitute a SalI site). The DNA of one of these clones, pED366, was purified further and characterised by TaqI digestion followed by polyacrylamide gel electrophoresis (Fig. 4.5). It can be seen that pED366 contains a TaqI restriction fragment not present in pBR322, but which comigrates with the 150bp TaqI fragment of pED360. pED366 does not contain the 315bp and 312bp TaqI fragments of pBR322 as a consequence of their replacement during its construction.

To confirm that the oriT site was on the 150bp TaqI fragment,

the mobilisation of pED366 was measured from JC6310 (pUB307, RSF1010) to ED24 (Table 4.1). Although pED366 also contains the pBR322 oriT site, it does not encode any mobility functions and so mobilisation from this oriT would not be expected (Young and Poulis, 1978). Again, the mobilisation was less efficient than for the original HaeII clone, pED361, but because of the relatively high efficiency of mobilisation it must contain the minimum sequence essential for initiation of mobilisation at oriT.

An integral part of mobilisation is the successful re-establishment in the recipient of a double-stranded plasmid after single-stranded transfer. This process requires recircularisation and complementary strand synthesis. The three unrelated plasmids R6K, pSC101 and RSF1010 all have their oriT and oriV sites closely associated and so Nordheim et al. (1980) suggested that complementary strand synthesis in the recipient was initiated from a primer synthesised in the donor, from oriV towards oriT, which was then transferred. However, this hypothesis was disproved for ColE1 (Finnegan and Sherratt, 1982), which also has its oriT and oriV sites in close proximity. They altered the position and orientation of oriT with respect to oriV and showed that this had no effect on transfer. One would have predicted, according to the model of Nordheim et al. (1980), that inverting oriT with respect to oriV would have prevented transfer, as the primer formed between oriV and oriT would be complementary to the non-transferred strand and would not be able to initiate complementary strand synthesis in the recipient.

pED366 and pED360 (Fig. 4.4) have their oriT fragments cloned in opposite orientations. As both of these clones are highly mobilised (Table 4.1) then this finding is contrary to the hypothesis of Nordheim et al. (1980) and in agreement with the data of Finnegan and Sherratt (1980). However, Covarrubias et al. (1981) showed that when the oriT site from pBR322 was cloned into the PvuII site of pBR328, it was functional in only one of the two orientations. One possible explanation for this is that as the PvuII site is in the Cm^R gene, then cloning oriT in the inverted orientation inactivates oriT as the nicked strand is the same as that actively transcribed from the chloramphenicol gene promoter. This could be tested by inactivating the promoter by mutation or deletion.

Fig. 4.6 Restriction analysis of pED367 in an 8% polyacrylamide gel.

Track	DNA	Restriction Endonuclease
1	pED825	MspI
2	pED360	TaqI
3	pED367	TaqI
4	pED367	EcoRI, HindIII
5	pED360	EcoRI, HindIII

Notes:

(i) The sizes of the MspI and TaqI fragments are marked in base pairs.

(ii) The TaqI fragment of 150bp is common to both pED36o and pED367. However, the two small TaqI fragments are not present and this is shown by comparing the sizes of the small oriT containing EcoRI-HindIII fragments of the two plasmids within which the TaqI fragments were cloned (Fig. 4.2).

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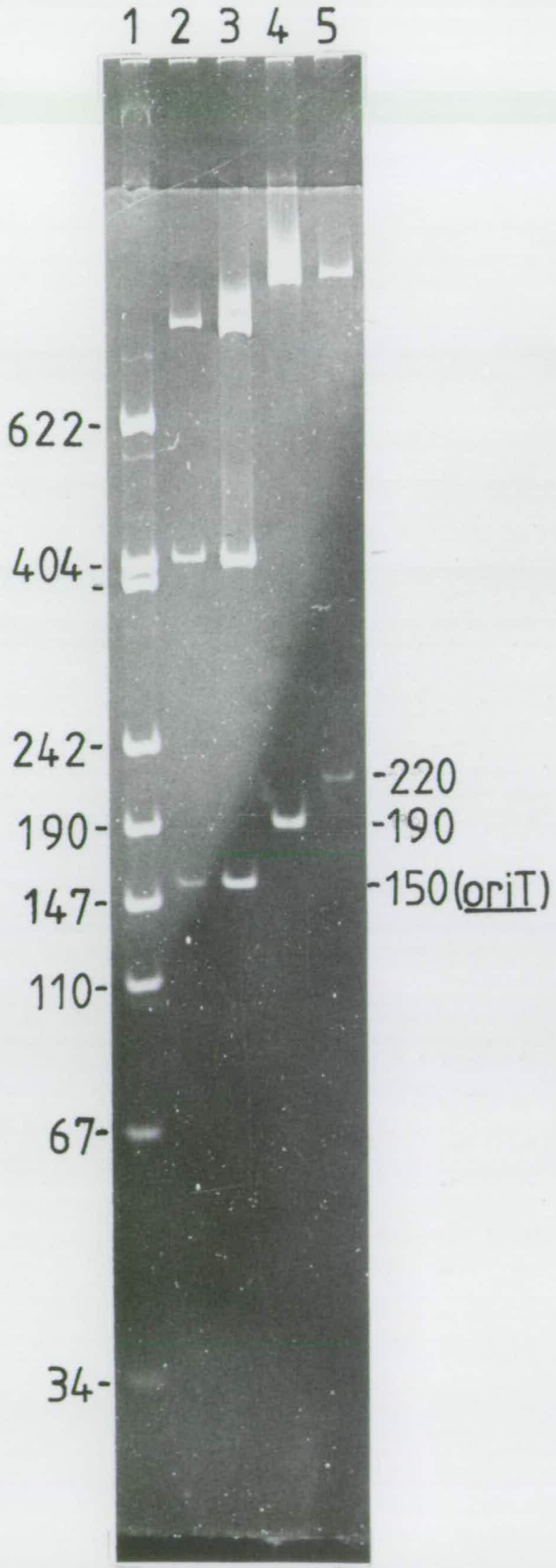
Fig. 4.7 Comparative restriction analysis of pED360 and pED367, showing that the oriT TaqI fragment is cloned in the same orientation in both plasmids.

Figure 4.7 has been omitted because the Sau3A used for the digestions has now been shown to be AluI (incorrectly supplied by manufacturer). Although the AluI digestions were similar, they could not conclusively confirm the orientation of the TaqI fragment. However, the digestions have been repeated using new Sau3A and this has confirmed the orientation to be as stated in the text.

FIG.4.6.



Everett and Willetts (1982) have shown that the oriT of F, when cloned in pBR322, is unstable, but only in one orientation and only in the presence of F. They showed that the traYZ genes caused this instability and suggested that the nicking at oriT was interfering with replication of the plasmid. No such instability was noted with the oriT clones of RSF1010 in the presence of RSF1010.

(iv) Re-cloning the oriT TaqI fragment from pED366 into pED825.

The 150bp oriT containing TaqI fragment was sub-cloned into the ClaI site of pED825 (Fig. 4.2) as in 4(b)(ii). DNA from the smallest recombinant plasmid, pED367, was purified and its DNA analysed by restriction enzyme digestion followed by polyacrylamide gel electrophoresis (Fig. 4.6). pED367 has the same oriT containing TaqI fragment as pED360 and pED366. However, pED367 does not contain the two small TaqI fragments present in pED360. The difference in size between the small EcoRI-HindIII fragments of pED360 and pED367 also demonstrates this (Fig. 4.6). As expected, pED367 is still mobilised efficiently (Table 4.1).

The orientation of the 150bp TaqI fragment in pED367 was shown to be the same as in pED360. A single Sau3A site, as determined from sequence data (Chapter 5), is located 138bp from the TaqI site closest to the HindIII site in pED360 (Fig. 4.4). A HindIII, Sau3A double digest of both pED360 and pED366 followed by gel electrophoresis showed that the HindIII-Sau3A fragments containing oriT from both plasmids co-migrated and therefore the oriT TaqI fragment of pED367 is in the same relative orientation as pED360 (Fig. 4.7).

(c) Reduction in Size of the oriT Clone, pED367, Using the Transposon Tn1725.

In order to define further the oriT site of RSF1010 the transposon Tn1725 was chosen to generate cis-dominant insertion mutants of pED367. pED367 is a particularly useful 'target' as there is very little non-essential DNA in the vector and so insertions into the small cloned fragment should be recovered relatively frequently. No such cis-acting mutants were ever found. However, the EcoRI sites present in the inverted repeats of Tn1725 (Fig. 4.8) were used to generate deletions from the insertions that were obtained and these

Fig. 4.9 Accurate estimation of the points of insertion of four Tn1725 insertions in pED367.

Track	DNA	Restriction Endonuclease
1	pED383	EcoRI, HindIII
2	pED383	EcoRI
3	pED381	EcoRI, HindIII
4	pED381	EcoRI
5	pED379	EcoRI, HindIII
6	pED379	EcoRI
7	pED377	EcoRI, HindIII
8	pED377	EcoRI
9	pED367	EcoRI, HindIII
10	pED825	MspI

(i) The fragment sizes are marked in base pairs. The sites of insertion have been calculated from this gel by measuring the size of the EcoRI-HindIII fragment. These are shown in Fig. 4.10, along with the insertion site of nine other Tn1725 insertions.

(ii) The 187bp EcoRI-HindIII oriT-containing fragment, from which the deletions are generated, is shown.

(iii) The gel is an 8% polyacrylamide gel.

FIG.4.9.

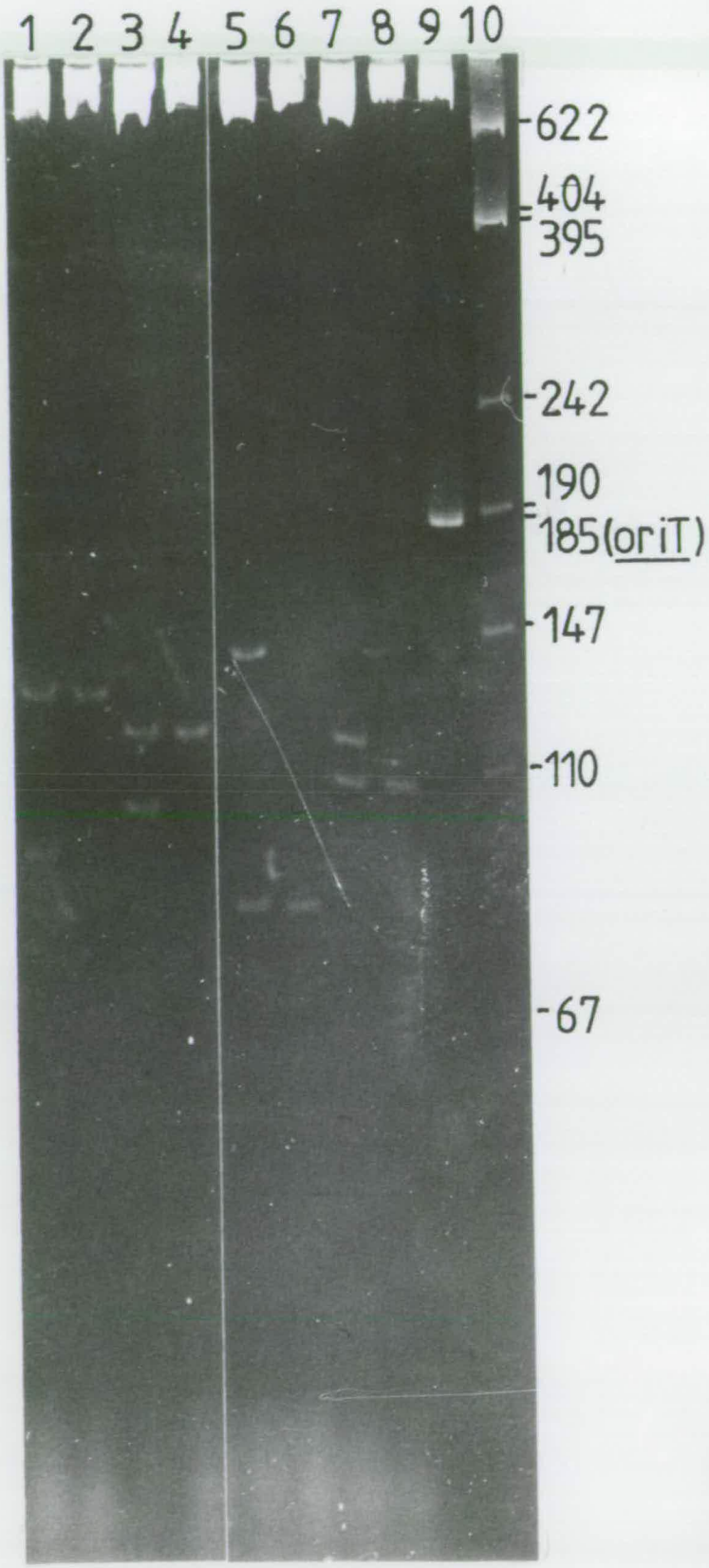


Fig. 4.8 Map of Tn1725 according to R. Schmitt (Unpublished).

Notes:

(i) Arrows at ends of map indicate the inverted repeats.

(ii) Key to restriction enzyme sites: E, EcoRI; P, PstI; H, HindIII; S, SmaI; Pv, PvuII.

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Fig. 4.10

(a) Detailed map of the EcoRI-HindIII fragment of pED367 showing the sites of thirteen Tn1725 insertions which were mapped as in Fig. 4.9. Each bar represents an insertion and those characterised further (see b) are named. The position of insertion has been calculated taking into account the 156bp within the inverted repeat of Tn1725 up to the EcoRI site.

(b) Map showing extend of pED367 retained in the deletions constructed from the four insertions. pED385 is a HindIII deletion from pED383 and contains 1.12Kb of Tn1725 DNA (see Fig. 4.8). The other four deletions are all EcoRI deletions and contain only 15bp of Tn1725. The ± indicates whether they are mobilised efficiently or not and therefore the presence or absence of the oriT site.

Key to restriction enzyme sites: These are the same as Fig. 4.8, with the addition: T, TaqI.

FIG.4.8

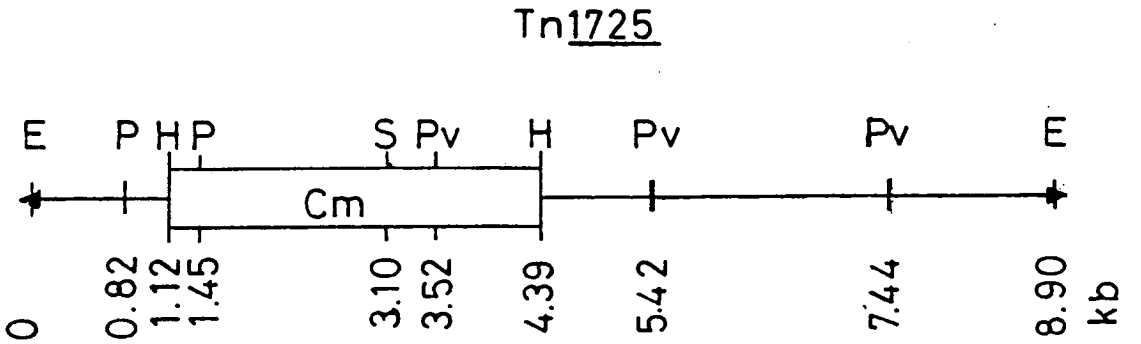
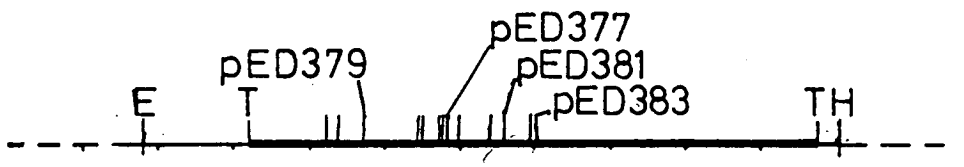


FIG.4.10

a)



b)

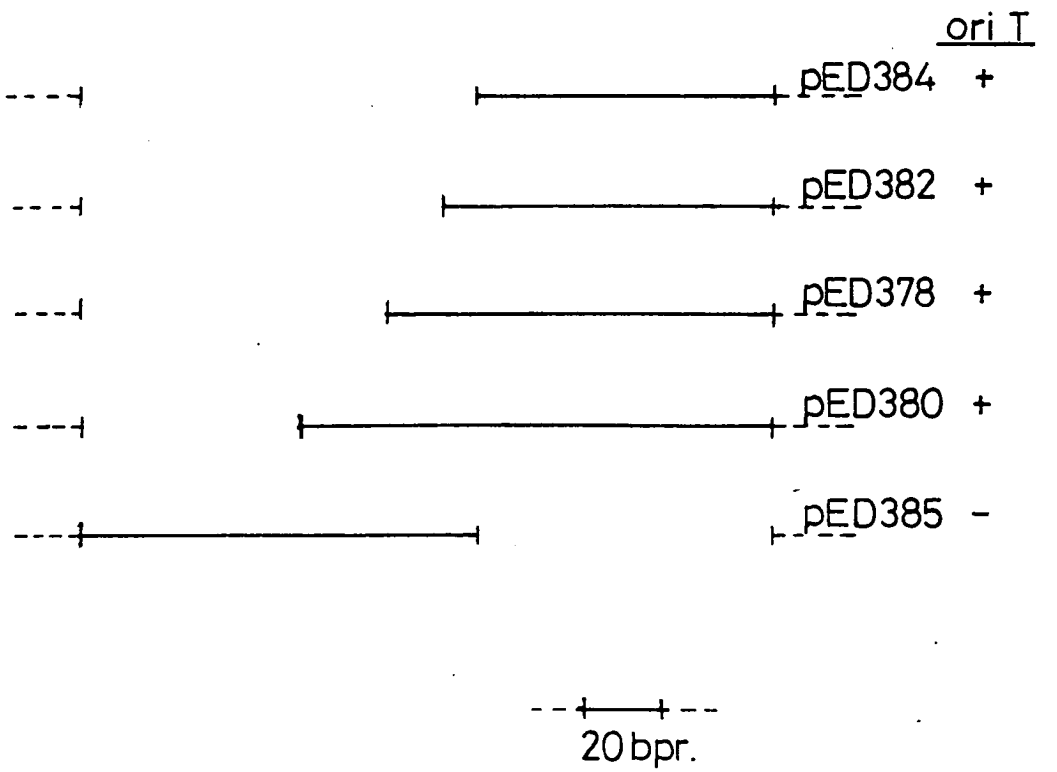


Table 4.2 Comparison of the mobilisation ratios of the Tn1725 insertions, and deletions created from them, to pED367.

Plasmid	Tn1725	Ratio	Distance of Insertion from HindIII Site (bp)
pED367	-	1	-
pED379	+	0.13	125
pED380	-	1.45	125
pED377	+	0.075	103
pED378	-	0.935	103
pED381	+	0.1	89
pED382	-	2.7	89
pED383	+	0.055	78
pED384	-	1.2	78
pED385	-	$<10^{-4}$	78

All matings were plate matings. The donor was JC310 (pUB307, RSF1010) and the recipient ED24. The contraselection was Spc^R. The ratios quoted are expressed in terms of the mobilisation frequency of pED367. This allows for easier comparison of the effects of the transposon and the deletions on mobilisation of pED367. The average mobilisation frequency of pED367 compared to the transfer frequency of pUB307 was 0.08.

reduced the minimum size of oriT$^+$ TaqI clone still further.

Tn1725 insertions were generated as in Chapter 2(h). DNA from twenty-four independently isolated insertion derivatives was screened by EcoRI, HindIII double digestion followed by agarose gel electrophoresis and thirteen were shown to have Tn1725 inserted within the EcoRI-HindIII fragment containing oriT. An accurate estimation of their site of insertion was determined by EcoRI and EcoRI, HindIII double digestion followed by electrophoresis in 8% polyacrylamide gels (Fig. 4.9). The sites of insertion for all the Tn1725 insertions in the oriT fragment are shown in Fig. 4.10. Four insertions, pED377, pED379, pED381 and pED383 were chosen for further characterisation.

The mobilisation frequency of each of the four insertions was measured from JC6310 (PUB307, RSF1010) to ED24 (Table 4.2). They transferred approximately ten times less efficiently than pED367. To determine whether this reduction was due to a partial inactivation of oriT or to an effect on oriT by the adjacent transposon, deletions of the transposon were made by taking advantage of its EcoRI sites.

The DNA of pED377, pED379, pED381 and pED383 were digested to completion with EcoRI. The DNA was then self-ligated in a relatively large volume (200µl) and recovered by transformation of JC6310 (pUB307, RSF1010). $Ap^R Cm^S$ transformants were picked and their DNA analysed by EcoRI, HindIII double digests followed by polyacrylamide gel electrophoresis (data not shown). The four deletions obtained from pED377, pED379, pED381 and pED383 were called pED378, pED380, pED382 and pED384 respectively and had a single EcoRI site. That is, two EcoRI fragments had been deleted, the transposon EcoRI fragment and the small EcoRI fragment from the vector EcoRI site to the transposon EcoRI site (Fig. 4.10).

The mobilisation of all four deletion derivatives was measured from JC6310 (pUB307, RSF1010) to ED24 (Table 4.2). All deletions have mobilisation frequencies equivalent to pED367. This would seem to imply that it was the presence of the transposon that was reducing the transfer frequencies of the original insertions. The fact that all four deletions are fully oriT$^+$ (as compared to pED367) demonstrates that the oriT site of RSF1010 is within the 80bp to the right of the EcoRI site in pED384.

This does not preclude the unlikely possibility of a second

oriT site to the left of the insertion in pED383. To show this to be incorrect a deletion was made from pED383 that removed the 80bp known to contain an oriT. It was made by deleting in vitro all the DNA from the HindIII site in the vector to the HindIII site in the transposon (Fig. 4.8). This deletion derivative, pED385, (shown in Fig. 4.10) fails to be mobilised from JC6310 (pUB307, RSF1010) to ED24 (Table 4.2). It is therefore concluded that there is no oriT site to the left of the insertion in pED383.

(d) oriT Specific Recombination

(i) Introduction

It is assumed, by analogy to F (see Chapter 1), that ColE1 transfer is initiated from a fixed origin, oriT, which allows transfer of a single-strand to the recipient where it is recircularised at oriT (Warren et al., 1978). The 60Kd relaxation protein becomes covalently attached to the 5' end of the nick (Lovett and Helinski, 1975) and so it has been hypothesised that recircularisation in the recipient is facilitated by this protein recognising the 3' end of the nick. This recircularisation event can take place independently of the RecA protein and of expression of the plasmid in the recipient (Warren and Clark, 1980), and is therefore a RecA independent, transfer dependent, site-specific recombination process.

Such a process has been demonstrated for F (Everett and Willetts, 1982), ColE1, ColK and related plasmids (Warren and Clark, 1980; Broome-Smith, 1980). ColE1 can form cointegrates with related plasmids during transfer and the site of cointegration maps very close to, or at, oriT (Warren and Clark, 1980; Broome-Smith, 1980). Furthermore, these cointegrates can be resolved into their component plasmids but only after being retransferred. These results were explained by the ligation of one 5' terminus of oriT of one molecule to the 3' terminus of oriT of another molecule during transfer and vice-versa. Resolution of the cointegrate occurs as a consequence of transfer being initiated from one of the two oriT sites in the cointegrate and then either a second nick occurring in the donor or the second oriT site being recognised and cut as it enters the recipient. These experiments therefore provide genetic evidence for nicking at oriT and its recircularisation during transfer.

Fig. 4.11 Example of a gel used to determine the resolution frequency of a multimer of pED360.

0.7% Agarose

Track	DNA
1	pUB307, RSF1010, pED360D
2	pED360D
3	pED360
4 - 12	Samples of DNA purified from Ap^R transconjugants

Notes:

(i) The DNA was purified by the method described in Chapter 2(d)(i) and consequently is contaminated with chromosomal DNA (chr) and RNA. As the plasmids were not digested they are present mainly in their covalently closed circular forms. The DNA prepared from the Ap^R transconjugants was compared to that from the donor strain (track 1) and to the other standards (tracks 2, 3) to calculate the efficiency of monomerisation.

(ii) pED360D represents DNA prepared from a strain carrying the dimeric (D) form of pED360.

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Fig. 4.12 Restriction endonuclease analysis of pED373.

1.1% Agarose

Track	DNA	Restriction Endonuclease
1	RSF1010	EcoRI
2	pED360	EcoRI
3	pED373	HindIII
4	EDλ7	HindIII
5	pED373	EcoRI
6	pED373	EcoRI, HindIII
7	pED360	EcoRI, HindIII
8	RSF1010	HaeII
9	pED373	HaeII
10	pED360	HaeII

Notes:

(i) DNA fragments sizes are given in kilcbases. The sizes other than those given for the EDλ7 digestion are calculated from this gel or as in the case of the vector fragments of pED360 are known from the sequence (Sutcliffe, 1978).

(ii) In track 6 an EcoRI-HindIII fragment is generated equivalent in size to that of pED360, therefore demonstrating that the point of integration in pED360 is in the smaller oriT containing EcoRI-HindIII fragment.

(iii) The loss of the 800bp HaeII fragment in pED373 also demonstrates that the point of integration is within this oriT containing fragment in RSF1010.

FIG.4.11.

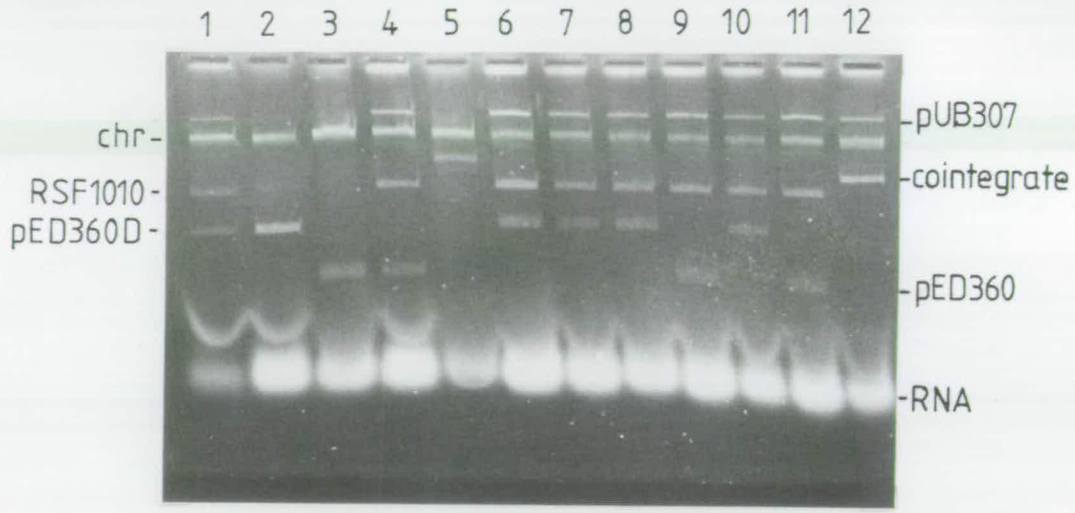
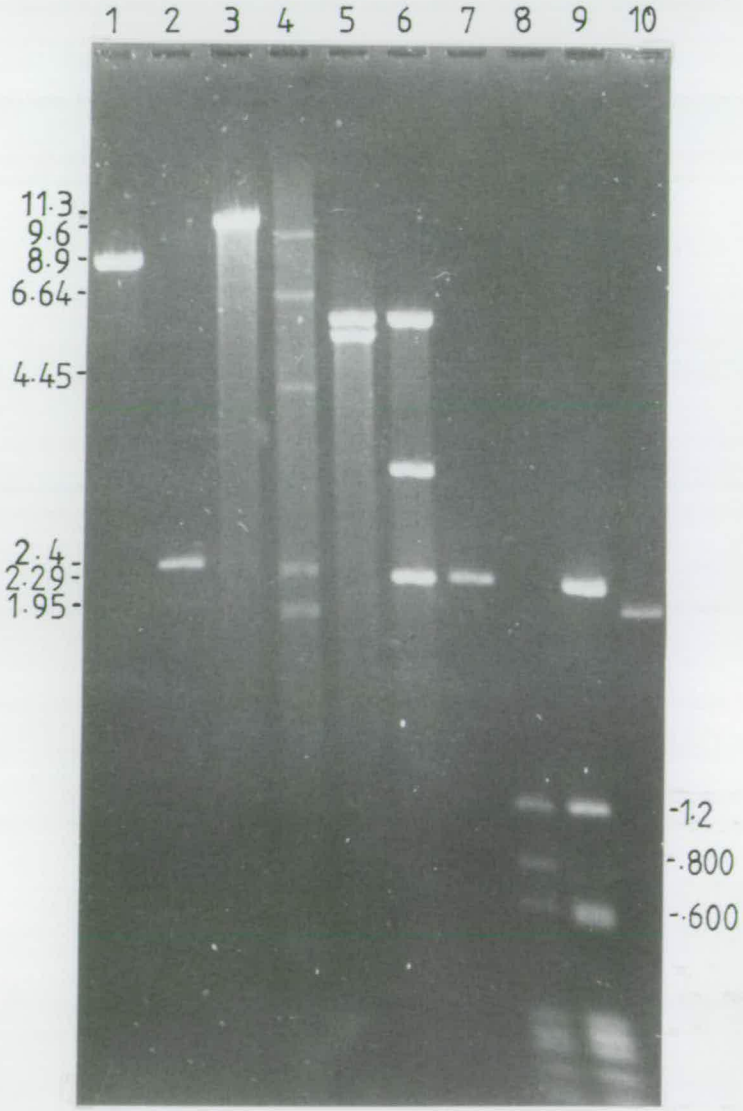


FIG.4.12.



(ii) Monomerisation of plasmid multimers

The simplest way to detect this site-specific recombination process is to screen for resolution of plasmid multimers from transconjugants in matings where the donor contains a plasmid multimer (Warren and Clark, 1980; Everett and Willetts, 1982).

A dimer of pED360 (oriT$^+$ TaqI clone) was isolated following transformation of the recA$^-$ strain JC6310 with pED360 DNA. Ap^R transformants were screened for plasmid DNA larger than the monomeric form of pED360. A dimeric form, pED360D, was identified and used to transform JC6310 (pUB307, RSF1010). This strain was then platemated with another recA$^-$ strain, ED3886. Ap^R transconjugants were selected and the DNA from 50 of them was screened by gel electrophoresis. An example of such a gel is seen in Figure 4.11. It can be seen that pED360 is present in the recipients as both dimers and monomers. The resolution of the dimer to monomer was transfer dependent; even after six days of subculturing of the donor strain no monomeric forms of pED360 were visible (data not shown). 56% of the transconjugants contained the dimer. In tracks 5 and 12 of Figure 4.11 there is no plasmid corresponding to either the dimeric or monomeric forms of pED360 yet both isolates were Ap^R. However, RSF1010 was migrating with an elevated size, consistent with an RSF1010::pED360 cointegrate. These cointegrates arose at a frequency of 10% of the transconjugants, a frequency very similar to those reported by Warren and Clark (1980) and Broom-Smith (1980).

(iii) Characterisation of the RSF1010::pED360 cointegrate

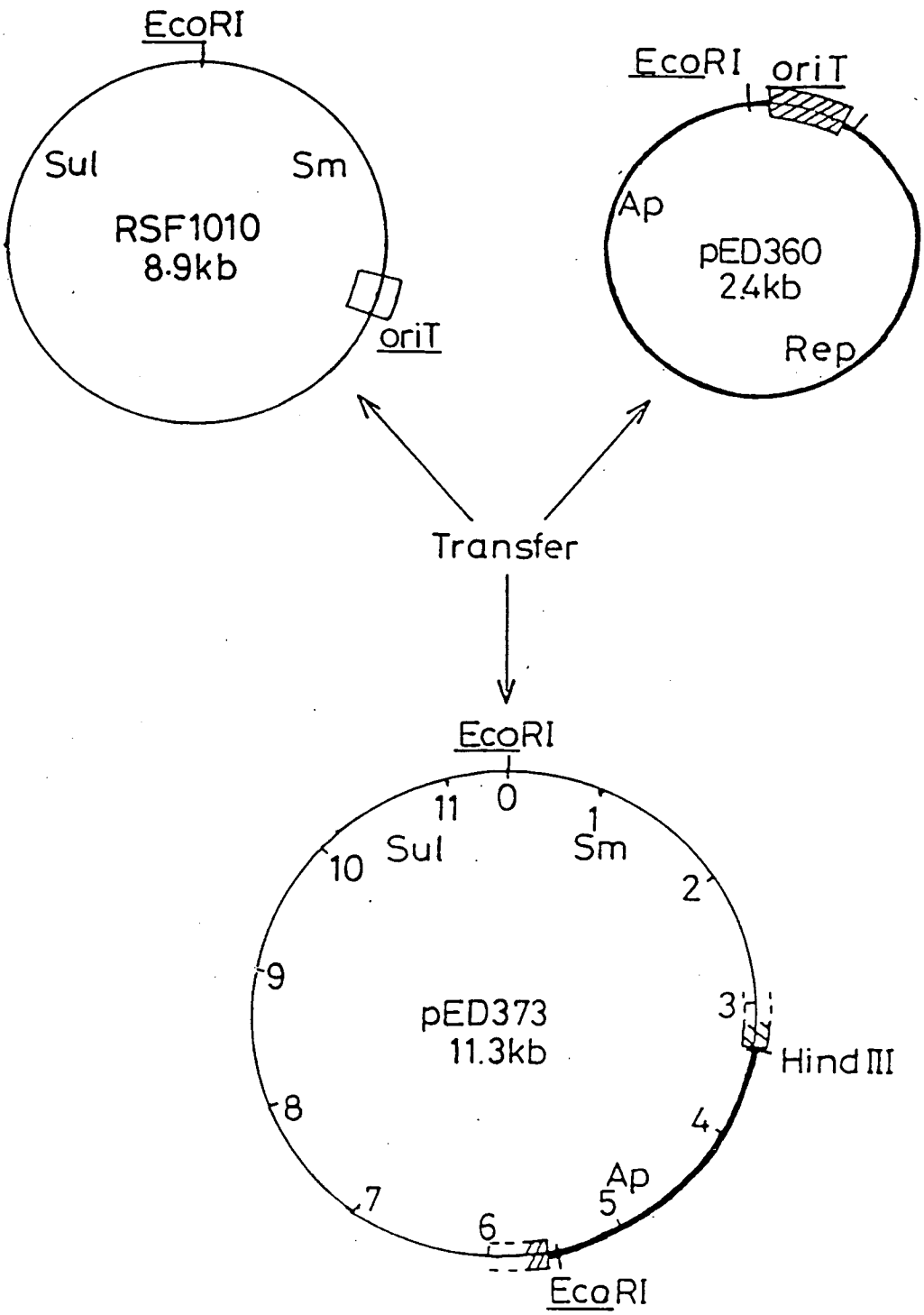
Four of the isolated cointegrates were identical on the basis of preliminary restriction analysis (data not shown). One cointegrate was chosen, pED373, and its DNA purified to allow further characterisation.

Gel electrophoresis of pED373 digested with HindIII (Fig. 4.12) resolved a single band, presumably a linear molecule, of slower mobility than RSF1010 and a size compatible with that of a cointegrate of RSF1010 and pED360. On digestion with EcoRI two fragments of 5.8Kb and 5.5Kb were produced showing that pED373 was a cointegrate of RSF1010 and a monomer of pED360. A dimer would have produced an extra EcoRI fragment equivalent in size to pED360. On digestion with both EcoRI and HindIII, the 5.5Kb EcoRI fragment was replaced by two

Fig. 4.13 Restriction endonuclease map of pED373 and its component plasmids.

The oriT regions are drawn as boxes. The location of the oriT region of RSF1010 is based on the fact that the EcoRI-HindIII fragment in pED373 is 3.3Kb and therefore the oriT site must be 3.3 ± 0.2Kb from the EcoRI site of RSF1010.

FIG.4.13


EcoRI
Sul
Sm
RSF1010
8.9kb
oriT
EcoRI
oriT
Ap
pED360
2.4kb
Rep
Transfer
EcoRI
11
0
1
Sul
Sm
10
2
9
3
pED373
11.3kb
Hind III
8
4
Ap
7
5
6
EcoRI

fragments of 2.24Kb and 3.3Kb. The generation of a fragment equivalent in size to the vector part of pED360 (2.24Kb) implies that the site of cointegration was within the oriT containing fragment of pED360. A restriction map of pED373 is presented in Figure 4.13. HaeII restriction analysis (Fig. 4.12) also revealed that the point of integration in RSF1010 was within the 800bp HaeII fragment, also known to contain oriT (Chapter 4(b)(i)), as when pED373 was digested with HaeII the 800bp HaeII fragment was absent and replaced by two novel fragments of 2.1Kb and 0.615Kb. The recombination event must therefore have occurred within 200bp of oriT in the clone pED360 and within 800bp of the same oriT in RSF1010. As this process is transfer dependent and the site of recombination is at, or very close to, oriT then it is best explained by the ligation of the 5' and 3' ends of the oriT sites of RSF1010 and pED360 (Fig. 4.13); this provides genetic evidence for nicking and religation occurring at the nick site during transfer.

(iv) Resolution of pED373

Resolution of the oriT cointegrates of ColE1 occurred at a similar frequency to their formation (Warren and Clark, 1980; Broome-Smith, 1980). The resolution of pED373 was demonstrated and its frequency determined as follows.

pED373 was used to transform ED3886 (pUB307) and plate-mated with JC6310. Transconjugants were screened as in Chapter 4(d)(ii). All Ap^R transconjugants were Sul^R, and out of 50 screened all but one contained only the cointegrate pED373; the exception also contained pED360. Ap^R transfer is therefore a consequence of pED373 transfer. However, 32% of the Sul^R transconjugants were Ap^S and contained only RSF1010. Many of the $Sul^R Ap^R$ transconjugants contained both RSF1010 and pED373, though this latter plasmid band was often fainter on the gel and this presumably reflected incompatibility between the two plasmids.

These data are in agreement with those previously reported (Warren and Clark, 1980; Broome-Smith, 1980) although the frequency of resolution is slightly higher. One anomaly is the lack of resolution of pED373 when Ap^R is selected. It is difficult to interpret this result further without more information on the direction of transfer and the possible role of incompatibility. One possibility is that one of

hybrid oriT sites is less active because of poor recognition or the effect of adjacent sequences. If the active oriT was oriented such that it transferred Sul^R first, then all Ap^R transconjugants would have to be Sul^R.

Finally, it should be noted that although these experiments were carried out using the TaqI clone pED360, which had the two extraneous TaqI fragments, it is highly unlikely that these could have been involved in this process as they could be deleted without affecting the mobilisation (N.B. pED367) and they map over 500bp away from oriT (Chapter 7).

(e) Cloning the oriT of RP1

RP1 mobilises RSF1010 very efficiently and in addition to supplying the mating pair system supplies at least one trans-acting product required for RSF1010 mobilisation (Chapter 3). The RP1 oriT region is therefore of particular interest, as it might be similar to that of RSF1010 or there may be a second site which is the recognition site for the RP1 conjugation system.

The oriT site of RP1 was cloned in a similar manner to that of RSF1010. Initially HaeII fragments were cloned using pED825 (Fig. 4.2) and plasmids carrying oriT selected by their mobilisation from JC6310 (pUB307) to ED24. The smallest clone isolated, pED374, contained a single HaeII fragment of approximately 700bp which was mobilised nine times more efficiently than pUB307 was transferred (Table 4.1). The oriT site was sub-cloned from pED374 using MspI. MspI restriction fragments were cloned into the ClaI site of pED825 and mobilisation ability selected as before. The smallest clone which was mobilisable, pED375, contained an MspI fragment of 130bp in size. This clone, although obviously containing oriT, was mobilised fifty times less efficiently than pED374 (Table 4.1) so part of a recognition sequence may be deleted. Recently, Guiney and Yakobson (1983) have cloned a 760bp HaeII fragment which contains both the oriT site of RP1 and also its relaxation nick site. This HaeII fragment is almost certainly the same as that cloned in pED374. Guiney and Yakobson (1983) have also sub-cloned oriT from this HaeII clone as a 112bp MspI fragment. Again, allowing for error in measurement, this is in agreement with the size of the MspI fragment cloned in pED375. Similarly, it was shown that the

112bp MspI fragment did not confer 100% mobilisation. This 112bp MspI fragment was sequenced by Guiney and Yakobson and will be compared to the oriT sequence of RSF1010 in Chapter 5.

(f) Summary

The oriT site of RSF1010, which includes the nick and recircularisation site and their protein recognition sites, has been cloned within a 150bp TaqI fragment. Deletion analysis of this clone has located oriT on an 80bp segment. This is the smallest oriT-containing fragment yet isolated and implies, that at least in RSF1010, the functional sequence required in cis for transfer is small. The oriT site of RP1 was cloned on an MspI fragment probably identical to a clone isolated by Guiney and Yakobson (1983).

The RSF1010 oriT sequence was able to carry out a site-specific recA-independent recombination that was dependent on transfer. This is the strongest genetic evidence that transfer involves nicking at oriT followed by recircularisation in the recipient after transfer. During this recombination process cointegrates could be formed between the oriT regions of two plasmids. This may reflect a role in nature - a process causing genome reassortment by a highly specific transfer-dependent process. The conjugative plasmid R6K has three origins of replication (α, β and γ) and associated with two of these (α and β) is an oriT (Kolter, 1981; Nordheim et al., 1980). It would be interesting to speculate that these multiple oriV and oriT sites in R6K arose by this recombination process. A simple, but by no means conclusive, way of testing this would be to screen for resolution of the plasmid.

Chapter 5

SEQUENCING OF THE oriT REGION OF RSF1010

(a) Introduction

As described in the previous chapter, the oriT site of RSF1010 has been located on a 150bp TaqI fragment cloned in pED825. This chapter describes the sequencing of this oriT region, which permits the comparison of the RSF1010 sequence with other known oriT sequences and the location of regions possibly involved in mobilisation.

(b) Sequencing pED360

pED360 has the oriT containing TaqI fragment cloned in the ClaI site of the vector pED825 (Fig. 4.4). The close proximity of the EcoRI and HindIII sites to the ClaI site allows easy manipulation of the cloned fragment. The sequencing method used was that described by Maat and Smith (1978) and modified by Sief et al.(1980) (Chapter 2 (j)(i)). The method requires a restriction fragment to be 5'-terminally labelled at one end, and the EcoRI and HindIII sites are ideally situated for this purpose. DNA linearised at the EcoRI (or HindIII) site was 5'- terminally labelled using γ-^{32}P-ATP as described in Chapter 2(e)(v). The DNA was then digested with HindIII (or EcoRI) to generate two fragments each labelled only at one end. The DNA was then fractionated on an acrylamide gel and the fragment containing oriT purified as in Chapter 2(e)(iii). The sequencing reactions were then carried out on the labelled DNA and the reaction products separated by electrophoresis through an 8% polyacrylamide sequencing gel. A typical example of such a gel after autoradiography is shown in Figure 5.1. The complete sequence of the clone pED360, as determined from each strand (i.e. both labelled ends), is shown in Figure 5.2. Sequencing data (Chapter 7) identified several mistakes in this sequence, the sequence presented here has been corrected.

(c) Discussion

(i) Defining a minimum oriT region.

As can be seen from the sequence there are four TaqI restriction

Fig. 5.1 An example of a sequencing gel used to determine the sequence of pED360.

Notes:

(i) The gel was an 8% polyacrylamide gel.

(ii) The sequencing reactions are in the order, from left to right, G,A,T,C, (forward reactions), G,A,T,C (backward reactions).

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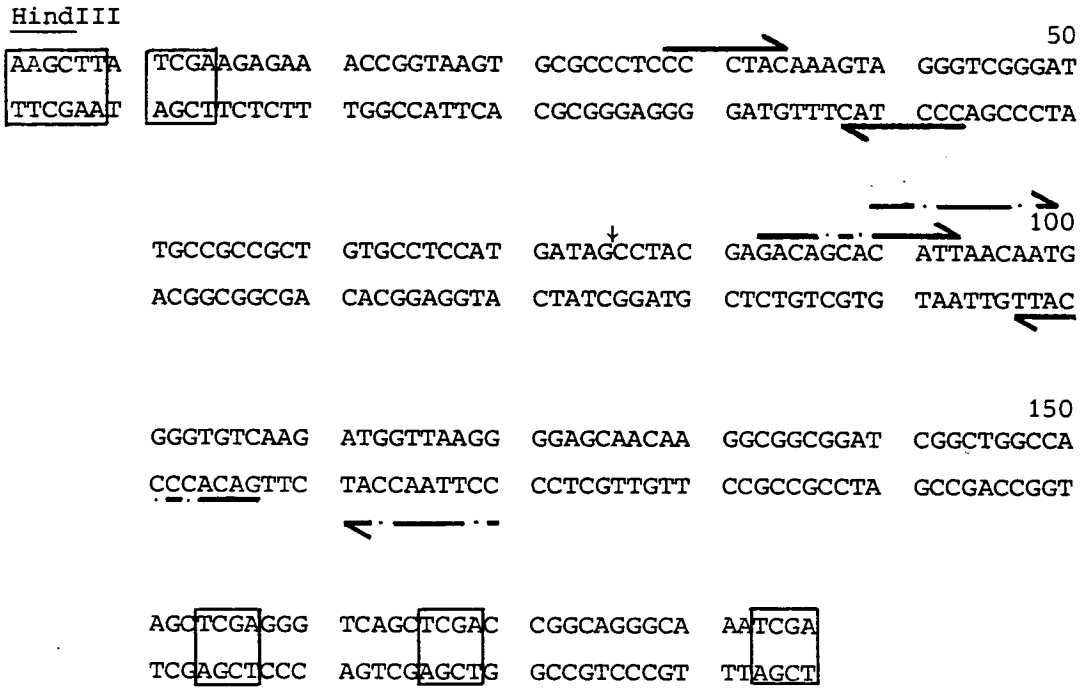
Fig. 5.2 Nucleotide Sequence of the oriT region.

The four TaqI restriction sites are boxed. The HindIII site of the vector, from which the deletion derivatives were measured is shown. The EcoRI site is 25bp to the right of the TaqI site at 184bp. The three regions of dyad symmetry, discussed in the text, are shown (mismatches are represented as gaps). The arrow at 75bp represents the beginning of the 80bp region from the HindIII site to the EcoRI site in the deletion derivative pED384, defined as the minimum oriT region.

Fig. 5.2


HindIII
50
AAGCTTA TCGAAGAGAA ACCGGTAAGT GCGCCCTCCC CTACAAAGTA GGGTCGGGAT
TTCGAAT AGCTTCTCTT TGGCCATTCA CGCGGGAGGG GATGTTTCAT CCCAGCCCTA
100
TGCCGCCGCT GTGCCTCCAT GATAGCCTAC GAGACAGCAC ATTAACAATG
ACGGCGGCGA CACGGAGGTA CTATCGGATG CTCTGTCGTG TAATTGTTAC
150
GGGTGTCAAG ATGGTTAAGG GGAGCAACAA GGCGGCGGAT CGGCTGGCCA
CCCACAGTTC TACCAATTCC CCTCGTTGTT CCGCCGCCTA GCCGACCGGT
AGCTCGAGGG TCAGCTCGAC CGGCAGGGCA AATCGA
TCGAGCTCCC AGTCGAGCTG GCCGTCCCGT TTAGCT

sites which would generate three fragments of 153, 18 and 12bp. The latter two fragments had not been detected previously on the acrylamide gels used, although the presence of a partial band above the 153bp TaqI fragment in certain gels (Fig. 4.3) had suggested that there may be a smaller fragment(s) totalling 30bp. The re-cloning of the large oriT containing TaqI fragment is described in Chapter 4(b)(iii) and this is now considered to be the minimum sequence required for oriT as defined by TaqI cloning.

Transposon insertions were used to create deletions within oriT (Chapter 4(c)). The largest of these deletions, pED384 (oriT$^+$), removed all but 80bp from the HindIII end of the oriT clone pED367 (which contains only the single 153bp TaqI fragment, Fig. 4.10). This allows the mapping of the nick site to these 80bp. oriT sites have been isolated on similarly small fragments e.g. RP1 oriT has been isolated on a 112bp fragment (Guiney and Yakobson, 1983) and indicates that oriT and its recognition site are relatively small.

(ii) Secondary structure in the sequence

The 'SEQ' computer programme (Queen and Korn, 1980) was used to analyse the sequence and locate regions of dyad symmetry. These are shown in Figure 5.2. Two of the three inverted repeat sequences lie outside the minimum region defined by the deletion derivative pED384, and therefore are not involved in oriT function. The third inverted repeat (28-43) is a perfect inverted repeat of 6bp with an intervening sequence of 3bp. As it is within the region defined for oriT it may be involved in oriT function.

(iii) Recognition sites

It has been suggested that the tranferred-strand of ColE1 (or pBR322) may be converted to a double stranded form by the initiation of complementary strand synthesis by a primer synthesised by the primosome (Kornberg, 1982) after binding to an n' site (Nomura et al., 1982; Zipursky and Marians, 1981). An n' site has been located on the nicked, and therefore putative transferred strand, and is not required for replication (Fig. 5.3b) (Oka et al., 1979; Van der Ende et al., 1983). In the recipient this n' site could serve for the assembly of the primosome complex. As the n' site is oriented such that it would be tranferred last, then recipient conjugal DNA synthesis

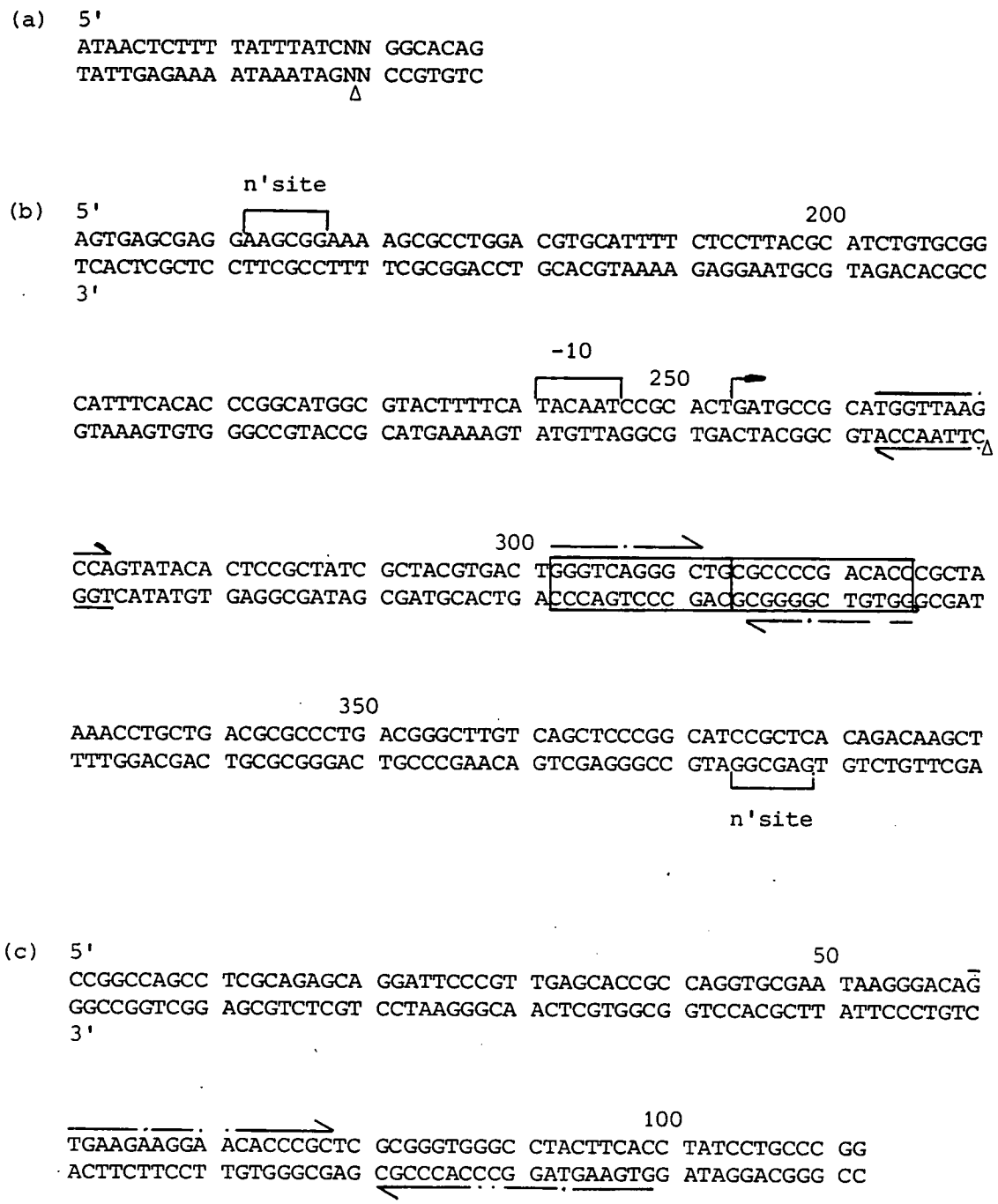
Fig. 5.3 oriT sequences used for comparison with RSF1010 oriT

(a) pSC101 (Nordheim, 1979) Unknown bases are designated (N) and the nic site is represented as a triangle (Δ).

(b) ColE1 (OKa et al. (1981)). The nic site is marked with a triangle (Δ), and the region of imperfect two-fold rotational symmetry surrounding it (Bastia, 1978). The boxed sequences are related to part of a large inverted repeat in the F oriT region (Thomson et al., 1983). The promoter located by Queen and Rosenberg (1981) is shown as are the two n' sites (Nomura et al., 1982; Soeller and Marians, 1982).

(c) RK2 (Guiney and Yakobson, 1983). The arrows show the location of the 18bp inverted repeat.

Fig. 5.3


(a) 5'
ATAACTCTTT TATTTATCNN GGCACAG
TATTGAGAAA ATAAATAGNN CCGTGTC
n'site
(b) 5'
200
AGTGAGCGAG GAAGCGGAAA AGCGCCTGGA CGTGCATTTT CTCCTTACGC ATCTGTGCGG
TCACTCGCTC CTTCGCCTTT TCGCGGACCT GCACGTAAAA GAGGAATGCG TAGACACGCC
3'
-10
250
CATTTCACAC CCGGCATGGC GTACTTTTCA TACAATCCGC ACTGATGCCG CATGGTTAAG
GTAAAGTGTG GGCCGTACCG CATGAAAAGT ATGTTAGGCG TGACTACGGC GTACCAATTC
300
CCAGTATACA CTCCGCTATC GCTACGTGAC TGGGTCAGGG CTGCGCCCCG ACACCCGCTA
GGTCATATGT GAGGCGATAG CGATGCACTG ACCCAGTCCC GACGCGGGGC TGTGGGCGAT
350
AAACCTGCTG ACGCGCCCTG ACGGGCTTGT CAGCTCCCGG CATCCGCTCA CAGACAAGCT
TTTGGACGAC TGCGCGGGAC TGCCCGAACA GTCGAGGGCC GTAGGCGAGT GTCTGTTCGA
n'site
(c) 5'
50
CCGGCCAGCC TCGCAGAGCA GGATTCCCGT TGAGCACCGC CAGGTGCGAA TAAGGGACAG
GGCCGGTCGG AGCGTCTCGT CCTAAGGGCA ACTCGTGGCG GTCCACGCTT ATTCCCTGTC
3'
100
TGAAGAAGGA ACACCCGCTC GCGGGTGGGC CTACTTCACC TATCCTGCCC GG
ACTTCTTCCT TGTGGGCGAG CGCCCACCCG GATGAAGTGG ATAGGACGGG CC

cannot be initiated until completion of transfer. Soeller and Marians (1982) define an essential sequence for the n' site by its ability to act as a template for rifampicin-resistant, dnaB, dnaC, and dnaG dependent DNA synthesis. The essential sequence was 5'-AAGCGG-3' and may be involved in stem loop structures (Shlomai and Kornberg, 1980). There are no such sites within the oriT fragment of RSF1010. If pED367 transfer were to initiate recipient complementary strand synthesis using n', it would have to do so via the n' nite in the vector (2462-2457 on the pBR322 map) and one would also have to postulate that it was that strand which was transferred.

Finnegan and Sherratt (1982) suggested that a cAMP-dependent promoter located 17bp upstream of oriT (Fig. 5.3b) may activate nic and initiate transcription of RNA in the donor, which could serve as a primer for complementary strand synthesis in the recipient. However, rifampicin treatment of donor cells, before mating, did not affect ColE1 mobilisation by F (Maule and Willetts, pers. comm.), which rules out this role of activation and primer formation in ColE1. Two possible promoter sequences were identified in RSF1010 (Siebenlist et al., 1980). These are on the same strand and one is completely outside the minimum oriT region ('-35', 109-104; '-10', 89-84). The second putative promoter sequence is located such that the '-10' region is just inside the minimum oriT sequence ('-35', 98-93; '-10', 74-69). More experimental evidence is needed to show that these sequences can act as promoters (see Chapter 7). It also needs to be determined whether these promoters are involved in transcribing the mob genes and/or activating transfer. This latter possibility could be determined by pre-treatment of donor cells, before mating, with rifampicin.

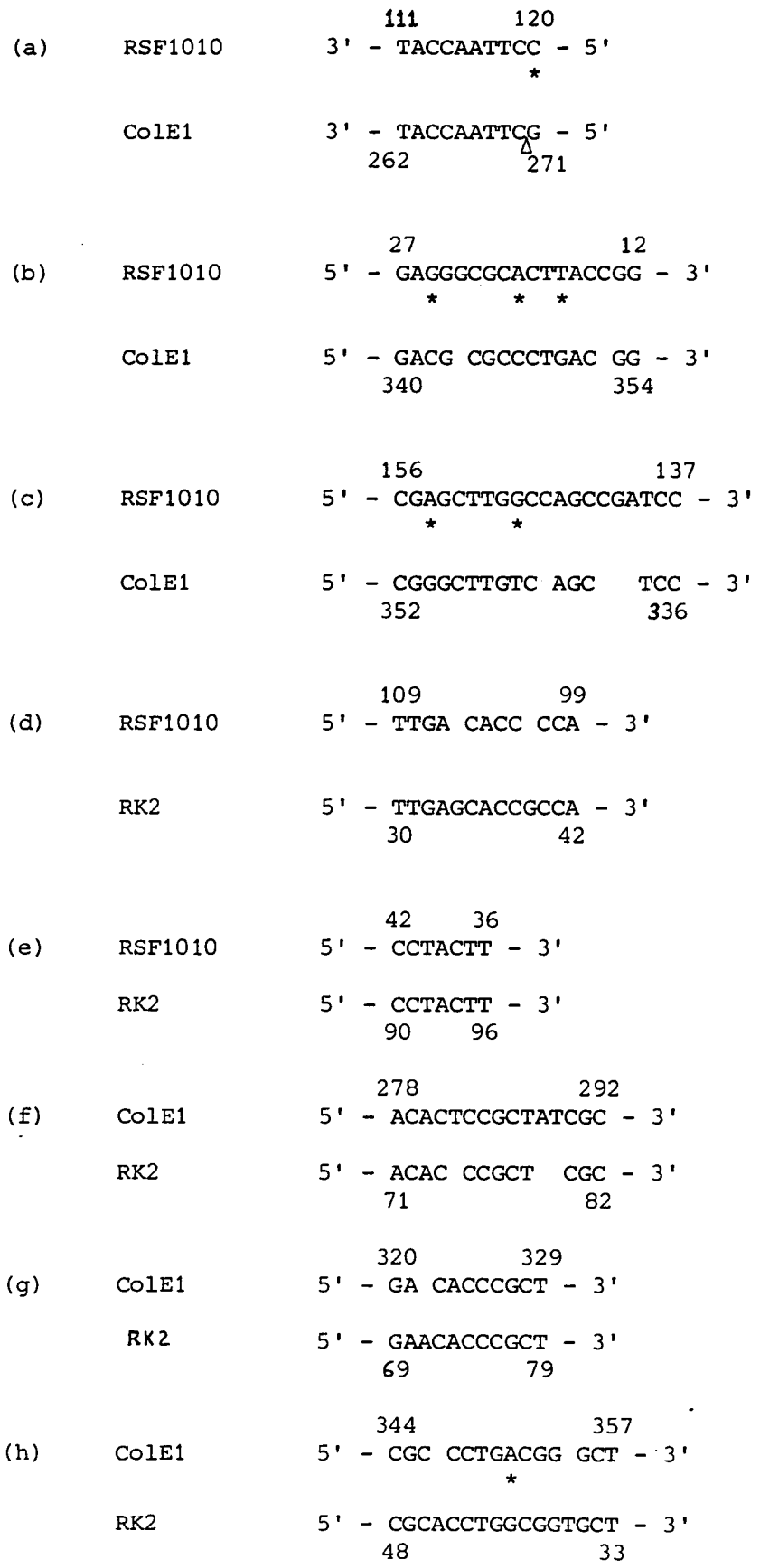
(iv) Comparison of the RSF1010 oriT sequence to other known oriT sequences

The sequences used for comparison with the RSF1010 oriT were: ColE1 (Bastia, 1978; OKa et al., 1979); pSC101 (Nordheim, 1979) and RK2 (Guiney and Yakobson, 1983). One might expect from such comparisons two different types of homology depending on the plasmids concerned. Firstly, one might expect homology between oriTs and their protein recognition sites, if they were recognised by the same conjugative DNA metabolism gene products. Secondly, it was evident from the data in Chapter 3 that there was a specific interaction between the non-

Fig. 5.4 Regions of Sequence Homology

(a) - (e) show regions of homology between RSF1010 and ColE1 or RK2. (f) - (h) show regions of homology between ColE1 and RK2. The numbers refer to the location of the sequences in Figure 5.3. Mismatches are represented as (*). The triangle is the ColE1 nick site (Δ).

Fig. 5.4


(a) RSF1010
111 120
3' - TACCAATTCC - 5'
ColE1
3' - TACCAATTCG - 5'
262 271
(b) RSF1010
27 12
5' - GAGGGCGCACTTACCGG - 3'
ColE1
5' - GACG CGCCCTGAC GG - 3'
340 354
(c) RSF1010
156 137
5' - CGAGCTTGGCCAGCCGATCC - 3'
ColE1
5' - CGGGCTTGTC AGC TCC - 3'
352 336
(d) RSF1010
109 99
5' - TTGA CACC CCA - 3'
RK2
5' - TTGAGCACCGCCA - 3'
30 42
(e) RSF1010
42 36
5' - CCTACTT - 3'
RK2
5' - CCTACTT - 3'
90 96
(f) ColE1
278 292
5' - ACACTCCGCTATCGC - 3'
RK2
5' - ACAC CCGCT CGC - 3'
71 82
(g) ColE1
320 329
5' - GA CACCCGCT - 3'
RK2
5' - GAACACCCGCT - 3'
69 79
(h) ColE1
344 357
5' - CGC CCTGACGG GCT - 3'
RK2
5' - CGCACCTGGCGGTGCT - 3'
48 33

conjugative plasmid and the conjugative plasmid - the mobilisation recognition system. If this occurred by recognition of a particular DNA sequence then one might expect a sequence homology between the non-conjugative plasmid and the conjugative plasmid which mobilises it. In F, ColE1 and CloDF13 a partially homologous region of dyad symmetry has been identified and tentatively suggested to be involved in the recognition, by a host- or plasmid-encoded protein, of a site necessary for mobilisation by the F conjugation system (Willetts and Wilkins, 1983) (Fig. 5.3b).

Several regions of homology were found between RSF1010 and ColE1 (Fig. 5.4(a-c)). The most interesting similarity was that between RSF1010 and the nine base pairs of ColE1 immediately to the 5' side of the relaxation nick site (Fig. 5.4(a)). This sequence might therefore seem an ideal candidate for the nick site in RSF1010, but as this region is deleted in pED384 (Fig. 4.10), then it cannot contain the nick site for RSF1010 mobilisation by IncP plasmids. Furthermore, unlike the ColE1 nick site, there is no two-fold rotational symmetry in this region of RSF1010, which might play a role in oriT recognition. Why should such a similarity exist? It seems more than just chance that such a sequence similarity should exist within these two small cis-acting regions, but until the oriT site of RSF1010 is accurately mapped it is difficult to suggest how it might be involved in oriT function.

The other two regions of similarity that are presented in Figure 5.4(b),(c) are partial homologies between RSF1010 and ColE1. However, both of these regions can be deleted in pBR322 (Finnegan and Sherratt, 1982), which is virtually identical to ColE1 in the oriT region, with only a small reduction in mobilisation frequency. The sequence in (c) can also be deleted from pED360 without any effect on mobilisation (Table 4.2) and the homology is therefore irrelevant. There was no homology to the oriT region of the non-conjugative plasmid pSC101 (Fig. 5.3(a)).

The sequence of the RK2 oriT site is shown in Figure 5.3(c) and two sequence homologies between this and the RSF1010 oriT sequence are presented in Figure 5.4(d),(e). One of these (e) is exactly homologous to part of the 18bp inverted repeat in RK2. Furthermore, these 7bp are also part of the only inverted repeat in the minimum

defined oriT sequence. It might be that this sequence is the recognition site of a protein necessary for mobilisation by the IncP plasmid transfer system. However, the regions of partial homology between ColE1, mobilised relatively efficiently by RK2 (Table 3.1), and RK2 were different to those identified with RSF1010, Figure 5.4 (f-h), although two of these (f) and (g) were partially homologous to the inverted repeat. The significance of these homologies therefore requires further investigation.

The actual site of the nick must be within the 80bp of RSF1010 in pED384 (Fig. 4.10, 5.2). By using these clones it should be possible to map the nick site and to characterise the direction, and strand, of transfer. In vitro methods used to map the nick site require isolation of a relaxation complex (Bastia, 1978; Nordheim et al., 1980), but attempts to isolate a relaxation complex of either RSF1010 or pED360 by the method of Nordheim et al. (1980) have so far proved unsuccessful. To avoid the problems of purifying relaxed DNA it may be possible to detect relaxation (nicking at oriT) in cleared lysate plasmid extracts (Chapter 2(d)(i)). A proportion of the plasmid molecules isolated from a cleared-lysate would be nicked at oriT, the two strands could then be separated in a denaturing gel, and the nicked strand detected using a single-stranded probe containing oriT. Such probes are available using the M13-containing oriT templates (Chapter 7). Accurate location of the nick site could be detected by using sequencing gels to separate the two DNA strands that had been pre-digested with a suitable enzyme.

Alternatively, an in vivo system similar to that described by Everett and Willetts (1980) could be used. This would involve the cloning of oriT into a suitable λ vector, followed by the purification of phage DNA from cells carrying RSF1010. A proportion of these cells would be nicked at the oriT site, by analogy to the F oriT system, and the location of this site could then be determined by separation of the fragments after denaturation by electrophoresis (Everett and Willetts, 1980; 1982). The direction and strand transferred could be identified by measuring the transfer frequencies of adjacent chromosomal markers after integrating the λ ($oriT^+$) phage into the chromosome (Rupp and Ihler, 1968; Ohki and Tomizawa, 1968).

Chapter 6

CHARACTERISATION OF THE MOBILISATION REGION OF RSF1010

(a) Introduction

The transfer regions of conjugative plasmids, especially the F-factor, have been extensively characterised (Willetts and Skurray, 1980). The twenty-two tra genes of the F factor have been conveniently sub-divided into functional groups (Chapter 1(b)). The two largest sub-groups are those involved in pilus synthesis and conjugal DNA metabolism. The genes involved in conjugal DNA metabolism show some plasmid specificity. Several different alleles of the genes required for nicking and triggering of transfer have been observed in the F-like plasmids (Willetts and Maule, 1979; Everett and Willetts, 1980). In contrast to this the products of the pilus synthesising genes and traN, G and D are interchangeable between F-like plasmids (Ohtsubo et al., 1970; Willetts, 1971; Alfaro and Willetts, 1972).

The non-conjugative plasmids ColE1 and CloDF13 do not require any of the known DNA metabolism genes of F, so far tested, for their mobilisation (Van de Pol et al., 1978; Willetts, 1980); these genes must be replaced in an overall functional sense by the non-conjugative plasmid's own mobilisation products. Complementation of mobilisation deficient mutants of ColE1 by ColK, but not ColE2 (Warren and Sherratt, 1977), and lack of complementation of mobilisation mutants of CloDF13 by ColE1 (Van de Pol, 1980), imply that the non-conjugative plasmids also have plasmid-specific DNA metabolism genes. Furthermore, as different plasmids have different oriT sequences (Chapter 5) then this would also imply they must have plasmid-specific conjugal DNA metabolism genes.

The regions of CloDF13 and ColE1 involved in mobilisation are of a similar size and orientation with respect to their oriV sites (Clark and Warren, 1979; Van de Pol, 1978; Veltkamp and Stuitje, 1981; Fig. 1.4). Three genes (B, Y and X) have been identified in CloDF13 as being involved in mobilisation and these encode proteins of molecular weights 62Kd, 16Kd and 11Kd respectively. Genes B and Y are thought to be transcribed in a single unit away from oriT, while X is transcribed towards oriT (Van de Pol, 1980; Veltkamp and Stuitje,

1981). In contrast to ColE1, CloDF13 cannot be isolated as a relaxation complex (J. Nijkamp, pers. comm.). However, the mechanism of mobilisation is assumed to be by a similar process to that postulated for ColE1 (Warren et al., 1978; Van de Pol, 1980; Veltkamp and Stuitje, 1981).

Preliminary complementation data has divided the ColE1 mobilisation-deficient (Mob^-) mutants into three complementation groups (Inselberg and Ware, 1979). However, the transcriptional organisation and the identity of the proteins encoded by these complementation groups has still to be determined (Chapter 1(c); Fig. 1.4a). Furthermore there is no direct evidence that the proteins present in the relaxation complex are ColE1 determined, although two proteins of similar molecular weight to the 16Kd and 11Kd relaxation components are absent or reduced in some relaxation deficient mutants (Dougan and Sherratt, 1977; Inselberg and Applebaum, 1978).

The mobilisation proteins are assumed to carry out the same nicking process to initiate transfer from oriT in vivo as is observed during the in vitro relaxation process. In addition, Inselberg and Ware (1979) isolated Mob^- ColE1 mutants capable of being relaxed. This implied that some mobilisation proteins have additional functions.

Data presented in Chapter 4(b)(i) demonstrated that additional functions were necessary in trans for the mobilisation of pED361, which carries the 800bp HaeII oriT fragment,other than those supplied by RP1. This has since been confirmed by other data (Bagdasarian et al., 1981a;b; 1982), which have located a region on the RSF1010 map involved in mobilisation, physically distinct from oriT (Fig. 6.6). Although no evidence was given to indicate that this region supplied a trans-acting function, it was assumed, by analogy to ColE1 and CloDF13, and by the demonstration that a trans-acting function was necessary for mobilisation of pED361, that this region is supplying a mobilisation function(s).

This chapter describes the cloning of genes involved in the mobilisation of RSF1010. Mutants isolated by transposon insertion, hydroxylamine mutagenisis and deletion have allowed a preliminary genetic analysis to be carried out and proteins which may be involved in mobilisation to be identified.

(b) The Cloning and Physical Characterisation of the Mobilisation Region of RSF1010

(i) The isolation of a Mob$^+$ clone

Chapter 4(b) describes the cloning of the oriT of RSF1010 into the HaeII vector pED825 (Fig. 4.2). All the clones isolated carried at least the oriT-containing 800bp HaeII fragment (cloned in pED361, Fig. 4.1). Several of these oriT$^+$ clones were tested for their ability to be mobilised in the absence of RSF1010. The smallest clone that was efficiently mobilised by pUB307 was pED350 (Fig. 4.1). HaeII restriction analysis (Fig. 6.3a, 6.5) revealed that pED350 contained seven HaeII restriction fragments of 800, 310, 220, 170, 130, 70 and 45bp. The 800bp fragment is that containing oriT. A 1.2Kb HaeII fragment is located anti-clockwise to the 800bp fragment in RSF1010 (Bagdasarian et al., 1982) and so, assuming that the clone is a simple partial digest fragment of RSF1010, pED350 must contain approximately 945 (1745-800) bp of RSF1010 to the clockwise side of the 800bp HaeII fragment. The DNA in pED350 therefore would extend from approximately 2.8Kb to 4.6Kb on the RSF1010 map (Fig. 1.1, 6.6). This is in agreement with the data of Bagdasarian et al. (1981; 1982) and has since been confirmed by heteroduplex analysis (data not shown) and sequence data (Chapter 7).

(ii) Construction of a restriction endonuclease map of pED350

A detailed restriction map of pED350 was constructed to facilitate the characterisation of deletion and insertion mutants which were later isolated.

The order of the seven HaeII fragments of pED350 was determined by the mapping technique of Smith and Birnstiel (1976). pED350 was particularly suitable for this technique as a detailed restriction map of the vector is known and the sizes of all the HaeII fragments within this clone are known, allowing the prediction of certain partial fragment sizes. The HindIII site of pED350 was end-labelled with α-^{32}P CTP as described in Chapter 2(e)(v) and then digested with EcoRI to generate two end-labelled fragments. One of these would be only 29bp and would not be resolved on an agarose gel. The second end-labelled fragment contained all but 29bp of the plasmid. The DNA was then subjected to HaeII digestion for increasing lengths of time before fractionating on a 1.5% agarose gel (Fig. 6.1).

Fig. 6.1 An Autoradiogram showing a 'Smith and Birnstiel' Analysis of pED350 to determine the order of the HaeII sites after electrophoresis in a 1.5% agarose gel.

	Track	DNA	Restriction Endonuclease		
(a)	1	pED825	TaqI		End-labelled after digestion
	2	EDλ7	HindIII		
	3	pED350	HaeII	for 15'	End-labelled at the HindIII site, followed by EcoRI digestion
	4	pED350	HaeII	for 30'	
	5	pED350	HaeII	for 45'	
	6	pED350	HaeII	for 60'	
(b)	1	pED350	HaeII	for 30'	- " -

Notes: (i) Sizes of fragments are marked in kb.

(ii) The size standards of (b) have been omitted but were the same as (a) and were labelled as in (a).

(iii) The heavily labelled band in (a) consists of two fragments resulting from end-labelled partial digests in both directions from the HindIII site, because of incomplete EcoRI digestion.

= = = = = = =

Fig. 6.2 Restriction Endonuclease Map of pED350

Notes: (i) Sizes of HaeII fragments within the clone (thicker line) are marked in base pairs.

(ii) HaeII sites are indicated by an H. The HindIII-HaeII distance is 206bp and the two HaeII sites in the vector are 370bp apart (Sutcliffe, 1978; Everett and Willetts, 1982).

FIG.6.1

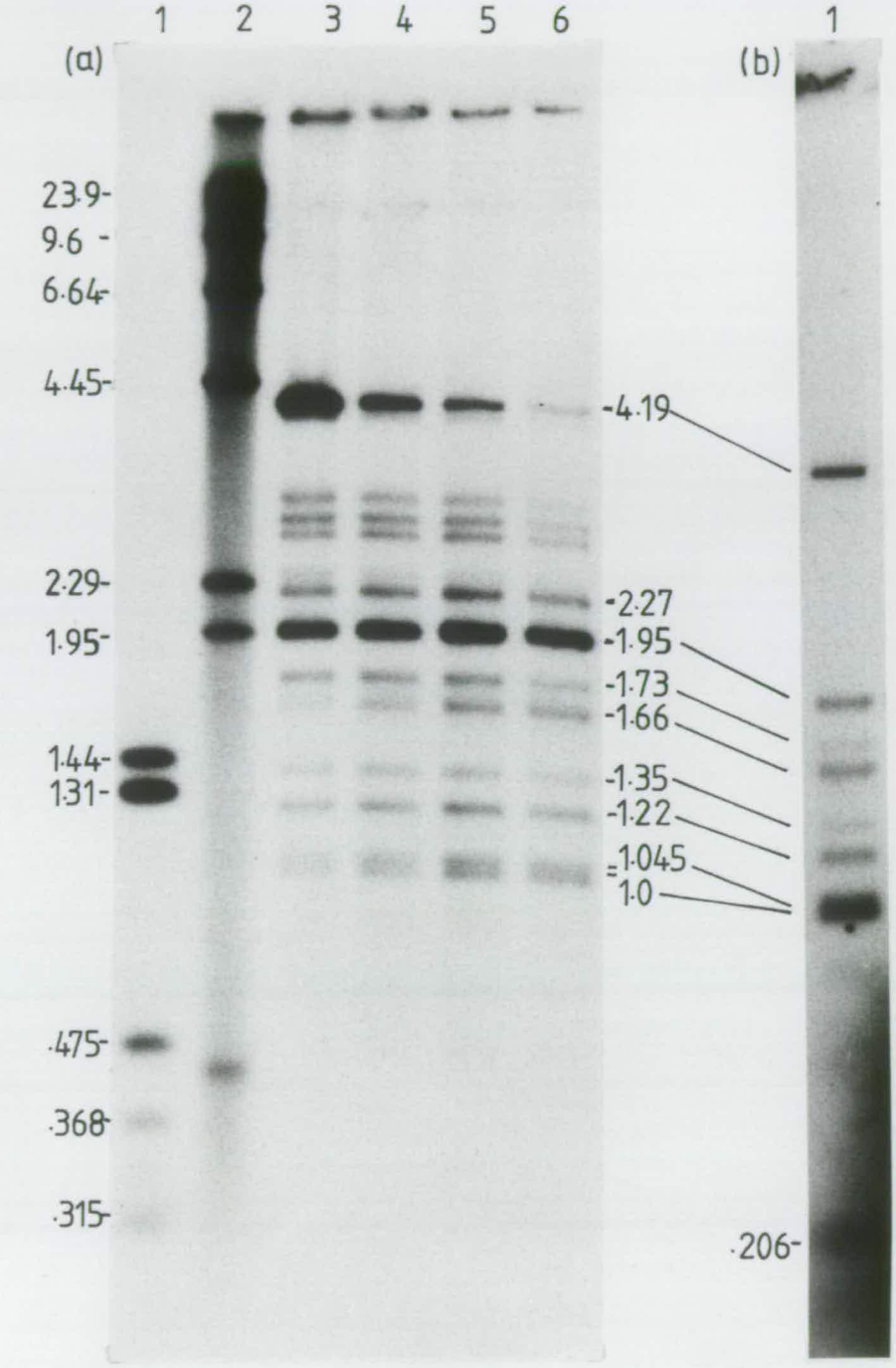
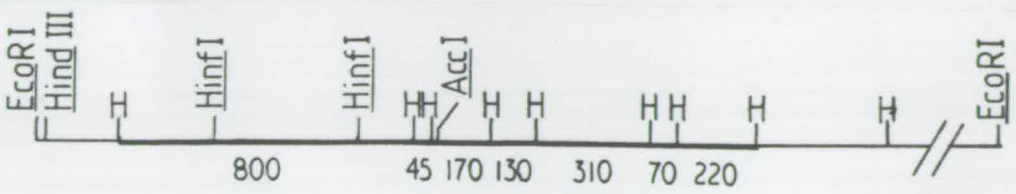


FIG.6.2 pED350



The smallest HindIII-HaeII fragment predicted would be 203bp. Although this is not resolved in Fig 6.1a it can easily be seen in the autoradiogram of a second independent experiment (Fig. 6.1b). As this HaeII site is that into which the RSF1010 DNA has been cloned then all the next seven end-labelled partial digestion fragments are due to HaeII sites within the cloned DNA. The largest predicted end-labelled partial digestion fragment is from the HindIII site to the HaeII site 370bp outside the HaeII clone. Unexpectedly, end-labelled fragments were found above this last predicted fragment. These can be explained by an incomplete EcoRI digestion allowing end-labelled HaeII partially digested fragments to be seen from an anticlockwise direction. This was confirmed by repeating the experiment and ensuring that EcoRI digestion was complete, and, as can be seen in Fig. 6.1b, none of these bands exist. Furthermore, the sizes of the bands due to the incomplete EcoRI digestion confirm the order of the HaeII fragments as estimated from the HindIII site. The HaeII restriction map of pED350 is shown in Figure 6.2. This has since been confirmed by sequence data (Chapter 7) except that one extra 10bp HaeII fragment was predicted. A fragment of this size would be impossible to detect in the polyacrylamide gels used or by the 'Smith and Birnstiel' analysis. The sizes of the HaeII restriction fragments were also in good agreement with the sequence data.

Bagdasarian et al. (1981) indicated the presence of an AccI restriction site at co-ordinate 3.7Kb in RSF1010 (Fig. 6.6). This would be within pED350. The presence of this site was confirmed and mapped by various restriction enzymes (e.g. HaeII, AccI double digest, Fig. 6.5) to 10bp from the end of the 170bp HaeII fragment closest to oriT (Fig. 6.2). This location has been confirmed by the sequence data (Chapter 7).

In agreement with Meyer et al. (1982b), two HinfI sites were also located in the mobilisation region. These two sites were mapped 390bp apart within the 800bp oriT clone by measuring the sizes of various double-digestion products (data not shown, Fig. 6.2). Again the location of these sites has been confirmed by sequence data.

(c) Isolation and Characterisation of Mob$^-$ Mutants

(i) Deletion mutants

Given the detailed restriction map of pED350 for the restriction

Fig. 6.3

(a) HaeII Restriction Analysis of the deletion derivatives of pED350.

Track	DNA	Restriction Endonuclease
1	pED825	MspI
2	pED350	HaeII
3	pED351	HaeII
4	pED352	HaeII
5	pED353	HaeII
6	pED354	HaeII

Notes: (i) Sizes are in base pairs.

(ii) Sizes of the HaeII fragments of pED350 are estimates from several gels and have been confirmed by sequence data.

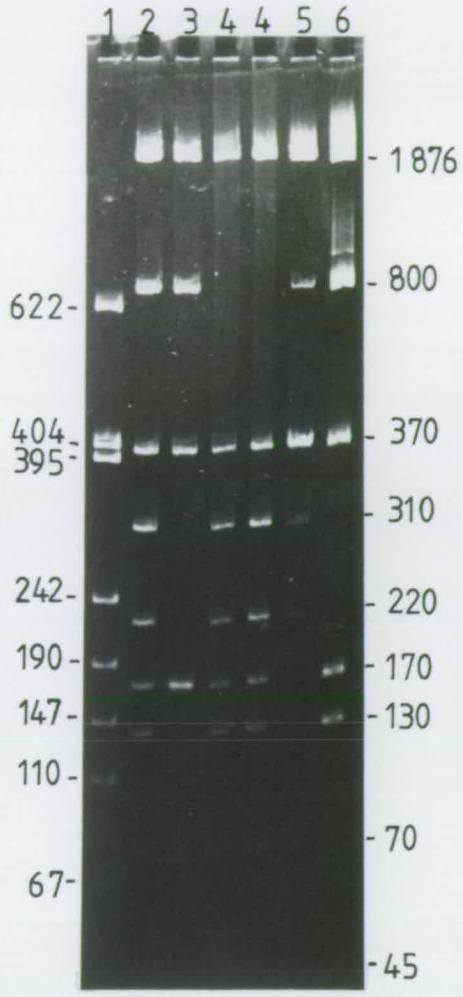
(iii) The gel was an 8% polyacrylamide gel.

(b) Detailed map of pED350 and its deletion derivatives summarising the data obtained from (a).

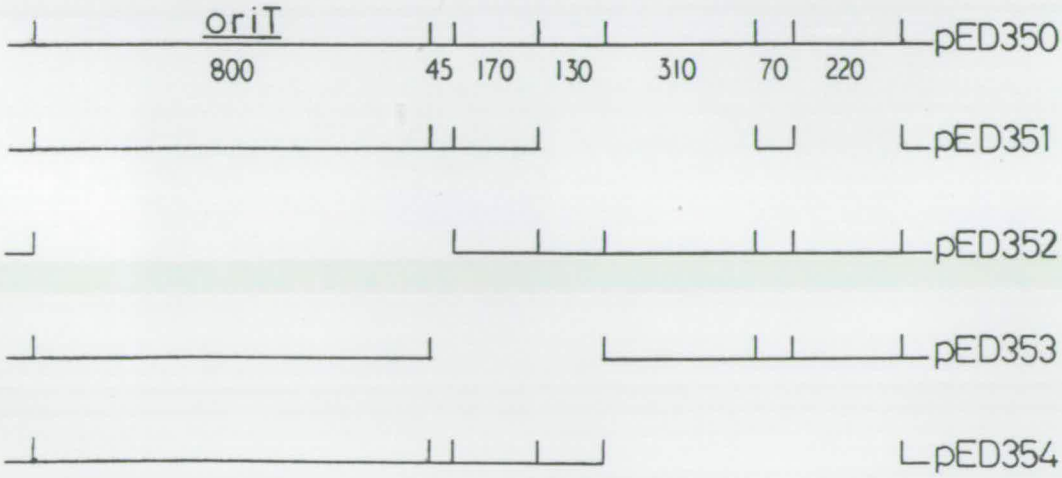
The figure shows only the HaeII sites (indicated as vertical bars) present in each of the clones. Fragment sizes are in base pairs.

FIG.6.3

(a)


1 2 3 4 4 5 6
1876
800
622
404
395
370
310
242
220
190
170
147
130
110
70
67
45

(b)


oriT
800
45 170 130 310 70 220
pED350
pED351
pED352
pED353
pED354

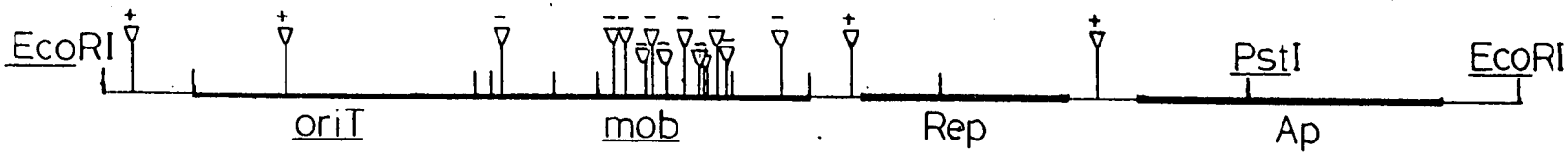
enzyme HaeII, one of the simplest ways to create mutants was by deleting HaeII fragments. pED350 was partially digested with HaeII before diluting the DNA and religating. The DNA was then used to transform JC6310 (pUB307) and Ap^R transformants were selected in liquid culture. This culture was then diluted into fresh Ap-broth and grown to mid-exponential phase before being used as a donor in a filter mating with ED24. The DNA of twenty-four Ap^R transconjugants were screened for a reduction in size. However, none were smaller than the original clone pED350 (data not shown), implying that all the HaeII fragments of pED350 are necessary to provide the Mob^+ phenotype. This was substantiated by screening DNA from twelve Ap^R transformants after plating out an aliquot of the liquid culture. Six out of twelve of these transformants contained DNA smaller than pED350. This DNA was purified and digested with HaeII before separating in an 8% polyacrylamide gel (Fig. 6.3a). One of the clones had an identical restriction pattern to pED825 (data not shown) while a second was identical in restriction pattern to the oriT clone pED361 (data not shown), these were discarded. The other four clones were different deletions of pED350 (Fig. 6.3a,b). When tested for mobilisation all four had a Mob^- phenotype (Table 6.1) which implied that some DNA essential for mobilisation had been deleted.

Table 6.1 The Mobilisation Ratios of pED350 HaeII deletion derivatives

Plasmid	Mobilisation Ratio[(a)]
pED350	1
pED351	$<2.6 \times 10^{-6}$
pED352	$< 4 \times 10^{-6}$
pED353	$<1.11 \times 10^{-6}$
pED354	$<1.1 \times 10^{-6}$

(a) The ratio of mobilisation frequency to transfer frequency of pUB307.
The donor was JC6310 (pUB307) and the recipient ED24. Spc^R was the contraselection.

Fig. 6.4 Map of pED350 showing the site of insertion and Mob phenotype of fifteen Tn1725 insertions.



Notes: (i) HaeII sites are shown as vertical bars.

(ii) The + or - indicates their ability to be mobilised. These were initially measured by 'spot matings', Chapter 2(c)(v) and confirmed by plate-matings.

(iii) The sites of these insertions are based on the sizes of EcoRI and EcoRI, PstI double digest fragments and are therefore only approximate.

Fig. 6.3b shows the restriction fragments present in the four clones. As the initial digestion was a partial digestion it is assumed that the order of the fragments is the same as in pED350, but this has not been confirmed.

pED352 does not contain the 800bp HaeII oriT fragment (nor the adjacent 45bp HaeII fragment) and therefore is not mobilisable. The other three clones are all oriT$^+$, as they carry the 800bp HaeII fragment, but are still Mob$^-$ implying that they have lost information essential for mobilisation. pED353 and pED354 contain deletions from opposite ends of this essential region up to the same site (Fig. 6.3b). As both are Mob$^-$ this demonstrates that a mobilisation gene must span this HaeII junction or that there are two mobilisation genes.

(ii) Transposon insertion mutants

Transposons have been extensively used to create insertion mutants (e.g. Rubens et al., 1976; Dougan and Sherratt, 1977; Barth et al., 1978b). They can cause both polar and non-polar effects and the site of insertion can be easily mapped. The transposon Tn1725 is particularly useful in this respect as independent isolates are easily obtained, Chapter 2(h), and they can be mapped by virtue of the EcoRI sites in the inverted repeats of the transposon. The EcoRI sites are also useful in that deletions of the whole transposon can be made in vitro and, as the EcoRI sites are only 15bp from the ends of the inverted repeat, this deletion leaves only 30bp of transposon DNA and 5bp of duplicated target DNA. The sequence of the inverted repeats has been determined (Schoffl et al., 1981; R. Schmitt, pers. comm.) and they do not contain any stop codons. The removal of the transposon by in vitro deletion therefore causes a frame-shift mutation.

Tn1725 transposon insertions were made into pED350, as described in Chapter 2(b). Both Mob$^+$ and Mob$^-$ insertion derivatives within the mobilisation region were desired as this allowed the limits and organisation of any genes to be determined. Thirty-six independent insertions were isolated and mapped by EcoRI and EcoRI, PstI double digestions followed by comparison of their restriction patterns after electrophoresis (data not shown). Eleven of the insertions mapped within the mobilisation region; all but one had reduced mobilisation (Fig. 6.4). The single insertion, pED416, which mapped within the oriT-containing HaeII fragment, was Mob$^+$. As this is so close to the

end of the HaeII clone it probably defines the limit of DNA essential for mobilisation, assuming oriT lies to the right of this insertion. All the other insertions mapped outside the oriT containing HaeII fragment, which suggests they are inserted in a gene or genes essential for mobilisation.

To simplify the analysis of this region, the transposon containing EcoRI fragment was deleted from six of the insertions. This was carried out by partial EcoRI digestion followed by dilution of the DNA and religation. The ligation was stopped by heating to 65°C, and the DNA was digested with SmaI. The transposon contains a single SmaI site and therefore any plasmids containing the transposon would be linearised, thus reducing their transformation efficiency. The digested DNA was used to transform JC6310 and transformants screened for DNA containing the appropriate deletion. Both types of EcoRI deletion were retained i.e. those deleted for the transposon EcoRI fragment and those deleted for both the transposon EcoRI fragment and the small plasmid EcoRI fragment (Fig. 6.4). The larger deletions, though not described further in this thesis, may well prove useful in complementation analyses. Each of the partial deletions was analysed by PstI, HindIII double digestion to confirm that they were indeed partial digestion products rather than re-constituted plasmids with the EcoRI fragments in the reverse orientation.

The remaining transposon EcoRI site was then accurately mapped by comparing the mobilities of various HaeII and EcoRI, HaeII restriction fragments after electrophoresis in an 8% polyacrylamide gel (Fig. 6.5). The HaeII fragment which contains the EcoRI site can be detected as it has a reduced mobility (equivalent to the 35bp insertion) when compared to the corresponding fragment in pED350. This larger fragment, on digestion with EcoRI, produced two novel fragments (one of which was too small to be seen in pED401 and pED405) which, when measured, allowed the mapping of the EcoRI site within that fragment. AccI, EcoRI double digestions (data not shown) allowed the orientation of the EcoRI site within the HaeII fragment to be determined. Tracks 12 and 13 in Figure 6.5 show the HaeII digestion products of a plasmid (pED410) still containing the transposon. The transposon was later deleted to give the plasmid pED411. Although difficult to discern from the gel the transposon is probably inserted within the 220bp

Fig. 6.5 Comparative HaeII restriction analysis of pED350 and pED350::Tn1725 deletion derivatives used to map the insertion sites.

Track	DNA	Restriction Endonuclease
1	pED825	MspI
2	pED350	HaeII, AccI
3	pED350	HaeII
4	pED401	HaeII
5	pED401	HaeII, EcoRI
6	pED403	HaeII
7	pED403	HaeII, EcoRI
8	pED405	HaeII
9	pED405	HaeII, EcoRI
10	pED407	HaeII
11	pED407	HaeII, EcoRI
12	pED410	HaeII
13	pED410	HaeII, EcoRI
14	pED409	HaeII
15	pED825	MspI

Notes: (i) Sizes are in base pairs. The gel was 8% polyacrylamide.

(ii) On digestion with EcoRI the 1876bp HaeII vector fragment is replaced by two fragments 235bp and 1641bp in size.

(iii) Only the HaeII restriction analysis of pED409 is shown, however other data (not shown) imply that the EcoRI site is approximately 40bp from one end of the 170bp HaeII fragment.

(iv) All the digests were carried out on pED350::Tn1725 derivatives from which the transposon containing EcoRI fragment had been removed, except pED410. However, the transposon was later deleted to give the derivative pED411.

FIG.6.5

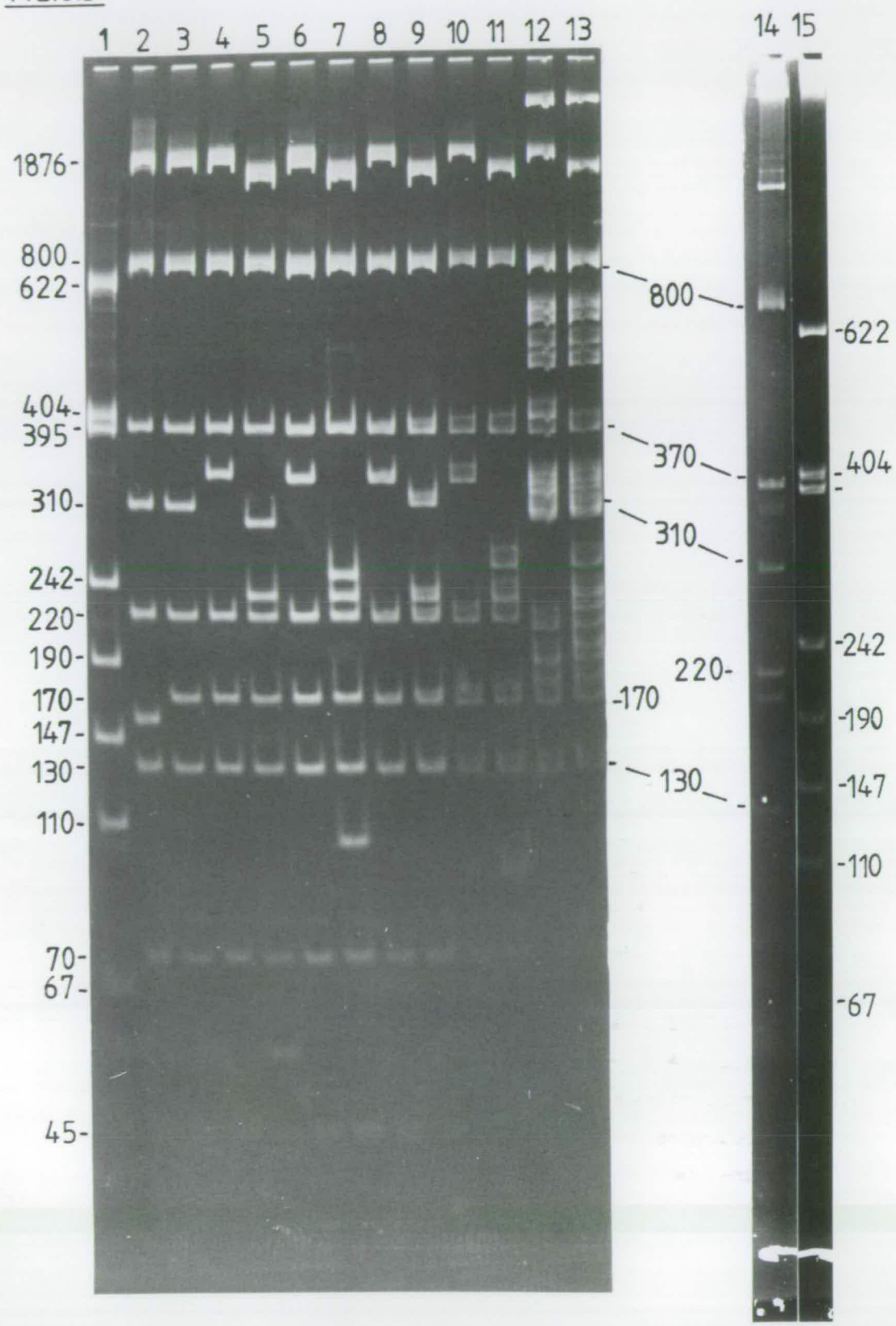

1 2 3 4 5 6 7 8 9 10 11 12 13
14 15
1876-
800-
622-
404-
395-
310-
242-
220-
190-
170-
147-
130-
110-
70-
67-
45-
800
370
310
220-
170
130
-622
-404
-242
-190
-147
-110
-67

Table 6.2 Mobilisation Ratios of pED350::Tn1725 deletion derivatives compared to pED350.

Plasmid	Ratio
pED350	1
pED401	7.8×10^{-5}
pED403	1.7×10^{-4}
pED405	3.2×10^{-3}
pED407	2.7×10^{-3}
pED409	3.5×10^{-3}
pED411	4.8×10^{-4}
pED412	8.4×10^{-4}

All matings were plate matings. The donor was JC6310 (pUB307) and the recipient ED24. Contraselection was for Spc^R. The ratios quoted are expressed in terms of the mobilisation frequency of pED350. The average mobilisation ratio of pED350 compared to the transfer frequency of pUB307 was 1.0.

All the deletions contained only the 30bp insert of the transposon up to the EcoRI site, except for pED412 which still contains the whole transposon.

HaeII fragment, as this fragment is absent from both tracks. This was later confirmed by measuring the sizes of the AccI, EcoRI double-digestion products which placed the insert 15bp inside the 220bp HaeII fragment. The EcoRI site in the deletion derivative pED409 has only been mapped to the 170bp HaeII fragment. The sites of insertion of three of the plasmids, pED401, pED405 and pED407 have since been confirmed by sequencing (Chapter 7) and the site of insertion, as mapped above, has been shown to be extremely accurate (± 5bp). Figure 6.6 summarises the sites of insertion of all the derivatives.

The mobilisation frequencies of these insertion mutants were measured to show that deleting all but 35bp of the insert had no effect on the mutant phenotype (Table 6.2). All the mutants retain the Mob^- phenotype. The insertion mutant pED412, which contains the whole transposon was also accurately mapped by sequencing (Fig. 6.6) and its reduced mobilisation frequency is also given (Table 6.2).

(iii) Hydroxylamine mutagenesis

Point mutants are an extremely useful form of mutation for complementation analyses as there are few of the polar effects which are often associated with transposon insertions. Hydroxylamine was used to induce mutations in RSF1010 (Humphreys et al., (1976; 2(i)). This modifies cytosine so that it pairs with adenine rather than guanosine. After several independent experiments and the screening of several thousand colonies only two Mob^- mutants were obtained. The mobilisation frequency of these two mutants is given in Table 6.3. When the Ap^R Mob^+ clone pED350 was mutagenised two independent Mob^- mutants pED358, pED359 were also isolated and their mobilisation frequencies are also given in Table 6.3.

Table 6.3 The Mobilisation Ratio[(a)] of Hydroxylamine Mutants

Plasmid	Replicon	Mobilisation Ratio
pED355	RSF1010	$< 10^{-6}$
pED357	RSF1010	$< 10^{-6}$
pED358	pED350	$< 10^{-4}$
pED359	pED350	$< 10^{-7}$

(a) Mobilisation Ratio is the ratio of the mobilisation frequency to transfer frequency.

All matings were plate matings. The donor was JC6310 (pUB307) and the recipient ED24.

There is no obvious explanation why RSF1010 should be so difficult to mutagenise. One possibility may be that as replication and mobilisation functions are so closely mapped in RSF1010 (Meyer et al., 1982a,b; Bagdasarian et al., 1982) that creating mutants in a mobilisation gene may affect replication. Indeed pED357 DNA was always purified in low yields (data not shown). In pED350 derived mutants the plasmids would replicate using pBR322 replication functions.

The mutations isolated and described above were used to carry out a preliminary complementation analysis and to detect proteins synthesised by the mobilisation genes.

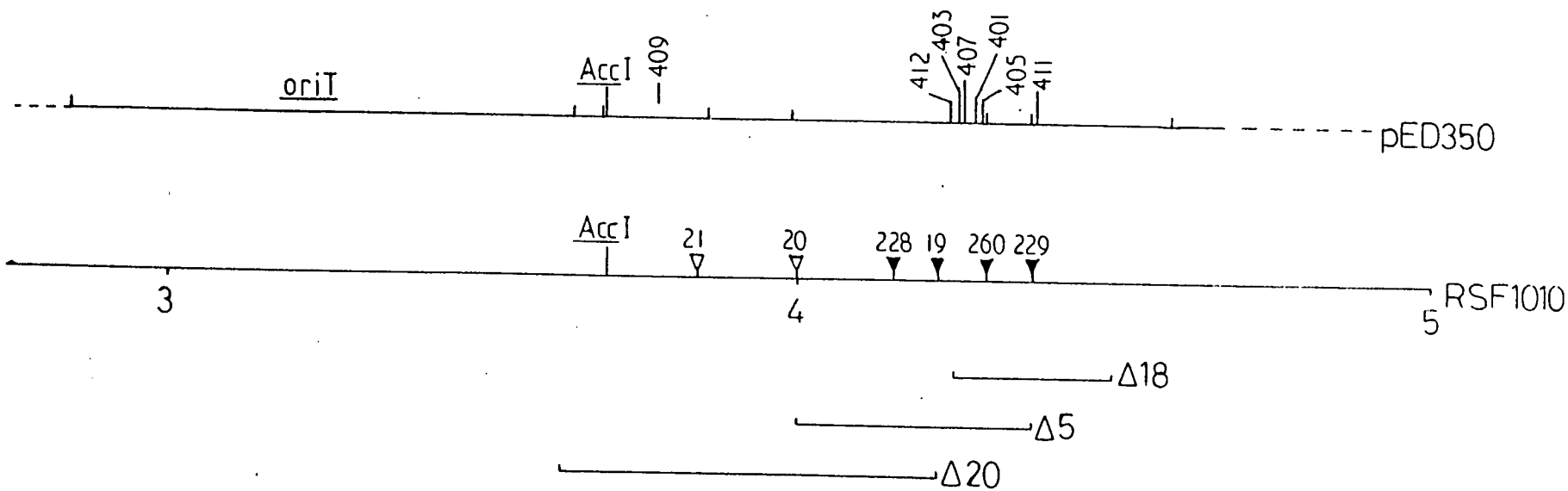
(d) A Preliminary Complementation Analysis of the Mobilisation Region of RSF1010

(i) Introduction

To avoid incompatibility between Mob$^-$ mutants of pED350, Mob$^-$ mutants of RSF1010 were obtained. These RSF1010 mutants were kindly supplied by Prof. M. Bagdasarian and their construction has been described (Bagdasarian et al., 1982). The location of the Tn3 insertion mutants and extent of the deletion mutants used in the complementation analysis (Table 6.4) is shown in a detailed map of the mobilisation region between RSF1010 co-ordinates 2.7Kb and 5Kb (Fig. 6.6). Immediately above the map is a detailed map of the mobilisation region cloned in pED350. The two maps have been aligned by their AccI sites. The insertion mutants of both plasmids are in good agreement as to the location of one particular mobilisation function.

One insertion mutant, pED409, was located at least 300bp away from the main group of insertion mutants. Interestingly two Tn3 insertion derivatives of RSF1010, pMMB20 and pMMB21, that map between the Mob$^-$ insertion in pED409 and the other major Mob$^-$ insertion group, are Mob$^+$ (M. Bagdasarian pers. comm.). This suggests that there are at least two genes involved in mobilisation. The two insertion derivatives, pMMB20 and pMMB21, exhibit a reduced host range specificity (hrs) and cannot be mobilised or transformed into Pseudomonas, though they can be mobilised normally in E. coli x E. coli matings. It may be that hrs is a replication function required in Pseudomonas. The deletions RSF1010 Δ5 and RSF1010 Δ20 in addition to being Mob$^-$ are hrs$^-$ (M. Bagdasarian, pers. comm.).

Fig. 6.6 A detailed map comparing the mobilisation regions of RSF1010 and pED350.



The two maps have been aligned with respect to their AccI sites.

The map of RSF1010 is that between co-ordinates 2.7Kb and 5Kb (Fig. 1.1; Bagdasarian et al., 1982). The Tn3 insertion derivatives of RSF1010, pMMB20 and pMMB21 are mob^+ hrs^- (open triangle ▽) (M. Bagdasarian, pers. comm.). The deletions RSF1010Δ5 and RSF1010Δ20 are mob^- hrs^-, while RSF1010Δ18 is mob^- hrs^+ (M. Bagdasarian, pers. comm.). All other Tn3 insertions of RSF1010 are mob^- (filled in triangle ▼) (Bagdasarian et al., 1982)

The map of pED350 shows only the mobilisation region. The vertical bars indicate HaeII sites. All the insertion derivatives marked have been accurately mapped, except that in pED409 which has only been located within the 170bp HaeII fragment. The insertion derivatives have all been deleted for the transposon-containing EcoRI fragment except pED412 and all are mob^- (Table 6.2).

Table 6.4 A summary of the complementation analysis carried out between pED350 insertion and point mutants and RSF1010 insertion and deletion mutants.

	pED350 mutants					
RSF1010 mutants	pED350	pED401	pED403	pED405	pED409	pED359
RSF1010						+
pKT228	+		-		-	-
pKT229	+		-		-	-
pKT260	+		-		+	+
pKT19	+				+	+
RSF1010Δ18	+	-	-	-	+	+
RSF1010Δ5	+	-	-	-	-	-
RSF1010Δ20	+	-	-	-	-	-

Complementation is indicated by +, and represents a mobilisation ratio of 0.04 or greater compared to pUB307 transfer. Lack of complementation is indicated by -, and indicates mobilisation ratios not significantly different to their ratios without the complementing plasmid (approx. 10^{-5}). The complementation was always measured in terms of Sul^R transfer, as the pKT (Tn3) derivatives were Ap^R (like pED350). However, in the deletion derivatives Ap^R transfer was also measured and the complementation was always reciprocal. The sites of insertion and extent of the deletions are shown opposite in Figure 6.6. pED401, 403, 405, and 409 are all pED350::Tn1725 derivatives in which the transposon containing EcoRI fragment has been deleted. pED359 is a hydroxylamine-induced point mutant of pED350. Blank spaces represent complementation tests not carried out.

(ii) The complementation analysis

To demonstrate that all the RSF1010 mutants were recessive, each derivative was used to transform JC6310 (pUB307, pED350). These constructs were then used as donors in plate matings with ED24. Each mutant was complemented by pED350 (Table 6.4). The reciprocal complementation was not carried out as the RSF1010 deletions were efficiently complemented, demonstrating that only recessive mutations are present within the region in which Tn1725 is inserted.

The pED350::Tn1725 insertion mutant derivatives that contained only the EcoRI site were used to transform JC6310 (pUB307) that contained each of the RSF1010 mob$^-$ derivatives. These constructs were then used as donor, in plate matings with ED24. The mobilisation ratio of each plasmid was determined. The mobilisation ratios of the non-conjugative plasmids are comparable because pUB307 consistently transferred at 50%. pED359 was the only hydroxylamine-induced mutant used because transformation using pED355 and pED357 (the RSF1010 mutants) into several E. coli strains (including JC6310) proved impossible (data not shown), again implying that they may be replication mutants. They were therefore omitted from the analysis. Table 6.4 summarises the data.

The most striking data are those obtained with pED409 and pED359. These two plasmids complement pKT260, pKT19 and RSF1010 Δ18. This confirms the mapping data which suggested that there are two genes involved in mobilisation. One gene is defined by the mutations pED409 and pED359 while the second gene is defined by the major cluster of insertions and the deletion RSF1010 Δ18. Consistent with this hypothesis the insertion derivatives pED401, pED403, pED405 did not complement any of the RSF1010 mutants.

pKT228, pKT229 and RSF1010 Δ5, did not complement pED409 and pED359. These mutations may be having a polar effect on the second gene or a third gene. As a consequence, in complementation tests a positive result is of greater importance than a negative result. The positions of the mutations in pED350 have been accurately mapped and several sequenced. However, the insertions and deletions of RSF1010 have only been positioned by heteroduplex and restriction analysis (M. Bagdasarian, pers. comm.) which is less accurate and may explain why RSF1010 Δ5 does not complement pED409 and pED359; perhaps the

Fig. 6.7 Autoradiogram of an SDS - (10-20%) gradient polyacrylamide gel used to compare the proteins produced from pED350 and its mutant derivatives in minicells.

Track	DNA in minicell
1	pED825
2	pED350
3	pED401
4	pED403
5	pED405
6	pED407
7	pED409
8	pED412

Notes:

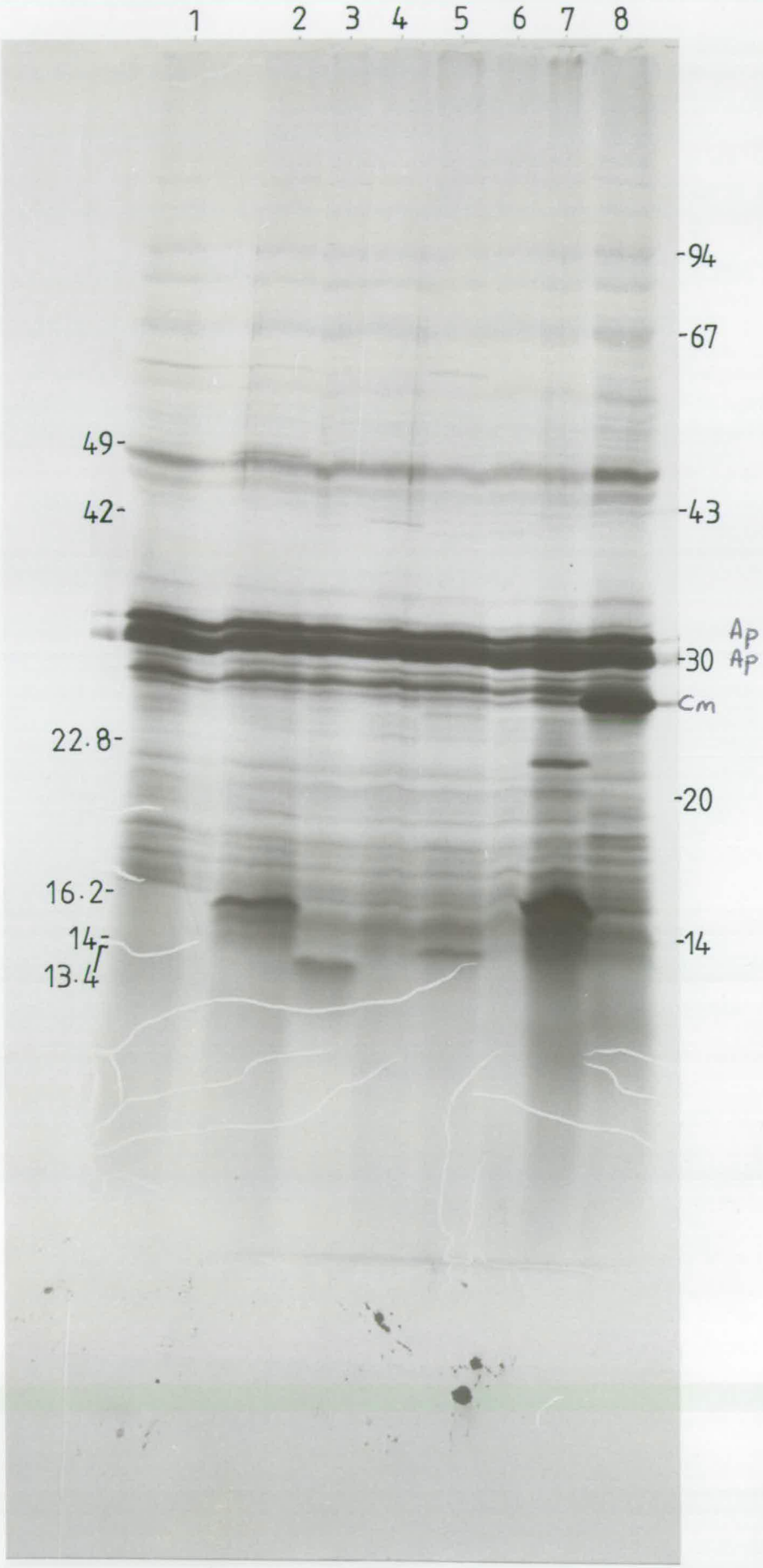
(i) Standards used were: Phosphorylase b (94Kd); Bovine Serum Albumin (67Kd); Ovalbumin (43Kd); Carbonic Anhydrase (30Kd); Soybean Trypsin Inhibitor (20Kd); α Lactalbumin (14Kd)

(ii) The novel proteins are marked and their sizes are estimated from this gel and are consistent with other measurements.

(iii) The two fainter bands below the 16Kd protein were not consistently seen in other gels (data not shown). They may therefore be either degradation products of the larger proteins or correspond to some of the other open-reading frames identified in Chapter 7.

(iv) The two novel proteins synthesised from pED409 are produced in greater quantities than those synthesised from pED350, this is discussed in Chapter 7.

(v) The β-lactamase products present in all tracks are marked (Ap) and are consistent with sizes previously reported (Dougan et al., 1979). The novel band in track 8 is the chloramphenicol gene product (Cm) of 24Kd (Altenbuchner et al., 1983), present because this plasmid still contains the entire Tn1725.


1
2
3
4
5
6
7
8
-94
-67
49-
42-
-43
Ap
Ap
-30
Cm
22·8-
-20
16·2-
14-
13·4
-14

deletion may extend into the second gene. Finally, BamHI restriction analysis of pKT260 revealed that it has two BamHI sites and was larger than predicted from a Tn3 insertion in RSF1010 i.e. it appears to have duplicated some of the transposon DNA. The complementation data, however, implies that this duplication has not affected its mobilisation properties.

(e) An Analysis of Proteins Produced from pED350

pED350 and its mutant insertion derivatives pED401, pED403, pED405, pED407, pED409 and pED412 were used to transform the mini-cell producing strain DS410. The proteins produced from these mini-cells were labelled using [^{35}S] methionine as described in Chapter 2(f). They were then separated in SDS-polyacrylamide gels followed by auto-radiography (2(g)). Figure 6.7 shows a typical autoradiogram.

Two novel polypeptides were produced from the Mob^{+} plasmid pED350 with approximate molecular weights of 51Kd and 16Kd. The 51Kd protein was absent from all the mutant plasmid tracks but instead, in some cases, a second smaller protein was present. In the four derivatives pED401, pED403, pED405 and pED407 (Fig. 6.6) a novel polypeptide of approximately 42Kd was seen. A polypeptide of 23Kd could be clearly detected in mini-cells carrying pED409. These data suggest that the 51Kd protein was encoded by DNA which spans the site of insertion in pED409 and the site of insertions in pED401, 403, 405 and 407. The insertion in pED412 would also be expected to be within the coding sequence of this protein because of its location. However, no truncated form of the 51Kd protein could be seen. The most likely explanation for this is that the protein was hidden by the antibiotic resistant proteins, and, as the site of insertion is between the other insertions, then one would predict an intermediately sized protein (approx. 30Kd). As the insertion in pED409 produced the smallest truncated protein then it was concluded that the N-terminal end of the protein was closest to this insertion i.e. the gene is transcribed from left to right on the pED350 map (Fig. 6.6). In addition an estimate can be made as to the limits of the start of translation of this protein if one assumes that the insertion in pED409 causes immediate cessation of translation rather than a frame-shift followed by a stop codon. Taking the average molecular weight of an amino-acid

as 110 daltons then there are (25,000 ÷ 110) amino acids in the truncated protein. This would be encoded by approximately 620bp of DNA. Thus it can be concluded that the translational start must be within the 800bp oriT-containing fragment and the protein is not a fusion product with any vector protein.

The second novel protein of 16Kd was also affected in mini-cells containing the plasmids pED401, 403, 405, 407 and 412; as these plasmids also synthesise a truncated form of the 51Kd protein, it can be concluded that the 16Kd and 51Kd proteins are encoded by overlapping regions of DNA. The possibility that the 16Kd protein is a processed form of the 51Kd protein can be precluded because the insertion mutant pED409 still synthesises the 16Kd protein while truncating the 51Kd protein. pED401, 403, 405, 407 and 412 all synthesise either a novel protein smaller than 16Kd or no 16Kd protein. The presence or absence of this protein presumably reflects the position of the nearest stop codon in the new reading frame or in the case of pED412, which still contains the transposon, the nearest stop codon within the transposon. Furthermore, the insertions in pED401 and pED405 map very close to one another (20bp) and they are the only ones producing a novel polypeptide presumably reflecting termination at the same stop codon.

(f) Discussion

This chapter describes the cloning and identification of genes involved in the mobilisation of RSF1010. PED350 contains 1760bp of RSF1010 extending from approx. 2.7Kb to 4.5Kb on the RSF1010 map (Fig. 6.6). Contained within this DNA was all the necessary information for efficient mobilisation of pED350, including oriT. A region of similar size and position has been found in RSF1010 (Bagdasarian et al., 1981a; 1982). This is not in agreement with the data of Meyer et al. (1982a; b) who used the apparently identical plasmid R1162.

Meyer et al. (1982a) describe the isolation of a HaeII deletion derivative of R1162, pMS14. This 1.8Kb deletion would extend, according to their data, from one of the HaeII sites close to the second HinfI site in pED350 (Fig. 6.2) through all the Mob^- insertions (except perhaps the insertion in pED409) and so should be Mob^-. However, it is reported to be Mob^+ (Meyer et al., 1982a). If this is true then two explanations exist, either their mapping data is very inaccurate or that a contaminating plasmid in their strains is supplying the

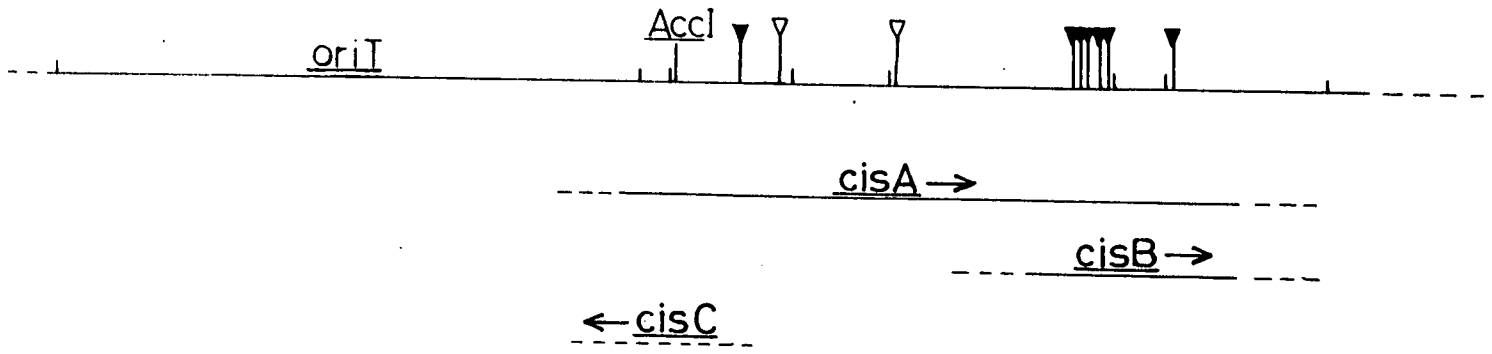
mobilisation functions. Furthermore, they defined the mobilisation region to be within co-ordinates 2.3 - 3.6Kb in RSF1010 using sets of partial deletions. This would be too small and incorrectly positioned. Also many of the deletions they used to define this region would be *oriT*$^-$, which they fail to mention, and as a consequence it would be impossible for those plasmids to be mobilised.

pED350 has been developed as a vector to allow transposon mutagenisis and mobilisation of cryptic non-conjugative plasmids in Gram-negative bacteria (G. Warren, pers. comm.). pED350 has been used because although it should be capable of being mobilised into a variety of Gram-negative bacteria, other than *E. coli*, it cannot replicate in them. A pED350::Tn derivative has been constructed to allow transposon mutagenisis. This plasmid when mobilised into its new host is quickly lost, however transposition into host DNA can be selected with the appropriate antibiotic. A second plasmid derivative has been constructed to enable mobilisation of cryptic non-conjugative plasmids. This derivative has the entire mobilisation region and *oriT* cloned into a transposon. This transposon therefore confers on the DNA, into which it has integrated, the ability to be mobilised. These two types of derivative have been used to mutagenise and mobilise *Rhizobium* megaplasmids (G. Warren, pers. comm.). Similar vectors have been derived using the RP1 *oriT* site cloned in pBR322 (Simon *et al.*, 1983).

The complementation analysis and mapping of insertion mutants demonstrated that at least two genes are involved in mobilisation. The analysis of proteins synthesised by pED350 also showed at least two proteins were encoded by this region. These two proteins may both be involved in mobilisation, and be those encoded by the two complementation groups identified. However, the data, due to the lack of mutants in the rest of the cloned DNA, does not rule out other genes being involved in mobilisation whose protein products were not identified.

The RSF1010::Tn3 derivatives, pMMB20 and pMMB21 (Fig. 6.6), are Mob$^+$ (M. Bagdasarian, pers. comm.), yet the transposons are inserted within the gene coding for the 51Kd protein. As pMMB20 and pMMB21 are deficient in replication in *Psudomonas* (*hrs*$^-$) then this would suggest that the 51Kd protein is involved in replication. This agrees with the data of Meyer *et al.* (1982a; b) in locating replication functions in this region.

Fig. 6.8



A physical and genetic map of the mobilisation region of RSF1010 based on the data presented in this chapter. The restriction map of pED350 is that of the cloned DNA showing HaeII sites as vertical bars. Filled in triangles represent the Tn1725 transposon insertions resulting in a mob$^-$ phenotype in pED350. Open trianges represent the two mob$^+$ hrs$^-$ Tn3 transposon insertions in RSF1010. The two maps were superimposed by aligning their AccI sites.

Below the map is marked the tentative location of cisA and cisB as identified by mapping, complementation and protein analyses. The direction of transcription (arrow) of cisA is deduced from the sizes of truncated pCisA synthesised in mutants. The direction of transcription of cisB is less certain (see text). CisC is a hypothetical gene based on the fact that the two Tn3 insertions are mob$^+$ implying pCisA has a role in replication. As pED409 is mob$^-$, then the insertion in it must be located in a separate gene, cisC.

As the deletion derivative RSF1010 Δ18 is *hrs*$^+$ then this would suggest that only the N-terminal portion of the protein is required for replication in *Psudomonas*. Finally, if the 51Kd protein is not involved in mobilisation, then one has to postulate that the insertion in pED409 is in a third gene, presumably transcribed on the opposite strand, whose product was not identified in mini-cells. These possibilities will be discussed further in Chapter 7 but are summarised in Figure 6.8.

Most importantly the data enabled the two protein coding regions to be mapped and demonstrated that they were overlapping (Fig. 6.8). Although the direction of transcription of the gene (*cisA*) coding for the 51Kd protein (pCisA) can be determined it cannot be determined whether the gene (*cisB*) coding for the 16Kd protein (pCisB) is transcribed from the same strand. The only argument that appears to substantiate that it is transcribed from the same strand is that the three insertion mutants pED412, 403, and 407 did not produce any detectable truncated pCisB, whereas insertion mutants pED401 and pED405 produced polypeptides of about 14Kd. The map locations of the three former insertion mutants show that they would be promoter proximal if transcribed from the upper strand and therefore might be expected to produce smaller polypeptides. Conversely, if *cisB* is transcribed from the lower strand (i.e. opposite to *cisA*) then one would expect the three insertion mutants to produce proteins larger than those from pED401 and pED405. No larger proteins were detected, thus implying that *cisA* and *cisB* are located on the same strand. It cannot be determined from the available data if they are transcribed from the same promoter.

Several examples of overlapping genes exist for bacterio-phage (ϕX174 (Barrell *et al.*, 1976), λ (Shaw and Murialdo, 1980)) but only one example has been fully characterised in plasmids. The large conjugative plasmid ColIdrd-1 encodes a primase (*sog*) and it has a separate smaller translation product encoded by DNA specifying the C-terminal region of the *sog* gene. The data imply that there is a single transcriptional unit which is transcribed from a single promoter (Boulnois *et al.*, 1982). The function of the smaller product is unknown. Similarly, the IncP plasmid RP4 specifies a primase (*pri*) of 118Kd and a second smaller antigenically related polypeptide of about 80Kd

(Lanka and Barth, 1981). However, it has not been proven that the smaller polypeptide is an in-phase translation product of the pri gene as it has in ColIdrd-1. Interestingly these primases are both associated with conjugation and are thought to be involved in the synthesis of primers which would initiate complementary strand synthesis in the recipient and donor cells (Wilkins et al., 1981; Chatfield et al., 1982). RSF1010 has been shown not to produce a primase (Lanka and Barth, 1981) but it is interesting that functions involved in transfer have overlapping genes. Finally, as mentioned in the introduction, if ColE1 were to encode all the protein components involved in relaxation, then the only conceivable way for the mobilisation region to encode these proteins would be if the genes were overlapping.

Chapter 7

THE NUCLEOTIDE SEQUENCE OF THE MOBILISATION REGION OF RSF1010

(a) Introduction

The data accumulated from the physical and genetical analysis of the Mob^+ clone pED350 demonstrated the presence of two complementation groups involved in mobilisation and identified two proteins with overlapping coding sequences. Indirect evidence suggested that a third gene (*cisC*) was also involved in mobilisation which overlapped *cisA* (Fig. 6.8; Chapter 6). To confirm this data and to resolve the complementation ambiguities a more direct approach was taken which would elucidate further the genetic organisation of the mobilisation region of RSF1010; the nucleotide sequence was determined. This was expected to clarify the points alluded to above and identify every open reading frame which could potentially produce a protein involved in mobilisation. Such a comprehensive knowledge of the region would allow the isolation of further mutants in a more controlled fashion with the ability to predict their effect. Furthermore, by sequencing the *Tn1725* insertion mutants it was possible to determine their site of insertion, and most importantly, to interpret their effect on the overlapping proteins and any other proteins encoded by that region.

(b) The Sequencing Strategy

All the sequencing described in this chapter was done in collaboration with Dr G. Hatfull and was carried out at the M.R.C. Laboratory of Molecular Biology, Cambridge. To simplify the sequencing strategy the 3Kb *AvaI* fragment (2.2 - 5.2Kb, Fig. 1.1) from RSF1010 was sequenced, as this contains all the necessary information for mobilisation (Chapter 6). 30μg of RSF1010 was digested with *AvaI* and the products separated in an agarose gel by electrophoresis. The 3Kb *AvaI* fragment was excised, the DNA purified as in Chapter 2(e)(iii), and sonicated to generate DNA fragments of about 300bp in size that were randomly distributed throughout the *AvaI* fragment. These fragments were cloned into M13mp8 in preparation for sequencing (Chapter 2(j)(ii)). A typical sequencing gel is shown (Fig. 7.1).

Fig. 7.1 A typical example of a buffer gradient polyacrylamide sequencing gel.

Notes:

(i) There are seven sets of sequencing reactions loaded onto the gel in the order T, C, G, A.

(ii) The end of the M13 vector sequence and the beginning of the cloned DNA can be seen by identifying the SmaI site in the sequence. This can be seen at the base of each C track as four C residues (arrowed). All DNA after this base is RSF1010 DNA and was entered into the computer.

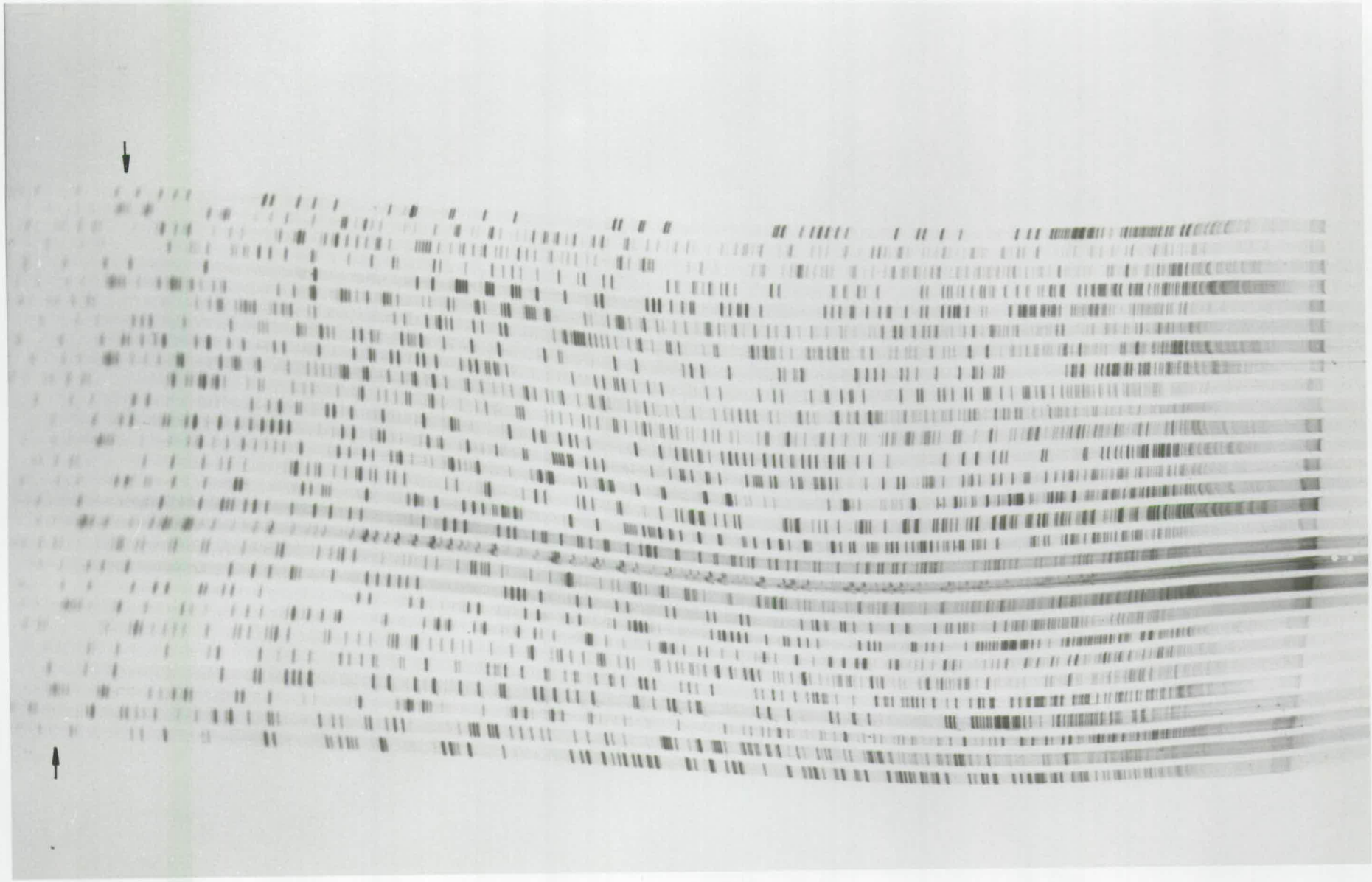


Fig. 7.2 The nucleotide sequence of the DNA cloned in the Mob^+ plasmid pED350.

Notes:

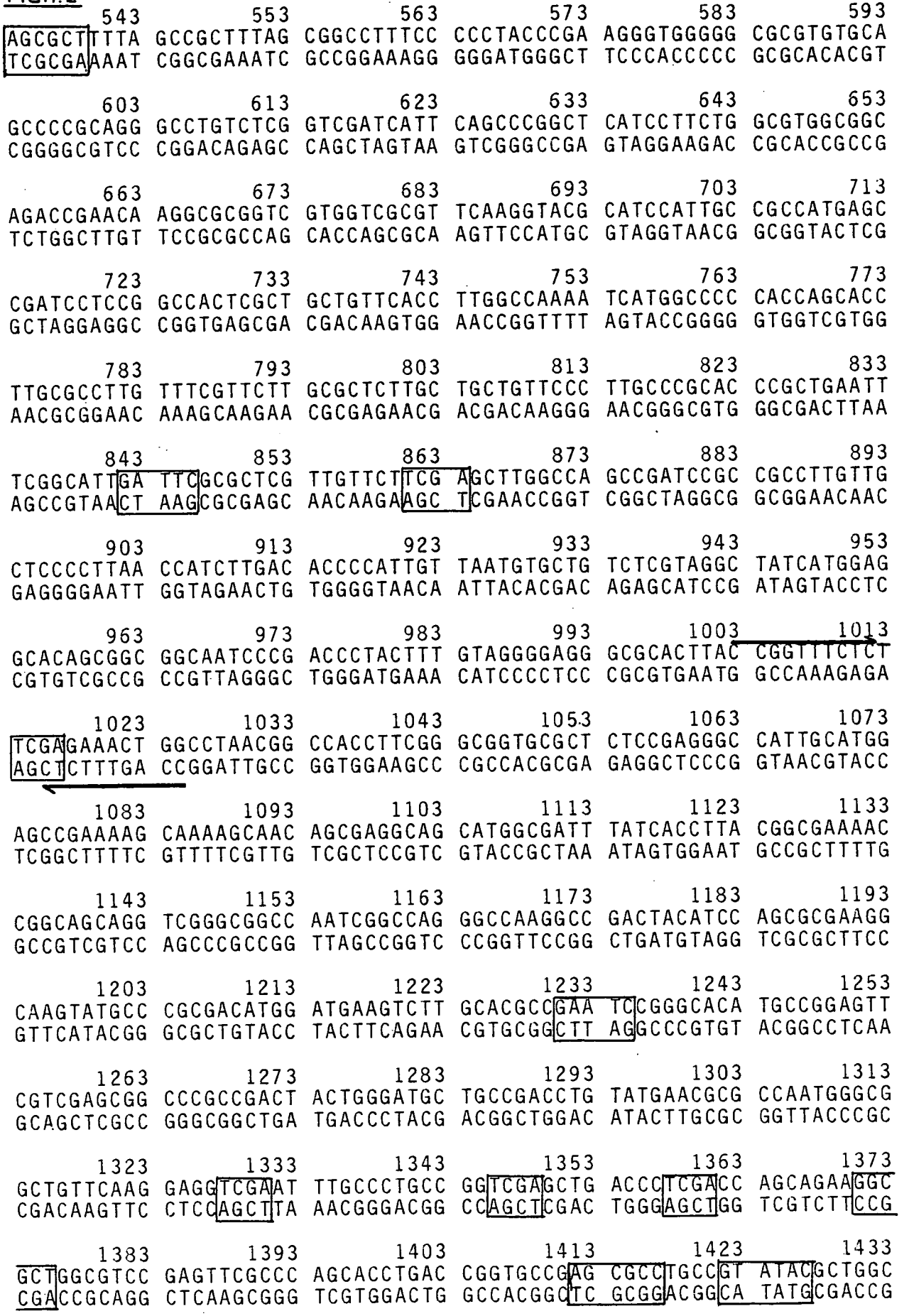
(i) Numbers refer to nucleotides from the AvaI site at 2.2Kb on the RSF1010 map (Fig. 1.1).

(ii) HaeII, AccI, HinfI restriction sites (Table 2.4) are boxed on both strands. Only the TaqI sites associated with oriT are shown.

(iii) Inverted repeat structures are indicated by horizontal arrows.

(iv) The sites of insertion of each of the transposon insertion mutants are indicated by vertical arrows.

FIG.7.2


543 553 563 573 583 593
AGCGCTTTTA GCCGCTTTAG CGGCCTTTCC CCCTACCCGA AGGGTGGGGG CGCGTGTGCA
TCGCGAAAAT CGGCGAAATC GCCGGAAAGG GGGATGGGCT TCCCACCCCC GCGCACACGT
603 613 623 633 643 653
GCCCCGCAGG GCCTGTCTCG GTCGATCATT CAGCCCGGCT CATCCTTCTG GCGTGGCGGC
CGGGGCGTCC CGGACAGAGC CAGCTAGTAA GTCGGGCCGA GTAGGAAGAC CGCACCGCCG
663 673 683 693 703 713
AGACCGAACA AGGCGCGGTC GTGGTCGCGT TCAAGGTACG CATCCATTGC CGCCATGAGC
TCTGGCTTGT TCCGCGCCAG CACCAGCGCA AGTTCCATGC GTAGGTAACG GCGGTACTCG
723 733 743 753 763 773
CGATCCTCCG GCCACTCGCT GCTGTTCACC TTGGCCAAAA TCATGGCCCC CACCAGCACC
GCTAGGAGGC CGGTGAGCGA CGACAAGTGG AACCGGTTTT AGTACCGGGG GTGGTCGTGG
783 793 803 813 823 833
TTGCGCCTTG TTTCGTTCTT GCGCTCTTGC TGCTGTTCCC TTGCCCGCAC CCGCTGAATT
AACGCGGAAC AAAGCAAGAA CGCGAGAACG ACGACAAGGG AACGGGCGTG GGCGACTTAA
843 853 863 873 883 893
TCGGCATTGA TTCGCGCTCG TTGTTCTTCG AGCTTGGCCA GCCGATCCGC CGCCTTGTTG
AGCCGTAACT AAGCGCGAGC AACAAGAAGC TCGAACCGGT CGGCTAGGCG GCGGAACAAC
903 913 923 933 943 953
CTCCCCTTAA CCATCTTGAC ACCCCATTGT TAATGTGCTG TCTCGTAGGC TATCATGGAG
GAGGGGAATT GGTAGAACTG TGGGGTAACA ATTACACGAC AGAGCATCCG ATAGTACCTC
963 973 983 993 1003 1013
GCACAGCGGC GGCAATCCCG ACCCTACTTT GTAGGGGAGG GCGCACTTAC CGGTTTCTCT
CGTGTCGCCG CCGTTAGGGC TGGGATGAAA CATCCCCTCC CGCGTGAATG GCCAAAGAGA
1023 1033 1043 1053 1063 1073
TCGAGAAACT GGCCTAACGG CCACCTTCGG GCGGTGCGCT CTCCGAGGGC CATTGCATGG
AGCTCTTTGA CCGGATTGCC GGTGGAAGCC CGCCACGCGA GAGGCTCCCG GTAACGTACC
1083 1093 1103 1113 1123 1133
AGCCGAAAAG CAAAAGCAAC AGCGAGGCAG CATGGCGATT TATCACCTTA CGGCGAAAAC
TCGGCTTTTC GTTTTCGTTG TCGCTCCGTC GTACCGCTAA ATAGTGGAAT GCCGCTTTTG
1143 1153 1163 1173 1183 1193
CGGCAGCAGG TCGGGCGGCC AATCGGCCAG GGCCAAGGCC GACTACATCC AGCGCGAAGG
GCCGTCGTCC AGCCCGCCGG TTAGCCGGTC CCGGTTCCGG CTGATGTAGG TCGCGCTTCC
1203 1213 1223 1233 1243 1253
CAAGTATGCC CGCGACATGG ATGAAGTCTT GCACGCCGAA TCCGGGCACA TGCCGGAGTT
GTTCATACGG GCGCTGTACC TACTTCAGAA CGTGCGGCTT AGGCCCGTGT ACGGCCTCAA
1263 1273 1283 1293 1303 1313
CGTCGAGCGG CCCGCCGACT ACTGGGATGC TGCCGACCTG TATGAACGCG CCAATGGGCG
GCAGCTCGCC GGGCGGCTGA TGACCCTACG ACGGCTGGAC ATACTTGCGC GGTTACCCGC
1323 1333 1343 1353 1363 1373
GCTGTTCAAG GAGGTCGAAT TTGCCCTGCC GGTCGAGCTG ACCCTCGACC AGCAGAAGGC
CGACAAGTTC CTCCAGCTTA AACGGGACGG CCAGCTCGAC TGGGAGCTGG TCGTCTTCCG
1383 1393 1403 1413 1423 1433
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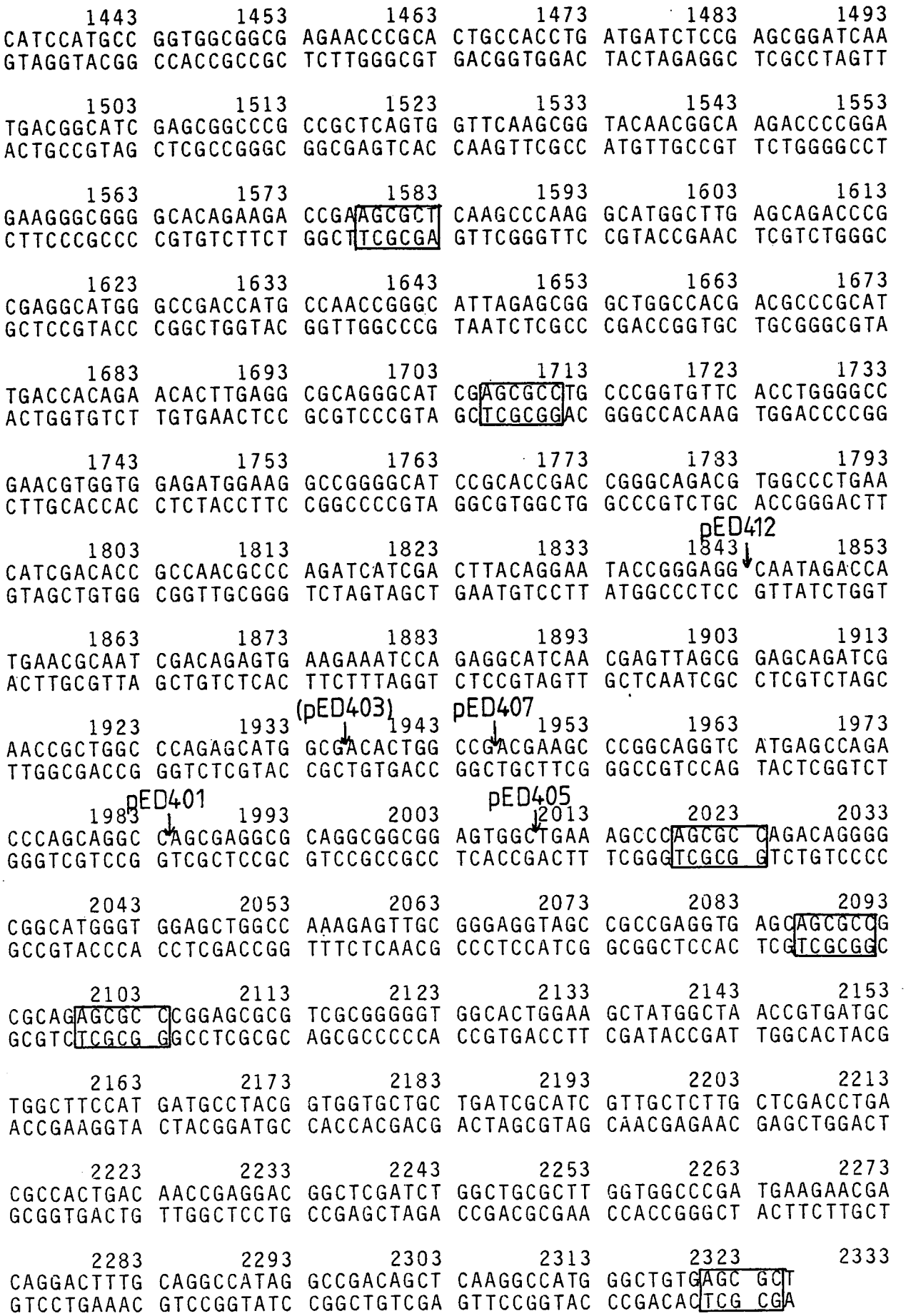
CGACCGCAGG CTCAAGCGGG TCGTGGACTG GCCACGGCTC GCGGACGGCA TATGCGACCG


1443 1453 1463 1473 1483 1493
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GTAGGTACGG CCACCGCCGC TCTTGGGCGT GACGGTGGAC TACTAGAGGC TCGCCTAGTT
1503 1513 1523 1533 1543 1553
TGACGGCATC GAGCGGCCCG CCGCTCAGTG GTTCAAGCGG TACAACGGCA AGACCCCGGA
ACTGCCGTAG CTCGCCGGGC GGCGAGTCAC CAAGTTCGCC ATGTTGCCGT TCTGGGGCCT
1563 1573 1583 1593 1603 1613
GAAGGGCGGG GCACAGAAGA CCGAAGCGCT CAAGCCCAAG GCATGGCTTG AGCAGACCCG
CTTCCCGCCC CGTGTCTTCT GGCTTCGCGA GTTCGGGTTC CGTACCGAAC TCGTCTGGGC
1623 1633 1643 1653 1663 1673
CGAGGCATGG GCCGACCATG CCAACCGGGC ATTAGAGCGG GCTGGCCACG ACGCCCGCAT
GCTCCGTACC CGGCTGGTAC GGTTGGCCCG TAATCTCGCC CGACCGGTGC TGCGGGCGTA
1683 1693 1703 1713 1723 1733
TGACCACAGA ACACTTGAGG CGCAGGGCAT CGAGCGCCTG CCCGGTGTTC ACCTGGGGCC
ACTGGTGTCT TGTGAACTCC GCGTCCCGTA GCTCGCGGAC GGGCCACAAG TGGACCCCGG
1743 1753 1763 1773 1783 1793
GAACGTGGTG GAGATGGAAG GCCGGGGCAT CCGCACCGAC CGGGCAGACG TGGCCCTGAA
CTTGCACCAC CTCTACCTTC CGGCCCCGTA GGCGTGGCTG GCCCGTCTGC ACCGGGACTT
pED412
1803 1813 1823 1833 1843 1853
CATCGACACC GCCAACGCCC AGATCATCGA CTTACAGGAA TACCGGGAGG CAATAGACCA
GTAGCTGTGG CGGTTGCGGG TCTAGTAGCT GAATGTCCTT ATGGCCCTCC GTTATCTGGT
1863 1873 1883 1893 1903 1913
TGAACGCAAT CGACAGAGTG AAGAAATCCA GAGGCATCAA CGAGTTAGCG GAGCAGATCG
ACTTGCGTTA GCTGTCTCAC TTCTTTAGGT CTCCGTAGTT GCTCAATCGC CTCGTCTAGC
(pED403) pED407
1923 1933 1943 1953 1963 1973
AACCGCTGGC CCAGAGCATG GCGACACTGG CCGACGAAGC CCGGCAGGTC ATGAGCCAGA
TTGGCGACCG GGTCTCGTAC CGCTGTGACC GGCTGCTTCG GGCCGTCCAG TACTCGGTCT
pED401 pED405
1983 1993 2003 2013 2023 2033
CCCAGCAGGC CAGCGAGGCG CAGGCGGCGG AGTGGCTGAA AGCCCAGCGC CAGACAGGGG
GGGTCGTCCG GTCGCTCCGC GTCCGCCGCC TCACCGACTT TCGGGTCGCG GTCTGTCCCC
2043 2053 2063 2073 2083 2093
CGGCATGGGT GGAGCTGGCC AAAGAGTTGC GGGAGGTAGC CGCCGAGGTG AGCAGCGCCG
GCCGTACCCA CCTCGACCGG TTTCTCAACG CCCTCCATCG GCGGCTCCAC TCGTCGCGGC
2103 2113 2123 2133 2143 2153
CGCAGAGCGC CCGGAGCGCG TCGCGGGGGT GGCACTGGAA GCTATGGCTA ACCGTGATGC
GCGTCTCGCG GGCCTCGCGC AGCGCCCCCA CCGTGACCTT CGATACCGAT TGGCACTACG
2163 2173 2183 2193 2203 2213
TGGCTTCCAT GATGCCTACG GTGGTGCTGC TGATCGCATC GTTGCTCTTG CTCGACCTGA
ACCGAAGGTA CTACGGATGC CACCACGACG ACTAGCGTAG CAACGAGAAC GAGCTGGACT
2223 2233 2243 2253 2263 2273
CGCCACTGAC AACCGAGGAC GGCTCGATCT GGCTGCGCTT GGTGGCCCGA TGAAGAACGA
GCGGTGACTG TTGGCTCCTG CCGAGCTAGA CCGACGCGAA CCACCGGGCT ACTTCTTGCT
2283 2293 2303 2313 2323 2333
CAGGACTTTG CAGGCCATAG GCCGACAGCT CAAGGCCATG GGCTGTGAGC GCT
GTCCTGAAAC GTCCGGTATC CGGCTGTCGA GTTCCGGTAC CCGACACTCG CGA

The sequences were entered into the computer using a digitiser device and the information was processed by the GELINZ programme (Staden, unpublished). As more sequence was obtained the new gel readings were compared to the existing sequences present in the database using the automatic programme DBAUTO (Staden, 1982). This compiles overlapping gel readings until one contiguous sequence has been obtained. This sequence was then searched, using the ANALYSEQ programme (Staden, unpublished), to detect open reading frames and restriction sites.

Determination of the complete sequence of the AvaI fragment from a randomly cloned library generates, as a corollary, a large amount of sequence duplication. However, this is not a disadvantage, as it means that each base is sequenced several times and, as a consequence, the sequence is very reliable. The sequence presented in Figure 7.2 is that contained within the oriT$^+$ Mob$^+$ HaeII clone, pED350 and therefore contains all the necessary information for mobilisation.

In addition to sequencing the mobilisation region, the sites of insertion of four Tn1725 insertion mutants were also determined. Their DNA was cloned into the EcoRI site of M13mp8. This took advantage of the fact that an EcoRI site was left in each of the in vitro transposon deletions made from Tn1725 insertion mutants (Fig. 6.5). This meant that each plasmid, on digestion with EcoRI, gave two EcoRI fragments. To ensure that the transposon EcoRI site would be that sequenced, four recombinants from each transfection were sequenced. In this way the sites of insertion of the transposons in pED401, pED405 and pED407 were determined (Fig. 7.3). A similar procedure was followed for pED412 which still contained the entire transposon. The sequence was only obtained in one orientation for each plasmid and therefore the unlikely possibility that a deletion had occurred from the other end of the inverted repeat could not be dismissed. However, the fact that each insertion has the predicted effect on proteins pCisA and pCisB (see discussion), and that the initial mapping by measuring restriction fragments (Fig. 6.5) did not detect any abnormally small fragments, makes this unlikely.

(c) Discussion

(i) Introduction

The length of the AvaI fragment is 2886bp. The sequence confirmed the previous mapping of all restriction sites and Tn1725 insertion sites

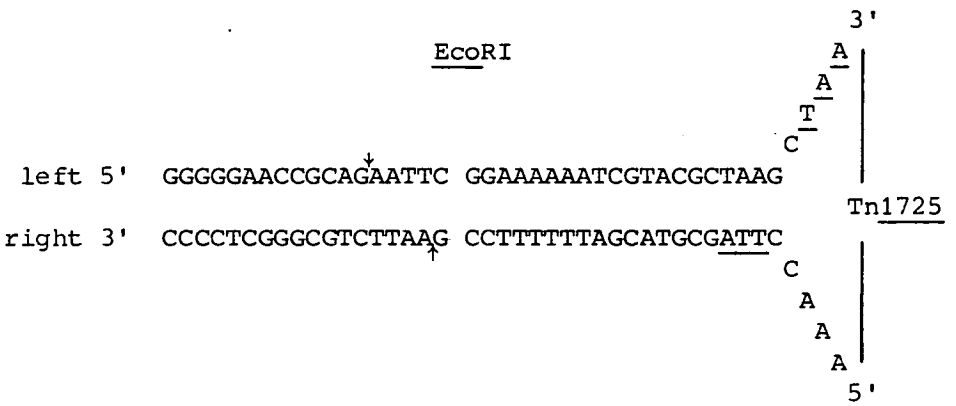
Fig. 7.3 Example of a sequencing gel used to determine the site of insertion of a Tn1725 generated insertion mutant, pED405.

Notes:

(i) The gel was a 6% polyacrylamide buffer gradient gel and the tracks are TCGA for each of the identical templates.

(ii) The Tn1725 sequence and the 5bp direct repeat are marked.

(iii) The sequence of the Tn1725 inverted repeat is shown below (R. Schmitt, pers. comm.). The TAA stop codons (underlined) outside the end of the left inverted repeat and at the end of the right inverted repeat (on the complementary strand) cause truncation of pCisA and the potential protein initiating at 2163bp. The EcoRI site is marked (↓). It is the sequence to the left of this site which is remaining in the in vitro deletions of the transposon made from the EcoRI site.



DR
Tn1725

to within 20bp. These sites are shown in Figure 7.2. The sizes of the HaeII fragments, as determined from the sequence, were 838, 313, 222, 166, 128, 68, 41, 12bp and are in excellent agreement with the sizes previously estimated (Chapter 6).

(ii) oriT of RSF1010

Chapters 4 and 5 describe the isolation and sequencing of the oriT-containing TaqI fragment. The data presented here confirm the sequence and locate oriT with respect to the mobilisation region. As in many of the transfer systems so far characterised oriT is terminally located with respect to the mob or tra genes e.g. F (Willetts, 1972), RP1 (Guiney and Helinski, 1979), R46 (Brown, 1981), ColE1 (Warren et al., 1978), CloDF13 (Van de Pol, 1978). If the analogy is extended further then RSF1010 transfer would be oriented such that the mob region is transferred last as is the case for F and RP1, and ColE1 (assuming linear transfer from the 5' end of the relaxation nick site).

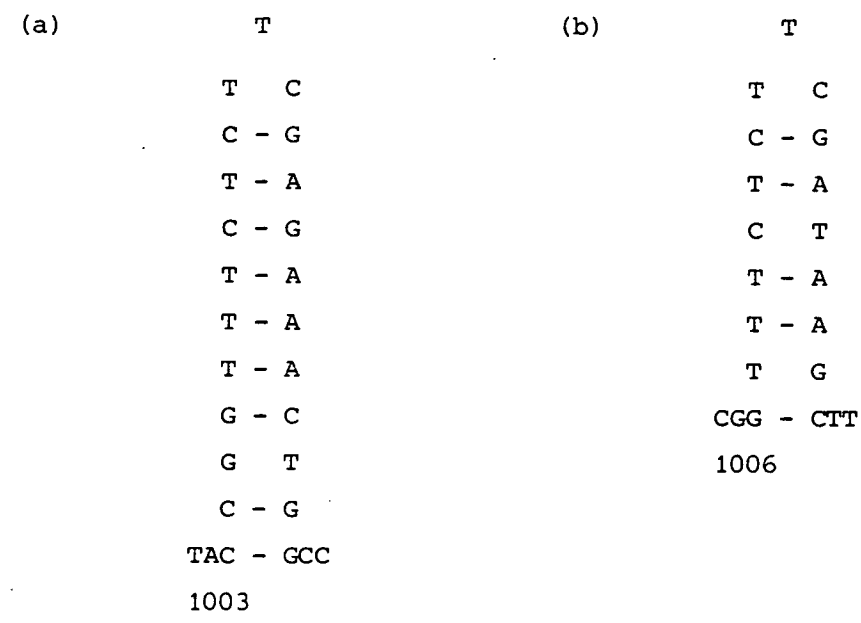
In the initial cloning of oriT on TaqI fragments it was found from sequence data that three fragments had been cloned. The two smaller fragments of 18bp and 12bp were shown to be dispensable for oriT function and not contiguous with the larger 153bp TaqI fragment in RSF1010 (Chapter 4 (b)(iii)).

This was confirmed by the sequence as the large fragment is located between 862 - 1015 (from the AvaI site, Fig. 7.2) while the two smaller fragments are contiguous and located over 300bp away at 1329 - 1347 - 1359bp within the 800bp HaeII fragment. This confirmed their irrelevance for oriT function.

Chapter 4 described the mapping of oriT to within 78bp of the HindIII site in the oriT$^+$ TaqI clone pED367 (Fig. 4.10). The TaqI site adjacent to this HindIII site is that located at 1015bp and oriT must be within 78bp of this. pED367 was not mobilised as efficiently as RSF1010 or pED361 (800bp HaeII clone, Table 4.1). One possible explanation for this is that adjacent vector sequences may be interfering with oriT or alternatively, part of a sequence necessary for efficient mobilisation has been lost. As the complete nucleotide sequence surrounding oriT is known, this second possibility can be considered by examining in more detail the sequence adjacent to these 78bp known to contain oriT.

Interestingly, a 10bp inverted repeat was found which crosses the

Fig. 7.4 Possible inverted repeat in RSF1010 and its possible alternative in the oriT TaqI clones.



(a) Sequence spanning oriT TaqI site in RSF1010.

(b) Possible alternative inverted repeat in pED360, pED366 and pED367 using part of the vector sequence to complete the inverted repeat.

Note:

(i) The TaqI site (TCGA) spans part of the interjacent sequence and the upper part of the second repeat.

TaqI site. This repeat (1003 - 1012, 1016 - 1025; Fig. 7.4a) contains only one mis-match and the TaqI site forms part of the interjacent sequence and the beginning of the second inverted repeat. If this inverted repeat was involved in the transfer process then disruption of the repeat at the TaqI site (by cloning as in pED360 and pED367) would be predicted to abolish transfer. However, both pED360 and pED367 were mobilised efficiently, albeit ten to twenty fold less than RSF1010 (Table 4.1). The sequence in the vector adjacent to the TaqI site, closest to oriT, was therefore examined to see if it could compensate for the deletion of the second repeat. A smaller (7bp), less perfect, but intriguingly similar, inverted repeat was detected (Fig. 7.4b). The 7bp inverted repeat and 3bp interjacent sequence would be in an analogous location to the 10bp RSF1010 inverted repeat, with respect to oriT. This shorter inverted repeat might play the same role as the RSF1010 inverted repeat and the reduction in mobilisation of pED367 is a consequence of the slight imperfection and smaller size of this inverted repeat. The pED367 inverted repeat contains four of the six base pairs of the HindIII recognition and cutting site (Fig. 5.2) and so even in pED366, with the inverted oriT TaqI fragment (Fig. 4.4), the HindIII-TaqI inverted repeat sequence is maintained.

If the inverted repeat were involved in recognition of oriT by a protein it may be that only one of the sequences is sufficient for recognition and that having the inverted repeat (as in RSF1010) only serves to increase the efficiency of transfer. This could be resolved relatively easily. If the TaqI fragment were recloned into a ClaI site such that a 'pseudo' inverted repeat could not be formed then one would expect this clone not to be mobilisable if both repeats were required. Alternatively, deletions could be made using the enzyme Bal31 (Legerski et al., 1978). This enzyme has an exonuclease activity which can be used to make progressive deletions into double-stranded DNA. Bal31 was used in this way to make deletions from the HindIII site into the TaqI fragment of pED367. Preliminary data showed that removal of the HindIII site abolished transfer but the extent of these deletions has still to be determined (Derbyshire and Hatfull, data not shown). However, it is hoped that by mapping the end-points of the deletions a more accurate estimate can be made as to the location of oriT and the role of the inverted repeat.

Fig. 7.5 Autoradiogram showing sequence anomalies around oriT TaqI site.

Notes:

(i) This is a 6% polyacrylamide buffer gradient gel.

(ii) The two outside sets of four tracks (TCGA) show the oriT sequence and the TaqI site is indicated.

(iii) Sequence B is from the upper strand in Fig. 7.2, while A is from the lower strand.

(iv) The compressions in A and B are arrowed, while the pile-ups in B are bracketed.


A
B
TCGA
TCGA

The only sequences that were difficult to determine were those containing direct and indirect repeated sequences. This was very obvious in this inverted repeat region of oriT. Figure 7.5 is an autoradiogram which shows two sequences, covering both strands, of oriT. Both sequences have regions which are difficult to interpret, especially the 'top' strand sequence (B). These anomalies are due to 'pile ups' and 'compressions'. The former are caused by secondary structures occurring in the template, especially after a run of G residues, which prevents the polymerase passing and as a consequence leads to termination in all four reactions and therefore bands in all four tracks (Bankier and Barrell, pers. comm.). Compressions are a consequence of secondary structures occurring at the 3' end of newly synthesised DNA causing it to move with an altered mobility and to be seen as compressed or co-migrating bands (Bankier and Barrell, pers. comm.). The pile ups (B only) and compressions (A and B) are found only in the region surrounding the oriT TaqI site. The synthesised strand in B, the top strand in Figure 7.2, shows both compressions and pile-ups before the TaqI site and all are within the inverted repeat. The newly synthesised strand in A, the bottom strand in Figure 7.2, shows a compression after the TaqI site also within the inverted repeat. There are two further pile-ups in B at 1051 - 1052bp and 1062 - 1064bp, which may be due to the G-C sequences on the template strand forming secondary structures and termination of complementary strand synthesis.

These inverted repeats need not form secondary structures in the double-stranded DNA to be functional for oriT. In Chapter 5 many inverted repeat structures were shown to be present among oriT sequences (see also Willetts and Wilkins, 1983) and in F two oriT point mutants have been located within inverted repeat sequences (Thompson et al., 1983). The inverted repeat described in RSF1010 might therefore be the recognition or nicking site of a nicking (relaxation) protein or alternatively be involved in the recognition of the mating pair system of the conjugative plasmid (Chapter 3).

In Chapter 5 the possible mechanisms of complementary strand synthesis in both donor and recipient after transfer were considered. Two n' sites have been detected in both ColE1 and pBR322 close to oriT but on opposite strands (Zipursky and Marians, 1980; Nomura et al., 1982). As both can be deleted without affecting plasmid replication

(Nomura et al., 1982; Van Der Ende et al., 1983), they are not involved in the synthesis of primers for vegetative replication. The n' site on the transferred strand has therefore been hypothesised to be the primosome assembly site for initiation of primers for complementary strand synthesis in the recipient (Nomura et al., 1982). The n' site on the non-transferred strand may prime the synthesis of the replacement strand (Willetts and Wilkins, 1983).

The hexanucleotide 5' - AAGCGG - 3' may be the concensus recognition sequence of n' (Soeller and Marians, 1982) and although none were found within the oriT TaqI fragment of RSF1010, two were detected outside this sequence. One sequence within the Mob^+ clone pED350 is located at (550-545), while the second is on the opposite strand just outside the displayed sequence (483-489). However, more experimental evidence is required to prove that they can act as primosome assembly sites, before their roles in replication and transfer can be considered. This could be carried out using the M13 templates, from the sequence library, as a substrate for in vitro conversion from the single-stranded to double-stranded form by the primosome complex (Nomura et al., 1982). As pED367 is efficiently mobilised it must use a different system to prime complementary strand synthesis after transfer, as it does not contain these n' sites (Chapter 5). However, the plasmid might adapt to different mechanisms for priming DNA synthesis depending on their availability. This would be an advantage to the broad-host-range plasmids as presumably not all hosts would supply the necessary functions for one particular mechanism.

(iii) Mobilisation of RSF1010

The sequencing data, apart from allowing the identification of all open reading frames, determined the site of insertion of four Mob^- Tn1725 insertion derivatives pED401, 405, 407 and 412 (Fig. 7.2; 7.3). pED401, 405 and 407 have no transposon EcoRI fragment, only a 35bp insertion (30bp and 5bp duplication), and are therefore frame-shift mutants. pED412 still contains the whole transposon which is known to have polar effects (R. Schmitt, pers. comm.). The effects of the insertions in pED409 and pED403 which were not sequenced, can be accurately predicted. The insertion in pED403 was mapped 10bp to the AccI side of the insertion in pED407 (Fig. 6.5; 6.6) and as there are no stop codons between these two insertions, then they will have

Fig. 7.6 The sequence and translational reading frames of the mobilisation region (in pocket at back of thesis).

Notes:

(i) All six translational reading frames are given using the amino-acid abbreviation according to Dayhoff (1973). Stop codons are indicated by an asterix. Open reading frames are coloured yellow and their corresponding Shine-Dalgarno sequences green. The two-possible overlapping promoters for *cisA* and *cisB* are marked in blue.

(ii) *Hae*II, *Acc*I and the *oriT*-associated *Taq*I fragments are boxed on both strands.

(iii) The insertion sites, as determined by sequencing and restriction mapping, for the pED350::Tn*1725* deletion mutants are arrowed. The actual site of insertion of Tn*1725* in pED409 in the 170bp *Hae*II fragment is not known and is not marked.

the same frame-shift effect on a protein. The insertion in pED409 was mapped to 40bp from either end of the 170bp HaeII fragment and so both possible insertion sites were considered for effects on proteins. pED350 was shown to synthesise a protein of 51Kd (pCisA) which is encoded by DNA spanning all the insertions (Chapter 6). In agreement with this, an open reading frame (starting at 1105bp, phase 3) preceded by a ribosome-binding site homology (Shine and Dalgarno, 1975; Steitz, 1979; Stormo et al., 1982) extends through all the insertion mutants (Fig. 7.6, in pocket at the back of this thesis). In pED350 this open reading frame extends 128bp into pED825 and terminates at the stop codon TGA (2479, in the pBR322 sequence, Sutcliffe, 1979). This protein would have a predicted molecular weight of 49.39Kd (av. mol. wt of an amino acid = 110 daltons), which is in excellent agreement with that estimated for pCisA. Furthermore the four insertion mutants pED409, pED403, pED405 and pED407 would cause a frame-shift from phase 3 to phase 1 stopping at TGA (2264bp). This would produce a truncated protein of 43.8Kd (Table 7.1). This again is in excellent agreement with the sizes estimated from protein gels (Fig. 6.7). This confirms that this open reading frame is that identified as cisA, which codes for pCisA, in Chapter 6.

The insertion mutant pED412 still carries the complete transposon and so translation of the 51Kd protein was predicted to stop within the transposon sequence, such that a truncated protein with a size similar to the β-lactamase and chloramphenicol acetylase proteins was produced (Chapter 6). The sequencing data (Fig. 7.6) reveals that this insertion would cause termination of translation at the TAA stop codon in the transposon but just outside its inverted repeats (see legend to Fig. 7.3). This truncated protein would be 28.7Kd in size (Table 7.1) and would almost certainly be masked by the ampicillin and chloramphenicol antibiotic resistance products. The insertion pED409 which contains only the extra 35bp would cause a frame-shift from phase 3 to phase 1 (Fig. 7.6). Depending on the site of insertion this could cause cessation of translation at the TGA stop codon (1472bp) or at the TAG stop codon (1646bp). The predicted truncated protein sizes for these would be 13.4Kd or 19.8Kd (Table 7.1). The measured size was 25Kd and this agrees best with the predicted size of 19.8Kd implying that pED409 has the EcoRI insert close to the HaeII site at 1580bp.

Table 7.1 The predicted effects of the Tn1725 insertion mutant derivatives on open reading frames identified in the sequence.

		PLASMID						
		pED350	pED401	pED403 (c)	pED405	pED407	pED409 (c)	pED412
(a)	Putative Protein							
	phase 1 (pCisB)	15.07	7.04	5.4	7	5.4	15.07	15.07
	phase 3 (pCisA)	51	43.8	43.8	43.8	43.8	19.8 13.4	28.7
	phase 6	12.32	8.58	8.58	8.58	8.58	12.32	12.98
	phase 6 (pCisC)	22.1	22.1	22.1	22.1	22.1	5.4 14.4	22.1
(b)	pCisA	49	42.5	43.8	41.8	42	22.8	-
	pCisB	16.2	13.4	-	14	-	16.2	-

(a) The actual protein sizes predicted from the sequence in Kd assuming the average molecular weight of an amino-acid is 110 daltons.

(b) Sizes below the line are those actually measured from the gel (e.g. Fig. 6.7). Dashes indicate no novel protein detected.

(c) pED403 has not been sequenced but its site of insertion is known to be 10bp to the left of the insertion site in pED407 and as there are no stop codons between the two sites, then the insertions must have the same effect. pED409 has only been mapped to either end of the 170bp HaeII fragment. Both possible frame-shift effects are given when necessary.

(d) Although pED412 does not have the transposon insertion within the cisB reading frame, it is located very close to the start codon and so probably prevents transcription into the gene.

pCisA in pED350 contains 42 amino-acids at its C-terminal end coded from pBR322. In RSF1010 this open reading frame continues to the end of the AvaI fragment with no translational stop signals (data not shown) and therefore pCisA would be at least 596 amino acids long (m. wt >65Kd). This will be discussed later.

It was postulated in Chapter 6 that cisB was contained within cisA and that the mutants pED401, pED403, pED405, pED407 and pED412 would have insertions within it. An open reading frame beginning at 1853bp (phase 2) with a good ribosome binding site homology, passes through all these insertions, except pED412, before terminating at the TGA stop codon (2264bp). This would encode a protein of 137 amino acids (m. wt 15.1Kd) which is very similar to that measured for pCisB (16Kd). Although pED412 does not have the transposon insertion within the coding region it is inserted only 9bp upstream from the start site and, as the transposon has polar effects (R. Schmitt, pers. comm.), it would presumably terminate transcription and thereby not produce pCisB. The mutants pED401, pED403, pED405 and pED407 would truncate pCisB by frame-shift to phase 2 (Fig. 7.6, Table 7.1). The 5Kd protein produced from pED403 and pED407 was presumably not detected on the gels used, but truncated forms of pCisB can be detected, synthesised by pED401 and pED405 with a similar size to those predicted (Fig. 6.7, Table 7.1).

These data confirm the location of cisB within cisA and show that they are not translated from the same reading frame. It cannot be determined whether they are translated from the same mRNA or if both genes have their own separate transcriptional units. There is one more open reading frame (phase 2) found on this upper strand (Fig. 7.6). This could code for a protein of 11Kd in size (100 amino acids). Translation would initiate at the ATG (948bp), within the oriT-containing TaqI fragment, and terminate at TGA (1215bp). This open reading frame has a short ribosome binding site homology but there is no direct evidence for or against its existence.

On the opposite strand there are four, non-overlapping, open reading frames of significant size (summarised in Fig. 7.7). One of these would begin with a valine residue (2128bp) and continue for 76 amino-acids (1900bp). This open reading frame is not preceded by a ribosome binding site and so is probably not used.

A protein of 12.3Kd (112 amino-acids) could be produced from

FIG.7.7

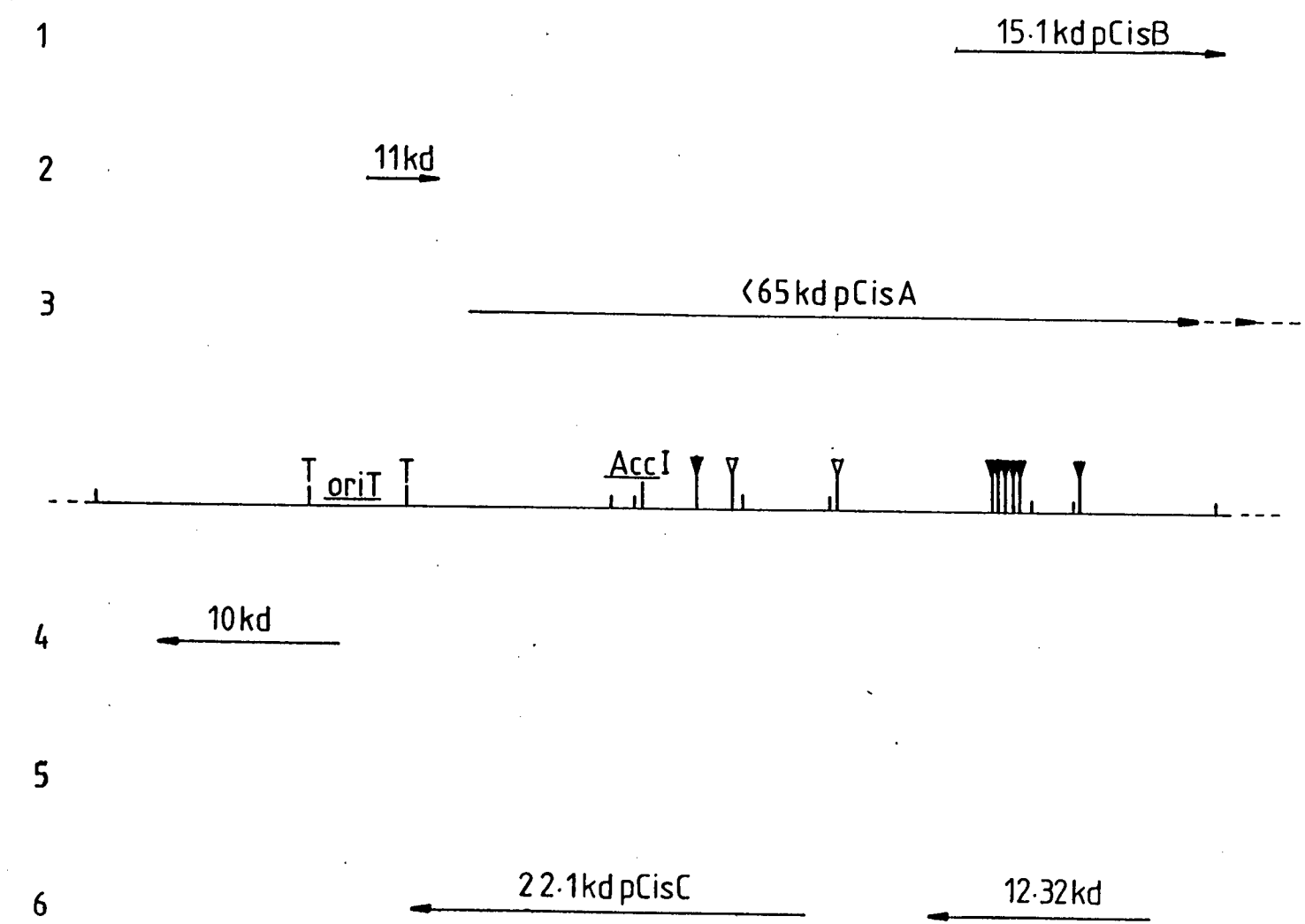

phase
1
15·1kd pCisB
2
11kd
3
<65kd pCisA
oriT
AccI
4
10kd
5
6
22·1kd pCisC
12·32kd

Fig. 7.7 A map summarising all the open reading frames of the mobilisation region.

Each open reading frame is indicated as a horizontal arrow (Fig. 7.6). The predicted sites of the proteins that would be encoded are marked above each reading frame. The reading phases are the same as those used in Figure 7.6. The map of the mobilisation region is the same as that presented in Figure 6.6 and Figure 6.8. The HaeII sites are marked as vertical bars. The transposon insertions are indicated as triangles; Tn1725 Mob^- insertion mutants of pED350 are marked as filled triangles; Tn3 insertion mutants of RSF1010 which are mob^+ hrs^- are open triangles (M. Bagdasarian, pers. comm.). The two TaqI sites (T), between which oriT is located, are marked.

between the start codon at 2163bp and the stop codon at 1827 (phase 6, Fig. 7.6). This open reading frame has a potential ribosome binding site and would cover the sites of all insertions affecting pCisB and is also of a similar size to pCisB. However, the effects of the insertions are not consistent with those predicted (Table 7.1), eliminating this open reading frame as a possible alternative candidate for pCisB.

A second open reading frame in phase 6 could code for a protein of 201 amino acids, 22.1Kd (1632 - 1027bp). This putative gene would cover the site of insertion of pED409 and is therefore potentially equivalent to cisC, alluded to in Chapter 6 (Fig. 6.8). However, no protein of this size was detected from mini-cells and the mob$^+$ Tn3 insertion in pMMB21 (Fig. 6.8) appears to map within the gene. This does not necessarily rule out the possibility that this is a gene involved in mobilisation as the mapping of the Tn3 insertions is not as accurate as the sequence mapping of the Tn1725 insertion mutants and so the Tn3 insertion in pMMB21 may be located outside this gene. Furthermore, protein patterns obtained from minicells carrying the same plasmids often show great variability (Meagher et al., 1977; Dougan and Sherratt, 1977; Inselberg and Applebaum, 1978; Collins, 1979) and so this protein may not be detected in the system used. However, more data is needed before it can be said this is cisC. More mutants are especially needed; unfortunately most of these would be within cisA. The final open-reading frame from the lower strand could potentially code for a 10Kd protein of 94 amino-acids (907 - 625bp). This has a ribosome binding site homology.

The confirmation of these open reading frames as genes requires the mapping of further mutants and transcriptional units. A preliminary indication that an open reading frame is transcribed may be acquired by screening the DNA sequence for promoter sequences. Many promoter sequences have been compared and homologies identified about 10bp and 35bp upstream of the mRNA start site. A concensus sequence has been defined for each of these homologous regions and can be used to search for promoters (Rosenberg and Court, 1979; Sienbenlist et al., 1980). Promoter-like sequences were not found near the translational start site for cisB but two possible promoter sequences were identified for cisA, these were 150bp upstream of the translational start codon

(Fig. 7.6). The first putative promoter has a perfect -35 homology to the concensus sequence (TTGACA) at 909 - 914bp but a poorer -10 homology (TGCTGT) 14bp downstream. The second promoter-like sequence has an excellent -10 homology (TATCAT) at 944 - 949 but a poorer -35 homology (TTGTTA) 18bp upstream (Fig. 7.6). Each promoter sequence has a purine base located just downstream from the -10 region which could be the initiation point of the mRNA. The location of these promoters is only speculative but could be confirmed in two ways using the M13 clones of these regions that were sequenced. The first method would be to use overlapping clones of this region to make strand-specific probes. These could be used to 'protect' mRNA, in S1 mapping experiments, prepared from cells containing RSF1010 or pED350 (Berk and Sharp, 1978). The second way, which would demonstrate their in vivo activity and strength, would be to use the M13 clones to prepare fragments of DNA. These could be cloned, using adjacent M13 restriction sites, into plasmids capable of detecting promoters e.g. the pKO-vector system (McKenney et al., 1981).

None of the other open reading frames are preceded by an obvious concensus promoter sequence. If these open reading frames were transcribed then transcription may be from a promoter external to the AvaI fragment or it may be a weak promoter, hence its deviation from the concensus promoter sequence and poor expression of these putative proteins in mini-cells. As many of the promoters are within other reading frames then the promoter sequence may be limited by the effects on the amino-acids in these open reading frames.

If one of the two promoters alluded to above did initiate transcription of cisA (and perhaps cisB, as no obvious promoter sequences were detected for this gene) from within the oriT-containing TaqI fragment, then transcription may be autoregulated by the mobilisation gene products. These transcripts would almost certainly have to be transcribed across the oriT site or alternatively at least across the inverted repeat structures postulated to be involved in protein recognition (see Chapter 7(c)(ii)). If the oriT recognition/nicking proteins were oriT bound this might prevent transcription and synthesis of more pCisA and the mobilisation protein pCisB. After transfer or replication the oriT may not be protein bound allowing transcription and production of these proteins. This is perhaps why only small

amounts of these proteins are detected (Fig. 6.7). In Figure 6.7 it is also noticeable that the truncated form of the 51Kd protein appears to be present in larger amounts from pED409 than its native and other truncated forms. It was suggested earlier that this could be due to instability of this protein. However, an alternative suggestion could be that pED409 is mutated in an oriT recognition/nicking gene (perhaps cisC) which allows increased transcription of cisA and cisB. This hypothesis could be tested by measuring the promoter activity in a pKO-vector system (McKenney et al., 1981) in the presence or absence of these putative products and their mutants.

(iv) Replication of RSF1010

Regions involved in replication of RSF1010 are known to be dispersed throughout the genome (Bagdasarian et al., 1982; Meyer et al., 1982a; 1982b). However, certain replication functions associated with incompatibility (Meyer et al., 1982a), copy number (Rubens et al., 1976; Meyer et al., 1982a) ability to survive in Pseudomonas (hrs) (Bagdasarian, pers. comm.; Barth et al., 1981), and the origin of replication oriV (de Graaf et al., 1978) all map close to the mobilisation region. This has made it difficult to interpret the functions of the potential genes.

cisA was shown from the sequence data to extend past the AvaI site and would therefore code for a protein of over 65Kd in size. Two mob^+ insertion mutants, pMMB20 and pMMB21, which map within this gene (Fig. 7.7), indicate that it is almost certainly involved in replication as these mutants have lost the ability to replicate in Pseudomonas (M. Bagdasarian, pers. comm.). A similar insertion mutant has been isolated in the same region by Barth et al. (1981). Further evidence that this gene is involved in replication is that replication functions have been mapped to the region clockwise of the mobilisation genes (Bagdasarian et al., 1982; Meyer et al., 1982a). The Bal31 deletions (RSF1010Δ18, Δ5, Δ20, Fig. 6.6) constructed from the BamHI site in pKT260 (Fig. 6.6) were asymmetric implying an essential function for replication in E. coli was adjacent to the Mob region (Bagdasarian et al., 1982). As pCisA is involved in replication in Pseudomonas and its coding sequence extends out through this region essential for replication in E. coli then pCisA would seem a prime candidate for a replication protein required in both E. coli and Pseudomonas.

Apart from pED409, all the mob^- insertion mutants which map within

cisA also map within cisB or would be predicted to have polar effects on cisB. The insertion in pED409 also maps within cisC (Fig. 7.7). This suggests that cisB and cisC are mobilisation genes while cisA is a replication gene. However, no pCisC was detected in mini-cells and although the open-reading frame had a closely associated ribosome-binding site, there was no obvious promoter sequence (Fig. 7.6). A less likely alternative to explain the effect of the insertion in pED409 would be that pCisA is a multi-functional protein. This seems doubtful as pED409 is complemented by mutants in cisB which, like pED409, synthesise a truncated pCisA. To overcome this, one would have to postulate that the truncated form of pCisA is able to function in mobilisation.

The region 300 - 400 (not presented), like the oriT region, contained several sequence anomalies because of repeated sequences and these have still to be clarified. However, it is apparent that there are several long direct repeats (4 x 22bp). These may be associated with oriV as it has been mapped to this region (de Graaf et al., 1978; Bagdasarian et al., 1982) and oriV regions often contain direct and indirect repeated sequences e.g. RK2 (Stalker et al., 1981) F (Murotsu et al., 1981), R100 (Rosen et al., 1979). Once this sequence has been confirmed experiments can be carried out to define the oriV region still further by using DNA prepared from the M13 sequence library. Regions involved in replication, incompatibility and copy number in both E. coli and other Gram-negative hosts could be investigated. Similar properties have been investigated using the broad-host-range plasmid RK2 (Thomas, 1981; Thomas et al., 1981; 1982; Stalker et al., 1981), but the smaller size and availability of clones covering the whole region make the experiments with RSF1010 considerably easier.

FINAL SUMMARY

The data presented in Chapter 7 has confirmed and substantiated the work of previous chapters. It has allowed the mapping of *oriT* which, as in other conjugative systems, is terminally located with respect to its mobilisation genes. A 10bp inverted repeat was located across a *TaqI* site used for cloning *oriT* and this might be a recognition or nicking site of a mobilisation protein. The slight reduction in mobilisation of *oriT TaqI* clones might be explained by compensation for the loss of this 10bp indirect repeat by a smaller less perfect 7bp indirect repeat involving adjacent vector sequences.

The open reading frames of the two genes *cisA* and *cisB* were identified and the predicted protein sizes for their products were in excellent agreement with those measured experimentally. The Mob^- insertion mutants demonstrated that *cisB* is a mobilisation gene. Two Mob^+ insertion mutants mapped within *cisA*. As these mutants are deficient in replication in *Pseudomonas* (M. Bagdasarian, pers. comm.) and as *cisA* extends out of the mobilisation region into a region required for replication in *E. coli*, then *cisA* is probably a replication gene. One Mob^- insertion mutant, pED409, mapped within *cisA* but outside *cisB*. This might imply that pCisA is multifunctional (mobilisation and replication). However, a more likely explanation is that a third gene *cisC*, overlapping *cisA*, has been mutated. An open reading frame which codes for a 22Kd protein, was identified on the opposite strand to *cisA*. This overlapped *cisA* and the insertion in the mutant pED409. No protein of 22Kd was identified in minicells, and so until more mutants have been isolated and characterised the existence of this gene cannot be confirmed. The overlapping nature of the replication and mobilisation genes probably selects against mutations in this region which is essential for the plasmids survival and dissemination.

The two genes *cisA* and *cisB* are transcribed from the same DNA strand and as no distinct canonical promoter sequence for *cisB* was detected, it may be that pCisA and pCisB are derived from a single mRNA translated from different initiation sites. There are two possible promoter sequences for this common mRNA, both within the *oriT TaqI* fragment. Transcription from these promoters might be auto-regulated by the mobilisation proteins binding to *oriT*.

The accurate mapping of these mobilisation genes makes it feasible to clone them into over-expression vectors (e.g. Backman and Ptashne, 1078; Remaut *et al.*, 1981; 1983). The *cisB* gene would be relatively easy to clone using the *Eco*RI site immediately before its initiation codon in pED412. Over-production of these proteins would allow their purification and analysis. This could lead to the development of an *in vitro* system for studying the initiation and metabolism of transfer. The DNA binding affinity of these proteins could be measured and their recognition sites determined by DNA 'footprint' experiments. Furthermore, the cloning of the *oriT* region on a small fragment makes these experiments more feasible and would allow the characterisation of nicking and religating ability of the mobilisation proteins. Thus the data presented in this thesis have provided a comprehensive and sound base from which the mechanism of DNA transfer of non-conjugative plasmids can be characterised.

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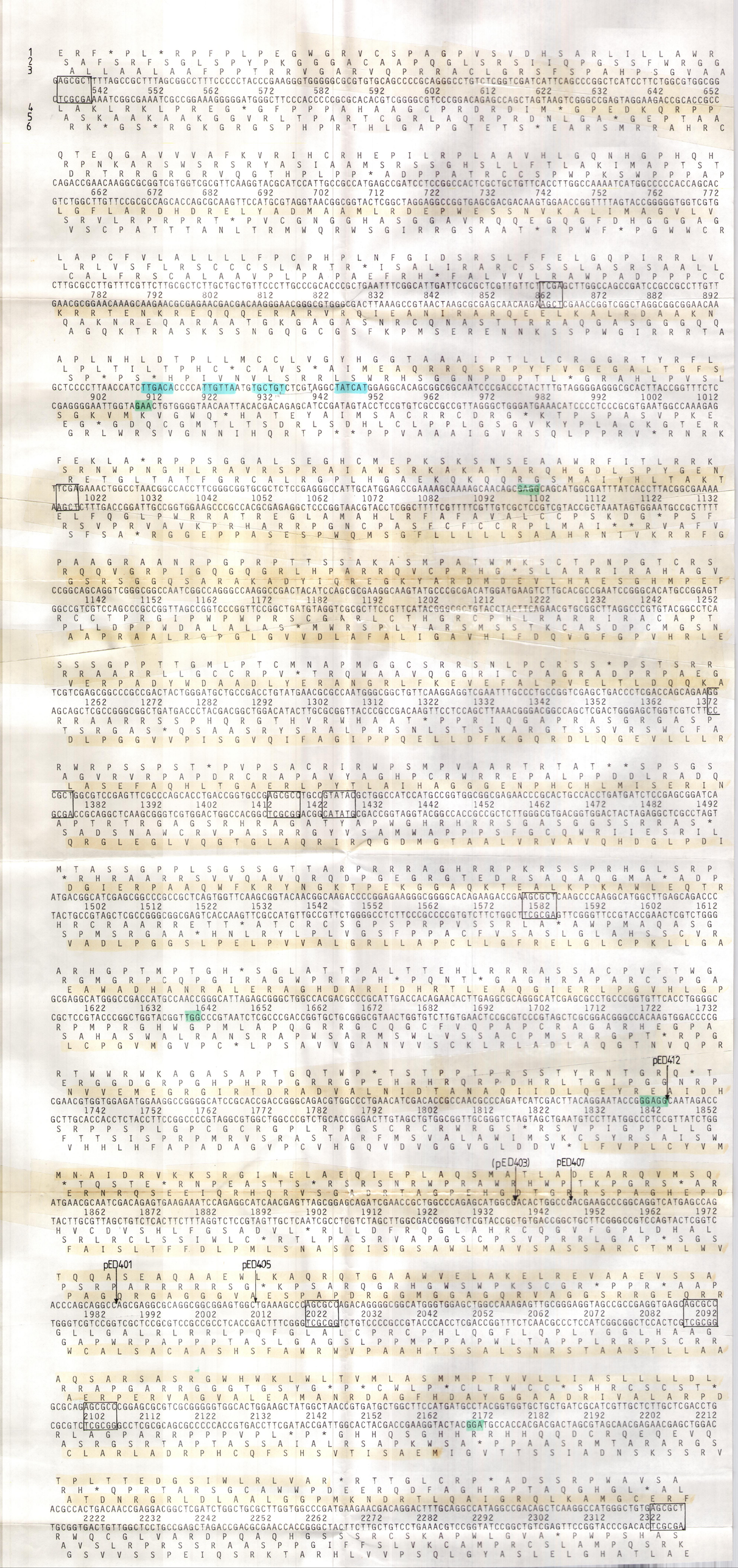
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