THE ROLE OF DNA PRIMASES

SPECIFIED BY PLASMIDS RP4 AND ColIb-P9

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For my mother and father

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ABBREVIATIONS

A	Absorbance
Ap	Ampicillin
BLA	BBL trypticase agar
bp	Base pair(s)
BTL	BBL trypticase top layer agar
CCE	Crude cell extract
CDS	Conjugative DNA synthesis
Cm	Chloramphenicol
cpm	Counts per minute
DMA	Difco methionine assay medium
DMSO	Dimethyl sulphoxide
2-dG	2-deoxyguanosine
dNTP	Deoxynucleoside triphosphate
dpm	Disintegrations per minute
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
HEPES	N-2-hydroxyethylpiperazine-N-2'-ethane-sulphonic acid
kb	Kilobase
kD	Kilodalton
Km	Kanamycin
LB	Luria broth
NA	Nutrient agar
Nal	Nalidixic acid
NB	Nutrient broth
PB	Phosphate buffer
PEG_{6000}	Polyethylene glycol 6000
pfu	Plaque forming unit
RI	Refractive index
Rif	Rifampicin
rNTP	Ribonucleoside triphosphate
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SGC	Salts-glucose-Casamino acid medium
Sm	Streptomycin sulphate
Su	Sulphonamide
Tc	Tetracycline
TCA	Trichloroacetic acid
Tris	Tris[hydroxymethyl]-aminomethane
Triton-X100	Octyl phenoxy polyethoxyethanol
UV	Ultraviolet

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CHAPTER ONE

INTRODUCTION

1.1. Perspective

Since the discovery that bacterial genes could be transmitted between intimately associated cells (Lederberg and Tatum, 1946a; 1946b; Tatum and Lederberg, 1947), the process of plasmid-mediated conjugation has been the focus of much research. Conjugation is a highly specific process which results in the transfer of DNA between bacterial cells by a mechanism that requires cell-to-cell contact. This process is specified by plasmid genes and many plasmids from a wide variety of bacterial species have been shown to be conjugative (Jacob et al., 1977). Since the conjugative process can allow transmission of genes both between populations of a single species, and between widely differing genera, it is of major importance as a mechanism for gene exchange between bacteria. As such, conjugation has a number of biological, clinical and technological implications.

Plasmids from well over twenty incompatibility (Inc) groups (Datta, 1979) have been shown to encode a conjugation system (Jacob et al., 1977). With the exception of the members of the IncF-group and the I-complex (Hedges and Datta, 1973), plasmids of different incompatibility groups share little DNA homology (Guerry and Falkow, 1971; Grindley et al., 1973; Falkow et al., 1974; Anderson et al., 1975; Roussel and Chabbert, 1978; Gorai et al., 1979). Since a large proportion of the coding capacity of many plasmids may be dedicated to conjugation, it may follow that there are as many distinct conjugative systems as there are incompatibility groups (Achtman and Skurray, 1977). This model receives support at the functional level, since transfer genes of one system are rarely able to complement transfer mutants from a system encoded by a plasmid of a different Inc group (Alfaro and Willetts, 1972).

Studies using mainly plasmids F (IncFI) and R64 ($IncI_1$) have lead to a fair understanding of the genetic and molecular processes of the conjugative transfer of DNA. Such studies have demonstrated that some, and maybe all, plasmids are transferred as single-stranded DNA. Therefore, in order to become established in the recipient cell, synthesis of DNA complementary to the transferred strand must occur in order to reform the plasmid duplex. Much of the previous work concerning the

factors required for complementary strand synthesis has involved the use of $IncI_1$ plasmids. These studies revealed that a novel DNA primase, encoded by the plasmid, is of central importance in this process. Furthermore, the analysis of other plasmid-encoded primases has revealed that these enzymes may also be important in the establishment of some plasmids in a wide variety of bacterial hosts (Lanka and Barth, 1981).

The main aim of this thesis was to explore further the important role of plasmid primases in the establishment of the transferred plasmid strand in the recipient cell. The two plasmids used for this study were ColIb-P9 ($IncI_1$) and RP4 (IncP; Figure 1.1). ColIb-P9 primase represents the best characterised plasmid primase, both structurally and functionally, and although the primase of the broad host range plasmid, RP4 has also been extensively studied, much remains to be discovered about the function of this enzyme. Therefore, in response to the interest of I.C.I. which supported this work through a CASE studentship, much of the work in this thesis will relate to the involvement of the RP4 plasmid primase in the conjugative process of the plasmid, and to the effect of strain and species variation on this.

It should be noted that plasmids in the $IncI_1$ group, ColIb-P9, R64 and R144 are closely related as revealed by restriction analysis and DNA-DNA hybridisation studies (Chabbert et al., 1978; Guerry and Falkow, 1971; Falkow et al., 1974). More recent data has shown that this homology may include at least some of the transfer genes (Dalrymple et al., 1982; Wilkins et al., 1985). Similarly, the plasmids RP4, RP1, R68, RK2 and R18 have been shown to be very similar by heteroduplex analysis and restriction cleavage analysis (Burkardt et al., 1979; Stokes et al., 1981). Therefore, unless indicated otherwise, it will be assumed that results found for one representative of these groups will also apply to other members of that group.

1.2. Bacterial conjugation

The conjugation systems of many conjugative plasmids are normally repressed, as shown to be the case for ColIb-P9 (Monk and Clowes, 1964). Clark and Adelberg (1962) suggested that such transfer systems were self-regulated and were normally repressed, presumably by the action of a plasmid-encoded regulatory protein, or repressor (Meynell and Datta, 1967). A small proportion of the plasmids in a population would be able

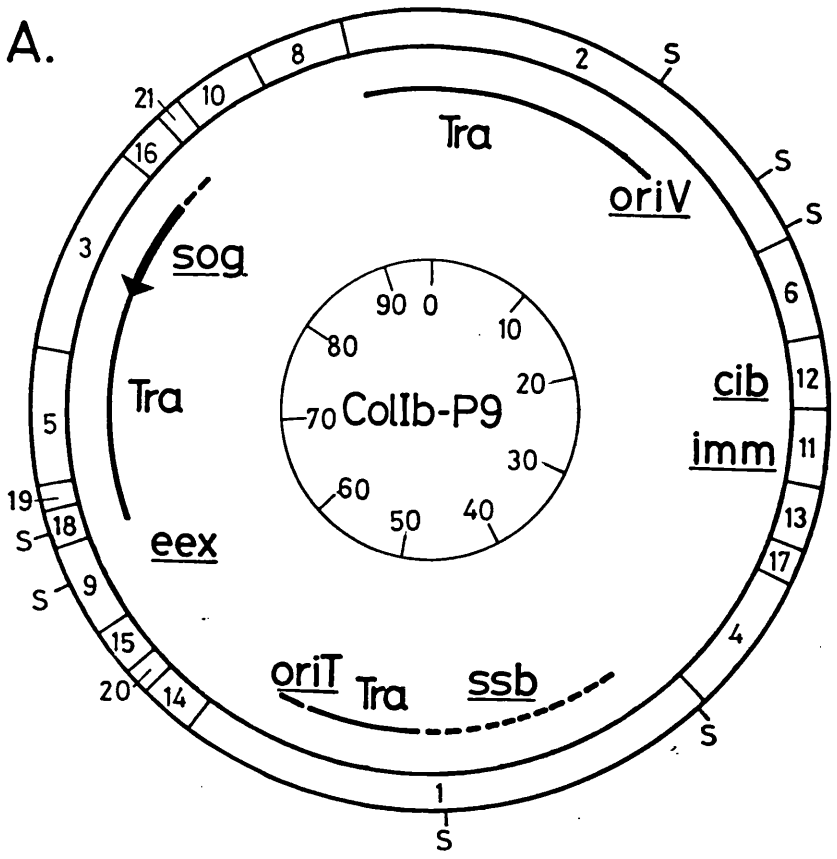
Figure 1.1 Physical and genetic map of plasmids ColIb-P9 and RP4

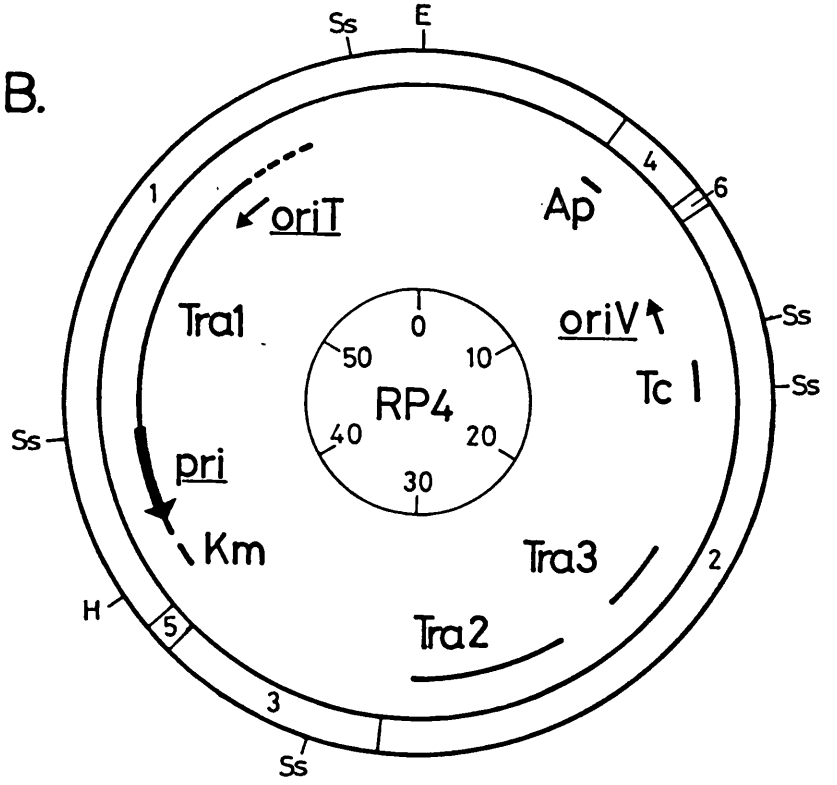
A. Map of $IncI_1$ plasmid ColIb-P9. The genetic map indicates the regions important for conjugative transfer (Tra), as determined by transposon mutagenesis (C.Rees, unpublished results). The dashed lines represent the regions thought also to be necessary for plasmid transfer, but which require further confirmation. The extent of the plasmid primase gene (sog) and the direction of transcription is indicated by the bold arrow. The regions containing the origin of transfer (oriT) and the genes specifying single-stranded binding protein (ssb) and the entry exclusion system (eex) are shown. The role of these genes is discussed in the text. The origin of vegetative replication and the genes required for plasmid replication are shown (oriV). Colicin-Ib synthesis and immunity are encoded by the cib and imm genes, respectively.

DNA fragments generated by EcoRI cleavage are indicated by the outer circle and SalI-cleavage sites (S) are shown. The inner circle gives the size of the plasmid in kilobases.

B. Map of IncP plasmid RP4. The genetic map indicates the three regions required for plasmid transfer (Tra; Barth et al; 1978). The direction of transcription and extent of the plasmid primase gene (pri) is represented by the bold arrow. The direction of transfer is indicated by an arrow from oriT and that of replication by an arrow from oriV. The genes encoding resistance to ampicillin (Ap), tetracycline (Tc) and kanamycin (Km) are shown.

Restriction sites (Lanka et al., 1983) are represented as follows: EcoRI (E), HindIII (H) and SstII (Ss). The outer ring shows PstI-cleavage fragments. The inner ring shows the size of the plasmid in kilobases.


A.
Tra
oriV
sog
Tra
ColIb-P9
cib
imm
eex
oriT
Tra
ssb
0
10
20
30
40
50
60
70
80
90
1
2
3
4
5
6
8
9
10
11
12
13
14
15
16
17
18
19
20
21
S


B.
oriT
Ap
Tra1
oriV
Tc I
RP4
pri
Km
Tra3
Tra2
0
10
20
30
40
50
1
2
3
4
5
6
E
H
Ss

to escape from this repression and transfer to recipient cells. The nature of such a system was established by the isolation of plasmid mutants in which the transfer system was permanently expressed (Meynell and Datta, 1967). The use of such drd mutants (Sasakawa and Yoshikawa, 1978) has greatly enhanced the understanding of plasmid transfer systems. It is worth noting that not all plasmids express such a repression system, and the conjugative genes of such plasmids, such as RP4, are constitutively expressed in E.coli (Bradley, 1980a; Datta et al., 1971). However, the small number of pili encoded by RP1 in Pseudomonas aeruginosa suggests that the mechanisms that control transfer gene expression of such broad host range plasmids may only be active in some species (Datta and Hedges, 1972; Olsen and Shipley, 1973).

The system which represses the transfer genes of most F-like plasmids, the FinOP fertility inhibition system (Meynell et al., 1968), is well characterised. This system, encoded by the finO and finP genes, negatively regulates the transcription of traJ, which acts to positively control transfer gene expression (reviewed in Willetts and Skurray, 1980). F itself is finO $finP^+$ and therefore constitutively expresses its conjugation system, but the provision of the relatively non-specific FinO protein from a closely related plasmid, allows the formation of the FinOP repressor which consequently reduces F transfer gene expression. In contrast to FinO protein, the finP gene product, which is proposed to be a small RNA molecule (Mullineaux and Willetts, 1985), is plasmid-specific in its inhibitory action (Willetts and Maule, 1985).

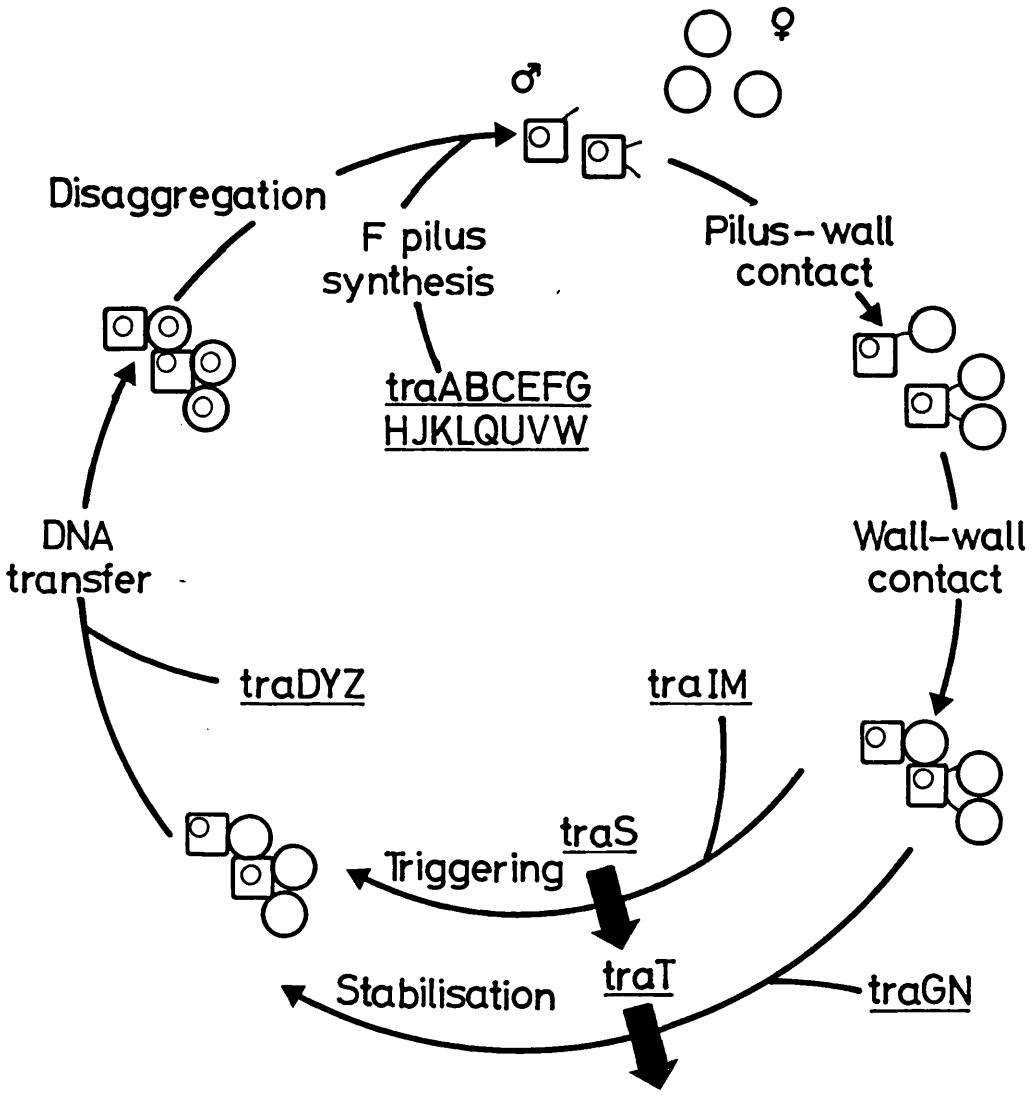
Much of the work concerning the sequence of events occurring during conjugation has been studied using F, and from this work a mating cycle has been proposed (Achtman and Skurray, 1977; Manning and Achtman, 1979) and is shown in Figure 1.2. This model proposes that, initially, mating cells become associated as a result of the action of the pilus. Plasmid-encoded factors then act to stabilise this association, so allowing the transfer of DNA to occur before the mating cells actively disaggregate. These stages will be considered in more detail below.

The establishment of cell-cell contact

As outlined above, cell-to-cell contact is a prerequisite for DNA transfer. At present, the physical nature of the interaction between mating cells is poorly understood. However, the various stages involved in the

Figure 1.2 Model of the F plasmid-mediated mating cycle

Adapted from Manning and Achtman (1979). Donor and recipient cells are represented by squares and circles, respectively, and the F conjugative plasmid is indicated by small circles within the cells. The F plasmid transfer genes (tra) essential for each stage of the mating cycle are shown, and in the case of traS and traT, the bold arrows indicate inhibition of the triggering and stabilisation stages, respectively. The role of the gene products, where known, is discussed in the text.


♂
♀
Disaggregation
F pilus synthesis
traABCEFG HJKLQUVW
Pilus-wall contact
DNA transfer
Wall-wall contact
traDYZ
traIM
traS
Triggering
Stabilisation
traT
traGN

interaction, in terms of genetics, are well documented. Most of this work has concerned the F conjugation system. Cell-to-cell contact is initiated by the action of the pilus. All conjugative plasmids so far tested encode pili (Bradley, 1980b), and it is widely assumed that pili are essential for conjugation (Ippen and Valentine, 1967; Harden and Meynell, 1972).

At least fourteen F tra genes have been determined to be involved in pilus synthesis. The F pilin subunit, which is 7kD in size (Moore et al., 1981a), is known to be glycosylated and phosphorylated before assembly (Brinton, 1971), and is specified by the traA gene in the form of a precursor with a 51 amino acid leader sequence (Frost et al., 1984). This pilin-precursor is thought to be processed in a reaction involving the traQ gene product (Moore et al. 1982; Ippen-Ihler et al., 1984). The roles of the other tra genes required for pilus synthesis are unclear, but are likely to be involved in the assembly of the pilus (Moore et al., 1981b). The cellular location of F-pilin and most of the tra gene products involved in pilus synthesis suggests that assembly occurs on or within the inner membrane (Manning and Achtman, 1979; Moore et al., 1981a; 1981b).

The precise role of the pilus is uncertain, but it is believed to stabilise an interaction between mating cells prior to the establishment of specific, more stable contacts (Achtman et al., 1978a). The F pilus receptor on the recipient cell surface remains to be identified; however, once attached, the F pilus retracts by some unknown mechanism to bring the donor and recipient cell walls into close contact (Ou and Anderson, 1970). The resultant unstable mating pair is then stabilised in a process which involves, in F, traG and traN proteins encoded in the donor cell and an LPS-OmpA protein complex on the recipient cell surface (Achtman et al., 1978a; Manning et al., 1981).

In contrast to F, very little is known about the composition, assembly or function of the pili encoded by ColIb-P9 or RP4. ColIb-P9 specifies two types of pili, flexible thin and rigid thick, which are morphologically and serologically distinct (Bradley and Coetzee, 1982; Bradley, 1983b; 1984). Using plasmids which were unable to synthesise thin pili, it was shown that the thick pili are essential for conjugation, whilst the thin pili, are also required for conjugation in liquid but not on solid medium. Presumably the thin pili act to stabilise mating cells in liquid medium (Bradley, 1984). As would be predicted from this, RP4, which only forms

thick rigid pili, is transferred between E.coli strains about 2000-fold less efficiently in liquid than on solid medium (Bradley et al., 1980).

Like F, stable mating pair formation in ColIb-P9-mediated conjugation is initially established by the pili which, following retraction, bring the donor and recipient cell walls into close proximity (Lawn and Meynell, 1972). It is known that the LPS component of the recipient outer membrane, but not OmpA protein, is involved in this process (Havekes et al., 1977), but it is unclear whether it acts as the pilus-receptor or is involved in stable mating pair formation (Manning and Achtman, 1979).

The transfer of DNA during conjugation

Once stable mating pairs are formed, then DNA transfer and conjugative DNA metabolism are triggered. Originally, it was thought that in F at least, the retracted pilus may act as the conjugation bridge, the axial hole being just large enough to accommodate single-stranded DNA (Brinton,1971; Ou and Anderson, 1970; Folkhard et al., 1979). However, the addition of sodium dodecyl sulphate, at high enough concentrations to depolymerise the F pilus, did not inhibit DNA transfer (Achtman et al., 1978b; Panicker and Minkley, 1985), and experiments designed to demonstrate DNA transfer through an extended pilus remained inconclusive (Ou and Anderson, 1970).

An alternative model envisioned that donor and recipient cell membranes fused locally in order to allow the transfer of DNA through some structure located at this point. Electron microscopy has lead to the belief that F pili are synthesised and act at sites where the donor cell inner and outer membranes are fused, at so called adhesion zones (Bayer, 1975; 1979). If this is the case, then the formation of stable mating pairs may involve the alignment of such adhesion zones and possibly the fusion of donor and recipient outer membranes (Willetts and Skurray, 1980). This model receives support from the observation that phage λ receptor sites, the lamB gene product which is located in the outer membrane (Randall-Hazelbauer and Schwartz, 1973), are exchanged bidirectionally as a result of conjugation (Goldschmidt and Curtiss, cited in Curtiss et al., 1977).

The concept of pilus assembly and DNA transfer at a transmembrane pore is currently popular. Envisioned is a donor membrane complex of polypeptides which would process the traA gene product and assemble the pilus at an

adhesion zone in the donor (Frost et al., 1984; Laine et al., 1985). The extended pilus, on recognising some feature of a recipient cell adhesion zone, would retract and so align the two adhesion zones and bring them into close contact (Bayer, 1975; 1979; Frost et al., 1984). Some interaction involving the recipient LPS-OmpA protein complex and the F traG and traN gene products in the donor, would then stabilise the mating pair so allowing the fusion of outer membranes and the generation of the hypothetical transmembrane pore. A single strand of plasmid DNA, presumably protected by single-stranded binding protein, could then pass through the pore possibly guided by a protein attached to the 5' terminus of the DNA (Marco et al. 1974; Kornberg, 1974). The motive force required to transmit the DNA would be generated by the unwinding of the duplex DNA in the donor by the traI protein (Abdel-Monem et al., 1983), which is anchored to the membrane, possibly at the adhesion zone, by the product of the traD gene (Panicker and Minkley, 1985).

In contrast to the wealth of evidence demonstrating that DNA is transferred between mating cells, the selective labelling of protein or RNA in donor cells has failed to show the transfer of any significant amount of radioactivity to the recipient cells during ColIb-P9 or F mediated conjugation (Silver and Ozeki, 1962; Silver, 1963; Silver et al., 1965). However, since the limit of resolution of these experiments was about 0.3% of the total protein or RNA present in the donor cell, the selective transfer of either component could not be excluded. This is in keeping with the proposal that certain factors involved with the conjugative process, such as plasmid primase, RNA primers, SSB or the 5'-terminal binding protein, may be supplied by the donor and selectively transferred to the recipient during conjugation (see later section). This aspect of conjugation will be discussed further in Chapter 6.

Once DNA transfer is complete, the mating cells disaggregate in an active process, possibly due to the synthesis of some factor encoded by the newly-immigrant plasmid. Such a factor may act to alter the surface of the recipient cell, so destabilising the cell-to-cell contacts (Achtman, 1977). In ColIbP9-mediated conjugation the demonstration that the amount of plasmid DNA transferred between mating cells was increased four fold when RNA or protein synthesis was inhibited in the recipient cells, suggests that some ColIb-P9-encoded polypeptide normally acts to limit the transfer of plasmid DNA (Boulnois and Wilkins, 1978). However, whether this product acts by causing active disaggregation, or by excluding the

entry of further plasmid strands was unclear.

Surface exclusion

Surface exclusion is a phenomenom, formally distinct from active disaggregation, which acts to limit conjugation between cells carrying plasmids which specify similar conjugation systems (Lederberg et al., 1952). Many exclusion groups have been elucidated, and only cells carrying plasmids of the same groups will be inhibited from transferring DNA (Willetts and Maule, 1974). The genes and products involved in the exclusion system of F and R144 ($IncI_1$+B) have been well characterised and will be discussed briefly here.

The F genes responsible for surface exclusion in F are traS and traT. The product of the traS gene (TraSp) is a 18kD polypeptide located in the inner membrane, whereas the traT protein (TraTp) is an outer membrane protein of 25kD which is transmembraneous (Achtman et al., 1977; Kennedy et al., 1977). TraTp is synthesised as a precursor which, in keeping with other outer membrane proteins, has a signal sequence to enable its export through the inner membrane (Minkley, 1984; Perumal and Minkley, 1984). Both precursor and mature forms of TraTp are lipoproteins by virtue of the addition of diglyceride and fatty acid moieties, however, the relevance of these residues is unclear (Perumal and Minkley, 1984).

The cellular location of both TraSp and TraTp corresponds to their observed roles. In F-mediated conjugation, the conjugative process can be blocked at two stages. TraTp blocks mating aggregate formation, a process termed surface exclusion (Achtman et al., 1977). It is unclear whether TraTp acts to block the recognition of the recipient cell surface by the F pilus, so preventing unstable mating pair formation (Achtman et al., 1977; Minkley and Willetts, 1984), or whether it acts to block the sites required for the stabilisation of the mating pairs, although it is known that ompA protein is not involved in either case (Eckerson and Reynard, 1977; Achtman et al., 1977; Manning and Achtman, 1979). Since variants in pilus structure have been found to usually correlate with corresponding variants in the surface exclusion system, it is possible that these two components interact to prevent pilus attachment (Willetts and Maule, 1985). In either instance, TraTp acts principally to prevent the formation of stable mating pairs.

In contrast, TraSp has a minor effect on mating aggregate formation, but greatly affects DNA transfer (Achtman et al., 1977). The mechanism of this process, called entry exclusion, is unclear, but the presence of TraSp in the inner membrane has prompted the suggestion that the triggering of donor conjugative DNA metabolism may be inhibited (Manning and Achtman, 1979).

Similarly the IncI plasmids have been shown to exhibit entry exclusion, and although this has been most intensely studied using R144, the ColIb-P9 gene has also been cloned (Wilkins et al., 1985). Although R144 and ColIb-P9 are reported to be in different incompatibility groups (Bird and Pittard, 1982), these two plasmids are in the same exclusion group (Hartskeerl et al., 1985a). Comparison of restriction endonuclease cleavage sites in the region around the exclusion genes of R144 (exc; Hartskeerl et al., 1983) and ColIb-P9 (eex; see Chapter 6), also suggested that this region may be common to both plasmids (Wilkins et al., 1985).

When present in recipient cells, the exc gene was shown to reduce the yield of R144 transconjugants approximately 100-fold (Hartskeerl et al., 1983; 1985a). Similarly, a 98% reduction in the formation of ColIb-P9 transconjugants was observed when recipient cells harboured the recombinant plasmid containing the eex gene, pLG252 (Wilkins et al., 1985). The exc gene was found to encode two polypeptide products of 13kD and 19kD, which are expressed from two separate promoters (Hartskeerl et al., 1983). Only the 19kD polypeptide is involved in R144 exclusion, this polypeptide being located on the periplasmic side of the inner membrane (Hartskeerl et al., 1985b). In keeping with this location, like F TraSp, the 19kD polypeptide is believed to act at a stage subsequent to mating pair formation, probably by excluding the entry of DNA (Hartskeerl and Hoekstra, 1984; 1985). What is not clear, and is of relevance to Chapter 6, is whether the exclusion system of R144, and therefore presumably ColIb-P9, acts prior to the formation of the hypothetical transmembrane conjugation bridge.

RP4 is also known to encode an exclusion system, but very little is known about the genetics or mechanism of the system. Like F, the RP1 exclusion system is reported to involve two genes, which are probably in the Tra3 region of the plasmid (Barth et al., 1978; P.Barth, personal communication, cited in Achtman et al, 1980; Figure 1.1).

1.3. Plasmid replication and host range

After the transfer of a plasmid into a cell, the proliferation of the plasmid depends on the ability of the host DNA replication system to recognise and replicate the plasmid DNA. The host range is therefore defined as the group of species in which the plasmid can replicate and be stably maintained. The host range is one of the principle differences between ColIb-P9 and RP4.

The host range of $IncI_1$ plasmids is apparently narrow, being restricted to species of Escherichia, Salmonella, Shigella and Klebsiella (Datta and Hedges, 1972; Jacob et al., 1977). Little is known about the genetical or molecular basis of $IncI_1$ plasmid replication. Following initiation, presumably at the origin of vegetative replication (oriV), which is located in the Sal-5 fragment (Figure 1.1; C.Rees, unpublished results), the primers for discontinuous DNA synthesis are probably formed by the primosome, as indicated by the requirement for dnaB protein in replication (Fenwick and Curtiss, 1973a; Wilkins and Hollom, 1974).

Rather more is known about the genetics of RP4 replication. This plasmid has a broad host range, and can be stably maintained in all Gram-negative bacterial species so far tested, with the exception of Bacteroides fragilis and Myxococcus xanthus (Datta and Hedges, 1972; Olsen and Shipley, 1973; Beringer, 1974; Cho et al., 1975, Jacob et al., 1977; Kulpa et al., 1983; Schwab et al., 1983; Breton et al., 1985; Guiney et al., 1985). In contrast to other plasmids, the loci important for the maintenance of RK2, and therefore RP4, are dispersed around the plasmid genome. Replication proceeds unidirectionally in a counterclockwise direction from oriV, which is located between coordinates 12.3 - 13.0 (Meyer and Helinski, 1977; Thomas et al., 1980; Figure 1.1). This region has been sequenced and shown to possess a putative promoter, suggesting that, like the E.coli chromosome, transcription of the origin by RNA polymerase may be a prerequisite to initiation (Stalker et al, 1981; Lother and Messer, 1981). It should however be noted that no oriV transcript has yet been identified.

Apart from oriV, RP4 replication in many species of bacteria requires only one plasmid locus, trfA which lies between coordinates 16.0 - 17.4 (Thomas et al., 1980; Shingler and Thomas, 1984; Schmidhauser et al., 1983; Schmidhauser and Helinski, 1985). However, replication in other bacterial

hosts requires some function encoded by a 3.1kb region between coordinates 52.5 and 55.6, in addition to oriV and trfA (Schmidhauser and Helinski, 1985). This region is known to carry the korA and korB genes, the products of which are thought to control the expression of trfA, and thus these genes are probably involved in the control of plasmid replication (Figurski et al., 1982; Thomas and Hussain, 1984). Transposon mutagenesis of this region of RP4 affected the stability of the plasmid in some, but not all hosts (Barth, 1979; Barth et al., 1984). Several other kil and kor genes have been discovered, but their role in plasmid replication is uncertain (Figurski et al., 1982).

The replication of the broad host range, mobilisable plasmid RSF1010, requires the products of three plasmid genes, repA, repB and repC, which are widely separated from the oriV sequence (Scherzinger et al., 1984). In vitro, the replication of RSF1010 (which is equivalent to R300B, Barth, 1979), is rifampicin-resistant and independent of the dnaB, dnaC and dnaG products. It was therefore speculated that the rep gene products may be responsible for the plasmid-specific initiation of RSF1010 replication (Scherzinger et al., 1984; Haring et al., 1985). Furthermore, since overproduction of repC resulted in increased plasmid copy number, it was suggested that this protein may act as a positive regulator. This model is in keeping with the ability of the repC gene product to bind tightly to the RSF1010 oriV region (Diaz and Staudenbauer, 1982; Scherzinger et al., 1984; Haring et al., 1985).

The factor limiting the host range of a plasmid is likely to be plasmid replication rather than conjugation, at least within the Gram-negative species of bacteria, since RP4, F and ColIb-P9 can all be transferred into hosts in which the plasmid is unable to be maintained as an autonomous replicon (Boulnois et al., 1985; Breton et al., 1985; Guiney et al., 1985). In the case of ColIb-P9, it was demonstrated that transposition of Tn5 can occur between the plasmid and the Pseudomonas putida chromosome, suggesting that a duplex plasmid molecule is formed in this host following the transfer of single-stranded DNA, even though the subsequent replication of the plasmid is not possible (Boulnois et al., 1985). Hence, it is likely that the factors required for conjugative DNA synthesis (CDS) and replication vary, and that those needed for CDS are provided by the donor cell. Considering the requirement for plasmid primase during ColIb-P9 and RP4-mediated conjugation, these enzymes are implied to be of importance in the establishment of the plasmid in the

recipient cell prior to the replicative process (Lanka and Barth, 1981; Chatfield and Wilkins, 1984). Before discussing conjugative DNA synthesis and the role of plasmid primase, some aspects of the E.coli priming systems will be considered.

1.4. Primer generation in E.coli

The replication of a double stranded DNA molecule requires primers at two stages; at initiation, as typified by plasmids such as ColEI, R1 and F, and during the discontinuous synthesis of the nascent strand growing in the 3' to 5' direction, i.e. the lagging strand (reviewed in Tomizawa and Selzer, 1979; Ogawa and Okazaki, 1980; Nossal, 1983). In E.coli, the activities of the two primer generating enzymes, DNA-dependent RNA polymerase and host primase, encoded by the dnaG gene, are not generally interchangable in vivo. RNA polymerase is required for the initiation of chromosomal replication (Lark, 1972) and also for the replication of some plasmids, such as ColEI (Itoh and Tomizawa, 1978), and phage, for example M13 (Brutlag et al, 1971). In contrast, the replication of other phage, such as G4 and ΦX174 requires initiation by host primase. The use of the single-stranded phage replication systems, and their reconstruction in vitro, has allowed an understanding of the priming reactions catalysed by the host enzymes, culminating in a model for the priming of discontinuous DNA synthesis (Kornberg, 1980; 1982).

RNA polymerase

RNA polymerase holoenzyme contains at least the subunits α, β, β' and σ in the form $\alpha_2\beta\beta'\sigma$ (Burgess, 1969; Kornberg, 1980). The σ subunit, attached to the core enzyme only during initiation, is responsible for strand-specificity (Harris et al., 1978). The β' subunit is thought to be involved in the binding of the DNA template, whereas the initiation and extension of chain growth concerns the β subunit (Zillig et al., 1970). The latter processes are inhibited by the action of rifampicin and streptolydigin respectively, both of which bind to the β subunit (Wehrli and Staehelin, 1971; Cassani et al., 1970). Consequently, rifampicin-resistant mutants have been shown to encode an altered β subunit that cannot bind the antibiotic (Wehrli and Staehelin, 1971).

Initiation of phage M13, and its close relatives fd and f1, requires the coating of the viral strand with SSB (Geider and Kornberg, 1974). This

exposes a unique region of potential secondary structure, resistant to melting by SSB, which is transcribed by RNA polymerase to form a 20-30 nucleotide primer (Geider and Kornberg, 1974; Geider et al., 1978; Gray et al., 1978). The primer is initiated 6 base pairs from the base of the stem structure and, as transcription proceeds towards the loop, the hairpin structure is presumably melted and the untranscribed strand of the stem structure bound by SSB. The termination of primer synthesis occurs when the RNA polymerase encounters this SSB-coated DNA at the top of the loop (Geider et al., 1978). The involvement of RNase H, and the two discriminatory factors α and β (Vicuna et al., 1977a; 1977b) are implicated in the formation of this specific primer on M13 template, but their roles are unclear since Kaguni and Kornberg (1982) suggest that the RNA polymerase subunit σ confers such specificity.

Initiation of chromosomal replication also requires the action of RNA polymerase at, or near, oriC both in vivo (von Meyenburg et al., 1978) and in vitro (Fuller et al., 1981). RNA polymerase is thought to initiate chromosomal replication (Hirose et al., 1983; Kaguni and Kornberg, 1984) and the specificity of this event is dictated by the action of RNase H (Kogoma et al., 1985). It is not clear however, whether the oriC transcript formed by RNA polymerase acts to prime DNA synthesis directly or, alternatively, to stabilise the separation of the strands so allowing other factors access to the origin region.

RNA polymerase also acts in the replication of plasmid ColEI by synthesising a transcript from a promoter 555 base pairs upstream from the origin, on the H (poly-U,G heavy) strand (Itoh and Tomizawa, 1980). The synthesis of this RNA species, termed RNAII, proceeds past the origin, and, after hybridising to the DNA at the origin, RNAII is cleaved by RNase H (Itoh and Tomizawa, 1982; Hillenbraud and Staudenbauer, 1982). This yields the 3'-hydroxyl group from which unidirectional DNA synthesis commences, initially catalysed by DNA polymerase I and subsequently by DNA polymerase III holoenzyme (Staudenbauer et al., 1978; 1979). The processing of RNAII is regulated by the transcription of a second RNA species, RNAI, from within the region encoding RNAII, but from the L strand. RNAI and RNAII are therefore complementary, and the hybridisation of the two species prevents RNAII associating with the origin DNA, so inhibiting primer formation (Tomizawa et al., 1981; Tomizawa, 1984). This RNAI:RNAII interaction is enhanced by the ColEI-specified rop protein (Tomizawa and Som, 1984).

Similarly, initiation of the IncFII plasmid R1 involves the transcription of a region adjacent to the origin by RNA polymerase (reviewed in Nordstrom et al., 1984). The transcription of a gene required for replication, repA (Uhlin and Nordstrom, 1978), is controlled by the synthesis of a small RNA species, termed CopA-RNA, which is transcribed by RNA polymerase from a region between repA and its promoter (Stougaard et al., 1981). Since CopA-RNA is transcribed in the opposite direction to repA, the former can hybridise to the repA message to prevent its translation (Light and Molin, 1983). However, the RNA primer required for initiation of DNA synthesis is not provided by RNA polymerase, since in the presence of repA protein, R1 derivatives can replicate in vitro in a rifampicin-resisitant process (Diaz and Ortega, 1984).

E.coli primase

In contrast to RNA polymerase, the other bacterial-encoded priming activity, primase, is involved both in initiation of replication and in the priming of lagging strand synthesis and is active as a monomer of molecular weight 65,563 daltons (Lupski and Godson, 1984). The dnaG gene, which maps at about 66 minutes on the E.coli chromosome (Bachmann, 1983), is under complex regulatory control and the amount of protein present in the cell is kept low (Lupski and Godson, 1984). The enzymatic activity of primase differs from RNA polymerase in several aspects; primase is able to form primers containing dNTPs as well as rNTPs (Wickner, 1977), in a rifampicin-resistant process, on a variety of DNA templates (Bouche et al., 1975; Schekman et al., 1972). Depending on the nature of the template, and the cofactors present, host primase can participate in at least three different priming systems.

The simplest of the processes involves the initiation of DNA synthesis on the viral strands of G4 and related phage (Bouche et al., 1975; 1978; Rowen and Kornberg, 1978a). The binding of SSB to the single-stranded DNA exposes a unique, intercistronic region, 135 bp long which contains two potential hairpin structures (Bouche et al., 1975; Sims et al., 1979; Sims and Dressler, 1978). This secondary structure is thought to be recognised by two molecules of primase, prior to the synthesis of a 29 nucleotide primer complementary to the phage origin (Wickner, 1978; Benz et al., 1980; Rowen and Kornberg, 1978a; Stayton and Kornberg, 1983). The primer is always initiated with an ATP residue, which is normally followed by GTP but may otherwise contain dNTP or rNTP residues (Bouche et al., 1978;

Rowen and Kornberg, 1978b). Primers of any length over 2-3 nucleotides may be extended by DNA polymerase III holoenzyme (Rowen and Kornberg, 1978b).

Primase can also participate in the non-specific priming of DNA synthesis on a wide variety of single-stranded DNA templates in the absence of SSB. In the presence of dnaB protein, a hexamer of 52,265 dalton subunits (Reha-Krantz and Hurwitz, 1978), primase can synthesise short RNA or RNA-DNA mixed primers at multiple sites on the DNA template (Arai and Kornberg, 1979; Arai et al., 1981a; Arai and Kornberg, 1981c). This generalised, non-specific priming is preceeded by the ATP-dependent binding of dnaB protein to the template. Due to the instability of this binding, the multiple primers are formed by the continual reinitiation of the complex (distributive) rather than by the processive movement of the complex along the DNA template (Arai and Kornberg, 1979; 1981a; 1981b). The nature of the dnaB:dnaG protein interaction is unclear, but the dnaB protein is believed to order the DNA into a conformation which can be recognised by primase in a manner analogous to the recognition of the hairpin structure formed by SSB-coated G4 phage template (Arai et al., 1981a; Arai and Kornberg, 1981b).

Since single-stranded binding protein (SSB) inhibits the generalised priming reaction of primase, its significance in vivo is likely to be minimal (Arai and Kornberg, 1981c). However, a specific priming process, also involving interaction between dnaB and dnaG proteins, has been described using SSB-coated ΦX174 viral DNA template in vitro. This serves as the current model for the priming of discontinuous DNA synthesis in E.coli (Kornberg, 1980; 1982).

The priming of ΦX174 viral strands requires the formation of a prepriming complex, called the preprimosome, to which primase binds to form the primosome. The preprimosome consists of at least six polypeptides (dnaB protein, dnaC protein and proteins n, n', n" and i) and is assembled at a specific site on the ΦX174 genome (Weiner et al, 1976; Wickner and Hurwitz, 1974; Schekman et al., 1975; Arai and Kornberg, 1981c; Arai et al., 1981b; Low et al., 1982; Shlomai and Kornberg, 1980b; 1980c). The assembly of these components is reviewed by Kornberg (1980; 1982), but of relevance to this thesis is the site at which assembly occurs. In the presence of SSB, a 55 nucleotide region of ΦX174 is specifically recognised and bound by a single molecule of the 76kD polypeptide n'

(Shlomai and Kornberg, 1980a; 1980c). Sequencing data and protection studies suggest that the n' recognition site has a complex tertiary structure which may involve the interaction of n' protein with two hairpin loops (Greenbaum and Marians, 1984; Soeller et al., 1984).

The binding of n' stimulates the assembly of the primosome which is then believed to migrate processively along the DNA template in a 5' to 3' direction (Arai and Kornberg, 1981d; 1981e). At specific, but as yet undetermined sequences, the dnaB protein is believed to induce changes in the structure of the DNA such that dnaG protein may recognise the template and form a short primer (Arai et al., 1981a). These primers are heterologous in size and base composition, but share a common adenine residue at the first position, usually followed by a purine. This suggests that primer synthesis tends to occur at 3'-T-Pyrimidine-5' sequences within the domain generated by dnaB protein (Ogawa et al., 1983). The processive nature of the primosome allows it to move with the replication fork, initiating lagging strand synthesis at appropriate intervals (Kornberg, 1982; Arai and Kornberg, 1981e). In keeping with this model, n' recognition sites have been found on the E.coli chromosome and near the replicative origin of several plasmids (van der Ende et al., 1983a).

The vegetative replication of F and $IncI_1$ plasmids has been shown to be dependent on dnaB protein (Marinus and Adelberg, 1970; Vapnek and Rupp, 1971; Fenwick and Curtiss, 1973a; Wilkins and Hollom, 1974). This, along with the discovery of a primosome assembly site near the ori2 replication origin of F (Imber et al., 1983; van der Ende et al., 1983a), has suggested that the discontinuous synthesis of the lagging strand may be primed by the primosome during vegetative replication. Similarly, it was found that ColEI, and its derivative pBR322, possessed two n' recognition sites, one on each strand, both of which were located near the origin of vegetative replication, oriV (Zipursky and Marians, 1980). When present in single-stranded DNA, the conversion to duplex plasmid DNA occurred by a rifampicin-resistant process dependent on dnaB, dnaC and dnaG proteins (Boldicke et al., 1981; Zipursky and Marians, 1981; Staudenbauer et al., 1979; Nomura et al., 1982). If small regions of the n' sites were deleted, the single stranded DNA was no longer able to act as template for DNA synthesis in vitro (Soeller and Marians, 1982). It therefore seemed likely that these two sites, rriA and rriB on the L and H strands of ColEI respectively, represented assembly sites for the primosome.

Surprisingly, the sequences of the n' recognition sites at rriA, rriB and of ΦX174 showed no significant homology (Marians et al., 1982). However, pancreatic DNase protection and methylation studies showed that all three sites had similar tertiary structure (Soeller et al, 1984; Abarzua et al., 1984; Greenbaum and Marians, 1985). Also the hexanucleotide sequence 5'-AAGCGG-3' common to all three sites appeared to be essential for n' recognition (Soeller and Marians, 1982). Thus the three-dimensional structure of the DNA appears to be of major importance in n' recognition.

Initially, the role of rriA and rriB was thought to be in ColEI replication. In this process the L strand is the template for discontinous, lagging strand synthesis and therefore, since the rriA site is downstream of oriV on the L strand, the primosome binding site would be single-stranded early during replication. Hence, the primosome could be assembled at rriA soon after initiation of replication, and proceed in a 5' to 3' direction along the L strand initiating the synthesis of the lagging (H) strand (Zipursky and Marians, 1980; 1981; Boldicke et al., 1981; Nomura et al., 1982).

Accordingly, the role of rriB on the H strand was assigned to the priming of ColEI leading strand synthesis, which was believed to be discontinuous (Zipursky and Marians, 1980; Sakakibara, 1978). However, the demonstration that ColEI leading strand synthesis was dnaG-independent (Staudenbauer et al., 1979), suggested that rriB was not involved in ColEI replication. Furthermore, the deletion of both rriA and rriB from pBR322 did not adversely affect vegetative replication of the plasmid (van der Ende et al., 1983b). The previous finding that deletion derivatives of ColEI lacking the region containing the rriB site were unable to be transmitted conjugatively (Warren and Sherratt, 1977; Warren et al., 1978) implied that the primosome assembly sites may have a role in conjugative DNA synthesis. It was suggested that the assembly of the primosome at rriB might allow the initiation of complementary (L) strand synthesis in recipient cells following conjugative transfer of the H strand (Zipursky and Marians, 1981; Boldicke et al., 1981; Nomura et al., 1982). Similarly, a primosome assembled at the rriA site during ColEI transfer might initiate replacement strand synthesis in the donor cell, given that the primosome could migrate along the L strand to form primers between rriA and nic (Willetts and Wilkins, 1984). If this model is correct, the rriB site would enter the recipient cell late during transfer, so complementary strand synthesis would probably occur subsequent to

recircularisation of the transferred strand. This is in contrast to the events assumed to occur during the transfer of large conjugative plasmids, where complementary strand synthesis is thought to occur concurrently with DNA transfer, and prior to recircularisation.

1.5. Conjugative DNA synthesis

The study of conjugative DNA synthesis has revolved around F, R538 (IncFII) and R64 ($IncI_1$), all of which transfer a single specific strand of plasmid DNA unidirectionally into the recipient cell. However, only in the case of F is this strand known to be transferred 5' terminus leading (Cohen *et al.*, 1968; Ohki and Tomizawa, 1968; Rupp and Ihler, 1968; Ihler and Rupp, 1969). Similarly, a specific single strand of ColEI DNA is transferred in this orientation (A.C.Boyd and D.J.Sherratt, personal communication), and like that of the conjugative plasmids, this strand is the heavier of the two in CsCl-poly(U,G) gradients (Vapnek and Rupp, 1970; 1971; Vapnek *et al.*, 1971; Warren *et al.*, 1978). ColEI is a mobilisable plasmid which, although it does not encode its own transfer system, can utilise that of conjugative plasmids in order to transmit its DNA. The reliance on the conjugative system by mobilisable plasmids will be discussed further in a later section.

It was shown that the strand of F transferred to the recipient was replaced by DNA synthesis in the donor cell, using the retained strand as template (Vapnek and Rupp, 1970; 1971), and that DNA complementary to the transferred strand was synthesised in the recipient (Ohki and Tomizawa, 1968; Vapnek and Rupp, 1970). $IncI_1$ plasmids and ColEI also transfer single-stranded DNA, so it may be reasonable to assume that similar events occur during the transmission of these plasmids. However, caution should be exercised in the extrapolation of the knowledge gained for F. For instance, although it has been established that RP4 is transmitted unidirectionally (Grinter, 1981; Al-Doori *et al.*, 1982), the polarity and strandedness of the transferred DNA remains to be determined and thus may be different to that observed during F transfer.

Origins of transfer

DNA transfer starts at a specified site where a single stranded nick is presumably introduced into the strand destined for transfer. Such origins of transfer (oriT) for several plasmids have now been cloned, including

ColIb-P9 (B.M.Wilkins, unpublished result), F (Thompson and Achtman, 1978; Johnson et al., 1981; Everett and Willetts, 1982). Deletion and restriction mapping established that the F oriT was the cis-acting nick site used to initiate conjugative DNA transfer (Willetts, 1972; Reeves and Willetts, 1974; Guyer and Clark, 1976; Thompson and Achtman, 1978; 1979). The sequence of the F and RK2 oriT regions has been determined (Thompson et al., 1984; Guiney and Yakobson, 1983).

Plasmid F can be isolated as a complex consisting of protein and super-coiled DNA. On treatment of this so-called relaxation complex with protein denaturing agents, it was shown that a specific strand of the plasmid was nicked (Kline and Helinski, 1971). By analogy with the relaxation complex of ColEI (see later section) the F relaxation nick site may correspond to oriT, but attempts to demonstrate this proved unsuccessful.

The use of λ::oriT phage and Flac tra point mutants showed that the products of the traJ, traY and traZ genes were required for nicking in vivo (Everett and Willetts, 1980). The traY and traZ products were proposed to act together to form an oriT-specific endonuclease which, since the traY and traZ proteins are located in the inner membrane and cytoplasm, respectively (see Willetts and Skurray, 1980), may act on the surface of the inner membrane. The traJ protein, as described earlier, positively regulates transcription of the F transfer genes (Willetts, 1977), and so may have an indirect role in nicking.

Thompson et al. (1984) showed that the F DNA could be nicked at several sites within the oriT region, although the three major sites were contained within a region only 5 base pairs in length. It is unclear whether the multiple nick sites occurred naturally in F, or were an artifact of the λ::oriT system used. The mechanism of nicking in F is uncertain, but one model suggests that the oriT is continuously nicked and ligated, and that the products of the traI and traM genes intervene to initiate strand separation once stable mating pairs are formed (Everett and Willetts, 1980; 1982). The location of the traM product in the inner membrane, and its ability to bind the oriT region further supports the model that traM is involved in the triggering process (Musgrave and Achtman, unpublished results cited in Thompson and Taylor, 1982; Thompson et al., 1984). Thus, it is apparent that the oriT region will be acted on by several proteins, and the presence of potential hairpin structures and of at least two essential nucleotides close to the major nick sites of

oriT, may indicate the presence of recognition sites in this region (Thompson et al., 1984). Such interactions between tra proteins and the oriT region have been demonstrated to be specific, even amongst closely related plasmids (Willetts and Maule, 1985).

The location of the F oriT sequence at the end of the transfer operon (Willetts, 1972) and the determination of the direction of gene transfer (Ohki and Tomizawa, 1968; Ihler and Rupp, 1969) has shown that the transfer operon of F is last to enter the recipient cell (Willetts, 1972). Likewise, the oriT region of RP4 is also similarly situated with respect to the conjugation genes (Grinter, 1981; Al-Doori et al., 1982). Although the exact site of the nick, or the endonuclease responsible for nicking RP4 oriT are unknown, DNA-protein relaxation complexes have been isolated and shown to produce a strand-specific nick at oriT when treated with protein denaturing agents (Guiney and Helinski, 1979; Guiney and Yakobson, 1983). The trigger for the nicking activity in vivo is unknown but the existence of a 19 bp inverted repeat, essential for oriT cleavage, suggests that some factor, possibly an endonuclease, binds to the RP4 oriT region (Guiney and Yakobson, 1983; Guiney et al., 1985).

Similarly, $IncI_1$ plasmids can be isolated as relaxable complexes between supercoiled DNA and protein (Clewell and Helinski, 1970; Kupersztoch-Portnoy et al., 1974). However, the site and the strand-specificity of the nicking reaction remains to be determined. The existence of a plasmid-encoded non-specific endonuclease activity has been demonstrated (B.M.Wilkins, unpublished data), but the role of this activity remains unclear. The ColIb-P9 oriT is located at one end of the transfer-related genes (C.Rees, unpublished results; Figure 1.1), but the direction of DNA transfer relative to the tra genes is unknown.

The best studied relaxation complex is that of the mobilisable plasmid ColEI, which may serve as a model for other plasmid systems. ColEI was found to have a region, designated bom, which was required in cis for mobilisation (Warren et al., 1978) and which contained the origin of transfer (Finnegan and Sherratt, 1982). There is also a region, named mob and comprising about one third of the genome, which encodes trans-acting factors necessary for ColEI transfer, in addition to the conjugative plasmid-encoded proteins (Dougan and Sherratt, 1977; Warren and Sherratt, 1977; Warren et al., 1978; Collins et al., 1978). A protein-DNA relaxation complex can be isolated for ColEI and has been shown to

comprise three polypeptides, of 60, 16 and 11kD (Blair and Helinski, 1975; Lovett and Helinski, 1975). All three polypeptides are encoded by the ColEI mob region (Dougan and Sherratt, 1977; Inselburg and Applebaum, 1978; A.C.Boyd and D.J.Sherratt, personal communication).

On treatment with protein-denaturing agents, the supercoiled ColEI DNA is relaxed (Clewell and Helinski, 1970) as a result of a specific single-stranded nick introduced at oriT by the trans-acting proteins (Bastia, 1978; Finnegan and Sherratt, 1982; Warren et al., 1978). On relaxation, the 16kD and 11kD polypeptides dissociate from the complex and the 60kD protein becomes covalently attached to the 5' terminus of the induced nick (Blair and Helinski, 1975; Guiney and Helinski, 1975; Lovett and Helinski, 1975). The mechanism by which the DNA is nicked is unclear, but one possibility is that the 60kD polypeptide possesses endonucleolytic activity which is repressed in the presence of other two polypeptides (Lovett and Helinski, 1975). The covalent attachment of the polypeptide to the 5' terminus may retain the energy of the phosphodiester bond and use it to ensure the efficient recircularisation of the plasmid in the recipient. This model receives support from the demonstration that ColEI DNA is transferred 5' terminus leading (A.C.Boyd and D.J.Sherratt, personal communication). A similar model for recircularisation has been proposed for the transfer of F (Everett and Willetts, 1982).

Nicking and ligation have been suggested to occur continuously at oriT (Everett and Willetts, 1980) and so nicking may not to require a trigger during conjugation. However, DNA transfer and conjugative DNA synthesis, which will obviously require the separation of the strands at oriT, are known to be triggered by the formation of stable mating pairs, in a process which may involve the F traM gene product (Ou, 1975; Kingsman and Willetts, 1978). Mutations in the F traI gene were also found to permit stable mating pair formation, but to inhibit conjugative DNA metabolism and DNA transfer (Kingsman and Willetts, 1978). Following the demonstration that the traI gene product was DNA helicase I, a DNA unwinding protein isolated from F-containing cells (Abdel-Monem et al., 1983), it is possible that DNA helicase I acts in concert with traM gene product to activate oriT and to separate the strands prior to DNA transfer. Interestingly, the traI or traM products are not required for ColEI mobilisation (Alfaro and Willetts, 1972, Willetts and Skurray, 1980) suggesting that even though conjugative and mobilisable plasmids may share the same route between mating cells, their conjugative DNA metabolism may

vary. This point will be emphasised further by results described in Chapter 7.

Donor cell conjugative DNA metabolism

Once initiated, DNA transfer usually occurs concurrently with the synthesis of a replacement DNA strand in the donor cell (DCDS) and of a complementary strand in the recipient cell (RCDS). However, the transfer of DNA is independent of both synthetic processes, demonstrating that DNA synthesis does not provide the motive force which drives DNA transfer (Sarathy and Siddiqi, 1973; Siddiqi and Fox, 1973). The inhibition of DNA transfer, but not of DCDS in F traG or traN mutants emphasises the independence of these two processes (Kingsman and Willetts, 1978).

In F-containing donor cells, conjugative DNA synthesis is mediated by DNA polymerase III (Kingsman and Willetts, 1978), the primary DNA polymerase of E.coli (Kornberg, 1980, 1982). All DNA polymerases so far discovered require a primer in order to provide a terminal 3'-hydroxyl group from which chain elongation may commence (Tomizawa and Selzer, 1979; Ogawa and Okazaki, 1980). The process by which such primers are provided for DCDS is unclear. Classically, the rolling circle model of DNA transfer (Gilbert and Dressler, 1968) predicted that the 3'-OH terminus of the nick would serve to prime replacement strand synthesis. However, the discovery that rifampicin blocked DCDS during both Flac and R64drd-11 transfer suggested that RNA polymerase, or an untranslated RNA species synthesised in response to the formation of mating pairs, was responsible for priming (Kingsman and Willetts, 1978; Fenwick and Curtiss, 1973b). In accordance with this suggestion, the non-transferred strand of F contains a potential promoter which may allow the synthesis of an RNA primer for DCDS. However, this promoter has not yet been demonstrated to be active in vivo (Thompson et al., 1984; Willetts and Wilkins, 1984).

In F, there is an n' recognition sequence about 100 bp from the major nick sites, where the primosome could be assembled on the strand retained by the donor to provide the primers required for DCDS (Thompson et al., 1984;Willetts and Wilkins, 1984). However, since primosome assembly could only occur towards the end of F DNA transfer when the site becomes single stranded, it is unlikely that DCDS is primed by this mechanism as DCDS and DNA transfer are thought to occur concurrently. The dnaB-independence of DCDS in the transfer systems of F and the $IncI_1$ plasmids also suggests

that the primosome is not active in the priming of this DCDS (Marinus and Adelberg, 1970; Vapnek and Rupp, 1971; Fenwick and Curtiss, 1973a; Wilkins and Hollom, 1974).

The requirements for DCDS in the RP4 transfer system remain obscure and the extrapolation of models for F and $IncI_1$ plasmid conjugation is not possible in the light of uncertainity surrounding the nature of the transferred RP4 DNA. Like many other conjugative plasmids, RP4 can enhance or suppress the thermosensitivity of dnaB mutants so implying that these plasmids specify a dnaB analogue (ban; Wang and Iyer, 1977; 1978). However, the existence of such products has yet to be proven.

Recipient cell conjugative DNA metabolism (RCDS)

In the recipient cell, the important processes are the synthesis of the complementary DNA strand and the recircularisation of the plasmid DNA. Neither process, in F or ColIb-P9 mediated conjugation, requires the expression of plasmid genes in the recipient cell (Hiraga and Saitoh, 1975; Boulnois and Wilkins, 1978), although the possibility exists that plasmid-encoded factors may be transferred to the recipient from the donor cell. This concept will be pursued throughout this thesis.

It is a widely held assumption that RCDS occurs prior to recircularisation. If this is the case for F, then since F DNA is transferred 5' terminus leading, it follows that the complementary strand will be synthesised discontinuously. Thus, RCDS could occur concurrently with the entry of the DNA into the recipient cell, so reducing the amount of potentially vulnerable single-stranded plasmid DNA. It is presumed that the transferred strand will be protected by a coating of single-stranded binding protein (SSB). The E.coli SSB is absolutely required for DNA synthesis in vivo, as shown by temperature-sensitive mutants (Glassberg et al., 1979; Meyer et al., 1979). The observation that such mutants could be partially suppressed by the presence of F (Kolodkin et al., 1983) led to the cloning of the F ssb gene (Chase et al., 1983). A survey of other conjugative plasmids showed that several, including RP4 and R64, displayed regions of homology with the F ssb gene (Golub and Low, 1985), and similar work has shown ColIb-P9 also to have an homologous region (C.Howland, unpublished results).

The role of these plasmid-encoded SSB proteins is unknown. Derivatives of

F which do not carry the ssb gene can replicate normally (Kolodkin et al., 1983), but the maintenance of such plasmids has not been tested in strains harbouring a mutant host ssb gene. The ability of both R64 and R64drd-11 to complement the E.coli ssb-1 mutation (Golub and Low, 1985), implies that the gene is not coordinately regulated with the transfer genes, however this does not preclude its involvement during conjugation.

For at least F and ColIb-P9drd-1, DNA polymerase III has been shown to be responsible for the synthesis of the DNA complementary to the transferred strand in the recipient cell (Wilkins and Hollom, 1974). The priming of this process in F can apparently be catalysed by RNA polymerase. However, the rifampicin-resistance of RCDS in dna^+ recipients may indicate that host primase can also fulfil this role (Wilkins and Hollom, 1974). By way of contrast, RCDS in $IncI_1$ plasmid-mediated conjugation was rifampicin-resistant and could occur in either dnaB or dnaG recipient strains, implying that neither recipient host priming system was involved in the priming of RCDS (Wilkins and Hollom, 1974; Boulnois and Wilkins, 1978; 1979). Subsequently, it was shown that, although the donor cell did not provide either of the host priming systems for RCDS (Boulnois and Wilkins, 1979), a plasmid-encoded priming activity was supplied to the recipient from the donor cell during conjugation. The nature and role of this activity will be discussed in a the next section.

1.6. Plasmid-encoded DNA primases

The existence of plasmid-encoded primases was indicated by the observation that $IncI_1$ plasmids ColIb-P9, R64 and R144 could partially suppress the temperature-sensitive host primase mutation dnaG3 in E.coli (Wilkins, 1975). The ability to suppress the dnaG3 allele was increased if the plasmids carried a drd mutation, which derepresses the expression of the transfer genes, and was further increased by a second, plasmid-borne mutation which caused further enhancement of transfer gene expression (Wilkins, 1975; Sasakawa and Yashikawa, 1978). This implied that the primase gene product was likely to have a role in conjugation. This concept will be pursued throughout this thesis.

A wide variety of plasmids from many, but not all, incompatibility groups, have now been shown to specify a DNA primase activity capable of stimulating DNA synthesis on single stranded fd or M13 viral DNA template in vitro (Lanka and Barth, 1981; see Willetts and Wilkins, 1984). It

should be noted that template specificity of some plasmid primases may preclude the use of such phage templates. Hence, this assay system may result in the mis-classification of some plasmids as primase-negative. Notable amongst the plasmids thought not to encode a primase is F. Caution must therefore be exercised in extrapolating the models concerning the conjugative DNA metabolism of F to plasmids which encode a primase.

The plasmid primase genes so far studied fall into three distinct classes, typified by those of ColIb-P9 ($IncI_1$), RP4 (IncP) and R16 (IncB). These three primase genes do not share significant DNA homology nor do the products exhibit antigenic cross-reaction (Dalrymple, 1982; Dalrymple et al, 1982; Lanka and Barth, 1981). However, the grouping of plasmids based on their primase gene shows no correlation to that based on incompatibility or pilus type (Coetzee et al., 1982; Datta, 1979; Bradley, 1980a). Indeed, even primase genes carried by plasmids within the I-complex of incompatibility groups (I_1, I_1+B, B, K; Hedges and Datta, 1973), which exhibit significant DNA sequence homology (Grindley et al., 1973; Falkow et al., 1974), can be divided on the basis of homology with either the ColIb-P9 or R16 gene (Dalrymple et al., 1982; Dalrymple and Williams, 1982).

$IncI_1$ plasmid primase gene

The primase gene locus of ColIb-P9 has been termed sog (suppression of dnaG; Boulnois and Wilkins, 1979). The primase gene carried by ColIb-P9, R64 and R144 share significant sequence homology, and the immunological cross-reactivity and similar apparent molecular weights of the products specified suggests that the three genes are related (Dalrymple, 1982; Dalrymple et al., 1982; Wilkins et al., 1981). The primases of these three plasmids will therefore be considered together.

The sog gene is located on an 8.0kb EcoRI fragment (Eco-3 according to Uemura and Mizobuchi, 1982) which has been cloned into pBR325, the resultant recombinant being termed pLG215 (Bolivar, 1978; Wilkins et al., 1981). Two sog polypeptide products, of apparent molecular weight 240kD and 180kD, are immunologically cross-reactive, and consequently may share a common amino-acid sequence (Wilkins et al., 1981; Boulnois et al., 1982). This is consistent with the idea that the polypeptides are translated from separate in-frame initiation sites on a single messenger RNA transcript, which is itself initiated from a promoter outside the

Eco-3 fragment (Boulnois et al., 1982). Deletion analysis of the sog gene, using pLG215-derivatives pLG226, pLG228 and pLG229, showed that transcription was from left to right in Figure 1.3, and that the 180kD polypeptide corresponded to the C-terminal region of the larger protein (Boulnois et al., 1982). The confinement of the primase moiety to the N-terminal region of the 240kD polypeptide, suggests that the protein may be multifunctional. The role of the rest of the larger polypeptide, or the related 180kD protein is unknown, but work described in Chapter 7 implies an involvement in conjugation.

Previously, the primase activity of R64 primase had been attributed to a 140kD polypeptide (Lanka et al., 1979). However, the absence of this polypeptide in an immunoassay of whole cell lysates suggested that this was a degradation product of the 240kD polypeptide formed during the purification of the sog proteins (E.Lanka, personal communication, cited in Willetts and Wilkins, 1984).

The sog gene products were purified by virtue of their ability to bind single-stranded DNA-agarose (Lanka et al., 1979). In vitro, these polypeptides were able to prime DNA synthesis in a rifampicin-resistant process on a wide variety of phage templates (Lanka et al., 1979). In assays using fd, G4 and ΦX174 DNA templates, plasmid primase was able to substitute for RNA polymerase, host primase and at least the dnaB, dnaC and dnaG protein components of the primosome, respectively (Lanka et al., 1979). Similarly, in vivo, ColIb-P9 primase can substitute for dnaG primase, allowing extensive colony formation, as well as the replication of $IncI_1$ and ColEI plasmids and phage λ, in a temperature-sensitive dnaG host strain (Wilkins, 1975; Wilkins et al., 1981). The dnaB and dnaG-independence and rifampicin-resistance of the priming activity, both in vivo and in vitro, suggests that plasmid primase acts in a novel manner, not involving the normal host priming machinery (Wilkins and Hollom, 1974; Lanka et al., 1979; Chatfield et al., 1982).

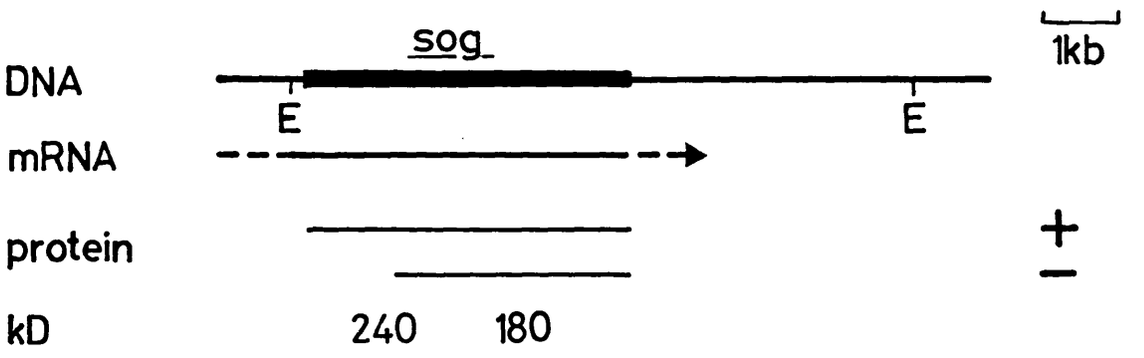
$IncI_1$ plasmid primase is antigenically unrelated to dnaG primase and has been shown to form primers of differing composition (Wilkins et al., 1981). The sog primase forms functional oligoribonucleotide primers in vitro, of between 2 and about 10 nucleotides long, which have either cytidine or CMP at the 5' terminus, usually followed by an AMP residue (E.Lanka, personal communication, cited in Willetts and Wilkins, 1984). These primers can be extended by DNA polymerase III holoenzyme in vitro in

Figure 1.3 Expression of ColIb-P9 and RP4 primase genes

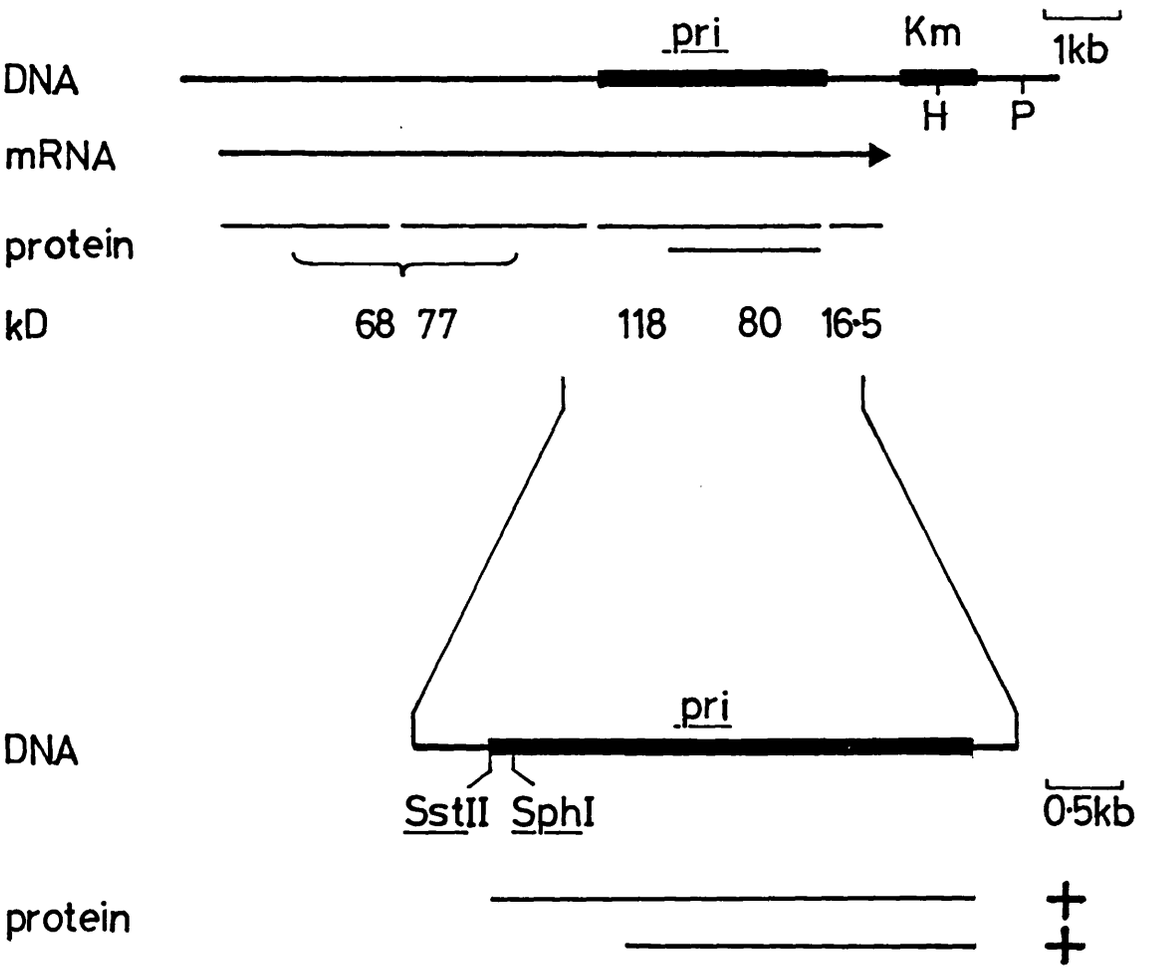
The approximate extent of the ColIb-P9 primase gene (sog) is represented by the solid box. The extent of the RP4 primase (pri) and kanamycin resistance gene (Km) are similarly indicated. The position of the transcriptional units containing the primase genes, and the direction of transcription, are shown by the arrows. The sizes of the proteins encoded by these regions are indicated in kilodaltons (kD). The ability or inability of the primase gene products to specify primase activity are represented by + and -, respectively.

Restriction cleavage sites are shown as follows: EcoRI (E), HindIII (H) and PstI (P).

ColIb-P9


sog
1kb
DNA
E
E
mRNA
protein
+
−
kD
240
180

RP4


pri
Km
1kb
DNA
H
P
mRNA
protein
kD
68
77
118
80
16·5
pri
DNA
SstII
SphI
0·5kb
protein
+
+

the presence of single-stranded binding protein (Lanka and Furste, 1985). In contrast, the primers formed by dnaG primase may contain dNTP and rNTP residues and are initiated by a 5' purine nucleotide (Rowen and Kornberg, 1978b; Arai and Kornberg, 1981c). The unique ability of plasmid primases to recognise the terminal sequence 3'-dG-dT-5' of linear single-stranded DNA, and to synthesise a primer complementary to this sequence, may be important in their proposed role in conjugative DNA metabolism (see later section).

IncP plasmid primase genes

The RP4 plasmid primase gene (pri), is similar to that of ColIb-P9 in several aspects. The two polypeptides encoded by pri, of 118kD and 80kD, show antigenic cross-reactivity and are therefore sequence-related (Lanka and Barth, 1981). Like sog, deletion analysis of pri has shown the polypeptides to be the products of two in-phase translational starts on a common transcript, the starts being on either side of the SphI site shown in Figure 1.3 (Lanka et al., 1984). Removal of the 139bp SstII-SphI fragment prevented the synthesis of the 118kD polypeptide, but not the 80kD protein, suggesting that the smaller protein is homologous to the C-terminal region of the larger (Lanka et al., 1984). In contrast to sog, both polypeptides exhibited primase activity, implying that the moiety responsible was within the C-terminal region of the polypeptides (Lanka and Barth, 1981). In keeping with this, Tn7 insertions into this region, such as that in pRP1 (Barth and Grinter, 1977), inactivated primase activity (Lanka and Barth, 1981).

Initial work using RP4::Tn7 derivatives positioned the primase gene at the end of the Tra1 operon (Lanka and Barth, 1981). Subsequent cloning of the gene has shown it to be 3.2kb in length, shorter than the sog gene, and to occupy the 40.3-43.5 region (Lanka et al., 1984) on the 60kb RP4 map (Lanka et al., 1983). The pri gene is penultimate in a transcriptional unit of at least four genes, the expression of which is probably dependent on a promoter at coordinate 48.6kb where a RNA polymerase binding site has been found (Lanka et al., 1984). The start codon and Shine-Dalgarno sequence (Shine and Dalgarno, 1975) for the pri gene have been determined (Lanka et al., 1984), and the almost perfect 5'-GGGAGGT-3' sequence 8 base pairs from the ATG start codon may explain the abundance of the pri polypeptides, which constitutes about 0.5% of the soluble cellular protein (Lanka and Barth, 1981). Promoter-proximal to the primase gene are two

genes encoding polypeptides of 68kD and 77kD of unknown function, and promoter-distal is a gene specifying a 16.5kD polypeptide (Lanka et al., 1984; Figure 1.3).

The expression of the primase gene from a promoter some 5kb distant, results in the inhibition of primase activity by Tn7 insertions mapping apparently outside the primase gene, presumably due to the polar effect of the insertions (Lanka and Barth, 1981). However, some insertions within TraI, and outside the primase transcriptional unit, also decrease primase activity suggesting that the expression of pri may be influenced by factors involved in the conjugative transfer of the plasmid.

RP4 primase has been shown to initiate a similarly wide diversity of phage DNA templates and to form primers of similar composition to the ColIb-P9 primase in vitro (Lanka and Barth, 1981; Lanka and Furste, 1985). However, the ability of RP4 primase to initiate DNA synthesis on these templates was 10-100 fold less efficient than the sog primase (Lanka and Furste, 1985). This observation is reflected in vivo by the poor suppression of the host primase mutation, dnaG3, by RP4-specified primase (Lanka and Barth, 1981; Lanka et al., 1984). However, apparently as a result of a mutation within the primase gene, the pri polypeptides are capable of dnaG suppression with a high efficiency, as seen in the recombinant pJF107 (Lanka et al., 1984; Lanka and Furste, 1985). A mutant of the related plasmid R68.45 also caused enhanced dnaG3 suppression (Ludwig and Johansen, 1980). Similarly, a spontaneous deletion of 1.5kb from the cloned sog gene, resulted in the enhancement of dnaG suppression by the truncated polypeptides specified by the resultant plasmid, pLG214 (Wilkins et al., 1981).

IncB plasmid primase genes

The IncB plasmid primase determinant has been cloned and encodes two polypeptides of 240kD and 175kD, which like those determined by sog and pri, are thought to be derived from overlapping genes (Dalrymple and Williams, 1984). Deletion analysis, and transposon mutagenesis suggests that, in contrast to sog and pri, the N-terminal region of the polypeptides, containing the moiety responsible for primase activity, is shared (Dalrymple and Williams, 1984). The R16 primase can prime DNA synthesis in a rifampicin-resistant process on M13 viral template in vitro, and can substitute for host primase in initiation of chromosomal

synthesis *in vivo* (Dalrymple *et al.*, 1982).

Role of plasmid primases

Clearly from the nature of the reaction catalysed by plasmid primases, their role can be assumed to be involved in some aspect of plasmid DNA metabolism, either during replication or conjugation. The investigation of these aspects has involved the use of primase$^-$defective RP4 and ColIb$^-$P9 derivatives, some of which will be used in this thesis and so will be briefly described here.

Barth and Grinter (1977) isolated and mapped a large number of transposon Tn*7* insertion mutants of RP4, including those used in this study, pRP1, pRP2 and pRP26. The insertion carried by pRP1 mapped to the primase gene and an *in vitro* assay of primase activity confirmed that this derivative was primase$^-$defective (Lanka and Barth, 1981). The Tn*7* insertion in pRP26 maps to the end of the major transfer operon, Tra1, in keeping with the observed transfer-deficiency of this plasmid (Barth *et al.*, 1978). However, the reason for the unusually high primase activity of extracts from pRP26-harbouring strains remains unclear (Lanka and Barth, 1981). The plasmid pRP2 carries a Tn*7* insertion which does not effect the transfer or maintenance functions (Barth *et al.*, 1978; Lanka and Barth, 1981), and by this virtue is used as a control plasmid for the experiments described in this thesis.

In plasmid maintenance studies, the primase$^-$defective RP4::Tn*7* derivatives suffered a 0.1-0.4% loss from both *E.coli* and *S.typhimurium* hosts, relative to Pri$^+$ plasmids (Lanka and Barth, 1981). This however, was considered to represent a minor role for RP4 primase in the resolution of replicative intermediates, rather than suggesting that plasmid primase was responsible for the priming of vegetative replication (Grinter and Barth, 1985). In contrast to this, it was found that RP4 primase was required for efficient transconjugant formation in some, but not all, intergeneric matings (Lanka and Barth, 1981). Using *E.coli* W3110T$^-$ donor cells, the yield of transconjugants using Pri$^-$ RP4 derivatives was normal in matings with six of the eleven recipient species tested, including *E.coli* and *Pseudomonas* species. However, transconjugant formation was reduced by as much as 98% when mated with five other species, which included *S.typhimurium*, *Proteus* and *Klebsiella* species (Lanka and Barth, 1981). Similarly, the same recipient species showed a decreased yield of

transconjugants when S.typhimurium donors of RP4::Tn7 Pri^{-} derivatives were used.

In a separate study, Pseudomonas aeruginosa donors of R18 (IncP) pri::Tn7 mutants also showed normal transconjugant yields using E.coli, Shigella flexneri , Klebsiella pneumoniae and most Pseudomonas species as recipients, and a depressed yield when S.typhimurium recipients were used (Krishnapillai et al., 1984). However, in contrast to other Pseudomonas species, the use of Pseudomonas stutzeri recipient cells caused a severe depression in the transconjugant yield. The reason why this Pseudomonas species should be so different is unclear.

From these studies it was suggested that, since variation in Pri^{-} plasmid transfer deficiency was dependent on the recipient species rather than the donor, that the plasmid primase may be involved in recipient cell conjugative DNA metabolism. It was suggested that, if RP4 transfers a single strand of plasmid DNA, RP4 primase may act to ensure the efficient priming of complementary strand synthesis in bacterial species encoding a host priming system which is unable to efficiently recognise the transferred RP4 template (Lanka and Barth, 1981).

The role of sog primase was investigated using a primase^{-}defective derivative of ColIb^{-}P9 called pLG250. This plasmid was constructed by replacing the Sog^{+} gene carried by pLG221, a derivative of ColIb-P9drd^{-}1 harbouring a copy of Tn5 in the colicin^{-}Ib gene (cib; Boulnois, 1981), with the mutant sog^{-}217 allele (Chatfield et al., 1982). The sog^{-}217 mutation inactivates primase activity without affecting the size of the sog polypeptides (Chatfield et al., 1982).

Plasmid maintenance experiments indicated that sog primase was not essential for vegetative plasmid replication. However, during matings with either E.coli or S.typhimurium recipient strains, the Sog^{-} plasmid formed 71 and 87% fewer transconjugants respectively, compared to the equivalent mating using pLG221. Measurements of conjugative DNA synthesis (CDS) in recipient and donor cells during such matings suggested that the role of plasmid primase was to initiate both replacement strand synthesis in the donor cell and complementary strand synthesis in the recipient (Chatfield et al., 1982). This implied that although plasmid primase was not essential for the transfer of ColIb^{-}P9, it played a major role in the conjugative process.

In complementation tests using the Sog^{+} conjugative plasmid pLG250 and the non-mobilisable plasmid pLG214, it was shown that the priming of recipient CDS was mediated by plasmid primase supplied by the donor cell (Chatfield et al., 1982). Furthermore, functional tests demonstrated that plasmid primase, encoded by a non-mobilisable, active sog gene in the donor cell, was transferred to the recipient cells (Chatfield and Wilkins, 1984; Wilkins et al., 1985). This is believed to be the first report that plasmid-encoded polypeptides are specifically transmitted between mating cells during conjugation and the physical studies demonstrating the transmission of these polypeptides is presented in Chapter 6.

1.7. Aims of this work

While it is clear from this introduction that plasmid primases are amongst the best characterised products of ColIb-P9 and RP4, a number of aspects of their physiological function require further investigation. The work reported in this thesis addresses some of the deficiencies in the understanding of the role of these enzymes during conjugation. This firstly concerns the hypothesis of Lanka and Barth (1981) that RP4 primase functions in the recipient cell to promote the establishment of the newly-transferred plasmid. One of the important questions evoked by this hypothesis concerns the source of the RP4 primase for this purpose. Is the enzyme synthesised in the recipient cell following the transmission of DNA, or can it be provided by the donor cell? This was investigated by cloning the RP4 primase gene and determining whether the conjugation deficiency of a Pri^{-} RP4 derivative could be rescued by the presence of the Pri^{+} recombinant plasmid in the donor or recipient cell.

The results, described in Chapter 4, indicate that the RP4 primase functions analogously to ColIb-P9 primase (Chatfield et al., 1982). In view of the functional similarity of the two primases, both in vivo and in vitro, complementation experiments were devised to test the specificity of the plasmid primases during conjugation. These studies are recorded in Chapter 5.

On the basis of a functional test, Chatfield and Wilkins (1984) proposed that ColIb-P9 primase is transferred between conjugating cells. However, these experiments were unable to resolve whether the polypeptide was transmitted in a native or processed form. Chapter 6 describes the development of a method for the physical detection of transmitted

polypeptides and shows that both those encoded by the sog gene were amongst a specific group of donor-specified proteins retained by recipient cells following conjugation.

The finding that the 180kD sog polypeptide is transmitted during conjugation, together with the complete absence of information concerning the role of this protein, prompted a genetic investigation of the function of the polypeptides encoded by the C-terminal region of the sog gene. This work is described in Chapter 7.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Bacterial strains and plasmids

Descriptions of bacterial strains and plasmids used in this thesis are given in Tables 2.1 and 2.2. BW103 was isolated as a Thy^+ recombinant of BW85, constructed in a 30 min mating with KL16-99 donors (*thy*$^+$, *recA1*; Clarke *et al.*, 1973) at 30°C. The recombinant was chosen for its sensitivity to ultraviolet light and the recombinant-defective phenotype confirmed in test matings with HfrH (Merryweather *et al.*, in press). The strains of C600 and SL329 used as recipients in filter matings were spontaneous nalidixic acid-resistant mutants, selected on nutrient agar plates containing 50$\mu g ml^{-1}$ nalidixic acid. In matings where donor cells carried colicin-producing plasmids, a C600 strain resistant to colicin was used. This derivative was isolated by plating a colicin-producing strain in a soft nutrient agar overlay on a nutrient agar plate and incubating overnight. After removing the soft agar layer and treating with chloroform to kill the colicin-producing cells, a lawn of C600 cells was spread onto the plate. Spontaneous colicin-resistant mutants produced colonies after overnight incubation.

2.2. Media and chemicals

Liquid growth medium for bacterial strains was nutrient broth 'E' (London Analytical and Bacteriological Media Limited., 12.5$g l^{-1}$) or Luria broth (LB; tryptone [Difco], 10$g l^{-1}$; yeast extract [Difco], 5$g l^{-1}$; NaCl, 5$g l^{-1}$). A salts-glucose-Casamino acids medium (SGC) contained Na_2HPO_4, 42mM; KH_2PO_4, 22mM; NH_4Cl, 18mM; NaCl, 8.5mM; glucose, 22mM; $CaCl_2$, 0.1mM; $MgSO_4$, 1mM; thiamine HCl, 3μM; Casamino acids (Difco), 2.5$g l^{-1}$. Solid growth medium was nutrient broth 'E' containing 15$g l^{-1}$ agar (Sterilin). Minimal agar was SGC containing 15$g l^{-1}$ agar except the Casamino acids were replaced by amino acids appropriate to the auxotrophic requirements; leucine, 0.3mM; proline, 0.17mM; methionine, 0.13mM; threonine, 0.16mM. BBL trypticase agar (BLA) contained 10g BBL trypticase, 5g NaCl and 15g agar per litre distilled water, and BBL trypticase top layer agar (BTL) was the same but contained only 7$g l^{-1}$ agar. Iso-sensitest agar and broth (Oxoid) was used when sulphonamide was the selective antibiotic. Where required by auxotrophic strains, thymine was added to 0.16mM in all media.

Table 2.1 Bacterial Strains

Strain	Description or genotype	Source or reference
E. coli K-12		
BW40	*dna*$^+$ *leu-6 proA2 thr-1 argE3 his-4 thi-1 uvrB5 ara-14 mtl-1 xyl-5 galK2 lacY1 tsx-33 rpsL31 tdk-1*	Wilkins *et al.* (1971)
BW84	*dnaG3 leu thyA deoB rpsL* ColI^r	Boulnois *et al.* (1979)
BW85	*dna*$^+$ *leu thyA deoB rpsL* ColI^r	Boulnois *et al.* (1979)
BW86	*dnaG3 leu thyA deoB rpsL Δ(chlA-uvrB)* ColI^r	Boulnois and Wilkins (1979)
BW89	*dna*$^+$ *leu thyA deoB rpsL Δ(chlA-uvrB)* ColI^r	Boulnois and Wilkins (1979)
BW96	*dna*$^+$ *leu deoA deoC tdk rpsL rpoB* ColI^r	Boulnois and Wilkins (1979)
BW97	*dna*$^+$ *leu thyA deoB rpsL Δ(chlA-uvrB) gyrA* ColI^r	Boulnois and Wilkins (1979)
BW103	*dna*$^+$ *leu deoB rpsL recA1* ColI^r	This work (section 2.1.)
W3110T$^-$	*thyA36*	Bachmann (1972)
HH27	*thyA36 recA1*	P.T.Barth (I.C.I., Runcorn)
C600 [1]	*thr leu thi lacY1 supE44*	Bachmann (1972)
M105	*dnaG3 thr-1 leu-6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 rpsL31 tsx-33 supE44*	Derived from C-2360 Harris *et al.* (1978)
N1205	*recB21 recC22 sbcB15 thi-1 proA2 thr-1 leu-6 argE3 his-4 lacY1 galK2 ara-14 xyl-5 mtl-1 rpsL31*	R.Lloyd (Nottingham University)
BC1304	*thyA polA1 end arg dnaB1304 dnaC201*	Schuster *et al.* (1977)
CSH26ΔF6	*ara thi Δ(lac-pro) Δ(recA-srl)F6 rpsL*	M.E.Jackson (Leicester University)

Table 2.1 continued

Strain	Description or genotype	Source or reference
E.coli K-12		
DS410	*lacY1 minA minB rpsL malA ara* Azi^r Sup^o	M.E.Jackson (Leicester University)
KL16-99	Hfr KL-16 *recA1*	Clarke *et al.* (1973)
Salmonella typhimurium LT2		
SL329 [1]	*trp*	Lanka and Barth (1981)

Genotypic symbols are defined in Bachmann (1983).

$ColI^r$ is the phenotypic symbol for resistance to colicin Ib-P9.

[1] C600 was made nalidixic acid-resistant and resistant to colicin Ib-P9 and SL329 was made nalidixic acid-resistant as described in section 2.1.

Table 2.2 Plasmids

Plasmid	Description or genotype	Source or reference
IncI, plasmids		
ColIb-P9drd-1	Derepressed for transfer	Dowman and Meynell (1970)
ColIb-P9drd-2	Derepressed for transfer	Laboratory stock
pLG221	ColIb-P9drd-1 cib::Tn5 Sog^+ Km^r	Boulnois (1981)
pLG250	pLG221 sog-217 Km^r	Chatfield et al. (1982)
pLG270	ColIb-P9drd-1 tra::Tn5 (Eco-9)[1] Sog^+ Km^r	C.Rees (unpublished work)
pLG264	ColIb-P9drd-2::Tn5 sog-262 Km^r	This work
pLG265	ColIb-P9drd-2::Tn5 sog-263 Km^r	This work
pC2	ColIb-P9drd-2::Tn5 (Sal-5)[1] Sog^+ Tra^+	Laboratory stock
IncP plasmids		
RP4	Km^r Ap^r Tc^r	Datta et al. (1971)
pRP1	RP4 pri::Tn7 (40.8kb)[2] Pri^+ Km^r Ap^r Tc^r Tp^r Sm/Sp^r	Barth and Grinter (1977)
pRP2	RP4::Tn7 (15.3kb)[2] Pri^+ Km^r Ap^r Tc^r Tp^r Sm/Sp^r	Barth and Grinter (1977)
pRP26	RP4 tra::Tn7 (49.7kb)[2] Pri^+ Km^r Ap^r Tc^r Tp^r Sm/Sp^r	Barth and Grinter (1977)

Table 2.2 continued

Plasmid	Description or genotype	Source or reference
Vector plasmids		
pBR328	Ap^r Tc^r Cm^r	Soberon *et al.* (1980)
R300B	IncQ Sm^r Su^r	Barth *et al.* (1981)
pGSS33	IncQ Ap^r Tc^r Cm^r Sm^r	Sharpe (1984)
pUC12	Ap^r	Messing (1983)
Recombinant plasmids		
pLG215	pBR325Ω[Sog^+, EcoRI 8.0kb] Ap^r Tc^r	Wilkins *et al.* (1981)
pLG214	pBR325Ω[Sog^+, EcoRI 8.0kb Δ1.4kb] Ap^r Tc^r	Wilkins *et al.* (1981)
pLG226	pBR325Ω[Sog^+, EcoRI 8.0kb]Δ4.4kb Ap^r	Boulnois *et al.* (1982)
pLG228	pBR325Ω[Sog^+, EcoRI 8.0kb]Δ6.7kb Ap^r	Boulnois *et al.* (1982)
pLG229	pBR325Ω[Sog^+, EcoRI 8.0kb]Δ7.7kb Ap^r	Boulnois *et al.* (1982)
pLG262	pLG215::Tn*5* *sog*-*262* Ap^r Km^r Tc^r	This work
pLG263	pLG215::Tn*5* *sog*-*263* Ap^r Km^r Tc^r	This work
pLG252	pBR325Ω[Eex^+, EcoRI 3.4kb] Ap^r Tc^r	Chatfield *et al.* (1982)
pLG2009	pED825Ω[*oriT*, TaqI ~860bp] Ap^r	Wilkins *et al.* (1985)
pLG218	pBR325Ω[Sog^- Am, EcoRI 8.0kb] Ap^r Tc^r	Chatfield *et al.*, 1982

Table 2.2 continued

Plasmid	Description or genotype	Source or reference
pAM3	pBR328Ω[Pri$^+$, HindIII ~15kb, 8.4kb, 2.75kb] Ap^r Cm^r	This work
pAM3-4	pBR328Ω[Pri$^+$, HindIII ~15kb, 2.75kb] Ap^r Cm^r	This work
pLG260	pBR328Ω[Pri$^+$, HindIII 13.9kb] Ap^r Cm^r	This work
pLG261	R300BΩ[Pri$^+$, PstI 12.3kb] Km^r	This work
pJF107	pBR325Ω[Pri$^+$, HindIII 14.5kb]Δ10.6kb Ap^r Cm^r	Lanka et al. (1984)
pJF107Δ32	pBR325Ω[Pri$^+$, HindIII 14.5kb]Δ13.7kb Ap^r	Lanka et al. (1984)

Plasmid nomenclature follows the guidelines of Novick et al. (1976).
Abbreviations are ampicillin, Ap; chloramphenicol, Cm; kanamycin, Km; streptomycin, Sm; spectinomycin, Sp; sulphonamide, Su; tetracycline, Tc; trimethoprim, Tp. Superscript r and s represent resistance and sensitivity to antibiotics, respectively.

[1] Bracketed symbol represents ColIb-P9 restriction fragment carrying Tn5 (C.Rees, unpublished results; Uemura and Mizobuchi, 1982).

[2] Bracketed figure represents coordinate of Tn7 insertions (Barth and Grinter, 1977; Lanka et al., 1984).

For the serial dilution of cells or the resuspension of colonies, sodium phosphate buffer (PB, Na_2HPO_4, 50mM; KH_2PO_4, 22mM; NaCl, 68mM; $MgSO_4$, 0.4mM) was used. Lambda buffer contained 6mM Tris-HCl (pH 7.2), 10mM $MgSO_4$ and 50μgl^{-1} gelatin in distilled water.

Where strains harboured recombinant plasmids based on vectors derived from pBR322, Tc (7.5 μgml^{-1}) or Ap (25 μgml^{-1}) was used to select for the presence of the plasmid, unless otherwise stated. Other antibiotics were generally used at the following concentrations: Cm, 25μgml^{-1}; Km, 100μgml^{-1}; Nal, 50μgml^{-1}; Rif, 100μgml^{-1}; Sm, 200μgml^{-1}; Su, 500μgml^{-1}. All antibiotics were purchased from Sigma except Sm (Glaxo) and Su (Oxoid), and solutions were sterilised before use either by dissolving in a solution of 50% ethanol, or in the case of Km, Cm and Su, by filtration through cellulose nitrate discs (Whatmans, 25mm diameter, 0.45μm pore size).

Radiochemicals [2-^{14}C]thymine, (2.0GBqmmol^{-1}; 1.85MBqml^{-1}); [methyl-^{3}H]thymine, (1.92TBqmmol^{-1}; 37MBqml^{-1}); [methyl-^{3}H]-thymidine 5'-triphosphate, (1.74TBqmmol^{-1}; 37MBqml^{-1}); [^{35}S]methionine, (45TBqmmol^{-1}; 555MBqml^{-1}) were purchased from Amersham International.

All chemicals used were of at least AR grade and, unless otherwise stated, were purchased from Sigma.

2.3. Strain storage and characterisation

Bacterial strains were kept on nutrient agar (NA) plates containing thymine and antibiotics as appropriate, and stored at 4°C. For long term storage, sterile glycerol was added to cells grown overnight in nutrient broth (NB) to 25%, vortexed, then stored at -30°C. Bacteria were recovered from storage by gently thawing the vial and inoculating 3ml of NB with 50-100 μl of the stored culture. The phenotype of bacterial strains was confirmed using the following tests.

Auxotrophic mutants were streaked to single cells on minimal agar containing, as appropriate, thymine, thiamine and amino acids. Strains carrying antibiotic-resistance markers were tested either on NA plates containing the relevant antibiotics, or where convenient, by using Multodisks (30-44K, Oxoid).

Sensitivity of strains to bacteriophage was tested by cross-streaking a suspension of the bacteriophage with exponentially grown cultures of cells and incubating overnight.

To test for sensitivity to colicin Ib, the strains were spotted onto a NA plate and allowed to form small colonies overnight. After exposure to chloroform vapour for 10 min, the plates were overlaid with a light suspension of a colicin Ib-sensitive strain (BW40) in soft NA, and incubated overnight. A clear halo of lysis around the test colony indicated the production of colicin Ib.

Sensitivity to ultraviolet light was determined by exposure of a lightly-inoculated streak of cells on NA plates to UV light (wavelength, 254nm) from a Hanovia germicidal lamp over a range of $0-60 Jm^{-2}$. This was achieved by setting the dose rate to $2 Jm^{-2}s^{-1}$, using a Latarjet dosimeter, and partially shielding the streaked cells with a cardboard screen for time intervals appropriate to give the range of exposures. Streaks of control strains were always included on each plate. Plates were incubated overnight and the viability of the cells determined relative to the control strains.

Temperature-sensitive dnaG3 strains were tested by their ability to form colonies at the restrictive temperature, 40°C. This is described more fully in section 2.8.1.

2.4. Methods used in the construction of bacterial strains.

2.4.1. Bacterial matings

Strains containing $IncI_1$ plasmids were generally constructed as follows. Overnight cultures were diluted 1:100 in NB and grown to a density of about 2×10^8 cells per ml. Donor and recipient cultures were mixed in the ratio of 1:10 and gently shaken at the appropriate temperature, usually for 1 h. Donor strains for conjugative DNA synthesis experiments were constructed in 5 min matings to ensure that the resulting strains carried plasmids contained a drd mutation. Transconjugants were selected by plating on appropriate media.

The yield of RP4 transconjugants was very poor when the mating occurred in liquid, presumably because the aggregates were unstable. To

overcome this, matings were carried out on solid media, using either cellulose nitrate discs for the accurate assessment of transconjugant yield (see section 2.6.2.), or NA plates. Overnight cultures of donor and recipient cells were diluted 1:10 in NB. A wide streak of recipient cells was applied to a NA plate containing no selective reagents, allowed to dry, then cross-streaked with the donor cells. After overnight incubation at a temperature appropriate to the growth of both strains, the growth at the intersection was resuspended in 0.5ml PB and the cells streaked out for single colonies on selective agar and incubated overnight.

2.4.2. Transformation of bacterial strains

The method described here was found to be efficient for the transformation of bacterial strains by both conjugative and recombinant plasmids, and is based on that of Cohen *et al.* (1972). A 0.1ml volume of overnight culture was used to inoculate 10ml NB, and the culture was grown, with aeration, at the appropriate temperature. The growth was monitored by the absorbance at 600nm (A_{600}) using a spectrophotometer (Bausch and Lomb Spectronic 20; path length 10mm). Cells grown from an A_{600} of 0.05 to 0.35 were chilled on ice for 5 min then pelleted at 3700g at 2°C for 5 min, and resuspended in 5ml ice-cold 100mM $MgCl_2$. The cells were sedimented as before, resuspended in 5ml ice-cold 100mM $CaCl_2$, and kept on ice for 20 min. After centrifugation as before, the cells were resuspended in 1ml ice-cold 100mM $CaCl_2$.

For maximum transformation efficiency, the cells were stored overnight at 4°C. During this period the transformation efficiency increases by approximately five-fold (Dagert and Ehrlich, 1979), but decreases again after 24 h storage. Aliquots of 250µl cell suspension were added to about 0.5µg of plasmid DNA in a precooled Eppendorf tube and kept on ice. If the volume of DNA exceeded 10µl, then an equal amount of precooled 200mM $CaCl_2$ was added. After 1 h, the cells were heat-shocked at 42°C for 3 min without disturbing the cells, then returned to ice for a further 3 min. The cell suspension was used to inoculate 5ml NB, prewarmed to the appropriate temperature. Cultures were shaken for 1 h at 37°C, or 1.5 h at 30°C, as appropriate, before the cells were pelleted at 2000g for 7 min at room temperature. The cell pellet was resuspended in 0.2ml PB and plated onto selective media at 10^0-10^{-3} dilutions. This procedure usually gave about 10^4 transformants per ml for small plasmids and 10^2-10^3 per ml for larger plasmids such as ColIb-P9 or RP4.

2.4.3. Bacteriophage transduction

This method was adapted from Miller (1972). P1 transducing phage, P1 *c*Ts Cm^r, was prepared from the appropriate strain as described in section 2.5.1. Cells from 5ml overnight culture were pelleted at 2000g for 10 min at room temperature and resuspended in 5ml MC buffer ($MgSO_4$, 100mM; $CaCl_2$, 5mM). After aeration at the appropriate temperature for 15 min, 0.1ml of the cell suspension was added to each of five Eppendorf tubes. To the first four tubes, was added 0.1ml of increasing dilutions (10^0-10^{-3}) of P1 transducing phage, one dilution per tube, and to the fifth, 0.1ml NB. To a sixth tube was added 0.1ml of undiluted transducing phage suspension and 0.1ml MC buffer. All tubes were incubated at 30°C for 20 min, before pelleting the cells in tubes 1 - 5 at 12000g for 30 sec. The pellets were resuspended in 0.2ml citrate buffer (as for SGC, except the Casamino acids were replaced with amino acids appropriate to the strains' requirements, and sodium citrate, 0.86mM) and all tubes incubated at 30°C for 1 h. The whole volume of each tube was then plated onto agar plates selective for the transduced marker.

2.4.4. Purification of bacterial strains

After selection on appropriate media, a number of single colonies, usually four, were picked into 0.5ml PB and streaked for single cells, again on selective media, before incubation overnight at the appropriate temperature. A single colony of each isolate was picked and used to inoculate a 3ml overnight culture in NB. This culture was then subjected to the characterisation tests described in section 2.3.

2.5. Production of bacteriophage

2.5.1. Bacteriophage P1

This method was adapted from that of Miller (1972). A P1 phage, P1 *c*Ts Cm^r, which contains a temperature-sensitive mutation of the repressor gene, *c*, was used (Table 2.3). To prepare phage, an overnight culture of a strain lysogenic for phage P1 was grown at 30°C and used to inoculate 10ml NB to A_{600} of 0.05. This culture was grown, with aeration, at 30°C to A_{600} of 0.2, before being heat-shocked at 41°C for 10 min. The culture was incubated at 37°C with gentle aeration until the absorbance dropped below 0.05, indicating cell lysis. One ml chloroform was added and the

Table 2.3 Genotypes of bacteriophage

Phage	Description or genotype	Source or reference
P1	cTs Cm^r	Laboratory stock
λ::Tn5	λ cI857 b221 rex::Tn5 Oam	Dr.A.Boyd (Leicester Biocentre)

lysate was shaken vigorously and left to stand at 37°C for 15 min. The cellular debris was then pelleted at 7700g for 10 min at room temperature and the supernatant removed for storage at 4°C.

To titre the phage stock, an overnight culture of a sensitive strain was diluted in 10ml NB containing 5mM $CaCl_2$ and grown, with aeration, at the appropriate temperature from A_{600} of 0.05 to 0.4. 0.2ml aliquots of the culture were transferred into test tubes and to these was added 0.1ml of appropriate dilutions (10^{-2}–10^{-10}) of the transducing phage stock, diluted in NB containing 5mM $CaCl_2$. After 20 min at 30°C, 4ml of soft NA containing 5mM $CaCl_2$ was added and poured onto Z plates (NA containing 5mM $CaCl_2$ and 5.5mM glucose) before incubation overnight at 37°C.

2.5.2. Production of bacteriophage λ::Tn5

The λ bacteriophage used was λ cI_{857} rex::Tn5 b221 Oam (Table 2.3). An overnight culture of C600 was diluted 1:20 into 10ml NB and grown at 37°C, with aeration, to an A_{600} of between 0.6 – 0.8. Cells were pelleted at 2000g for 7 min and resuspended in 10ml Lambda buffer. To 0.1ml of the resultant plating cells was added 10^5 pfu of λ::Tn5. After a 10min adsorption period at room temperature, 4ml BTL containing 10mM $MgSO_4$ was added before overlaying on BLA plates. The plates were incubated overnight at 37°C, this temperature inactivating the cI repressor and allowing lytic growth. The overlay was removed and 0.5ml Lambda buffer was added and the soft agar macerated. After the addition of 0.5ml chloroform, the tube was left to stand at 37°C for 30 min, before the debris was pelleted at 7700g for 5 min at room temperature. The supernatant was stored in the presence of a small amount of chloroform added to prevent bacterial growth.

2.5.3. Other bacteriophage

Bacteriophage M13, Iα, PR64FS and T6 were taken from laboratory stocks. DNA of bacteriophage fd, used in the in vitro primase assay (see section 2.8.3.) was a gift from E.Orr.

2.6. Quantitative measurement of transconjugant yield

2.6.1. Matings in liquid media

This method was used to measure the yield of transconjugants generated by donors harbouring ColIb-P9 and its derivatives. Overnight cultures of the strains, diluted in 10ml NB, were grown with aeration from A_{600} of 0.05 to 0.35 (approximately 2.0 x 10^8 cells per ml) at the appropriate temperature. The donor and recipient cultures were then mixed in a ratio of 1:10 and 0.35ml of the mixed culture was added to a 150ml Erlenmeyer flask and gently swirled (100 rpm), at the required temperature, for 60 min. At the end of this period the cell suspension was violently shaken, either using a mechanical agitator or by vigorous vortexing, to separate the mating cells. After dilution in PB, appropriate dilutions were plated on selective media and incubated overnight. Undiluted 0.1ml samples of input cultures were always plated on the same media as controls.

2.6.2. Matings on solid media

Since the yield of RP4 transconjugants from liquid mating is low, a method involving mating on cellulose nitrate filters was used. Shaken overnight cultures of donor and recipient strains were diluted to an A_{600} of 0.35 in 10ml NB, and mixed in a ratio of 1:5. 1ml of this mixture was applied to a sterile cellulose nitrate disc (Whatmans, 25mm diameter, 0.45µm pore size) under gentle vacuum. The disc was then incubated for 3 h (see Chapter 4) on a pre-warmed NA plate containing thymine at 37°C or, in the case of a temperature-sensitive strain, at 30°C. The cells were washed into 5ml PB by vigorous vortexing, and diluted appropriately in PB. Cells were plated onto duplicate sets of NA plates containing Nal to select for the recipient strain, and either Km or Tc to select for the incoming conjugative plasmid. The choice or Km or Tc depended upon the antibiotic-resistance markers carried by any plasmids harboured by the recipient strain. Input cultures were always streaked onto the same media as controls. The plates were incubated overnight at 37°C before counting.

2.7. Preparation and manipulation DNA

2.7.1. Preparation of DNA

(A) Method based on the Triton-X100 lysis procedure.

Large amounts of recombinant and vector DNAs were isolated by a method based on that of Katz et al., (1973). A 25ml overnight culture of the plasmid-containing strain was grown in NB containing antibiotics appropriate to the maintanance of the plasmid. This culture was used to inoculate 1l of NB, also containing antibiotics, in a 2l baffled flask. The culture was grown to an A_{600} of 0.8 and plasmid DNA amplified (Clewell and Helinski, 1972) by the addition of either chloramphenicol ($170\mu g ml^{-1}$) or spectinomycin ($300\mu g ml^{-1}$), according to the sensitivity of the strain, and shaken overnight. The culture was split between to two 500ml polycarbonate tubes and cells pelleted at 4200g for 5 min at 4°C. The pellets were resuspended in a total of 11.2ml 730mM sucrose in 50mM Tris-HCl buffer (pH 8.0) and the cell suspension divided equally in two. To each part was added 0.94ml of freshly-prepared lysozyme solution ($10mg ml^{-1}$ in 50mM Tris-HCl, pH 8.0) and samples kept on ice for 15 min before adding EDTA (pH 8.0) to 20mM to form spheroplasts. After 15 min on ice, cleared lysates were prepared by the addition of 7.5ml lysis mixture (Tris-HCl, 50mM; EDTA, 62.5mM; Triton-X100, $20g l^{-1}$) and immediately centrifuging at 39000g for 40 min at 4°C.

The nucleic acid in the supernatant was precipitated by the addition of $100g l^{-1}$ PEG_{6000} and NaCl to 500mM and chilled overnight at 4°C. After centrifugation at 3000g for 10 min at 4°C, the pellet was resuspended in 1.1ml TES buffer (Tris-HCl, 50mM; EDTA, 5mM; NaCl, 50mM; pH 8.0) and transferred to an ultracentrifuge tube. The samples were underlaid with 4ml caesium chloride-ethidium bromide gradient solution before being centrifuged to equilibrium. The gradient solution contained $1g ml^{-1}$ CsCl (Fisons) and $0.5mg ml^{-1}$ ethidium bromide (Serva) dissolved in TES buffer. The refractive index, measured with a Bellingham and Stanley refractometer, was adjusted to within the limits of 1.3990 - 1.4000 using CsCl or TES buffer, and the gradients spun at 220000g for 12-20 h at 15°C. The DNA bands were viewed under UV light (wavelength 365nm) and the lower band, containing the plasmid DNA, was withdrawn using a hypodermic needle and syringe. To extract the ethidium bromide, samples were treated three times with equal volumes of propan-2-ol, saturated with an aqueous

solution of NaCl, prior to dialysis against three changes of 2l dialysis buffer (Tris-HCl, 10mM; EDTA, 1mM; pH 7.5) at 4°C over 20 h. The DNA was then concentrated as described in section 2.7.2.

(B) The method based on that of Uemura and Mizobuchi (1982)

This method is particularly useful for the isolation of plasmid DNA of large molecular weight. 1l of NB was inoculated with 20ml of overnight culture, and grown to an A_{600} of 0.8 - 1.2. The culture was split in two and the cells pelleted at 8300g for 10 min at 4°C. The cells were washed in 125ml 50mM Tris-HCl (pH 8.0), sedimented and finally resuspended in 12.5ml 730mM sucrose in 50mM Tris-HCl (pH 8.0). This suspension was further divided between two tubes and to each was added 1.25ml freshly-prepared lysozyme (5mgml^{-1} in 50mM Tris-HCl, pH 8.0). After 5 min on ice, EDTA (pH 8.0) was added to 25mM before standing on ice for a further 5 min. The resultant spheroplasts were lysed with 10ml lysis mixture (Tris-HCl, 50mM; EDTA, 62.5mM; Brij 58 (Ciba-Geigy), 10gl^{-1}; Sodium deoxycholate (Ciba-Geigy), 4 gl^{-1}; pH 8.0) and held on ice for 10 min before centrifugation at 39000g for 40 min at 4°C. The volume of the supernatant was measured before adding sodium N-lauroyl sarcosinate to 10gl^{-1} and incubated at 30°C for 10 min. The supernatant was then treated with NaCl and PEG_{6000} and centrifuged to equilibrium in caesium chloride-ethidium bromide gradients as described in section 2.7.1.(A), except the gradient solution also contained 18gl^{-1} sodium N-lauryl sarcosinate.

(C) Rapid mini-preparation method of Klein et al., (1980)

This method was used for the small-scale isolation of recombinant and vector plasmids. Cells from 5ml overnight culture in NB containing the relevant antibiotics were pelleted at 3600g for 5 min at 4°C, and resuspended in 1ml 50mM Tris-HCl (pH 8.0). This was divided between two Eppendorf tubes and 50µl of 10mgml^{-1} lysozyme, in 50mM Tris-HCl (pH 8.0), was added to each. After incubation at room temperature for 15 min, 0.5ml phenol mixture (100g phenol [BDH Chemicals Ltd.], 0.1g 8-hydroxyquinoline [BDH Chemicals Ltd.], 4ml isoamyl alcohol [Fisons] in 100ml chloroform [Fisons]; stored at 4°C under 10mM Tris-HCl, pH 7.5) was added. After gentle inversion and centifugation for 12 min at 12000g, the upper, aqueous phase was removed, avoiding any material deposited at the interface, and the DNA purified as described in section 2.7.2.

(D) Rapid mini-prep method of Birnboim and Doly (1979)

This rapid method was used to produce plasmid DNA from strains carrying recombinant or vector plasmids. It has the advantage over the method of Klein et al., (1980) in that DNA of large plasmids such as RP4 and ColIb-P9 can be isolated. Cells from 1.5ml overnight culture containing the relevant antibiotics were pelleted in an Eppendorf tube by centrifugation for 30 sec at 12000g. The pellet was resuspended in 100µl lysis buffer (Tris-HCl, 25mM; EDTA,10mM; glucose, 50mM; pH8.0) containing 1mgml^{-1} freshly-prepared lysozyme, and kept on ice for 10 min prior to the addition of 200µl alkaline-SDS solution (NaOH, 200mM; SDS, 35mM). After 5 min on ice, potassium acetate (pH 5.2) was added to a final concentration of 1M and mixed prior to standing on ice for 10 min. The mixture was centrifuged at 12000g for 3 min and the supernatant transferred to another Eppendorf tube, avoiding the pelleted debris. The plasmid DNA in the supernatant was rapidly treated with phenol and precipitated as described in section 2.7.2. The DNA was finally resuspended in 40µl distilled water. For recombinant or vector DNA, about 5µl was used for digests (~0.5µg) and 15µl for large conjugative plasmid DNA.

2.7.2. Purification of DNA

The removal of protein and other substances was often essential following the isolation of plasmid DNA from cleared lysates, or after treatment of DNA with various enzymes. This was achieved by treating the DNA with phenol mix (see section 2.7.1.C). If the volume of DNA was small, sterile distilled water was added to make a volume of about 200µl. An equal volume of phenol mix was added to the DNA solution in an Eppendorf tube, and shaken vigorously to form an emulsion. After centrifugation at 12000g for 3 min, the top, aqueous layer was removed, avoiding the debris at the interface, and the phenol treatment repeated. The aqueous layer was again removed and an equal volume of 24:1 chloroform:iso-amyl alcohol added and mixed prior to centrifugation for 2 min at 12000g. To the aqueous layer was added sodium acetate (pH 5.6) to 200mM and 2.5 volumes of absolute alcohol and the DNA allowed to precipitate in an ethanol-dry ice bath for 5 min. The DNA from the cleared lysate formed in the Birnboim and Doly mini-prep method was precipitated at room temperature for 5 min since precipitation in an ethanol-dry ice bath caused degradation of the DNA. The DNA precipitate was pelleted by centrifugation for 3 min at 12000g and

the supernatant discarded. The pellet was dissolved in 250µl 200mM sodium acetate (pH 5.6) and 625µl absolute alcohol was added, mixed vigorously before being re-precipitated as above. The pellet was finally washed in 1ml 70% ethanol, to remove excess salt, and dried under vacuum before dissolving in sterile distilled water. DNA samples were stored at -20°C in small aliquots. The concentration of DNA was measured by absorption at 260nm using a Cecil spectrophotometer, or by comparison with DNA of known concentration, in an agarose gel.

2.7.3. Manipulation of DNA

(A) Restriction endonuclease digestion of DNA

Digestion of DNA samples was performed according to the manufacturer's (BRL Inc.) instructions. Digests were carried out at 37°C for 30-60 min and contained DNA at a concentration of $\leq 0.5 mg ml^{-1}$ and 0.5-1.0 unit of the appropriate restriction enzymes per mg of DNA, unless stated. Spermidine was added to 4mM in all digests to enhance the activity of the enzymes (Bouche, 1981). In double digests, where the enzymes used could not tolerate the conditions of a single buffer, the enzymes were used in the presence of their compatible buffer and the DNA was ethanol precipitated and washed between the two digestions.

(B) Digestion of DNA with exonuclease Bal31

The enzyme Bal31 is an exonuclease which is able to digest double-stranded DNA and RNA in both 5' to 3' and 3' to 5' directions (Lau and Gray, 1979). Since RNA is recognised as a substrate for reaction, best results were obtained by removing RNA from the DNA before treating with Bal31, This was done by digestion with $10 mg ml^{-1}$ heat-treated RNase A in 300µl TE buffer (Tris-HCl, 10mM; EDTA, 1mM; pH 8.0) for 60 min at 20°C. The whole sample was then loaded on top of 4ml TES (Tris-HCl, 10mM; EDTA, 1mM; NaCl, 1M; pH 8.0) in an ultracentrifuge tube and the DNA pelleted by centrifugation at 192000g for 6 h at 20°C. The supernatant, containing the oligoribonucleotides, was discarded and the DNA pellet dissolved in 300µl distilled water.

Digests were carried out in a total volume of 50µl in Bal31 digestion buffer (Tris-HCl, 20mM; NaCl, 600mM; $MgCl_2$, 12mM; $CaCl_2$, 12mM; EDTA, 1mM; pH 8.1) at 30°C for 30 min and contained 0.5µg DNA, cleaved appropriately.

To obtain different rates of reaction, the amount of Bal31 added was varied. Reactions were stopped on ice by the addition of EGTA (Ethylene glycol-bis [β-aminoethyl ether]-N,N,N',N'-tetraacetic acid; Pelham and Jackson, 1976) to 40mM. The DNA was then either loaded directly onto an agarose gel or treated with phenol prior to end repair. The single-stranded ends of the DNA molecules created by Bal31 digestion were repaired using 1 unit of the Klenow fragment of DNA polymerase I (E.coli; Boehringer Mannheim) at 20°C for 30 min in Fill-in buffer (Tris-HCl, 50mM; $MgCl_2$, 10mM; DTT, 1mM; pH 8.0) containing dNTP's each at a final concentration of 80nM. The reaction was terminated by treatment with phenol and the DNA precipitated.

(C) Ligation of DNA fragments

Prior to ligation, fragments generated by restriction-endonuclease cleavage or Bal31 digestion were treated with phenol and ethanol precipitated. Vector DNA was usually treated with 1 unit per μg DNA calf-intestinal alkaline phosphatase (Boehringer Mannheim) for the final 20 min of the restriction-endonuclease digestion. The alkaline phosphatase was inactivated at 65°C for 15 min prior to treatment with phenol. The vector and fragment DNAs were ligated, using ~100 units T4 ligase (BioLabs), in a fragment-end ratio of 5:1, in a 50μl volume of ligation buffer (Tris-HCl, 50mM; $MgCl_2$, 10mM; DTT, 20mM; ATP, 1mM; pH 7.8) at 15°C for 20 h. The efficiency of the ligation was checked by comparison with input DNA fragments using agarose gel electrophoresis. The ligated DNA was used directly to transform competent cells.

2.7.4. Visualisation of DNA

DNA was viewed by electrophoresis in agarose gels (Aaij and Borst, 1972). Agarose gels were prepared by dissolving an appropriate percentage of agarose (HGT, Seakem) in running buffer (Tris-acetate, 40mM; EDTA, 1mM; ethidium bromide (Serva), 6μM). DNA samples were mixed with one-fifth volume of loading buffer (Tris-HCl, 80mM; glycerol, 100gl^{-1}; bromophenol blue, 0.01gl^{-1}; pH 6.8) before loading onto the gel which was submerged in running buffer. Electrophoresis was carried out at either 25V for 16-20 h, or 100V for 3-4 h. The DNA fragments were viewed using a short-wavelength UV transilluminator and photographed using a Polaroid Land camera loaded with Polaroid 52 film or a Nikon 35mm SLR camera containing Kodak AHU film. The DNA fragment sizes were measured by comparison with

molecular weight markers, such as λ x HindIII or pBR322 x Sau3A (Table 2.4).

2.7.5. Purification of DNA fragments using polyacrylamide gels

This method was particularly suitable for DNA fragments of less than 1kb in size and was taken from Maniatis et al., (1982). The cleaved DNA was electrophoresed in a polyacrylamide gel of the appropriate percentage, and stained in ethidium bromide (0.2μM) for 90 min. After removing the desired DNA fragment in a small slice of polyacrylamide, the slice was chopped into small pieces and placed in 1ml elution buffer (ammonium acetate, 0.5M; EDTA, 1mM; pH 8.0) and incubated at 37°C for 20 h with gentle mixing. The gel debris was pelleted at 12000g for 10 min, and the supernatant recovered. The gel fragments were washed with a further 500μl elution buffer and the DNA in the combined supernatants treated with phenol and ethanol precipitated. The molecular size markers used to measure the size of the DNA fragments were ΦX174 DNA cleaved with HaeIII (Table 2.4).

2.8. Measurement of DNA plasmid primase activity

2.8.1. Suppression of the dnaG3 mutation in vivo.

This method determines the ability of plasmid primases to suppress the temperature-sensitive mutation of the host primase gene, dnaG3, harboured by BW86. An overnight culture of the plasmid-containing BW86 strains, grown at 30°C, was diluted to give an absorbance A_{600} of 0.35 (~2.0 x 10^8 cells per ml) in NB. Cells were plated at appropriate dilutions onto prewarmed selective NA plates at 30°C and onto non-selective plates at 40°C, and incubated overnight. The index of colony forming ability was the proportion of colonies formed at 40°C compared to that at 30°C.

2.8.2. Measurement of DNA synthesis at 41°C.

This method was based on that of Wilkins et al. (1981). An overnight culture of the BW86 strain under test, in SGC containing 24μM thymine and 750μM 2-deoxyguanosine (2-dG), was diluted in 5ml of the same medium and the absorbance at A_{450}, measured using a Gilford 300-N spectrophotometer, was adjusted to less than 0.05. The cells were grown in a 50ml flask at 30°C in an orbital shaker until the absorbance (A_{450}) was approximately

Table 2.4 Fragment sizes of molecular weight markers

λ x HindIII[1]	pBR322 x Sau3A[2]		ΦX174 x HaeIII[3]
23.13	1374	75	1353
9.42	665	46	1078
6.56	358	36	872
4.36	341	31	603
2.32	317	27	310
2.02	272	18	281
0.56	258	17	271
	207	15	234
	105	12	194
	91	11	118
	77	8	72

[1] Sanger et al. (1982). Fragment sizes given in kilobases.

[2] Sutcliffe (1979). Fragment sizes given in base pairs.

[3] Sanger et al. (1978). Fragment sizes given in base pairs.

0.4. A 2ml sample was taken onto ice and the absorbance (A_{450}) measured. The remainder of the culture was incubated at 41°C for 5 min to inactivate host primase activity, before transferring 1.5ml to a second 50ml flask containing 1.5ml SGC plus 24μM thymine, 750μM 2-dG and 0.83MBq [methyl-^{3}H]thymine, prewarmed to 41°C and shaken. After 90 min, two 1ml aliquots of the labelled culture were removed into 2ml of TCA stock solution (trichloroacetic acid, 0.5M; thymine, 24μM) and chilled on ice for at least 30 min. The absorbance (A_{450}) of the labelled culture was also measured as before, and the increase in absorbance of the culture over the labelling period calculated.

The acid-precipitable radioactivity was determined by washing the samples onto cellulose nitrate membrane filters (Whatmans, 25mm diameter, 0.45μm pore size) presoaked in 160μM thymine. Each filter was washed ten times with 10ml boiling water and thoroughly air-dried. 1ml of non-aqueous scintillation fluid (5g 2,5-diphenyloxazole [PPO, Fisons] and 0.3g 1,4-di-2(4-methyl-5-phenyl oxazolyl)benzene [POPOP, Fisons] per litre of toluene [Fisons]) was added to each filter in a scintillation vial and the radioactivity measured in a Packard 3255 liquid scintillation spectrophotometer. The background retention of radioactivity by filters was measured by processing four 1ml aliquots of cell-free labelled samples in the same way.

The average background radioactivity was subtracted from the mean value of the radioactivity incorporated for each strain. The amount of DNA synthesis at 41°C was standardised by expressing the results as cpm incorporated per ml of labelled culture per unit A_{450} absorbance increase over the labelling time.

2.8.3. Measurement of DNA primase activity *in vitro*.

The activity of DNA primase was quantified *in vitro* by measuring the ability of the primase to prime DNA synthesis on single-stranded phage fd or M13 DNA templates (Lanka *et al.*, 1979). Crude cell extracts (CCE) of plasmid-containing strains were prepared by diluting an overnight culture 20-fold into 10ml NB and growing the culture at 30°C to an absorbance (A_{600}) of approximately 0.8. The cells were chilled and harvested by centrifugation at 12000g for 5 min at 4°C. The cell pellet was washed in 1ml 25mM HEPES buffer (pH 8.0) containing 50mM KCl and 1mM DTT before pelleting the cells as above. The pellet was resuspended in 200μl of

ice-cold spheroplast buffer (sucrose, 150mM; KCl, 100mM; DTT, 1mM; EDTA, 1mM; spermidine-HCl, 4mM; HEPES, 25mM; pH8.0) containing 1mgml^{-1} freshly prepared lysozyme and incubated on ice for 30 min. The cells were then lysed by adding Brij 58 to 2.5gl^{-1} and leaving on ice for a further 40 min. Samples were centrifuged for at least 30 min at 12000g at 4°C, the supernatant being removed avoiding the cellular debris, and immediately frozen in liquid nitrogen. The concentration of protein in the CCEs was determined by the method of Bradford (1976) as modified by Spector (1978).

Receptor extract for the primase assay was prepared from the dnaB, dnaC strain BC1304 according to a modification of the method of Lanka et al. (1979). A 50ml overnight culture was grown at 30°C and used to inoculate 1l TY broth (Tryptone (Difco), 8g; yeast extract (Difco), 5g; NaCl, 5g; per litre distilled water) and the cells grown with gentle shaking at 28°C until the absorbance (A_{600}) reached 1.0. The pH of the culture was monitored throughout and kept above pH 6.5 by additions of 5M KOH. The cells were harvested at 4200g for 10 min at 4°C and washed in 30ml 25mM HEPES buffer (pH 8.0) containing 50mM KCl. After centrifugation, as above, the pellet was resuspended in 1ml per gram cell pellet of 25mM HEPES buffer (pH 8.0) containing 50mM KCl before freezing in liquid nitrogen and storing at -80°C overnight. After quickly thawing the cell suspension, keeping the temperature below 15°C, 1/50th volume of 15mgml^{-1} lysozyme freshly prepared in 50mM EDTA (pH 8.0) was added. The cells were allowed to lyse on ice then immediately refrozen in liquid nitrogen. After thawing, the sample was centrifuged at 81000g for 30 min at 4°C and the pale brown supernatant quickly aliquoted into small tubes and stored under liquid nitrogen. When taken for use, the aliquots were only thawed sufficiently to yield the required volume of extract, before the remainder was immediately refrozen.

The activity of the plasmid-encoded primases was assayed in 25µl reactions containing the following: 25mM HEPES buffer (pH 8.0), 25mM KCl, 1mM DTT, 2mM spermidine, 200mM EDTA, 25µgml^{-1} Rif, 10mM magnesium acetate, 25µM cyclic adenosine 5'-monophosphate (cAMP), 25µM nicotinamide adenine dinucleotide (NAD), 2mM adenosine triphosphate (ATP), 17.5mM creatine phosphate, 3µg creatine phosphokinase, 125µM each of CTP, GTP and UTP, 12.5µM each of dATP, dCTP, dTTP and dGTP and 37kBq [methyl-^{3}H]-deoxythymidine 5'-triphosphate, 0.5µg single stranded fd or M13 DNA, 5µl receptor extract and an appropriate amount of CCE.

A range of dilutions of CCE were assayed to ensure that the reaction did not proceed to completion before the end of the incubation period (see Chapter 3). Reactions were carried out in duplicate in Eppendorf tubes at 30°C for 60 min, arrested by the addition of 250µl of stop mix (NaOH, 0.5M; SDS, 5gl^{-1}; sodium pyrophosphate, 100gl^{-1}; denatured salmon sperm DNA, 0.5µgml^{-1}) and kept at 20°C for 5 min before being incubated at 90°C for 3 min. After the addition of ice-cold TCA to a final concentration of 1.3M, the samples were left on ice for at least 30 min. Acid-precipitable radioactivity was determined by washing the samples onto Sartorius cellulose-nitrate membrane filters (25mm diameter, 0.45µM pore size) and flushing with 50ml distilled water. After air-drying, the radioactivity was determined (section 2.8.2.) and the primase activity calculated on the assumption that one unit of primase activity incorporated 1µmol of labelled dTMP into TCA-precipitable material (Wilkins et al., 1981). The primase activity was standardised relative to the amount of protein present in the CCE (in mg) added to the reaction.

2.9. Conjugative DNA synthesis in recipient cells

This method, adapted from Boulnois and Wilkins (1979), allowed the amount of conjugative DNA synthesis (CDS) occurring in recipient cells using the transferred plasmid DNA as template and the plasmid primase as the priming activity to be quantified. The theory of this method will be briefly described below.

BW96 (tdk, rpoB) donors of the conjugative plasmid are mated with UV-irradiated BW86 (dnaG3, thyA, ΔchlA-uvrB) recipient cells in the presence of Rif (100µgml^{-1}) and [2-^{14}C]thymine (18.5kBqµg^{-1}, 2µgml^{-1}) at 41°C. The donor tdk mutation inactivates thymidine kinase activity. This blocks the synthesis of deoxythymidine monophosphate from thymidine, thereby preventing the incorporation of radioactively-labelled thymine into donor DNA. The UV-irradiation of the recipient cells introduces pyrimidine dimers into the chromosome thereby preventing chromosomal replication masking the incorporation of label into the plasmid DNA. Therefore, under these mating conditions, the incorporation of radioactivity into DNA during conjugation predominantly reflects the synthesis, in the recipient cells, of DNA complementary to the transferred plasmid strand. Since the recipient host priming systems, RNA polymerase and primase, are inactivated by the presence of Rif and the mating temperature respectively, the synthesis of DNA in the recipient is

initiated predominantly by plasmid primase.

The Rif-treatment of the recipient cells also amplifies the amount of plasmid DNA transferred such that, over 60 minutes, each recipient cell receives the equivalent of about ten single strands of ColIb-P9 DNA (Boulnois and Wilkins, 1979). This results from the inhibition of transcription of plasmid genes in the recipient cells, so preventing the development of a plasmid-encoded transfer-limiting system (Boulnois and Wilkins, 1978; Hartskeerl et al., 1983).

BW96 donor strains and BW86 recipient strains were grown overnight in SGC at 37°C, and in SGC containing 160μM thymine at 30°C, respectively, using antibiotics appropriate to the retention of recombinant plasmids. The overnight cultures of the recipient strains were diluted in SGC containing 160μM thymine and 750μM 2-dG and grown at 30°C with aeration, from an A_{600} of 0.05 to 0.5. The cells were pelleted at 2000g for 10 min and resuspended in 20ml PB prior to irradiation with $400Jm^{-2}$ of UV light. Following centrifugation as above, the irradiated cells were resuspended in prewarmed (41°C) SGC containing 750μM 2-dG to an A_{600} of 0.05 (approximately 5×10^8 cells per ml). One ml was transferred into 150ml Erlenmeyer flasks at 41°C, containing 1ml SGC containing 750μM 2-dG and $400\mu g ml^{-1}$ Rif. After 10 min incubation, 0.7ml preconditioned [2-^{14}C]thymine (see below) was added to each flask.

Overnight cultures of donor strains were diluted and grown in SGC at 37°C with aeration, from an absorbance, A_{600}, of 0.05 to 0.35. The cultures were then prewarmed at 41°C for 5 min, before adding 2ml to the flasks containing the treated recipient cells (Time, t = 0). The flasks were gently swirled at 100 rpm at 41°C for 60 min, during which time duplicate 0.5ml samples were taken at 10 min intervals into 1ml TCA stock solution (section 2.8.2.). After overnight incubation on ice, the radioactivity in the acid-precipitable material was determined as described in section 2.8.3.

As controls, the donor and recipient strains were incubated separately in the manner described above, making up the volume with SGC or SGC containing thymine and 2-dG as appropriate. The amount of radioactivity incorporated into the control strains was subtracted from that obtained for the matings.

The label was preconditioned (Boulnois and Wilkins, 1978) by mixing the [2-^{14}C]thymine with BW40 cells, grown in SGC at 37°C to an A_{600} of 0.2, in a ratio of 1:6 and incubated at 37°C for 30 min. The cells were then removed by filtration and an equal volume of SGC containing 750µM 2-dG and 200µgml^{-1} Rif was added.

2.10. Visualisation of polypeptides

2.10.1. Minicells

The minicell-producing strain DS410 was used. A 50ml overnight culture was used to inoculate 400ml NB containing appropriate antibiotics, which was shaken at 37°C for at least 20 h to maximise the cell density. The culture was centrifuged at 680g for 5 min to remove most of the mother cells, and the supernatant recentrifuged at 11000g for 15 min to pellet the minicells. The pellet was resuspended in 3ml minimal medium (section 2.2.) and layered on top of a sucrose gradient. Gradients were prepared by freezing 30ml of gradient mixture (minimal medium containing 200gl^{-1} sucrose) in tubes at -30°C, then thawing overnight at 4°C prior to use. The loaded gradients were centrifuged at 3000g for 20 min and the top two-thirds of the resultant central band of minicells was removed avoiding the larger mother cells. The minicells were pelleted at 20000g for 10 min and resuspended in 1ml minimal medium before being loaded onto a second sucrose gradient. The gradients were centrifuged as above and the top three-quarters of the central minicell band removed. Following pelleting, the minicells were resuspended in 200µl minimal medium containing 1gl^{-1} Difco methionine assay medium (DMA). At this stage, 50µl aliquots of minicells in 30gl^{-1} glycerol could be stored at -30°C. If stored, the glycerol was removed prior to labelling by washing the minicells twice in 1.5ml minimal medium and resuspending in 50µl minimal medium containing DMA.

To label the polypeptides, 50µl minicells were incubated at 37°C for 60 min prior to the addition of 1.85MBq [^{35}S]methionine. The incubation was continued at 37°C for 30 min before methionine was added to 1.4mM as a chase, for 5 min. The minicells were harvested by centrifugation at 12000g for 2 min and resuspended in 60µl PB before adding an equal volume of SDS-PAGE sample buffer (Table 2.5). Labelled polypeptides were analysed using SDS-polyacrylamide gels (section 2.10.6.)

2.10.2. Maxicells

The strain used in this expression system, CSH26ΔF6, carries a deletion removing the *recA* gene making the cells highly sensitive to UV light. The method was based on that of Sancar *et al.* (1979), who found that a low UV dose would cause extensive chromosomal degradation in *recA* strains, whereas high copy-number plasmids in such strains would be less likely to receive a UV-induced lesion and would mostly remain intact.

Plasmid-containing CSH26ΔF6 strains were grown overnight in K medium (minimal medium containing 180μM proline) containing appropriate antibiotics. The overnight cultures were diluted 1:100 into 10ml K medium and grown at 37°C, with aeration, to an A_{450} of 0.5 (approximately 2 x 10^8 cells per ml). 2.5ml of the culture was irradiated with 3.7Jm^{-2} of UV light and 2ml of the irradiated culture was transferred to a foil-covered tube and shaken at 37°C. After 60 min, cycloserine was added to a final concentration of 0.2$mgml^{-1}$ to kill any growing cells. After overnight incubation, a further aliquot of cycloserine was added and the cells incubated for 60 min at 37°C, before harvesting at 12000g for 3 min. The pellet was washed in 1ml minimal medium containing DMA and resuspended in 1ml of the same medium. After 60 min incubation at 37°C, 1.11MBq of [^{35}S]methionine was added and the polypeptides labelled for 60 min at 37°C before the addition of methionine to a final concentration of 1.4mM and incubation for 5 min. The cells were pelleted at 12000g for 5 min and the pellet resuspended in 50μl PB and 50μl SDS-PAGE sample buffer.

2.10.3. Fractionation of maxicells

Maxicells were prepared and labelled as described in section 2.10.2., finally resuspending the cells in 450μl minimal medium containing DMA. A 50μl sample of the labelled cells was removed and kept as the "total" sample. The remaining 400μl were added to 8ml ice-cold 10mM sodium phosphate buffer (pH 7.2) and sonicated, at 6μm amplitude using a 19mm end diameter probe in a 150 Watt MSE ultrasonic disintegrator, for 3 x 30 sec, alternating with 30 sec on ice. The sonicated samples were centrifuged at 4400g for 20 min at 4°C and the supernatant immediately removed to an ultracentrifuge tube. The tubes were filled with sodium phosphate buffer (pH 7.2), and centrifuged at 224000g for 2 h at 4°C. Both the supernatant and the pellet were retained. The supernatant was removed, ice-cold TCA added to 0.5M and kept on ice for at least 30 min before centrifuging at

3000g for 5 min at 5°C. The resultant pellet, which contains the cytoplasmic and periplasmic fractions, was drained and resuspended in 200µl sodium phosphate buffer (pH 7.2).

The pellet from the ultracentrifugation was resuspended in 100µl sodium N-lauryl sarcosinate (5gl^{-1}) by freezing at -20°C and vortexing whilst thawing. After 30 min at 20°C the resuspended membrane was transferred to an ultracentrifuge tube and centrifuged at 110000g for 2 h at 5°C. The supernatant, which contains the solubilised inner membrane fraction, was removed to a separate tube. The pellet, which contains the outer membrane fraction, as resuspended in 100µl sodium phosphate buffer (pH 7.2) as described above.

To each fraction an equal volume of SDS-PAGE sample buffer was added and loaded onto a SDS-polyacrylamide gel in the ratio of 1:2:2:2 to represent approximately equivalent amounts of the total:cytoplasmic:inner membrane: outer membrane fractions, respectively.

2.10.4. Osmotic shock of maxicells

This method, based on that of Nossal and Heppel (1966), was used to separate the periplasmic fraction from the other cellular fractions. Maxicells were prepared and labelled as described in section 2.10.2. Half the labelled cells were pelleted, resuspended in 20µl PB and retained as the "total" sample. The remainder of the cells were pelleted at 12000g for 3 min and gently resuspended over 10 min in 125µl 30mM Tris-HCl (pH 8.0) containing 1mM EDTA and 0.6mM sucrose. The cells were then centrifuged as above at 4°C, and osmotically shocked by gently resuspending in 310µl ice-cold water over 10 min at 4°C. After centrifugation at 12000g for 4 min at 4°C, the pellet, which contains the membrane and cytoplasmic fractions, was resuspended in 20µl PB and the supernatant was concentrated by adding ice-cold TCA to 0.6M and keeping on ice for 30 min before centrifuging at 12000g for 1 min. The resultant pellet was drained and resuspended in 15µl PB. An equal volume of SDS-PAGE sample buffer and, where necessary, enough saturated Tris-HCl solution to restore the blue colour, was added to each sample before loading 10µl onto a SDS-polyacrylamide gel for analysis.

2.10.5. Whole cell protein labelling

Overnight cultures of strains in DMA were diluted 100-fold in the same medium and grown to an absorbance A_{450} of 0.2 (~2 x 10^8 cells/ml) at the appropriate temperature. 1ml culture was diluted in 1ml fresh DMA medium and 2.8MBq [^{35}S]methionine added. After 30 min incubation at a temperature appropriate to the growth of the cells, methionine was added to 0.7mM as a chase for 10 min. Cells were pelleted at 12000g for 3 min and washed in 10mM sodium phosphate buffer (pH 7.2) before resuspending the cells in 50µl PB and 50µl SDS-PAGE sample buffer.

2.10.6. SDS-polyacrylamide gel electrophoresis

(A) Preparation and running of gels

The procedure used was based on that of Laemmli (1970), using a Raven vertical gel kit (IN/96). The buffers for the separating gel (buffer A) and the stacking gel (buffer B) were made according to the recipes in Table 2.5 and the pH adjusted using concentrated hydrochloric acid. The acrylamide solution (Table 2.5) was treated with a small amount of activated charcoal for 60 min, filtered through 2 layers of Whatman No.1 filter paper, and stored in the dark at 4°C. Ammonium peroxydisulphate (Eastman Kodak Co., New York) was freshly prepared as a 10mgml^{-1} solution in distilled water.

Solutions were mixed, avoiding aeration, adding the TEMED (N,N,N',N'-tetramethylethylenediamide, Eastman Kodak Co.) immediately before pouring the gel. Gels were 1 mm thick and consisted of a 10% polyacrylamide plug, a separating gel of the appropriate percentage and a 7% polyacrylamide stacking gel of at least 1 cm distance between the sample wells and the separating gel (Table 2.6). All samples were boiled with an equal volume of SDS-PAGE sample buffer (Table 2.5) for 2 min prior to loading. To avoid lateral spread of the samples, unlabelled protein prepared from 0.5 A_{600} units of cells, was loaded into the outside gel slots. Electrophoresis was carried out in running buffer (Table 2.5) at 25mA from an LKB 2197 power pack until the dye front entered the gel plug. Gels were then fixed by gently swirling for 30 min in destain (Table 2.5) prior to fluorography.

Radioactively-labelled molecular weight markers (Amersham) consisted of a

Table 2.5 Composition of SDS-polyacrylamide gel solutions

Solution	Component	Concentration
Acrylamide (44:0.8)	Acrylamide	440gl^{-1}
	Bis-acrylamide	8gl^{-1}
Buffer A	Tris-HCl pH 8.8	750mM
	SDS	2gl^{-1}
Buffer B	Tris-HCl pH 6.8	250mM
	SDS	2gl^{-1}
Running Buffer	Tris-HCl pH 8.3-8.6	25mM
	SDS	1.9gl^{-1}
	Glycine	192mM
Sample Buffer	Tris-HCl pH 6.8	62.5mM
	SDS	20gl^{-1}
	Glycerol	100gl^{-1}
	2-mercaptoethanol	50gl^{-1}
	Bromophenol blue	a pinch
Destain	Glacial acetic acid	100gl^{-1}
	Propan-2-ol	250gl^{-1}

Table 2.6 Composition of SDS-Polyacrylamide gels

Component	Plug	Separating gel			Stacking
	10%	8.5%	10%	13%	gel 7%
Acrylamide 44:0.8	12.5 ml	4.83 ml	5.68 ml	7.38 ml	3.3 ml
Buffer A	25.0 ml	12.5 ml	12.5 ml	12.5 ml	
Buffer B					10 ml
Water	10.75 ml	6.72 ml	5.88 ml	4.2 ml	6.7 ml
Ammonium peroxydisulphate	1.75 ml	0.88 ml	0.88 ml	0.88 ml	0.5 ml
TEMED	140 µl	70 µl	70 µl	70µl	40µl

[2-^{14}C]methylated protein mixture of myosin (~200 kD), phosphorylase B (100 and 92.5 kD), bovine serum albumin (69 kD), ovalbumin (46 kD), carbonic anhydrase (30 kD) and lysozyme (14.3 kD). 1 kBq of this mixture, with an equal volume of SDS-PAGE sample buffer, was loaded onto gels.

(B) Fluorography and autoradiography of gels

Fixed gels were gently swirled for 60 min in two changes of 300ml DMSO and for a further 60 min in 300ml 220gl^{-1} PPO (2,5-diphenyloxazole) dissolved in DMSO, before swirling the gel in 300ml water for 30 - 60 min. The gel was dried onto Whatman 3MM chromatography paper using a Biorad slab gel drier (model 224) and the dried gels placed in a cassette with a sheet of Kodak X-Omat RP X-ray film and exposed at -80°C. After exposure, the cassette was thawed and the film developed using Kodak DX-80 developer, a 1% glacial acetic acid stop and Kodak FX-40 fixer.

2.11. Analysis of donor-encoded polypeptides retained by recipient cells during ColIb-specified conjugation

The donor-specified polypeptides retained by recipient cells during conjugation between phage T6-sensitive BW96 donor cells and T6-resistant BW40 recipient cells were visualised using the method based on that used by Chatfield and Wilkins (1984). Overnight cultures of recipient and donor strains, grown at 37°C in SGC and SGA (SGC except Casamino acids were replaced with a mixture of all amino acids excluding methionine), respectively. The overnight cultures of donor and recipient strains were diluted in 10ml of the same media to an absorbance A_{600} of 0.05 and grown, with aeration at 37°C, to A_{600} of 0.5 and 0.3, respectively. The recipient cells were centrifuged at 2000g for 10 min before being resuspended in 20ml PB and irradiated with 400Jm^{-2} of UV light. The cells were pelleted as above and resuspended, to an absorbance A_{600} of 0.35, in SGC containing 24µM thymine and 200µgml^{-1} Rif. The cells were allowed to stand for 10 min before use, to inactivate RNA polymerase. Once grown, 2ml of the donor strain cultures was transferred to prewarmed 150ml Erlenmeyer flasks at 37°C containing 2.78MBq of [^{35}S]methionine and shaken at 100 rpm for 15 min prior to the addition of methionine to 0.7mM as a chase for 5 min. 1.5ml of the labelled culture was then transferred to a fresh 150ml flask at 37°C, containing 1.5ml irradiated, Rif-treated recipient cells, and conjugation allowed to occur for 30 min.

After this time, the donor cells were selectively lysed with T6 phage as described previously (Wilkins et al., 1971). 2.5ml mating mixture was transferred to a polycarbonate tube containing 10^{11} pfu T6 phage. The phage had previously been irradiated with $80Jm^{-2}$ of UV light, to prevent the formation of host-range mutants, and resuspended in 0.5ml T2 adsorption buffer (minimal medium containing $10gl^{-1}$ gelatin and $20\mu gml^{-1}$ tryptophan). Donor cell lysis was allowed to occur for 5 min at 37°C before the addition of Tris-HCl (pH 8.0), DNase and RNase A to 50mM, $150\mu gml^{-1}$ and $100\mu gml^{-1}$, respectively. After a further 5 min incubation, protease K was added to $50\mu gml^{-1}$ and EDTA to 5mM. After 20 min at 37°C, Brij 58 was added to $5mgml^{-1}$ and the lysate left on ice for 5 min. 1ml BW40 carrier cells, which had been grown to A_{600} of 0.5 in SGC and irradiated with $500Jm^{-2}$ UV light, was added to encourage the recipient cells to form a pellet and the samples were centrifuged at 5900g for 5 min at 4°C. The pellet was washed three times in 2.5ml PB before being finally resuspended in 50µl PB and an equal volume of SDS-PAGE sample buffer. The donor-encoded labelled polypeptides retained by the recipient cells were analysed on SDS-polyacrylamide gels. The controls used in this experiment are discussed in Chapter 6.

CHAPTER THREE

CONSTRUCTION AND CHARACTERISATION OF PLASMIDS

3.1. Introduction

My study of the role of plasmid primase has involved tests for complementation between a primase-defective mutant of the conjugative plasmid and a cloned primase gene, both in the donor strain as well as in a variety of recipient strains. As discussed in Chapter one, the primase-defective derivatives of RP4 and ColIb-P9 required for such a study were available, as was the cloned ColIb primase gene, but the RP4 primase gene had not previously been cloned. The cloning of the gene is the subject of this chapter.

3.2. Choice of cloning vector

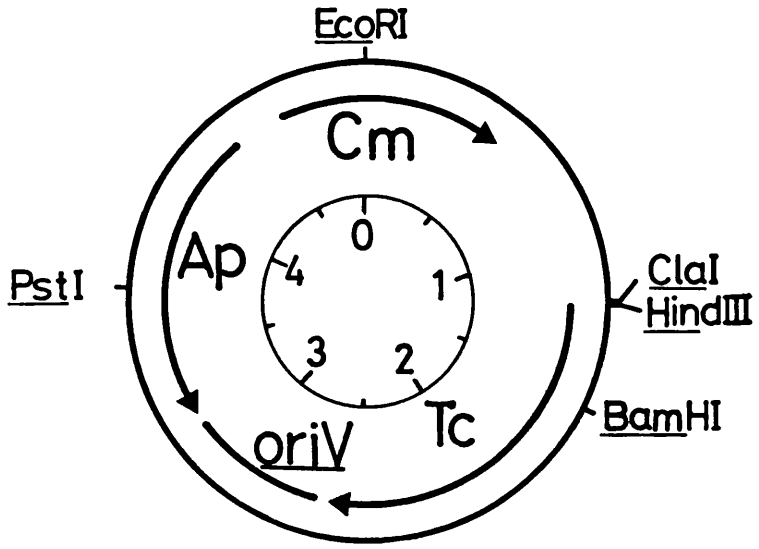
Lanka and Barth (1981) observed that the transfer-deficiency of a primase-defective (Pri$^-$) derivative of RP4 depended on the species used as recipient in the mating. Therefore to study complementation, it would be necessary to clone the RP4 primase gene into a vector capable of being maintained in a wide diversity of bacterial species. The plasmid R300B, having a broad host range and being maintained stably in all Gram-negative bacterial species, was suitable (Barth and Grinter, 1974; Barth et al., 1981). This IncQ plasmid, approximately 8.6kb in size, has a moderately high copy number of ~10 (Barth and Grinter, 1974) and has convenient restriction endonuclease cleavage sites for cloning, many of which fall within the genes encoding resistance to sulphonamide and streptomycin (Figure 3.1).

For complementation tests in the donor, it was important to use a non-mobilisable vector to prevent the transfer of the active primase gene into the recipient cells during mating. The R300B plasmid is mobilised very efficiently by RP4 (Barth et al.,1981), and with moderate efficiency by ColIb-P9 (Hedges and Datta, 1972; Guerry et al., 1974; Willetts and Crowther, 1981; Chapter 7) making it unsuitable for use in donor strains. However, the vector pBR328 is very poorly mobilised by RP4 and ColIb-P9, presumably because it lacks the ColEI nic site thought to be the origin of transfer site used in mobilisation (Warren et al., 1978; Soberon et al., 1980). This vector encodes resistance to three antibiotics, including Cm

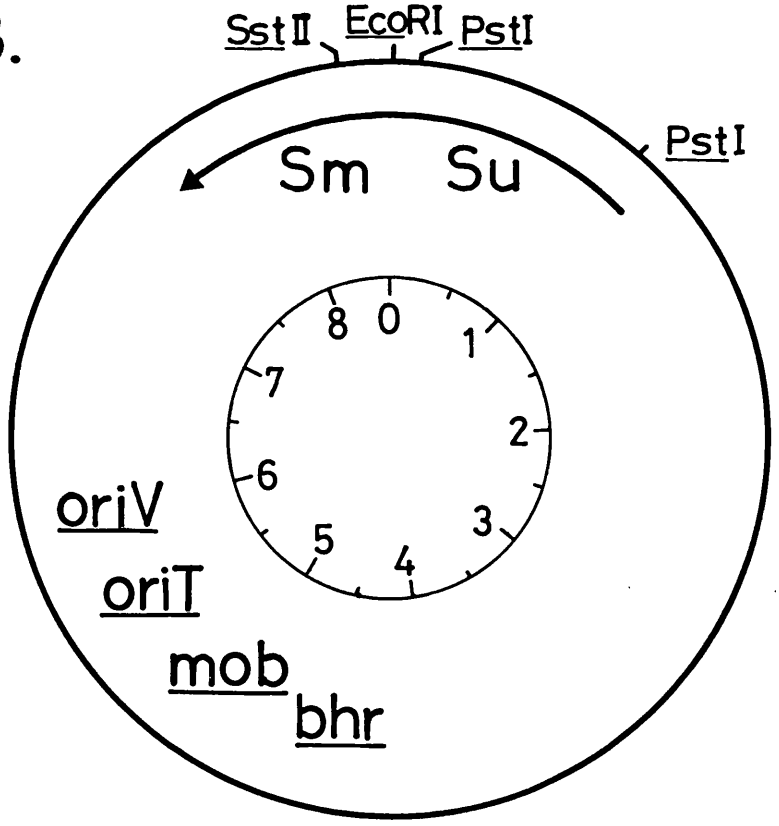
Figure 3.1 Restriction cleavage maps of vector plasmids

Restriction maps of plasmids pBR328 (A; Soberon *et al.*, 1980) and R300B (B; Barth *et al.*, 1981). Only those sites appropriate to the work in this thesis are included. The inner circle represents the size of the plasmid, in kilobases (kb). The direction of transcription and extent of antibiotic resistance genes are represented by arrows. The origin of vegetative replication (*oriV*), the origin of transfer (*oriT*), the mobilisation genes (*mob*) and the genes responsible for broad-host range (*bhr*) are also indicated.

A.


EcoRI
Cm
Ap
PstI
ClaI
HindIII
BamHI
oriV
Tc
0
1
2
3
4

B.


SstII
EcoRI
PstI
PstI
Sm
Su
oriV
oriT
mob
bhr
0
1
2
3
4
5
6
7
8

which would allow the vector and its derivatives to be distinguished from RP4 when present in the same strain.

3.3. Source of primase gene for DNA cloning

RP4 has very few known restriction endonuclease cleavage sites in the regions essential for the transfer and maintenance of the plasmid, including the region containing the primase gene (see Lanka *et al.*, 1983). By using a series of RP4::Tn*7* derivatives (Barth and Grinter, 1977), the cloning of the primase gene could be achieved by using restriction sites within Tn*7* (Gostu-Testi *et al.*, 1983). At the time of this work, the position of the primase gene had been narrowed down to a 5kb region of RP4 within which Tn*7* insertions caused a severe reduction in primase activity, presumably by insertional inactivation or polar effect (Lanka and Barth, 1981). A derivative of RP4 carrying Tn*7* at a position just clockwise of this region, pRP26, specified abnormally high primase activity in an *in vitro* assay (Lanka and Barth, 1981). The high activity encoded by this derivative would facilitate the selection of the desired recombinant clones and would be ideal for use in complementation tests. This RP4 derivative was therefore used as the source of the primase gene. The transposon insertion in pRP26 was originally mapped to coordinate 42.35 on the 56kb RP4 map (Barth and Grinter, 1977; Lanka and Barth, 1981), but was later repositioned to coordinate 49.7 (see later section) on the 60kb map (Lanka *et al.*, 1983).

3.4. Cloning the RP4 primase gene into the vector pBR328

The strategy involved the insertion of the *Hin*dIII fragment carrying the primase gene from pRP26 into the *Hin*dIII site of pBR328. Use of this site enabled the promoter of the Tc^r gene to express the primase gene, should its native promoter be absent from the RP4 fragment. The Cm^r recombinants obtained would be screened for sensitivity to Tc and for the ability to suppress the temperature-sensitive mutation in the host primase gene (*dnaG3*) harboured by the strain BW86. At the restrictive temperature (40°C), the mutant bacterial strain fails to form colonies, but plasmid primase can suppress this mutation (Wilkins, 1975; Lanka and Barth, 1981), so providing a useful screening procedure.

The published restriction endonuclease cleavage maps predict that pRP26, cleaved by *Hin*dIII, would produce four fragments of ~55, 9.8, 2.55 and

2.1kb; the 9.8kb fragment carrying the primase gene (Barth and Grinter, 1977; Barth, 1979; Lanka and Barth, 1981; Gosti-Testi et al., 1983). However, HindIII digestion of this plasmid yielded fragments of ~50, ~15, 2.7 and 2.2kb (Figure 3.2), indicating that the fragment carrying the primase gene was larger than predicted. As explained in a later section, this was due to the imprecise sizing of the RP4 DNA fragments by Barth and Grinter (1977).

Cleaved fragments generated by HindIII digestion of pBR328 and pRP26 were ligated and used to transform BW86. Cm^r transformants were selected at 30°C (permissive temperature for BW86) and allowed to form small colonies. These were replica plated, and screened for sensitivity to Tc, and for the ability to form colonies at 40°C (Ts^+). Transformants of this phenotype were purified on agar containing Cm. Many of the colonies that appeared Ts^+ on the replica plates were not able to grow at 40°C when purified. The reason for this is unclear, but may reflect cell density. Plasmid DNA prepared from purified clones that remained Tc^s Ts^+ was used to transform BW86. Most transformants retained the ability to form colonies at 40°C, thereby discounting the possibility of reversion of the host mutation. One such recombinant, pAM3, was choosen for further study.

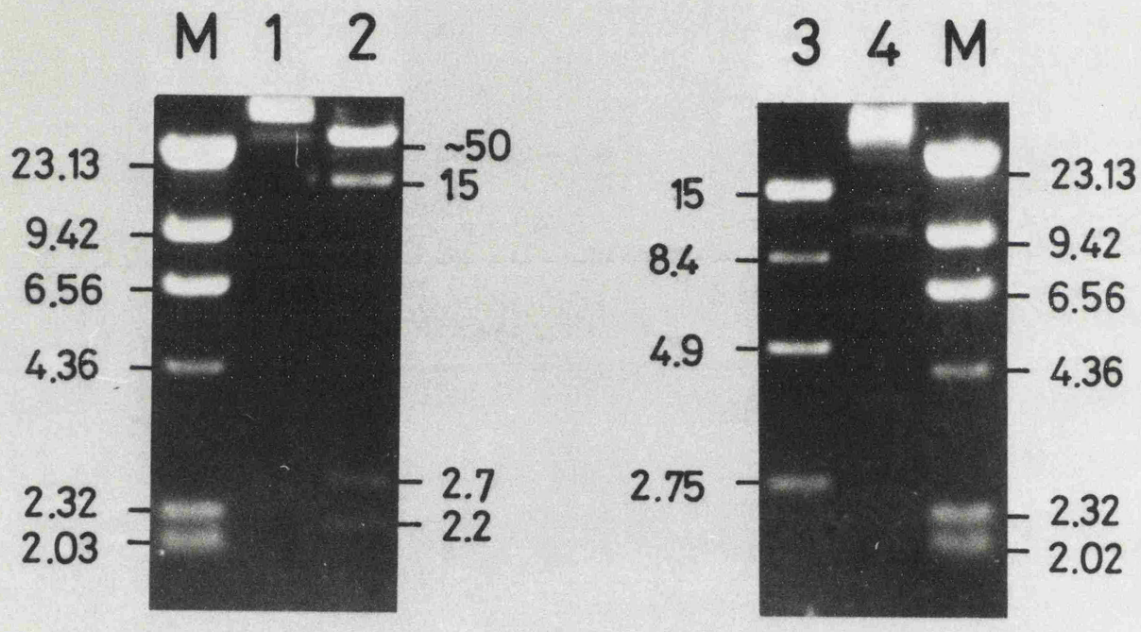
Plasmid pAM3 DNA was digested with HindIII and the generated fragments were compared with those derived from HindIII-digested pRP26 (Figure 3.2). The digest of pAM3 DNA yielded four fragments, the vector fragment (4.9kb), two fragments of ~15 and 2.75kb, which also appeared in the digest of pRP26 DNA, and an unexpected fragment of 8.4kb. The origin of the 8.4kb fragment was unclear. One possibility was that it was a deletion product of the ~15kb fragment, and that pAM3 DNA was a mixture of two subpopulations of plasmid. This theory was substantiated by the disproportionate brightness of the vector fragment (4.9kb) relative to the other fragments. In an attempt to separate the two plasmid populations, pAM3 DNA was used to transform BW86. All the resultant transformants were of the phenotype Ap^r Cm^r Tc^s, but only 10% suppressed the dnaG3 mutation. Four recombinant plasmids isolated from transformants which suppressed dnaG3 were shown to yield fragments of ~15, 4.9 and 2.75kb, but not 8.4kb, when digested with HindIII. One such recombinant was named pAM3-4. Thus it was concluded that the pAM3 preparation was a mixed population of plasmids which carried either the 8.4kb or ~15kb HindIII fragment, and only those with the larger fragment were shown to carry an active primase gene.

Figure 3.2 HindIII-generated restriction fragments of pRP26 and pAM3

Mobility of DNA fragments through 0.7% agarose. Preparation of DNA samples and digestion with restriction endonucleases are described in section 2.7. Lanes, loaded with approximately 0.5µg of DNA, contain:

(1) Uncleaved pRP26
(2) pRP26 x HindIII
(3) pAM3 x HindIII
(4) Uncleaved pAM3

Molecular weight markers (lane M) are λ x HindIII DNA fragments. The sizes of the fragments are indicated in kilobases (kb).


M 1 2
23.13
9.42
6.56
4.36
2.32
2.03
~50
15
2.7
2.2
3 4 M
15
8.4
4.9
2.75
23.13
9.42
6.56
4.36
2.32
2.02

In order to remove the unwanted 2.75kb fragment derived from Tn*7* from pAM3-4, the plasmid was digested with *Hin*dIII and ligated with *Hin*dIII-cleaved pBR328 DNA. The ligated DNA was used to transform BW86, and Ap^r Cm^r Tc^s Ts^+ transformants were isolated as described above. Digestion with *Hin*dIII of DNA prepared from such recombinants, revealed some which carried only the desired fragment of ~15kb, as well as the vector fragment. One such recombinant was named pLG260. The mapping of cleavage sites for *Eco*RI, *Hin*dIII, *Sst*II, *Bgl*II, *Pst*I and *Bam*HI determined that the DNA fragment cloned was 13.9kb in size and was bounded at one end by Tn7 DNA (Figure 3.3). The mapping also showed that the primase gene was orientated such that it could not be expressed from the promoter of the Tc^r gene, suggesting that a promoter within the cloned fragment was responsible for the expression of the primase gene.

3.5. Cloning the RP4 primase gene using R300B as vector

The broad host range vector plasmid, R300B, was used to enable the RP4 primase gene to be maintained in many host bacterial species. Since this plasmid carries no *Hin*dIII cleavage site (Figure 3.1), a different cloning strategy to that used for pLG260 was required. This involved the ligation of the *Pst*I fragment carrying the primase gene from pRP26 into *Pst*I-cleaved R300B. The *Pst*I fragment derived from pRP26 carries the kanamycin-resistance gene, which would allow the easy selection of the desired recombinant, thereby overcoming the problem of 'false-positives' encountered when using suppression of the *dnaG3* lesion in BW86 as the selection for pLG260.

Plasmid pRP26 cleaved with *Pst*I, was predicted to generate seven fragments (~32, ~20, 8.1, 5.8, 2.5, 0.8 and 0.6kb), the 8.1kb fragment carrying the *pri* gene. The digest (Figure 3.5) shows five of these fragments (~30, ~22, ~12.5, 6.4 and 2.8 kb), the smallest two being too faint to see. As observed in the *Hin*dIII digest of pRP26, the fragment thought to carry the primase gene was larger than expected. R300B DNA was cleaved with *Pst*I, yielding the expected 7.8 and 0.8kb fragments, and treated with alkaline phosphatase in an attempt to limit the inclusion of the small fragment into the recombinants, and to prevent self-ligation of the vector. The cleaved vector and pRP26 DNA fragments were ligated and used to transform BW86 before allowing the Km^r transformants to form small colonies at 30°C. Twelve of the resultant colonies were replica plated before overnight incubation at 40°C. Plasmid DNA, prepared from those

Figure 3.3 Restriction analysis of plasmid pLG260

A. Mobility of restriction fragments of pLG260 through 0.7% agarose. Fragment sizes are indicated below, C. Each lane contains approximately 0.5μg of DNA. Numbers to right indicate molecular weight (kb) of λ x HindIII DNA fragments in lane M.

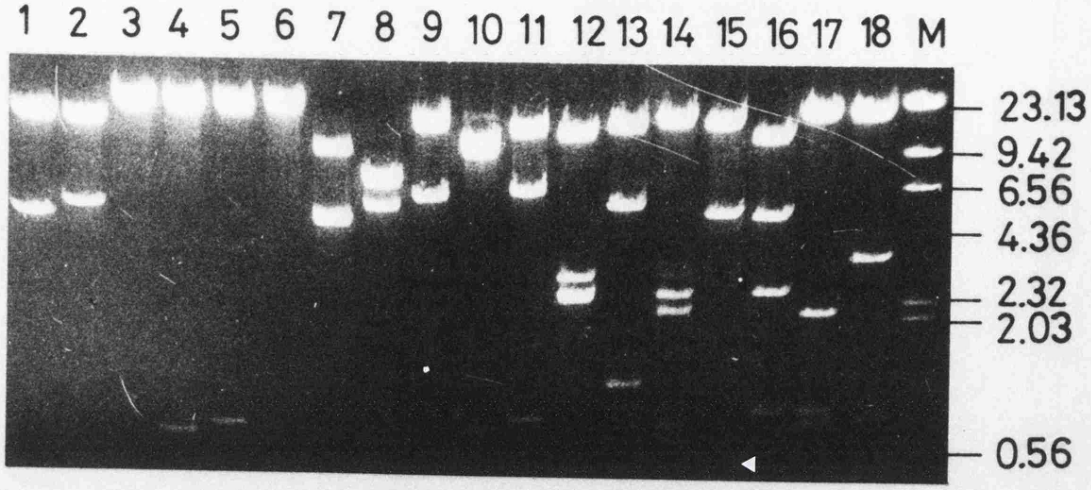
B. Restriction map of plasmid pLG260 derived from results shown in A. The bold line shows the extent of pBR328 vector DNA and light line indicates pRP26 DNA inserted fragment. Inner circle shows scale in kilobases and arrows represent the extent and direction of transcription of the genes indicated. Dotted arrows indicate the extent of disrupted genes. The RP4 primase gene (*pri*), origin of vegetative replication (*oriV*) and fragment of transposon Tn*7* are shown.

C. Molecular weights of DNA fragments shown in A.

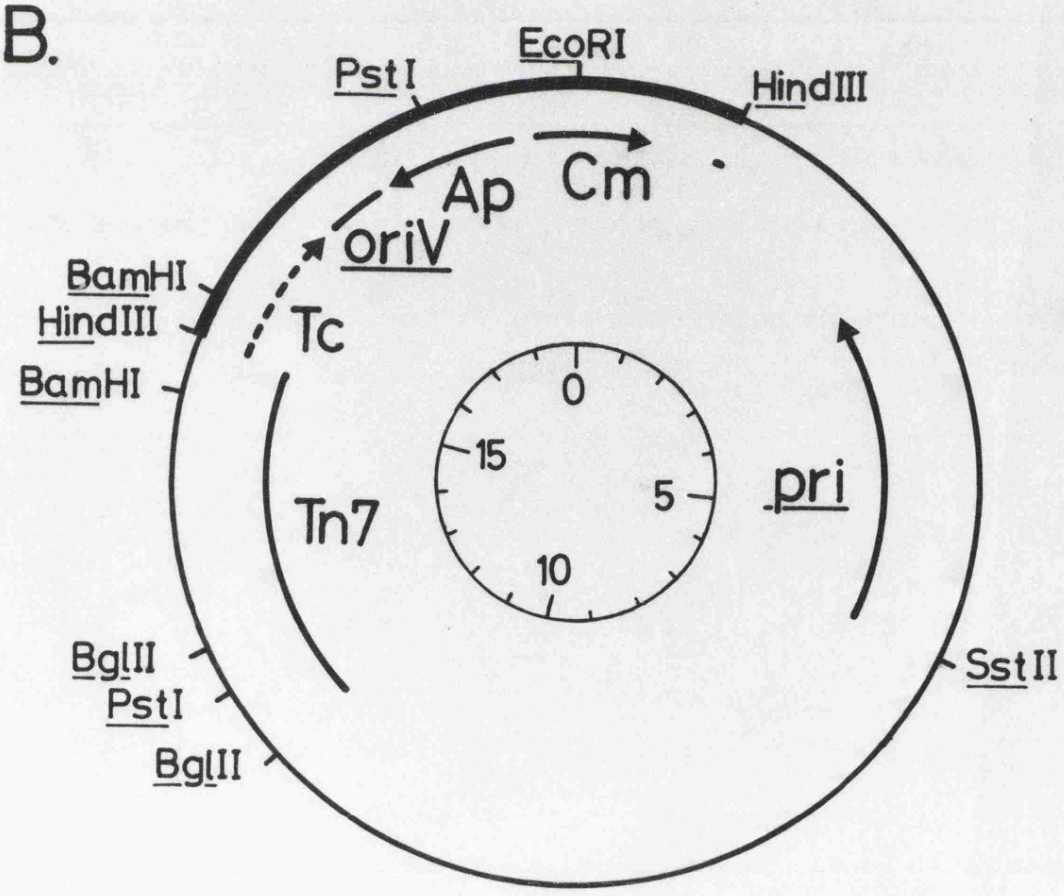
Enzyme	HindIII	PstI	EcoRI	BamHI	BglII	SstII	SstII HindIII	SstII PstI	SstII EcoRI
Lane	1	2	3	4	5	6	7	8	9
	~14.0	~13.5	~19.0	~18.0	~18.0	~19.0	9.0	6.7	~13.0
	4.8	5.2		0.7	0.8		4.8	6.3	5.6
							4.6	5.0	

Enzymes	SstII BamHI	SstII BglII	PstI HindIII	PstI EcoRI	PstI BamHI	PstI BglII	BglII HindIII	BglII BamHI	BamHI EcoRI
Lane	10	11	12	13	14	15	16	17	18
	~9.5	~12.0	~11.0	~12.0	~14.0	~13.0	~11.0	~16.0	~16.0
	8.7	5.8	2.82	5.0	2.5	4.8	4.7	2.2	3.3
	0.7	0.8	2.5	1.2	2.15	0.5	2.55	0.8	0.8
			2.35		0.8	0.3	0.8	0.7	

A.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 M
23.13
9.42
6.56
4.36
2.32
2.03
0.56

B.


PstI
EcoRI
HindIII
Ap
Cm
oriV
BamHI
HindIII
BamHI
Tc
0
15
5
10
Tn7
pri
BglII
PstI
BglII
SstII

isolates able to suppress the dnaG3 mutation, was shown to carry the ~12.5kb PstI fragment believed to contain the primase gene. The cleavage map of one such Km^r Ts^+ recombinant, named pLG261, was determined (Figure 3.4). The 12.3kb insert was orientated such that the promoter of the Su⁻Sm operon could allow expression of the primase gene, but it is likely that the primase gene is expressed from an RP4-derived promoter (see previous section).

3.6. Characterisation of plasmids pLG260 and pLG261

The restriction endonuclease cleavage maps of pLG260 and pLG261 showed that the HindIII, SstII, BglII and PstI sites have the some relative positions within the fragment cloned, indicating that the plasmids carried approximately the same region of RP4 DNA. However, both the cloned PstI and HindIII fragments were about 4.0kb larger than expected from the published maps (Barth and Grinter, 1977; Barth, 1979; Lanka and Barth, 1981; Gostu-Testi et al., 1983). To establish that the DNA used in the cloning was bona fide pRP26 DNA, it was compared with plasmid DNA isolated from an independently obtained strain harbouring pRP26, and with two other RP4 derivatives, pRP46 and pRP76, which have Tn7 insertions mapping close to that of pRP26 (Barth and Grinter, 1977). The two independently obtained isolates of pRP26 showed no differences in fragment size, indicating that the correct plasmid was used for the cloning (Figure 3.5).

The coordinates of the Tn7 insertion in pRP26, pRP46 and pRP76 were found to be 49.7, 52.7 and 51.2kb respectively, based on the 60kb RP4 map (Lanka et al., 1983). The relative order of these three insertions is in agreement with Barth and Grinter (1977), but the actual coordinates differ by between 4.7 and 7.4kb. This difference can be reconciled by comparison of the RP4 map published by Barth and Grinter (1977) and that of Lanka et al. (1983). This reveals several small differences, including an additional 4.7kb immediately clockwise of the primase gene. This difference can be partially attributed to the Tn7 map used by Barth and Grinter (1977), which shows only two HindIII sites rather than three (Gostu-Testi et al., 1983). This explains why the fragments cloned from pRP26 were larger than originally expected. A recent analysis of the primase gene (Lanka et al., 1984) also maps the Tn7 insertion carried by pRP26 to the same coordinate as calculated here and positions the primase gene 6.5kb from this insertion, between coordinates 40.3-43.5kb.

Figure 3.4 Restriction analysis of plasmid pLG261

A. Restriction map of plasmid pLG261 derived from results shown in B. The bold line shows the extent of R300B vector DNA and the light line indicates pRP26 DNA inserted fragment. Inner circle shows scale in kilobases and arrows represent the extent and direction of transcription of genes. Dotted arrows indicate the extent of disrupted genes. The RP4 primase gene (*pri*), origin of transfer (*oriT*), broad host range genes (*bhr*) and fragment of transposon Tn*7* are shown.

B. Molecular weights of DNA fragments obtained by restriction enzyme cleavage of pLG261.

Enzyme	PstI	EcoRI	SstII	HindIII	BglII
	~12.5	~20.0	~14.0	~20.0	~20.0
	7.8		5.8		

Enzyme	PstI SstII	PstI BglII	PstI HindIII	SstII BglII	SstII HindIII
	7.5	~12.0	~11.0	7.9	~14.0
	6.5	7.8	7.8	5.8	4.6
	5.9	0.5	1.0	5.8	1.2
	0.5				

A.

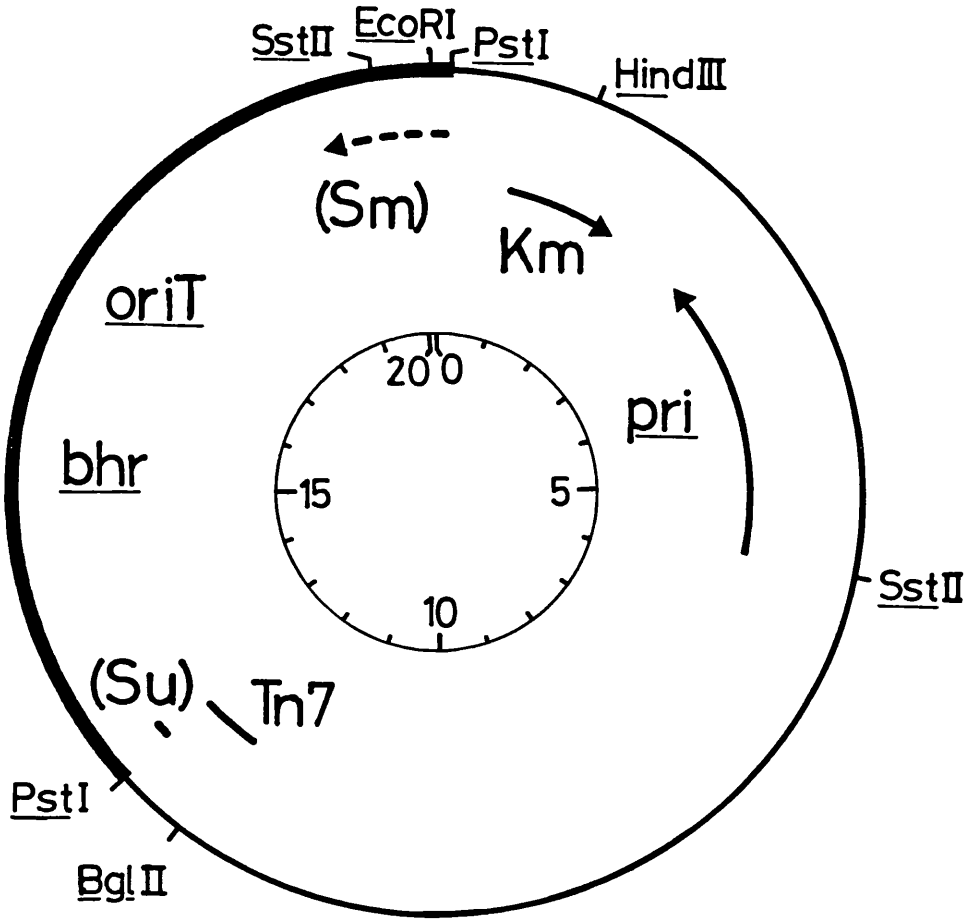

SstII
EcoRI
PstI
HindIII
(Sm)
Km
oriT
pri
bhr
20
0
15
5
10
SstII
(Su)
Tn7
PstI
BglII

Figure 3.5 Comparison of restriction fragments derived from RP4::Tn*7* derivatives

A. RP4::Tn*7* derivative plasmid DNA was cleaved with restriction enzymes and electrophoresed through a 0.5% agarose gel. Fragment sizes are indicated below, C. Each lane contains approximately 0.5µg of DNA. Numbers (kb) to the left indicate molecular weights of λ x *Hin*dIII DNA fragments in Lane M.

B. Restriction map of plasmid RP4 between coordinates 37 and 54, according to Lanka *et al.* (1983). The positions of the Tn*7* insertions in RP4 derivatives pRP26, pRP46 and pRP76 are derived from results shown in A and are represented by triangles. For clarity, the relevant features of Tn*7* are shown beneath and to the same scale. The genes encoding resistance to streptomycin/spectinomycin and trimethoprim are represented by Sm/Sp and Tp, respectively. The arrows represent the extent and direction of transcription of the genes indicated. The primase gene (*pri*) is part of the transcriptional unit shown by the dotted arrow (Lanka *et al.*, 1984).

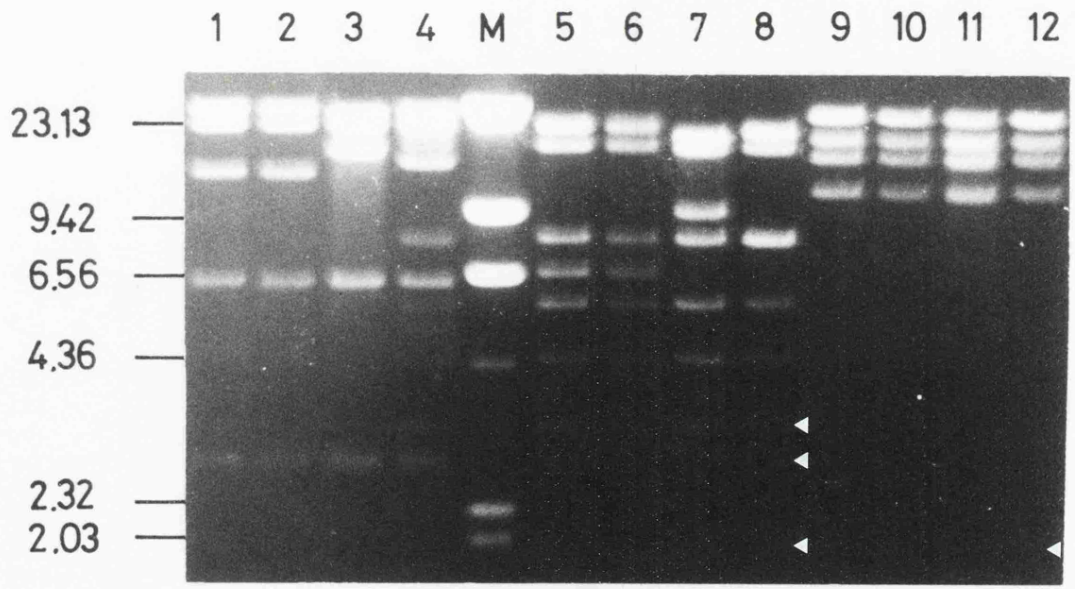
C. Molecular weights of DNA fragments shown in A.

Plasmid	pRP26	pRP26N	pRP46	pRP76	pRP26	pRP26N	pRP46	pRP76
Enzyme	*Pst*I	*Pst*I	*Pst*I	*Pst*I	*Pst*I *Sst*II	*Pst*I *Sst*II	*Pst*I *Sst*II	*Pst*I *Sst*II
Lane	1	2	3	4	5	6	7	8
	~32.0	~32.0	~28.5	~31.0	~23.0	~23.0	~19.5	~21.0
	~22.0	~22.0	~23.0	~23.0	~17.5	~17.5	~17.0	~17.0
	~12.5	~12.5	~16.5	~14.5	8.2	8.2	9.6	8.1
	6.4	6.4	6.4	6.4	6.6	6.6	8.1	8.1
	2.8	2.8	2.8	2.8	5.7	5.7	5.7	5.8
					4.4	4.4	4.4	4.4
					3.2	3.2	3.2	3.2
					2.8	2.8	2.8	2.8
					1.9	1.9	1.9	1.9

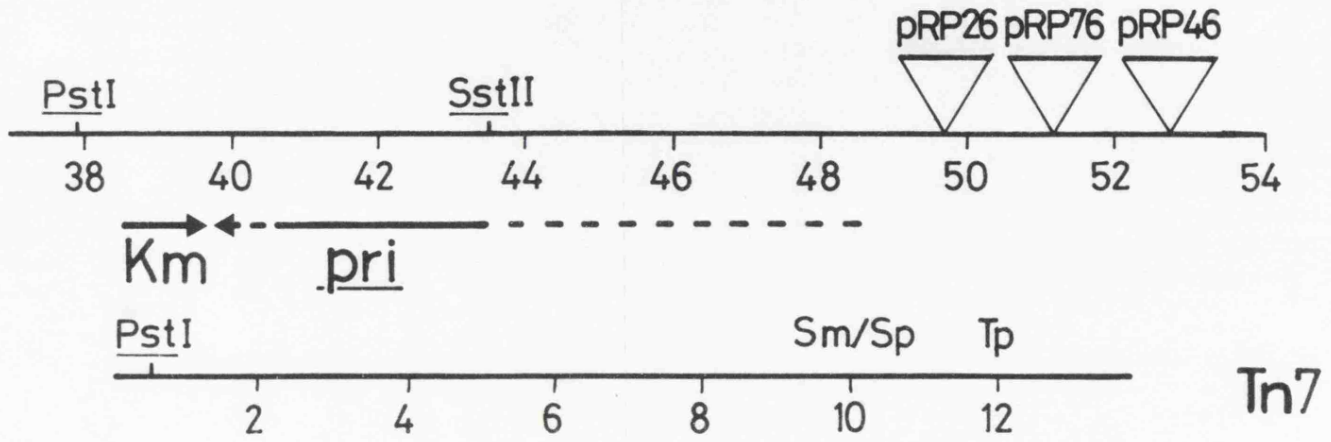
Plasmid	pRP26	pRP26N	pRP46	pRP76
Enzyme	*Sst*II	*Sst*II	*Sst*II	*Sst*II
Lane	9	10	11	12
	~28.0	~28.0	~28.0	~28.0
	~19.5	~19.5	~19.5	~19.5
	~15.5	~15.5	~15.5	~15.5
	~11.5	~11.5	~11.5	~11.5
	1.95	1.95	1.95	1.95

Plasmids pRP26 and pRP26N represent separately derived sources of the plasmid (see text).

A.


1 2 3 4 M 5 6 7 8 9 10 11 12
23.13
9.42
6.56
4.36
2.32
2.03

B.


pRP26 pRP76 pRP46
PstI
SstII
38 40 42 44 46 48 50 52 54
Km
pri
PstI
Sm/Sp
Tp
2 4 6 8 10 12
Tn7

The activity of the primase specified by pLG260 and pLG261 was measured using three assay systems. The amount of chromosomal DNA synthesis promoted by plasmid primase in a dnaG3 mutant can be assessed *in vivo*, either by measuring the incorporation of a radioactively-labelled thymine, or less directly, by measuring the ability of plasmid primase to suppress the temperature-sensitive host primase mutation so allowing the formation of colonies at the restrictive temperature. Alternatively, an *in vitro* assay system based on the ability of the plasmid primase to initiate complementary DNA synthesis on a single-stranded phage template can be used.

The *in vitro* assay is a more sensitive, but potentially more difficult method. For accuracy, the amount of plasmid primase must be the limiting factor in the reaction and it is therefore necessary to dilute the primase-containing crude cell extract (CCE) to achieve this. A pilot experiment was performed using the CCE prepared from BW86 harbouring pLG260. The amount of radioactivity incorporated was plotted against µg protein added to the reaction. This shows that RP4 primase began to saturate the system if more than 1µg protein was added, as shown by the non-linear section of the graph (Figure 3.6), and therefore 0.5µg protein in the CCE was used as the standard amount added to the assay. Some difficulties were experienced in the preparation of active receptor extracts, which resulted in poor reproducibility using this assay. Thus, subsequent to the characterisation of pLG260 and pLG261, the two *in vivo* assays, which were easier to perform and offered greater reproducibility, were used routinely.

The two recombinants, pLG260 and pLG261 are shown to specify a high primase activity, irrespective of the assay system used (Table 3.1). The slightly higher primase activity specified by pLG260, relative to pLG261, is considered to result from the difference in copy number between the vector plasmids used. It is therefore concluded, from both restriction mapping and the assays of primase activity, that pLG260 and pLG261 carry the IncP plasmid primase gene. The RP4 primase gene encodes two polypeptides of 118 and 80kD, both of which specify primase activity (Lanka and Barth, 1981; Lanka *et al.*, 1984). To confirm that the region of RP4 cloned in my experiments specified these two polypeptides, the minicell-producing strain, DS410, was transformed to Cm^r with pLG260. The strain specified five polypeptides of 117, 84, 74, 61 and 31kD (Figure 3.7), of which the two largest are very similar in size to those found to

Figure 3.6 In vitro DNA synthesis on phage fd DNA in response to increasing amounts of plasmid primase activity in the CCE

The assay system is described in section 2.8.3. CCE was prepared from strain BW86(pLG260) and was diluted 2, 5, 10, 25, 50, 75 and 100-fold in CCE dilution buffer (HEPES, 25mM; KCl, 50mM; EDTA, 2mM; DTT, 1mM; pH 8.0). 2.5µl of each dilution was added to a separate reaction mixture. The amount of radioactivity incorporated by each reaction is shown plotted against the amount of protein (µg) in the CCE added to the reaction. See text for intrepretation of results.

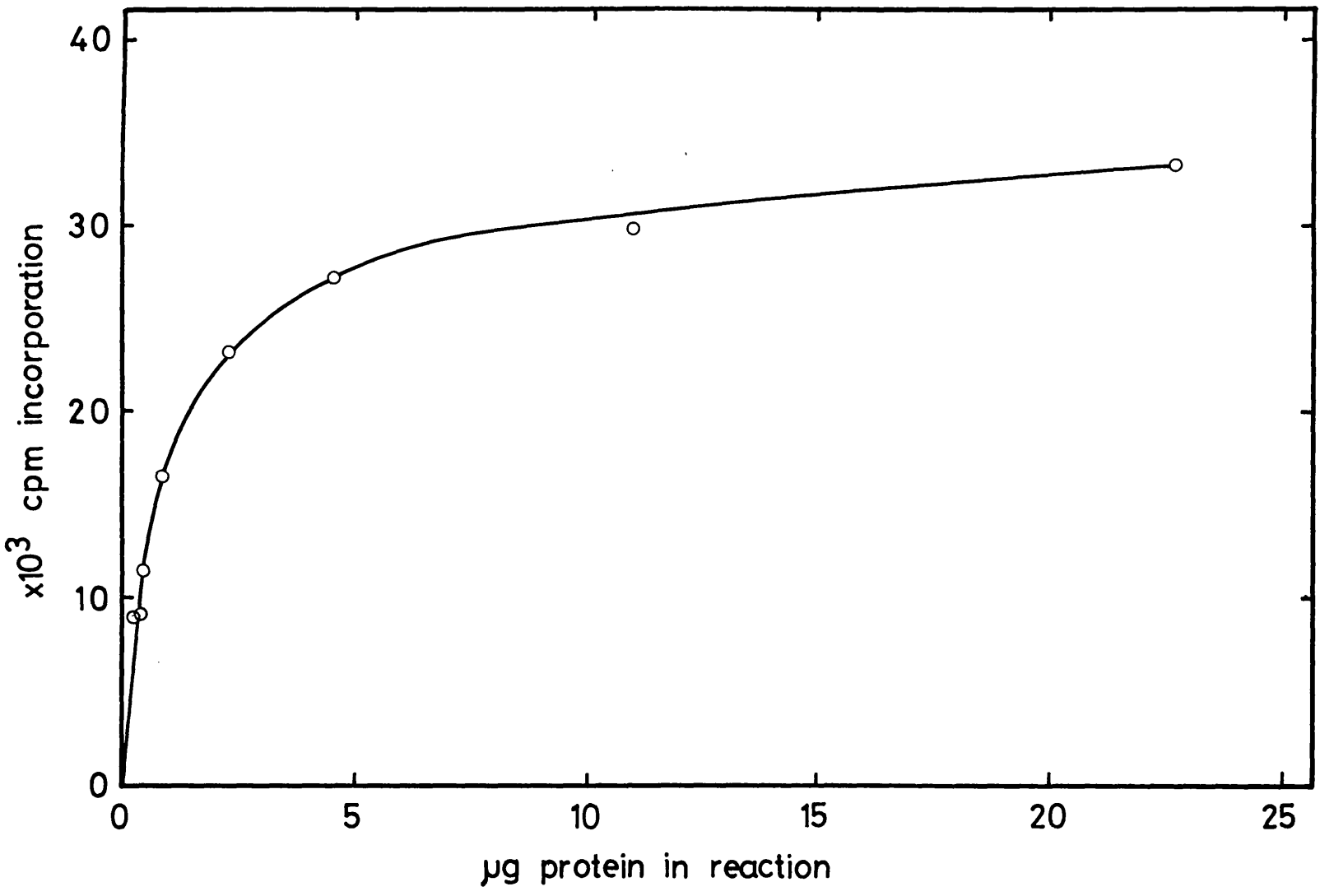

40
30
20
10
0
x10³ cpm incorporation
0
5
10
15
20
25
µg protein in reaction

Table 3.1 Measurement of primase activity encoded by plasmids in strain BW86

Plasmid	Colony forming ability[1]	DNA synthesis at 41°C[2]	Plasmid primase activity[3]
-	< 10^{-7}	9.8	< 1
ColIb-P9drd-1	4.2 x 10^{-2}		
pLG221	2.2 x 10^{-1}		
pLG250	2.9 x 10^{-7}		
pLG215	1.5 x 10^{-4}	116.2	
pRP1	5.5 x 10^{-8}	12.0	2.8
pRP2	2.2 x 10^{-6}	82.3	9.5
pRP26	3.0 x 10^{-5}	100.1	13.8
pLG260	5.9 x 10^{-1}	208.0	117.4
pLG261	1.3 x 10^{-2}	136.9	81.0
pBR328	< 10^{-7}	10.0	
R300B	< 10^{-7}		

[1] Colony formation of BW86 strains at 40°C relative to 30°C (section 2.8.1.).

[2] Radioactivity incorporated by DNA synthesis in BW86 strains. Measured in units of 10^3 cpm per ml per unit increase in absorbance at A_{450} (section 2.8.2.).

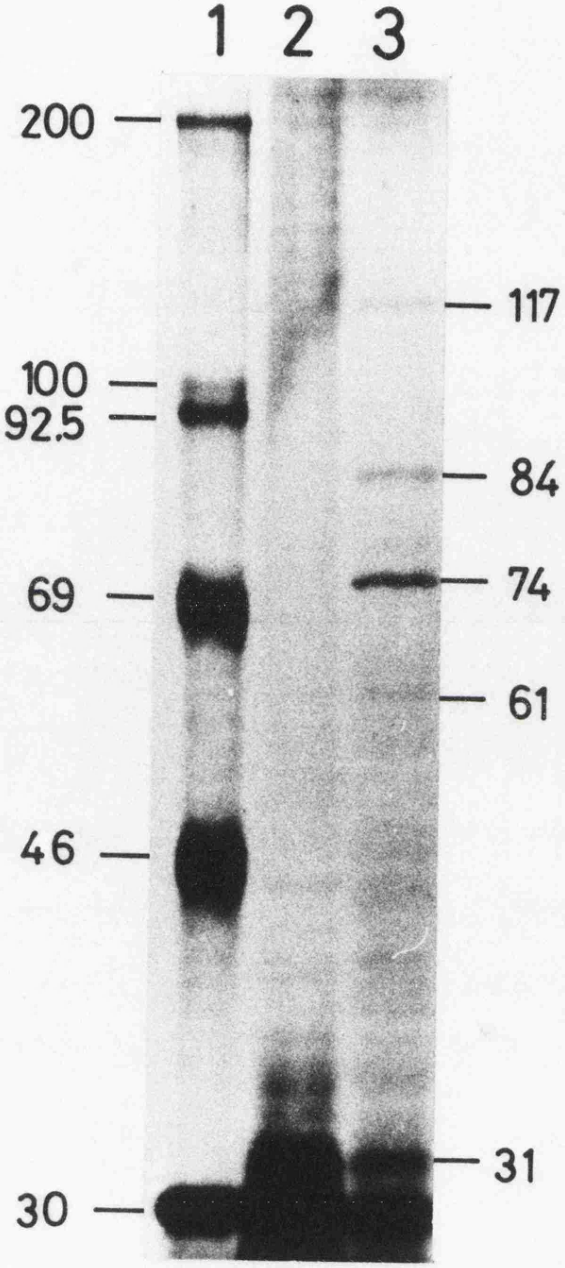
[3] Radioactivity incorporated by DNA synthesis on phage fd single-stranded template at 30°C by crude cell extracts of BW86 strains *in vitro*. Units of primase activity are milliunits per mg added CCE protein (section 2.8.3.)

Figure 3.7 Polypeptides detected in the DS410 minicell strain

Labelled polypeptides were identified by autoradiography following electrophoresis through a 10% SDS-polyacrylamide gel. The molecular weights of labelled polypeptide markers (lane 1) are shown to the left. Sizes of plasmid-encoded polypeptides are given to the right.

The plasmids harboured by the minicell strain are as follows:

Lane	Plasmid
2	pBR328
3	pLG260


1
2
3
200
100
92.5
69
46
30
117
84
74
61
31

copurify with primase activity (Lanka and Barth, 1981; Lanka *et al.*, 1984). The 74 and 61kD polypeptides specified by pLG260, may correspond to the 77 and 68kD polypeptides found to be encoded by two genes upstream of *pri* in the same transcriptional unit (Lanka *et al.*, 1984). The 31kD polypeptide is thought to be β-lactamase.

3.7. Summary

The primase gene from a RP4::Tn*7* derivative, pRP26, which encodes a high activity of primase, has been cloned into two vectors, pBR328 and R300B, on a *Hin*dIII and *Pst*I fragment, respectively. Analysis of the two resulting recombinants, pLG260 and pLG261, has shown that approximately the same region of RP4 DNA had been cloned into each vector, and that this region was larger than that described by Barth and Grinter (1977). However, restriction analysis of the cloned fragment carrying the primase gene, and of pRP26, has allowed the region to be mapped more precisely, and the revised map of this region was later corroborated by Lanka *et al.* (1984). The activity of the primase specified by the two recombinants was measured by three different methods and shown to be greater than that encoded by the parent plasmid. Analysis of the polypeptides encoded by pLG260 revealed two polypeptides of similar size to those previously shown to copurify with primase activity (Lanka and Barth, 1981; Lanka *et al.*, 1984). Two other polypeptides encoded by genes in the same transcriptional unit as *pri* were also synthesised by pLG260. The evidence indicates that the RP4 primase gene has been cloned from pRP26 and that the two recombinants specify more primase activity than RP4 and its derivatives.

CHAPTER FOUR

ROLE OF RP4 PLASMID PRIMASE IN RP4-SPECIFIED CONJUGATION

4.1. Introduction

As discussed in Chapter 1, Lanka and Barth (1981) suggested that plasmid primases had a role in conjugation, presumably in some aspect of conjugative DNA synthesis. If RP4 plasmid DNA is passed from donor to recipient cells as a single strand, then DNA synthesis will be required both in the donor cell, to replace the transferred strand, and in the recipient, to form the complementary strand. It is possible, therefore, that plasmid primase could have a role in both hosts to form the primers required for the two types of conjugative DNA metabolism.

The objective of the experiments described in this chapter was to investigate whether RP4 plasmid primase was required in donor or recipient cells or both, during conjugation. This was done through complementation tests, using a Pri^{-} RP4 derivative and Pri^{+} recombinant plasmids in the donor and recipient cells. Since Lanka and Barth (1981) found that RP4 plasmid primase was only required in matings with some, but not all, recipient host strains, the role of primase in different recipients would be investigated. This required the use of the broad host range recombinant, pLG261 described in the previous chapter. However, in complementation tests involving the presence of the recombinant plasmid in the donor cell, it was important to ensure that the Pri^{+} gene was not transferred during conjugation. Thus the recombinant, pLG260, which is based on the non-mobilisable plasmid pBR328, was used.

Much of the chapter is devoted to the definition of suitable mating conditions, and the development of appropriate donor strains. Such studies revealed an unexpected variation in the ability with which different *E.coli* K-12 strains donated the Pri^{-} RP4 derivative.

4.2. Establishment of mating conditions

The yield of RP4 transconjugants produced when matings were performed in liquid media was very poor, so a method involving the stabilisation of mating aggregates on the surface of cellulose nitrate filters was used. The duration of the mating was chosen to limit secondary matings involving

the newly-formed transconjugant cells, yet also to allow the formation of a significant number of transconjugants. These factors were particularly pertinent to E.coli x S.typhimurium matings since these inter-specific matings form about 10^3 fewer transconjugants than do E.coli x E.coli mating presumably due to the activity of the restriction system in Salmonella (reviewed in Arber, 1974).

Test matings were performed to define good mating conditions. W3110T$^-$ donors harbouring pRP2 and S.typhimurium SL329 recipients were mated for 1, 3, 6 or 20 h before measuring the yield of Km^r transconjugants (Table 4.1). After 1 h, 2.3×10^3 transconjugants per ml were produced, this value being increased over 100-fold after 3 h mating. This increase was clearly larger than could be produced by the vegetative growth of transconjugants, showing that conjugation occurred for at least 3 h. However, after 6 and 20 h mating, the rate of increase in transconjugant yield began to decline, probably indicating that recipient cells were becoming limiting, and that the increase more was likely to result from transconjugant multiplication. Therefore, 3 h was chosen as the standard duration of matings (section 2.6.2.). This period allows significant formation of transconjugants, yet will not allow substantial multiplication of transconjugants.

In each complementation test, a control plasmid, pRP2, was mated under the same conditions as the test plasmid, pRP1. The role of the plasmid primase in the conjugative process is therefore represented by the ratio of pRP1:pRP2 transconjugants per ml. Each experiment was repeated at least three times and the average ratio of the mating efficiencies was calculated. The significance of differences between such ratios was judged at the 95% level.

4.3. Role of RP4 plasmid primase in conjugation

In order to ascertain the importance of the RP4 plasmid primase in the conjugative process, the yield of transconjugants produced by the primase-defective RP4 derivative, pRP1, was compared to that of a control plasmid, pRP2.

W3110T$^-$ donor cells harbouring pRP1 or pRP2 were mated with Nal^r mutants of E.coli C600 or S.typhimurium SL329 recipients. The yield of Km^r Nal^r transconjugants per ml (Table 4.2), was reduced by four to five orders

Table 4.1 pRP2 transconjugant yields in matings of varying duration

Strain		Mating	Yield transconjugants
Donor	Recipient	Time h	per ml mating mix
W3110T^{-}(pRP2)	C600	6	6.9×10^{9}
W3110T^{-}(pRP2)	SL329	6	8.0×10^{6}
W3110T^{-}(pRP2)	SL329	1	2.3×10^{3}
W3110T^{-}(pRP2)	SL329	3	2.6×10^{5}
W3110T^{-}(pRP2)	SL329	6	5.0×10^{6}
W3110T^{-}(pRP2)	SL329	20	3.2×10^{7}

Matings were carried out on cellulose nitrate filters as described in section 2.6.2.

Table 4.2 Yield of Pri$^+$ and Pri$^-$ RP4 derivatives in *E.coli* x *E.coli* and *E.coli* x *S.typhimurium* matings

Strain		Yield transconjugants	Ratio
Donor	Recipient	per ml mating mix	pRP1/pRP2[1]
W3110T$^-$(pRP1)	C600	7.2×10^8	0.59
W3110T$^-$(pRP2)	C600	1.2×10^9	
W3110T$^-$(pRP1)	SL329	2.9×10^3	0.01
W3110T$^-$(pRP2)	SL329	2.7×10^5	

[1] Yield of pRP1 transconjugants per ml mating mix as a fraction of that of pRP2.

of magnitude in the inter-specific mating relative to that between E.coli strains, presumably due to the restriction barrier present in the inter-specific mating. The ratio of pRP1:pRP2 transconjugants per ml revealed a significant deficiency in transconjugant yield in S.typhimurium recipients when the RP4 primase gene was inactivated. This result is in broad agreement with Lanka and Barth (1981).

However, in contrast to Lanka and Barth (1981), pRP1 was also shown to be slightly conjugation-deficient in W3110T$^-$ x C600 matings. This anomaly is thought to arise from the differing sensitivities of the mating methods used. Lanka and Barth used a method involving the spotting of various dilutions of donor strains onto a lawn of recipient cells spread on agar selective for the growth of transconjugants. This method allowed the identification of recipient species requiring plasmid primase for efficient transconjugant formation, but the more refined filter mating system used in this thesis allowed smaller differences in transconjugant yield to be resolved.

4.4. Effect of donor strain on pRP1 transconjugant yield

Having established that plasmid primase has an important role in the conjugative process, the action of the enzyme was investigated, initially by complementing the primase-defective RP4 derivative with the cloned active RP4 primase gene in the donor cell. Thus, W3110T$^-$ was transformed to Ap^r Cm^r using pLG260. However, cleavage with HindIII of plasmid DNA isolated from transformants revealed that the plasmids had all suffered severe deletions removing the inserted fragment carrying the primase gene, and some of the vector DNA. Similar difficulties in the transformation of W3110T$^-$ have been experienced elsewhere. This implied that, even if W3110T$^-$ could be transformed by pLG260, the plasmids harboured might carry deletions, some of which could remain undetected. Consequently, an alternative donor strain was sought.

To allow the detection of the presence of an active primase gene, the strain BW86 was used. This strain carries the temperature-sensitive mutation, dnaG3, originally isolated by Carl (1970). At the restrictive temperature, the host primase is unable to promote discontinous chromosomal synthesis, but the suppression of this by the primase of several plasmids, including RP4 (Lanka et al., 1984) and ColIb-P9 (Wilkins, 1975), enables plasmid primase activity to be simply assayed.

To assess the performance of BW86 as a donor strain, the relative transfer efficiency of pRP1 and pRP2 in matings between BW86 donors and C600 or SL329 recipient strains at 30°C was quantified. The results (Table 4.3) indicated that the relative yield of pRP1 transconjugants was reduced almost equally in the two matings, in contrast to the result with W3110T^{-} donor strains. This variation did not result from the different mating temperatures used as shown by matings between W3110T^{-} donors, harbouring pRP1 or pRP2, and C600 recipients at both 30°C and 37°C. Although the efficiency of pRP1 transconjugant formation relative to pRP2 from W3110T^{-} donors was slightly lower at 30°C (Table 4.3), the decrease was insufficient to explain the difference observed between the two donor strains.

An alternative explanation was that the *dnaG3* mutation carried by BW86 influenced the transfer efficiency of pRP1, even at the permissive temperature. Lark (1972) showed that the Okazaki fragments produced at 30°C by the *dnaG3* strain PC3 were larger than those of the wild type strain, implying that even at the permissive temperature, the mutant host primase was not fully active. To test this explanation, the strain BW85, a revertant of the *dnaG3* mutation (Boulnois *et al.*, 1979) was used as donor strain in matings with C600 recipient cells at 30°C. BW85 was also found to donate pRP1 with a low efficiency, relative to the control (Table 4.3), suggesting that the *dnaG3* mutation was not the cause of the reduced pRP1 transconjugant yield.

Similarly, BW86*dna*$^{+}$ donor strains, in which the *dnaG3* allele had been exchanged for the W3110T^{-} primase gene by P1 transduction, also yielded similarly low relative transfer deficiencies (Table 4.3). This showed that the replacement of the *dnaG3* mutant allele with the W3110T^{-} *dna*$^{+}$ gene did not dramatically alter the ability of the strain to donate pRP1. These results suggest that the differing ability of W3110T^{-} and the BW-pedigree strains to act as donors of pRP1 was not due directly to the different alleles of the host primase gene.

One of the ultimate objectives of this series of experiments was to determine whether pRP1 could be complemented by a Pri^{+} recombinant plasmid in the donor cell. To prevent recombination between the primase genes carried by the recombinant and conjugative plasmids in the donor strain, the *recA1* mutation was introduced into the *dnaG3* donor strain using the Hfr strain KL16-99. The F plasmid harboured by KL16-99 is integrated

Table 4.3 Effect of *E.coli* donor strains on relative yield of pRP1 transconjugants in *E.coli* x *E.coli* and *E.coli* x *S.typhimurium* matings

Strain		Mating	pRP1/pRP2
Donor[1]	Recipient	Temperature	transconjugants/ml[2]
BW86	C600	30°C	0.016
	SL329	30°C	0.06
W3110T^{A}	C600	30°C	0.48
	C600	37°C	0.59
BW85	C600	30°C	0.008
BW86*dna*$^{+}$	C600	30°C	0.004
	C600	37°C	0.002
BW103	C600	37°C	0.002
	SL329	37°C	0.067
C600	C600	37°C	0.16
	SL329	37°C	0.03

[1] Each donor strain carried either pRP1 or pRP2 as the conjugative plasmid.

[2] Yield of pRP1 transconjugants per ml mating mix relative to that of pRP2.

at 61.5 min and promotes the transfer of the chromosomal markers in a counterclockwise direction (Low et al., 1972). The acquisition of sensitivity to UV light would indicate the integration of the recA1 mutation in the transconjugants. Since BW86 is already UV-sensitive by virtue of a deletion which removes the uvrB gene, the UV-resistant homogenic strain BW84 was used as recipient in a 30 min interrupted mating with KL16-99 donor cells. Of 116 Leu$^+$ Sm^r transconjugants obtained, two were UV-sensitive, but both grew very poorly both in liquid media and on NA plates. This suggested that the combination of dnaG3 and recA1 mutations imposed a stress on the cells that was likely to effect adversely their ability to donate conjugative plasmids, so making this strain unsuitable as a donor strain.

Since limitation of inter-plasmidic recombination using a recA background was of greater importance than the ability to detect the presence of primase activity by dnaG3 suppression, the recA1 strain BW103 was used. The construction of this strain is described in section 2.1, but it is noted here that the strain was derived from BW85 and is of the same pedigree as BW86. To confirm that BW85, and therefore BW103, carried a bona fide wild-type dnaG gene, P1 transduction was used to transfer the gene to the strain PM105. This strain, a derivative of AB1157, harbours the dnaG3 mutation and a Tn10 insertion, and these two markers are cotranduced by P1 at a frequency of 84% (Harris et al., 1978). P1 transducing phage, prepared from BW85, was used to infect PM105 cells, selecting for colonies capable of growth at 40°C. Screening showed that 64% of the 719 colonies obtained were Tc^S, strongly suggesting that the Ts$^+$ phenotype carried by BW85 and its derivatives resulted from the reversion of the dnaG3 mutation, rather than from a suppressor mutation in a second gene. The strain BW103 was therefore used as the donor strain in the filter mating experiments.

Plasmids pRP1 and pRP2 were transferred from W3110T$^-$ into BW103, and the resultant strains used as donors in filter matings with E.coli C600 and S.typhimurium SL329 recipient strains. The relative yield of pRP1 transconjugants (Table 4.3) was low irrespective of the recipient strain used. The results are broadly comparable with data obtained with other BW-pedigree donor strains.

The contrasting efficiency with which W3110T$^-$ and BW-pedigree strains were able to donate pRP1 to E.coli recipients suggests that different donor

strains are able to suppress the requirement for plasmid primase with varying effectiveness. This observation was further substantiated by showing that matings between C600 donors, harbouring pRP1 or pRP2, and C600 recipients, gave a relative yield of pRP1 transconjugants intermediate to that obtained using W3110T^{-i} or BW- pedigree donor strains. The variation in pRP1 transconjugant yield gained from different donor and recipient strains will be discussed later in this chapter.

4.5. Complementation of primase-defective RP4 derivatives using a cloned RP4 primase gene in donor cells

To determine during which stages of conjugation plasmid primase is required, complementation tests using the primase-defective RP4 derivative and a cloned active RP4 primase gene in the donor cells were performed. Appropriate donor strains were constructed by transforming BW103 cells to Cm^r using pLG260 or its vector pBR328. None of the problems experienced in the transformation of W3110T^{-i} cells with pLG260 were encountered using BW103. The plasmids pRP1 and pRP2 were then transferred from W3110T^{-i} into both BW103(pLG260) and BW103(pBR328) and Km^r Cm^r transconjugants selected. To assess the ability with which pLG260 could complement the transfer-deficiency of pRP1, these strains were used as donors in filter matings with C600 recipient cells. This showed a dramatic increase in the relative transfer-efficiency of pRP1 from 0.002 to 0.63 in the presence of pLG260. No comparable effect was produced by pBR328 (Table 4.4). Since the transfer of pLG260 and pBR328 to the recipient cells was shown to be inefficient (Table 4.5), some factor encoded by the RP4 DNA fragment cloned in pLG260 and synthesised in the donor cell must be responsible for the complementation.

Since, as described in Chapter 1, the primase gene is the penultimate gene in a transcriptional unit (Lanka *et al.*, 1984; Figure 1.3), the insertional inactivation of the primase gene by Tn*7* in pRP1 is likely to have a polar effect on the expression of the promoter-distal gene. Consequently, the product of either gene could be responsible for the transfer-deficiency of pRP1, and since both genes are present on pLG260, it is impossible to distinguish their relative contribution. To do this, it was necessary to remove the promoter-distal gene from pLG260 and repeat the complementation experiment.

Table 4.4 Complementation of pRP1 in the donor strain by non-mobilisable vector and Pri$^+$ recombinant plasmids

Strain Donor[1]	Recipient	Mating Temperature	pRP1/pRP2 transconjugants/ml[2]
BW103	C600	37°C	0.002
BW103(pLG260)	C600	37°C	0.63
BW103(pBR328)	C600	37°C	0.004
BW103(pJF107)	C600	37°C	0.15
BW103(pJF107Δ32)	C600	37°C	0.19

[1] BW103 donor strains also contained either pRP1 or pRP2 as the conjugative plasmid.

[2] Yield of pRP1 transconjugants per ml mating mix relative to that of pRP2.

Table 4.5 Ability of RP4 derivatives to mobilise vector and Pri$^+$ recombinant plasmids

Donor strain[1]	Mobilisation frequency of non-conjugative plasmid
BW103(pRP1,pBR328)	1.38×10^{-5}
BW103(pRP2,pBR328)	2.78×10^{-7}
BW103(pRP1,pLG260)	7.6×10^{-4}
BW103(pRP2,pLG260)	1.6×10^{-5}

[1] Filter matings were performed at 37°C for 3 h with Nal^r C600 recipient cells. Transconjugants were selected with Nal and either Km or Cm to select for the conjugative or recombinant plasmids, respectively.

4.6. Removal of the promoter-distal end of the transcriptional unit carried by pLG260

Since no known restriction endonuclease cleavage sites map between the *pri* gene and the promoter-distal gene, an attempt to remove the latter gene from pLG260 was made using Bal31 exonuclease. Although this could be achieved most easily by cleaving pLG260 at the unique ClaI cleavage site within the vector DNA (Figure 3.1) and digesting in either direction using Bal31, such a digest would also remove part of the Cm^r gene, leaving Ap as the sole means of selection. This would be unsatisfactory because maintenance studies of pLG260 showed that only 59% of BW86 cells retained the plasmid after 20 hours incubation in NB containing Ap, compared to 92% when Cm was used as the selection.

Therefore a separate strategy was attempted. pLG260 was cleaved with EcoRI and HindIII before treating with a range of concentrations of Bal31 to give deletions of the required length (Figure 4.1.I). The single-stranded ends of the fragments were repaired and ligated with HindIII-cleaved pBR328 DNA, which was also end-repaired. However, although this scheme ensured that recombinants carried the Cm^r gene, the transformation of BW86 yielded no transformants harbouring plasmids containing Bal31-treated fragments. This failure probably resulted from the low efficiency of the end repair reaction, which would limit the ligation reaction.

By using vector DNA cleaved by a restriction endonuclease which creates blunt ends, the probability of cloning the Bal31-treated fragments should be increased. Since such restriction endonucleases all cleave pBR328 within the Cm^r gene, the polylinker from pUC12 (Messing, 1983; Figure 4.1.II), which carries a SmaI recognition site (5'-CCC|GGG-3'), was cloned into pBR328 as described in Figure 4.1. The resultant plasmid pBR328::pL, was cleaved with SmaI and ligated with end-repaired fragments of Bal31-treated pLG260 DNA. The DNA was used to transform BW86 to Cm^r Ap^r, but of the 768 colonies isolated, none could suppress the *dnaG3* mutation, indicating that none carried recombinants expressing the RP4 primase gene.

Therefore, the attempt to remove the gene encoding the 16.5kD polypeptide from pLG260 was abandoned in favour of using the newly-published plasmids pJF107 and pJF107Δ32 (Lanka *et al.*, 1984). These plasmids, provided by

Figure 4.1 Construction of deletion derivatives of plasmid pLG260

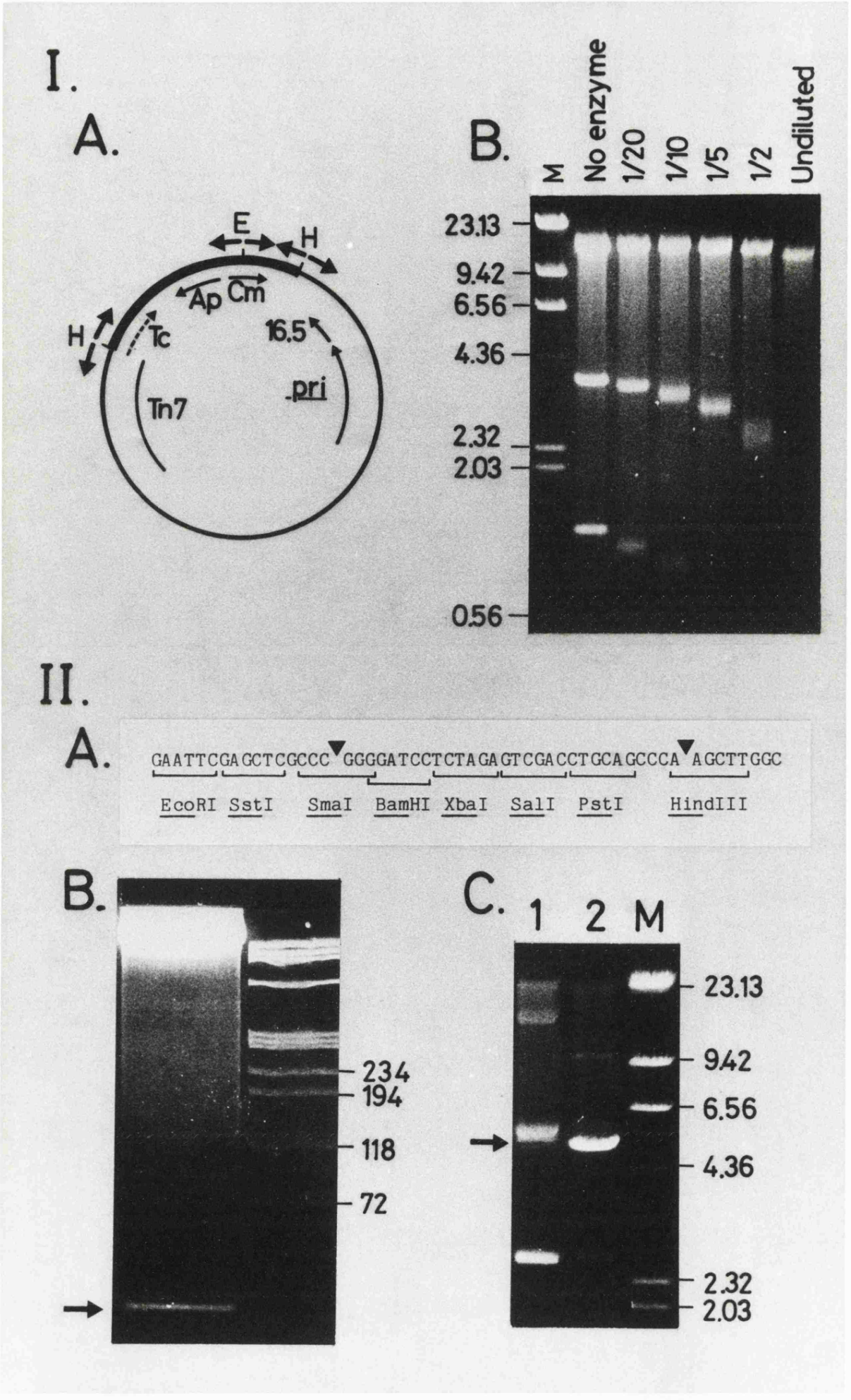
I. A. Map of pLG260 showing position of RP4 primase gene (*pri*) and the promoter-distal gene encoding the 16.5kD polypeptide (16.5). Other details are given in Figure 3.3. The direction of the Bal31 digestion of pLG260 DNA, cleaved by EcoRI (E) and HindIII (H), is indicated by the heavy arrows.

B. 5µg of EcoRI-HindIII cleaved pLG260 DNA in a volume of 25µl, was digested at 30°C for 15 min by 2µl each of various dilutions of Bal31 enzyme stock ($0.75u\mu l^{-1}$; BioLabs), as indicated above each lane. The reaction was stopped with EGTA and ~1µg of resultant DNA electrophoresed in 0.7% agarose. The numbers (kb) to the left indicate fragment sizes of λ x HindIII markers (lane M).

II. A. Sequence of the polylinker fragment carried by pUC12. Arrows represent cleavage sites of HindIII and SmaI.

B. 30µg pUC12 was cleaved with HindIII and SmaI, electrophoresed through a 10% polyacrylamide gel and the 34 base pair polylinker fragment (arrowed) was purified from the gel (section 2.7.5.). The molecular weights, in base pairs, of the ΦX174 x HaeIII marker DNA in the right lane are given to the right and in Table 2.4.

C. Following its isolation, the 34 base-pair polylinker fragment was ligated with HindIII-cleaved pBR328 and the DNA was used to transform BW86 to Cm^r Ap^r. Plasmid DNA was prepared from 12 isolates by the Birnboim and Doly method, treated with SmaI and electrophoresed through 0.7% agarose. Uncleaved DNA (Lane 1) indicates a recombinant lacking the polylinker fragment, whereas the appearance of a discrete fragment of molecular weight ~5.0kb (arrowed), shows a recombinant carrying the polylinker (Lane 2). SmaI-cleaved DNA of the latter recombinant was religated to remove multiple copies of the polylinker. The resultant recombinant (pBR328::pL) carries copies of the 34 bp polylinker orientated back-to-back, so recreating the SmaI recognition site between them.


I.
A.
E
H
Ap
Cm
H
Tc
16.5
Tn7
pri
B.
M
No enzyme
1/20
1/10
1/5
1/2
Undiluted
23.13
9.42
6.56
4.36
2.32
2.03
0.56
II.
A.
GAATTCGAGCTCGCCC▼GGGGATCCTCTAGAGTCGACCTGCAGCCCA▼AGCTTGGC
EcoRI SstI SmaI BamHI XbaI SalI PstI HindIII
B.
234
194
118
72
C.
1
2
M
23.13
9.42
6.56
4.36
2.32
2.03

E.Lanka, resemble pLG260 in carrying the RP4 primase gene from pRP26, but differ in that the promoter and the first two genes of the transcriptional unit have been deleted. In addition pJF107Δ32 carries a deletion which removes the whole of the promoter-distal gene in the transcriptional unit, and also the vector Cm^r gene. As explained previously, Cm was used to select for stable maintenance of pLG260, however, unlike pLG260, the pJF plasmids were relatively stably maintained, suffering only a 16% loss from a population of BW86 cells over 20 hours of growth in NB containing Ap. The pJF plasmids were found to encode a lower primase activity than pLG260 (Table 4.6). This presumably reflects the differing strengths of the promoters used to express the primase gene in the recombinants.

4.7. Determination of the factor responsible for the complementation of the pRP1 transfer-deficiency

BW103 was transformed to Ap^r by pJF107 or pJF107Δ32 and plasmids pRP1 and pRP2 were each transferred into these strains from W3110T$^-$. The resultant BW103 strains were used as donor strains in filter matings with C600 recipient cells. The results (Table 4.4) show that both recombinant plasmids were able to complement the transfer deficiency of the conjugative plasmid with approximately equal efficiency. This demonstrated that plasmid primase, and not the 16.5kD polypeptide encoded by the promoter-distal gene, was responsible for the increased transfer efficiency of pRP1.

4.8. Complementation of the primase-defective RP4 derivative by a cloned active primase gene in recipient cells

Lanka and Barth (1981) suggested that the role of plasmid primase could be to prime complementary strand synthesis in the recipient cell. To confirm that plasmid primase, or its products, acted in the recipient cell, complementation tests using the primase-defective RP4 derivative and an active primase gene in recipient strains were performed. Since both E.coli and S.typhimurium recipient strains were involved, the recombinant pLG261, based on the broad host-range vector R300B, was used.

The recombinant plasmid, pLG261 was mobilised into C600 and SL329 recipient strains, selecting for Km^r Nal^r colonies. These recipient strains were mated with W3110T$^-$, BW86, BW85 and BW103 donor strains, each harbouring either pRP1 or pRP2. The results showed that in most

Table 4.6 Plasmid-mediated suppression of the E.coli dnaG3 mutation

Plasmid	CFA[1]	DNA synthesis[2]
pLG260	5.9×10^{-1}	208.0
pJF107	3.7×10^{-3}	95.6
pJF107Δ32	5.1×10^{-5}	84.8

[1] Colony formation of BW86 strains at 40°C relative to that at 30°C (section 2.8.1.). BW86 = $<10^{-7}$.

[2] Radioactivity incorporated by DNA synthesis at 41°C in BW86 strains. Units of 10^3 cpm per ml per unit increase in absorbance at A_{450} (section 2.8.2.). BW86 = 9.8.

matings, the presence of pLG261 in the recipient strains significantly increased the relative yield of pRP1 transconjugants (Table 4.7). This clearly demonstrated that plasmid primase has a major role in the recipient cell during conjugation.

However, when BW103 donor strains were mated with C600(pLG261) recipient cells, no significant stimulation of the transconjugant yield was observed. The reason for this was unclear, but since the phenomenom was not observed with the closely related BW86 or BW85 donor strains, it is possible that the complementation of pRP1 by a primase gene in the recipient cell may require recA gene activity in the donor cell. To test this, a recA derivative of W3110T^{-}, HH27, was used to donate pRP1 and pRP2 to both C600 and C600(pLG261) recipient cells. The relative yield of pRP1 transconjugants from the two recipient strains did not differ significantly, in contrast to the result obtained when W3110T^{-} donor cells were mated with the same two recipients (Table 4.7). This implies that recA gene activity in the donor strain is required for some aspect of the RP4 conjugative process in the absence of plasmid primase.

4.9. Discussion

Lanka and Barth (1981) showed that primase-defective RP4 derivatives produced a low yield of transconjugants when mated from E.coli W3110T^{-} donor cells into some recipients, such as S.typhimurium, but transferred normally into other recipients, including E.coli. Similarly, the experiments described in this chapter using W3110T^{-} donor strains, showed that the transfer of the Pri^{-} RP4-derivative, pRP1 was deficient relative to the Pri^{+} control, when using S.typhimurium SL329 recipient cells. Furthermore, the increased sensitivity of the method used here showed a less pronounced, but equally reproducible deficiency in matings involving E.coli recipients. This indicated that some factor defective in pRP1 was required for the efficient formation of transconjugants in matings involving both recipient strains. Similarly, Chatfield et al. (1982), using derivatives of ColIb-P9drd-1 found that, although the transconjugant yield of a Sog^{-} plasmid was deficient in matings with both E.coli and S.typhimurium recipients, the effect was less pronounced with the former recipient strain.

Therefore, since the transposon insertion in pRP1 is known to inactivate plasmid primase activity (Lanka and Barth, 1981), it was proposed that

Table 4.7 Tests for complementation of pRP1 by broad host range Pri$^+$ recombinant plasmid in the recipient cell

Strain		Mating	pRP1/pRP2
Donor[1]	Recipient	Temperature	transconjugants/ml[2]
W3110T^{ts}	C600(pLG261)	37°C	0.84 (0.59)[3]
BW86	C600(pLG261)	30°C	0.28 (0.016)
BW85	C600(pLG261)	30°C	0.29 (0.008)
BW103	C600(pLG261)	37°C	0.008 (0.002)
BW86	C600(R300B)	30°C	0.003
BW103	C600(R300B)	37°C	0.025
W3110T^{ts}	SL329(pLG261)	37°C	0.37 (0.01)
BW86	SL329(pLG261)	30°C	0.58 (0.06)
BW103	SL329(pLG261)	37°C	0.29 (0.07)
W3110T^{ts}	SL329(R300B)	37°C	0.03
HH27	C600	37°C	0.31
HH27	C600(pLG261)	37°C	0.38

[1] Donor strains contained either pRP1 or pRP2 as the conjugative plasmid.

[2] Yield of pRP1 transconjugants per ml mating mix relative to that of pRP2.

[3] Bracketed figures show the relative transconjugant yield when recipient strains did not carry a plasmid.

this enzyme, like that of the $IncI_1$ plasmids, may be responsible for the efficient formation of transconjugants. Hence using pRP1 and the Pri^+ recombinant plasmids described in Chapter 3, the mode of action of RP4 primase in conjugation was investigated in complementation tests.

In the presence of an active primase gene in the recipient cell, it was shown that the yield of pRP1 transconjugants was significantly rescued in both E.coli x E.coli and E.coli x S.typhimurium matings. This indicated that plasmid primase has a role in the recipient cell. Furthermore, the provision of a non-mobilisable RP4 primase gene (pLG260) in the donor cell with pRP1 demonstrated that this requirement for plasmid primase in the recipient cells could be satisfied by enzyme specified in the donor cell. This model is in keeping with the finding for F and ColIb-P9, that conjugation does not require the expression of plasmid genes in the recipient cell (Hiraga and Saitoh, 1975; Boulnois and Wilkins, 1979).

During the course of this work, Lanka et al. (1984), in a detailed study of the expression of the RP4 primase gene, revealed that pri was one of four genes in a transcriptional unit, two genes being promoter-proximal and one promoter-distal, relative to pri. Since Tn7 insertions within this unit are polar (Lanka and Barth, 1981), the transposon in pRP1 would inactivate both the pri gene and the promoter-distal gene, which encodes a 16.5kD polypeptide (Lanka et al., 1984). Thus either gene product could be responsible for the deficiency of pRP1 transconjugant formation, and since pLG260 also encodes both products, the contribution of each in the rescue of the pRP1 transfer-deficiency could not be established using this plasmid.

To determine which gene product was responsible, the plasmids pJF107 and pJF107Δ32 were used in complementation tests with pRP1. These recombinants, like pLG260, carry the pri gene derived from pRP26, however a deletion of the DNA upstream of pri has removed the promoter and the two promoter-proximal genes from both plasmids (Lanka et al., 1984). In addition pJF107Δ32 carries a second deletion which removes almost all the DNA downstream of pri, so deleting the gene encoding the 16.5kD polypeptide. Thus by comparing the ability with which these two plasmids rescue pRP1 transconjugant yield, the relative involvement of plasmid primase and the 16.5kD polypeptide was established.

When present in the donor cell, both pJF107 and pJF107Δ32 were able to

complement the pRP1 transfer deficiency with approximately equal efficiency so demonstrating clearly that plasmid primase was involved in the rescue. Since the degree of complementation was some 3-4 fold less than that stimulated by pLG260, this may imply that the 16.5kD polypeptide has some role in the complementation. However, the primase activity specified by the pJF plasmids was less than half that observed for pLG260. Therefore, since plasmid primase has been shown to have a role in the stimulation of pRP1 transconjugant yield, it follows that the stimulation is likely to be less dramatic when using a plasmid specifying a lower activity of primase. The differing primase activity observed presumably results from the differing strengths of the promoters used to express the primase genes, which in the pJF plasmids is thought to be the pBR325 Pp promoter, rather than the endogenous RP4 promoter employed in pLG260 (Lanka et al., 1984; Stuber and Bujard, 1981).

Interestingly, neither pLG260 or the pJF Pri^+ plasmids could fully complement the transfer deficiency of pRP1, despite the fact that the recombinants specified at least as much plasmid primase as the control RP4 derivative, pRP2 (Tables 3.1 and 4.6). Since the Tn*7* insertion in pRP1 maps at the 3' end of the *pri* gene, the mutant may encode truncated polypeptides. Competition between such inactive *pri* polypeptides and primase specified *in trans* may explain the inability of the recombinants to complement fully. A similar effect is also seen during complementation tests involving the ColIb-P9 primase gene (Chatfield et al., 1982).

These complementation tests clearly show a requirement for plasmid primase in the conjugative process of RP4. Since the nature of the transferred RP4 DNA is unclear, the role of the primase remains uncertain. However, considering the reaction catalysed by primases, and by analogy with the ColIbP9-encoded enzyme, RP4 primase presumably acts to initiate the synthesis of DNA complementary to the transferred strand (Lanka and Barth, 1981; Chatfield et al., 1982). This could be achieved either by primers that are made in the donor cell and transferred with the plasmid DNA to the recipient cell, or as a result of transfer of the enzyme and subsequent primer synthesis in the recipient cell. In the absence of further data, these models cannot be distinguished.

It was noted that, even in the absence of plasmid primase, a significant number of pRP1 transconjugants were formed. Presumably therefore, by analogy with $IncI_1$ plasmids, the host primase can partially substitute for

plasmid primase in the recipient cell. As demonstrated by matings between W3110T⁻ donors and E.coli or S.typhimurium recipients, some host systems appear to be better able to substitute than others. Thus, plasmid primase appears to be important in the establishment of the plasmid in strains where the host priming system is able only to inefficiently recognise the plasmid as template (Lanka and Barth, 1981).

Similarly, in the absence of plasmid primase, different donor strains also effect the efficiency of conjugation. In E.coli x E.coli matings the pRP1 transconjugant yield, relative to pRP2, varied over the range 0.002 - 0.59, according to the donor strain used. The nature of this phenomenom is unclear, but cannot be attributed to either the mating temperature, or to the presence of the dnaG3 allele in the BW86 donor strains. An explanation may be offered if it is assumed that RP4 transfers single-stranded DNA. In this case, conjugative DNA synthesis in the donor cell (DCDS) would be required to replace the transferred strand. In the case of ColIb-P9, this process is primed almost exclusively by plasmid primase, although a host priming system is able to substitute poorly in the absence of sog primase. It is therefore tempting to suggest that RP4 primase may also have a role in DCDS, and that in its absence, donor cell priming systems may substitute. Thus, variation in specificity or expression of the substituting host systems may offer an explanation of the observation that W3110T⁻ donors contributed a relatively normal yield of pRP1 transconjugants, whereas C600 and BW- pedigree strains were unable to do so.

Interestingly, Hill and Harnish (1981) reported that W3110T⁻ carries a chromosomal inversion of the region 72-90 minutes. This may alter the expression of genes around the inversion end points. The proximity of one end to the dnaG3 gene, (67 minutes; Bachmann, 1983) may influence the expression or specificity of host primase, and therefore, in the absence of plasmid primase, may alter the ability of host primase to suppress the transfer-deficiency of pRP1. Similarly, the host ssb gene, mapping at 92 minutes, is close to one end of the inversion. Single stranded binding protein is envisaged to coat the DNA transferred during conjugation, although whether host- or plasmid-encoded SSB fulfils this role is unclear. Golub and Low (1985) reported that RP4 encodes a single-stranded binding protein, however, recent work in Leicester has been unable to demonstrate the presence of the RP4 ssb gene in hybridisation tests with a purified F ssb gene probe (C.Howland, unpublished results). Thus it is

possible that host SSB may be involved in the RP4 conjugative process, and consequently variation in the expression of the host ssb gene, or differing specificities of the protein, could effect pRP1 transconjugant formation.

Thus, the efficiency with which pRP1 is transferred in E.coli x E.coli matings appears to depend on both the donor and recipient strains. This strain variation is further emphasised by the inability of pLG261 in E.coli recipients to complement pRP1 transfer from BW103 donors whereas good rescue was obtained with S.typhimurium recipients harbouring pLG261. This may reflect a role for the recA gene product in some process involved in the transfer of pRP1 DNA between E.coli cells or the formation of transconjugant colonies. The nature of this phenomenon is unclear, but the recA gene product may be necessary for the expression of host-encoded factors required for conjugation in the absence of plasmid primase, presumably as a result of the SOS response. However, it is known that the host ssb gene is not regulated by such a mechanism (Villani et al., 1984).

The transfer efficiency of primase-defective R18 (IncP) derivatives in Pseudomonas matings has also been reported to vary according to the recipient strain used (Cowan and Krishnapillai, 1982; Krishnapillai et al., 1984). These examples illustrate the highly complex interaction occurring between host and plasmid processes during conjugation. It is however, important to stress that in the presence of active plasmid primase, the variability resulting from the different host systems is overcome. Thus, although the nature of this strain variability is unclear, the point of relevance to this thesis is that RP4 plasmid primase acts to promote the efficient transfer of plasmid DNA between most strains and species of Gram-negative bacteria.

This requirement for plasmid primase during conjugation is somewhat puzzling in view of the essential redundancy of RP4 primase in plasmid vegetative replication, where DNA synthesis is presumably initiated by host enzymes (Lanka and Barth, 1981). An explanation of this apparent contradiction is possible if it is assumed that, like F, a specific strand of RP4 is transferred 5' leading during conjugation. Since RP4 replication is unidirectional (Meyer and Helinski, 1977), it follows that only one strand will act as template for lagging strand synthesis. Since this requires the formation of multiple primers, presumably this strand will contain the sites required for recognition by the bacterial host

priming systems. If the model for ΦX174 replication can be extrapolated, then such sites may include the n' recognition site for assembly of the primosome (Shlomai and Kornberg, 1980a; 1980c), or alternatively, plasmid-encoded factors, not associated with conjugation, such as trfA may be involved. Since the other plasmid strand will be the template strand for leading strand synthesis, it would probably be deficient in such sites required for vegetative replication. Therefore, since the transfer of RP4 is opposite to that of plasmid replication (Meyer and Helinski, 1977; Grinter, 1981; Al-Doori et al, 1982), it follows that the proposed transferred strand would be that which acts as template for leading strand synthesis in vegetative replication. Subsequently, this strand should lack the recognition sites required for initiation of lagging strand synthesis by the host priming systems. Hence, plasmid primase would be required to ensure that the transferred strand is efficiently converted to duplex DNA in the recipient cell.

This model suggests that RP4 primase may have an important role in the host range of plasmids, by acting to ensure the efficient provision of duplex DNA which can be recognised by the recipient replicative process. Boulnois et al. (1985) implied that ColIb-P9 primase also acts to promote the conversion of transferred single-stranded plasmid DNA to duplex molecules, even in host species which were incapable of supporting plasmid replication. This suggests that RP4 and ColIb-P9 primase act in an analogous manner, and that this may represent a model for the role of other plasmid primases.

CHAPTER FIVE

SPECIFICITY OF PLASMID-ENCODED PRIMASES

5.1. Introduction

As described in Chapter 1, the products of the RP4 and ColIb-P9 plasmid primase genes do not display antigenic cross-reactivity either with each other or with the bacterial host primase. However, despite this difference, the two plasmid-encoded enzymes are able to form primers of similar composition *in vitro*, and can initiate DNA synthesis on a similarly wide diversity of templates, including the *E.coli* chromosome Lanka *et al.*, 1979; Lanka and Barth, 1981; Lanka and Furste, 1985). The two primases therefore appear to be functionally similar.

In the previous chapter, it was shown that RP4 plasmid primase has an important role in the conjugative transfer of RP4 DNA, probably in the initiation of complementary strand synthesis in the recipient cell. Similarly, ColIb-P9 primase has been demonstrated to have a role in conjugative DNA synthesis both in donor and recipient cells (Chatfield *et al.*, 1982). Furthermore, this requirement for plasmid primase in the recipient cell has been shown to be satisfied by active enzyme synthesised in the donor cell in both conjugative systems.

Since both RP4 and ColIb-P9 primases behave in so similar a manner, both *in vitro* and *in vivo*, the possibility that the two enzymes could substitute for one another during conjugation was investigated.

5.2. Complementation of a primase-defective RP4 derivative by the ColIbP9-encoded plasmid primase.

The filter mating method described in the previous chapter, was used to determine the efficiency with which ColIb-P9 primase could rescue the deficient transconjugant yield of pRP1. Since the major role for the RP4 plasmid primase is in the recipient cell, initial complementation tests were performed using the cloned *sog* gene, carried by pLG215, in the recipient strain. C600 recipient cells harbouring pLG215, were mated with BW103 donor strains carrying either pRP1 or pRP2, and the relative yield of pRP1 transconjugants was measured. The results showed that ColIb-P9 primase apparently could not significantly rescue the transfer of pRP1 (Table 5.1).

Table 5.1 Tests for complementation of pRP1 in the recipient strain by ColIb-P9 derivatives

Strain		pRP1/pRP2
Donor[1]	Recipient	transconjugants/ml[2]
BW103	C600	0.002
BW103	C600(pLG215)	0.008
BW103	C600(pLG221)	0.149
BW103	C600(pLG250)	0.021
BW86	C600	0.014
BW86	C600(pLG221)	0.078
BW86	C600(pLG250)	0.001
W3110T-	SL329	0.01
W3110T-	SL329(pLG221)	0.33
W3110T-	SL329(pLG250)	0.06

[1] Each donor strain carried either pRP1 or pRP2 as the conjugative plasmid.

[2] Yield of pRP1 transconjugants per ml mating mix relative to that of pRP2. Selection for transconjugants was Nal Km unless the recipient strain harboured an $IncI_1$ plasmid, when Nal Tc was used.

However, it was shown by Chatfield (1984), that the ability of the primase specified by pLG215 to suppress the dnaG3 mutation in BW86, was increased almost 40-fold when the strain harboured pLG250 as well as the recombinant, even though pLG250 itself encodes no active primase. This suggests that ColIb-P9 primase activity is enhanced when some other ColIb-P9-specified factor is provided. Thus, the inability of sog primase to rescue pRP1 transconjugant yield may result from the lack of such factors.

To test this possibility, pLG221, a Sog^+ derivative of ColIb-P9drd-1 was used in complementation tests. This plasmid harbours a copy of transposon Tn5 inserted into the gene specifying colicin Ib (cib; Boulnois, 1981), which does not effect the transfer or maintenance of the plasmid. As a control in the complementation tests, the plasmid pLG250 was used. This derivative of pLG221 is Sog^-, probably by virtue of a small deletion in the region encoding the primase moiety of the sog polypeptide (Chatfield et al., 1982). C600 strains, harbouring either pLG221 or pLG250 were used in matings with BW103 donor strains carrying pRP1 or pRP2. The findings indicated that the presence of pLG221 in the recipient strain results in a modest increase in the relative yield of pRP1 transconjugants from 0.2 to 15% (Table 5.1). ColIb-P9 primase is strongly implicated as the causative factor in this stimulation since no similar effect was seen when the primase-defective plasmid pLG250 was used. Similar results were obtained using BW86 donors, and also using $W3110T^-$ donor strains in matings with S.typhimurium recipients harbouring pLG221 or pLG250 (Table 5.1). Therefore, when provided in the recipient cell, sog primase appears to act in collaboration with other ColIb-P9-encoded factors, to modestly complement the pRP1 transfer-deficiency, both in E.coli x E.coli and E.coli x S.typhimurium matings.

Complementation tests were also performed to test whether the yield of pRP1 transconjugants could be stimulated by ColIb-P9 primase synthesised in the donor cell. To ensure that the active sog gene could not be expressed in the recipient cell, a non-transferable plasmid harbouring the sog gene was required. Since the sog primase requires plasmid-specified cofactors for efficient activity, a transfer-deficient conjugative plasmid, pLG270 was used. This ColIb-P9drd-2 derivative carries a Tn5 insertion in the Eco-9 fragment (Cath Rees, unpublished data; Figure 1.1), which severely disrupts the transfer of the plasmid, relative to the control plasmid, pC2 (Table 5.2). This mutation however, does not

Table 5.2 Properties of plasmid pLG270

Transfer efficiency of pLG270

Donor	Recipient	Transconjugants per ml[1]
BW86(pC2)[2]	BW97	1.0×10^{8}
BW86(pLG270)	BW97	4.0×10^{2}

[1] Yield of Nal^r Km^r transconjugants per ml mating mix. Liquid matings carried out at 30°C according to section 2.6.1.

[2] pC2 is a ColIb-P9*drd*-2 derivative carrying Tn*5* in *Sal*-5 fragment in a region inessential for plasmid transfer or maintenance (C.Rees, unpublished results).

Plasmid primase-mediated suppression of the *dnaG3* mutation

Strain	CFA[1]	DNA synthesis[2]
BW86(ColIb-P9*drd*-2)	6.5×10^{-1}	248.0
BW86(pLG270)	1.1	260.8

[1] Colony formation of BW86 strains at 40°C relative to 30°C (section 2.8.1.). BW86 = $<10^{-7}$.

[2] Radioactivity incorporated by DNA synthesis in BW86 strains. Measured in units of 10^3 cpm per ml per unit increase in absorbance at A_{450} (section 2.8.2.). BW86 = 9.8.

significantly effect the expression of the plasmid primase gene, as indicated by its ability to suppress the dnaG3 host primase mutation (Table 5.2).

To check that pLG270 did not interfere with the transfer process of the RP4-derivatives, the transfer frequency of pRP2 from donor strains harbouring pLG270 was compared to that in the control mating (Table 5.3). This showed that the yield of Tc^r transconjugants per ml, which represents the transfer efficiency of pRP2, was not significantly altered by the presence of pLG270. The yield of Km^r transconjugants per ml was slightly higher when donors harboured pLG270, possibly indicating a low level of pLG270 transfer. However, none of the 98 Km^r transconjugants tested were able to produce colicin-Ib, which is synthesised by pLG270 (Table 5.3). This indicated that less than 1% of pRP2 transconjugants contained the ColIb-P9 derivative, so making pLG270 a suitable Sog^+ plasmid for use in the donor cell during complementation tests.

Therefore, BW103 strains harbouring pLG270, and either pRP1 or pRP2 were used as donors in filter matings with C600 recipient cells. The results indicated that pLG270 was unable to stimulate the relative yield of pRP1 transconjugants (Table 5.4), implying that ColIb-P9 primase provided in the donor cells could not substitute for RP4 primase.

These results indicate that the proposed role for plasmid primase in the initiation of RCDS is a specific process in which non-homologous primases can only act inefficiently. Also, the total absence of complementation by a sog gene provided in the donor strain, suggests that the process by which priming activity is transferred between mating cells is very specific.

5.3. Complementation of Sog-defective ColIb-P9 derivatives by RP4 plasmid primase

In order to establish whether the ColIb-P9 conjugative system also has specificity for plasmid primase, the ability of RP4 primase to substitute for sog during ColIb-P9-mediated conjugation was tested. For these complementation tests, the measurement of transconjugant yield could not be used since primase-defective ColIb-P9 derivatives only formed 3-fold fewer transconjugants than the Sog^+ plasmid (Chatfield et al., 1982). However, Sog^- conjugative plasmids were 95% deficient in the

Table 5.3 Effect of a transfer-defective ColIb-P9 derivative on pRP2 transconjugant yield in E.coli x E.coli matings

Donor	Recipient	Nal^r transconjugants/ml[1] Tc^r	Km^r	ColIb^r[2]
BW86(pRP2)	BW97	1.2×10^8	1.2×10^8	
BW86(pRP2,pLG270)	BW97	9.3×10^7	9.5×10^7	0/98

[1] Yield of transconjugants per ml resulting from matings carried out according to section 2.6.1.

[2] Number of Nal^r Km^r transconjugants able to produce colicin-Ib, represented as a fraction of colonies tested. The ability of a transconjugant to form colicin-Ib indicates the presence of pLG270.

Table 5.4 Tests for complementation of pRP1 by ColIb^{a}P9 derivatives in donor cells

Strain		Relative yield of
Donor[1]	Recipient	transconjugants/ml[2]
BW103	C600	0.002
BW103(pLG270)	C600	0.004

[1] BW103 donor strains also contained pRP1 or pRP2.

[2] Yield of pRP1 transconjugants per ml mating mix relative to that of pRP2. Transconjugants were selected with Nal Tc after mating on filters for 3 h at 37°C.

incorporation of radioactivity during recipient conjugative DNA synthesis (RCDS; Chatfield et al., 1982). Hence, this sensitive system was used in complementation tests.

It has already been shown that ColIb-P9 primase is required to initiate conjugative DNA synthesis in both the donor and recipient cells and that such requirements can be satisfied by primase synthesised in the donor cell (Chatfield et al., 1982; Wilkins et al., 1985). These studies used a recombinant plasmid, pLG214, which carries a deletion derivative of the sog gene specifying truncated polypeptides. Since the truncated and wild-type polypeptides are known to differ greatly in their ability to substitute for the mutant dnaG3 host primase at 40°C (Wilkins et al., 1981), it was possible that the behaviour of the truncated polypeptides during conjugation may not be typical. Thus, if measurement of RCDS was to be used in complementation tests between Sog^- ColIb-P9 derivatives and Pri^+ plasmids, the ability with which wild-type sog primase could stimulate RCDS in this system had to be determined.

5.4. Complementation of the primase-defective ColIb-P9 derivative by the cloned primase genes of ColIb-P9 and RP4

Measurements of RCDS in BW86 recipient cells during matings with BW96 donors harbouring either pLG221 (Sog^+) or pLG250 (Sog^-), showed that, in keeping with the results of Chatfield et al. (1982), sog primase is a prerequiste for efficient recipient CDS, and that, in the absence of the enzyme, the incorporation of radioactivity into plasmid DNA is virtually abolished (Figure 5.1).

Over the course of several experiments, the level of recipient CDS in matings with BW96(pLG221) donors decreased from over 10000 cpm incorporated per hour per ml of mating mix, to only one tenth this value. At the same time, the strains showed decreased sensitivity to the I_1-specific phage PR64FS and Iα, suggesting a decrease in the proportion of plasmids in the population expressing the transfer genes. This problem was overcome by reconstructing the strains at regular intervals, using short mating times (section 2.4.1.).

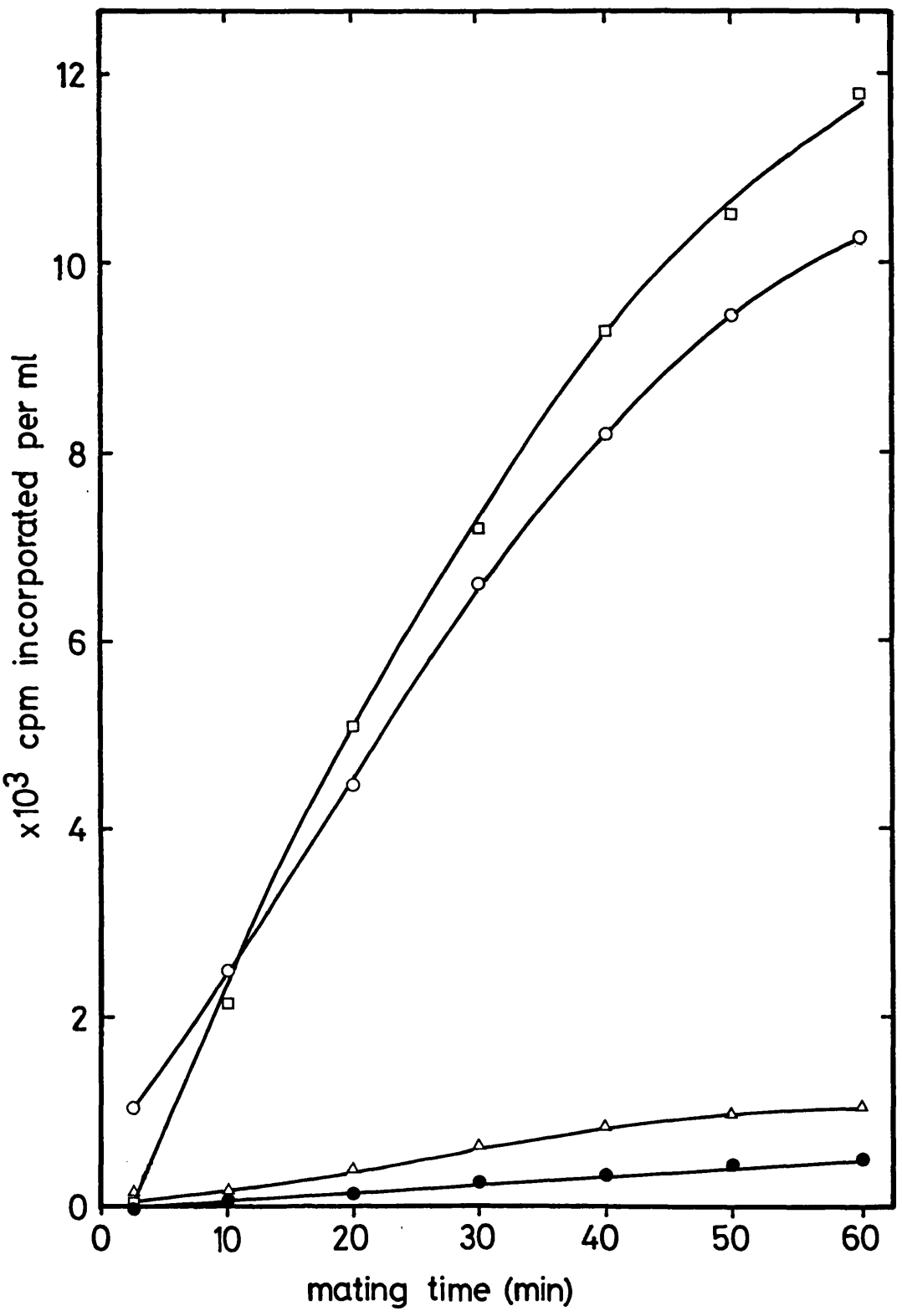
In order to optimise the detection of any effect caused by the Pri^+ recombinant, the complementation tests were performed with the recombinant plasmids present in both donor and recipient cells. The results showed

Figure 5.1 Conjugative DNA synthesis in *dnaG3* recipients during matings between strains harbouring Sog^+ and Pri^+ plasmids

BW96 donor and BW86 recipient strains were grown up as described in section 2.9. Recipient strains were irradiated with $400Jm^{-2}$ UV light prior to mating. Matings were performed at 41°C in the presence of $100\mu gml^{-1}$ Rif and $18.5kBq\mu g^{-1}$ [^{14}C]thymine for 60 min. Acid-precipitable radioactivity in samples taken from the mating mixtures was determined as described in 2.8.3. Donor and recipient strains were incubated separately and treated as for mating cultures. The radioactivity incorporated in the parental strains was subtracted from that obtained for the mating mixtures, and the resultant value plotted against mating time. Time was measured from the addition of the donor strains to the mating flasks. The data are the average of at least three experiments.

Matings were as follows:

(○) BW96(pLG221) x BW86
(●) BW96(pLG250) x BW86
(□) BW96(pLG250,pLG215) x BW86(pLG215)
(△) BW96(pLG250,pLG260) x BW86(pLG260)


12
10
8
6
4
2
0
x10³ cpm incorporated per ml
0
10
20
30
40
50
60
mating time (min)

that the presence of pLG215 (Sog^+) in both strains was able to fully complement pLG250, whereas pLG260 (Pri^+) was shown only to marginally increase the level of recipient conjugative DNA synthesis (Figure 5.1). This implied that RP4 primase, or its products, has only a very limited ability to substitute for *sog* primase.

As discussed previously, the ability of *sog* primase to stimulate the transconjugant yield of pRP1 depended on other ColIb-P9-specified factors. Thus, the ability of pLG260-specified RP4 primase to initiate DNA synthesis on the transferred ColIb-P9 plasmid strand may also depend on RP4-encoded cofactors. To answer this criticism, the primase-overproducing RP4 derivative, pRP26 was used in the donor cells. By virtue of the Tn*7* insertion in the major transfer operon, pRP26 is 100-fold transfer deficient in matings on solid media, relative to RP4 (Barth *et al.*, 1978). Also, in liquid media, IncP plasmids are transferred poorly, presumably due to the instability of the mating aggregates (Bradley *et al.*, 1980). The possibility that the stabilisation of mating aggregates by ColIb-P9 plasmids may allow the transfer of pRP26 was assessed by mating BW96 donors, harbouring pLG221 and pRP26, and BW97 recipient cells in liquid media. The yield of Tc^r transconjugants per ml, representing pRP26 transfer, was about 3×10^6 lower than the yield of transconjugants per ml selected by Km (Table 5.5). This indicated that pRP26 is not significantly transferred to recipient cell during matings in liquid media.

In control experiments, it was demonstrated that pRP26, when present in BW96 donor cells, was unable to stimulate any RCDS in the BW86 recipients. Similarly, the presence of pRP26 in donor strains harbouring pLG221 was shown not to significantly interfere with the amount of RCDS mediated by the $IncI_1$ plasmid (Figure 5.2). Therefore, the ability of pRP26-specified primase to substitute for ColIb-P9 primase was tested by measuring the amount of RCDS occurring in BW86 recipients. This showed (Figure 5.2) that pRP26, when present in the donor cells, was only able to stimulate the level of RCDS to about 20% of that produced by donor strains harbouring pLG221 and pRP26. A similar, moderate stimulation was observed when pRP26 was provided in both donor and recipient cells. This indicated that even in the presence of other plasmid-encoded factors, RP4 primase was only able to modestly substitute for the role of *sog* primase in RCDS.

Table 5.5 Frequency of transfer of pRP26 during ColIb-P9-mediated conjugation

Strain		Nal^r transconjugants/ml[1]	
Donor	Recipient	Tc^r	Km^r
BW96(pLG221, pRP26)	BW97	1.6×10^3	5.2×10^8

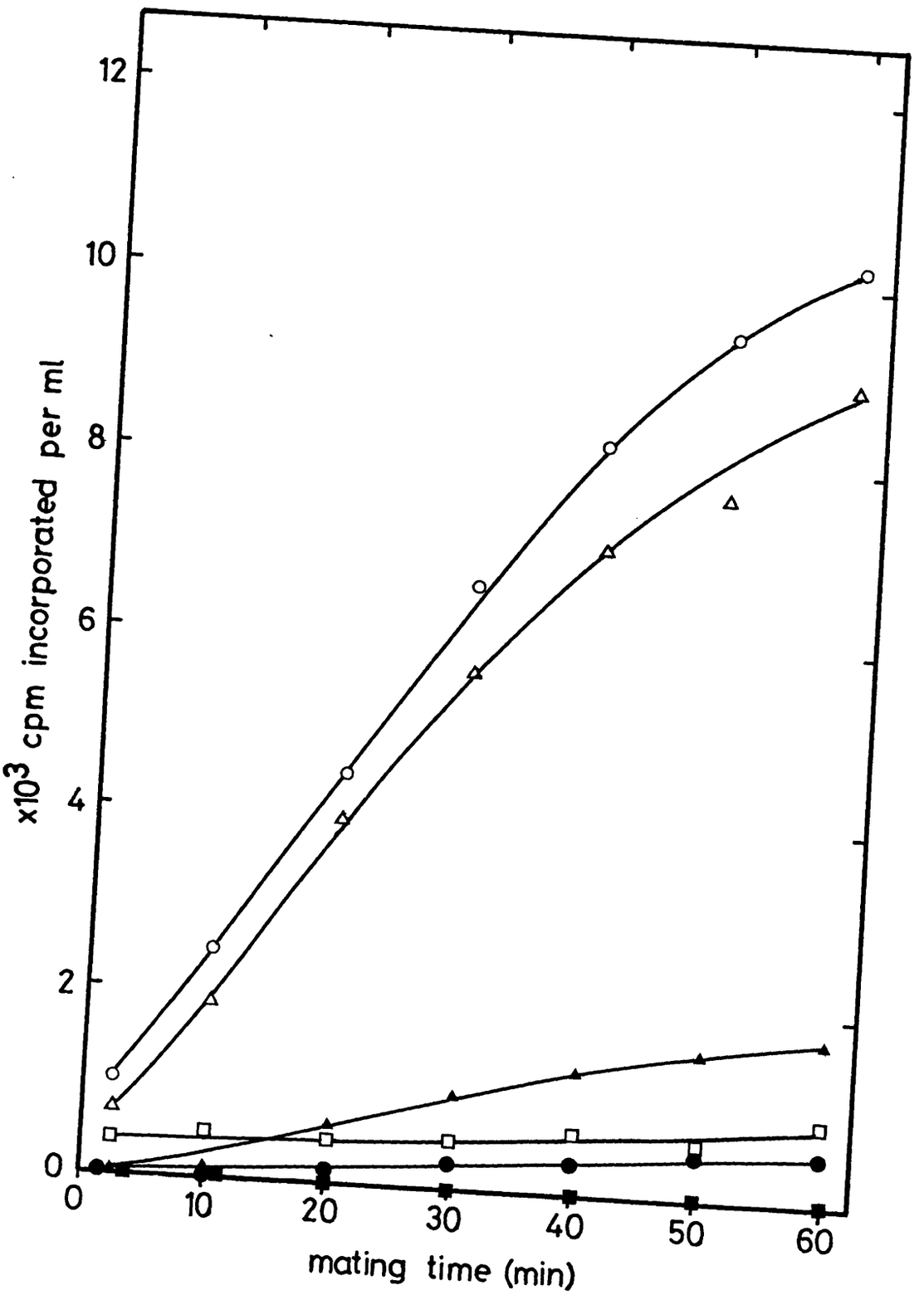
[1] Matings were carried out at 37°C in liquid medium for 1 h (section 2.6.1.) before plating transconjugants on media containing Nal and either Km or Tc.

Figure 5.2 Recipient conjugative synthesis during matings involving the Sog^{A} conjugative plasmid (pLG250) and the Pri^{+} transfer-deficient conjugative plasmid (pRP26)

Matings were performed and conjugative DNA synthesis detected as described in section 2.9. and Figure 5.1.

Matings were as follows:

(○) BW96(pLG221) x BW86
(△) BW96(pLG221,pRP26) x BW86
(●) BW96(pLG250) x BW86
(▲) BW96(pLG250,pRP26) x BW86
(□) BW96(pLG250,pRP26) x BW86(pRP26)
(■) BW96(pRP26) x BW86


12
10
8
6
4
2
0
0
10
20
30
40
50
60
x10³ cpm incorporated per ml
mating time (min)

5.5. Discussion

RP4 and ColIb-P9 plasmid primases are functionally similar *in vitro*, with regard both to the composition of primers formed and the variety of templates utilised. *In vivo*, both primases have been shown to be involved in recipient conjugative DNA metabolism, presumably in the initiation of complementary strand DNA synthesis (Lanka and Barth, 1981; Chatfield *et al.*, 1982; Lanka and Furste, 1985; Chapter 4). In the light of these similarities, the ability of the two primases to substitute for one another in conjugation was investigated. Results indicated that the conjugative process was only efficiently conducted in the presence of the homologous primase. This suggests specificity towards primases at some stage during conjugation.

The deficiency in transconjugant formation by a primase-defective RP4 derivative was shown to be complemented with moderate efficiency by the presence of a Sog^+ $IncI_1$ plasmid in the recipient cell. It is notable that, in order to have any activity in the non-homologous system, the *sog* primase required some as yet unidentified factor(s) encoded by ColIb-P9. However, even in the presence of these factors, the non-homologous primase was only able to complement with less than a quarter the efficiency of the cloned RP4 *pri* gene (Chapter 4). Similarly, the ability of *pri* primase to stimulate RCDS on the transferred ColIb-P9 strand was only a fifth that detected in the presence of *sog* primase.

These results imply that even when synthesised in the recipient cell, plasmid primases appear unable to efficiently recognise plasmid transferred by a non-homologous conjugative system. This might suggest that plasmid primases recognise specific sites on the transferred DNA, and that such sites on RP4 and ColIb-P9 DNA do not share extensive homology. This model however, appears to be in conflict with the results found *in vitro*, which suggest that both primases can initiate DNA synthesis on a similar variety of templates. This apparent anomaly may be explained by suggesting that, during conjugation, plasmid primases may act within a complex of plasmid-encoded polypeptides which is not essential for the activity of the primase during the replication of phage. Evidence for this model comes from the demonstration that purified *sog* and *pri* primase may initiate DNA synthesis on phage templates *in vitro* (Lanka *et al.*, 1979; Lanka and Barth, 1981), yet in contrast, for efficient activity during conjugation, plasmid primases require plasmid-encoded factors

(Chatfield, 1984; section 5.2). An analogy may thus be drawn here with the different activities of host primase on G4 phage DNA and when in the primosome.

In both the RP4 and ColIb-P9 conjugative process, the requirement for plasmid primase in the recipient cell can be satisfied by the synthesis of primase in the donor cell (Chatfield et al., 1982; Chapter 4). This suggests that primase, or its products, are transferred between donor and recipient cells during conjugation. In the case of ColIb-P9, Chatfield and Wilkins (1984) demonstrated by functional tests, that the sog primase itself was transferred. Therefore, it is possible that the specific 'priming complex' of polypeptides is transferred with the DNA to the recipient cell and recognises specific sites in order to initiate complementary strand synthesis on the transferred strand.

Hence, the inability of pri primase to efficiently act during ColIb-P9 mediated conjugation may result from two specificities. Firstly, the ColIb-P9 'priming complex' may be unable to efficently recognise non-homologous primases, so making the initiation of DNA synthesis by these enzymes ineffective. Secondly, even if pri primase is present in an RP4 'priming complex', as may be the case in complementation tests involving pRP26, the non-homologous complex may be unable to efficiently recognise the specific sites on the transferred ColIb-P9 DNA.

This 'priming complex' model may also explain the observation of Chatfield et al. (1982), that the cloned sog gene provided in donor cells cannot fully complement the deficiency in RCDS during the transfer of pLG250. The sog polypeptides encoded by pLG250 do not possess primase activity, but are thought to be of normal size (Chatfield et al., 1982). Therefore, a proportion of the 'priming complexes' assembled in donor cells harbouring pLG250 and pLG215 (Sog^+) would contain inactive primase, and thus be unable to perform the required role in the recipient cell. However, since the presence of pLG215 in the recipient cells is able to moderately complement pLG250, it must be hypothesised that inactive primase polypeptides in the transferred complex can be exchanged for active protein in the recipient cell. Such a 'priming complex' model would also encompass the observation that host priming systems are only able to substitute moderately well during the transfer of primase-defective ColIb-P9 plasmids (Chatfield et al., 1982).

A similar model can be hypothesised for the transfer of RP4 primase, or its products, during RP4-mediated conjugation. However, it must be stressed that there is no evidence to suggest that *pri* polypeptides are transmitted during conjugation. The total inability of pLG270, when present in the donor cell, to stimulate pRP1-transconjugant formation suggests that ColIb-P9 primase, or its products, synthesised in donor cells are unable to be transferred by the RP4 conjugative system. The formal possibility that the Tn*5* insertion in pLG270 may prevent the synthesis of some factor essential for the interaction of *sog* polypeptides with the RP4 conjugation system cannot be excluded. However, since the primase activity specified by pLG270 is able to efficiently substitute for mutant host primase in the formation of colonies, and in the synthesis of DNA at 41°C (Table 5.2), this objection seems unlikely.

The limited ability with which plasmid primases are able to act within non-homologous conjugative systems is in keeping with the model for the role of plasmid primases put forward in Chapter 4. This model suggests that plasmid primases are required to ensure the efficient initiation of RCDS on the transferred strand. It appears that this process is highly specific and can only be performed efficiently by the primase encoded by the homologous conjugative system. In ColIb-P9-mediated conjugation at least, it may be that the initiation of RCDS is performed by a complex of polypeptides, including plasmid primase, which is assembled in the donor cell and transferred to the recipient during conjugation. This model is further investigated in the next chapter.

CHAPTER SIX

ANALYSIS OF DONOR-SPECIFIED POLYPEPTIDES RETAINED BY RECIPIENT CELLS FOLLOWING ColIb-MEDIATED CONJUGATION

6.1. Introduction

The major implication of the work in the previous chapters is that, in order to initiate conjugative DNA synthesis in the recipient cells, the plasmid-specified priming activity synthesised in the donor cell must be transferred by some mechanism to the recipient cell. This could be achieved by either transmitting the enzyme directly to the recipient cell, where primers could be formed *de novo* on the template strand, or by generating primers in the donor cell on the transferred plasmid strand. Chatfield and Wilkins (1984) showed by functional tests, that during $IncI_1$ plasmid mediated conjugation, the enzyme itself was transferred. These experiments involved the measurement of DNA synthesis occuring in rifampicin-treated *dnaG3* recipient cells during matings with donor cells harbouring a Sog^- $IncI_1$ plasmid and a non-transferrable Sog^+ recombinant plasmid. This demonstrated that in 1 h matings, not only did DNA synthesis occur on the transferred plasmid strands, but also approximately a third of the *E.coli* DNA was replicated. Since no precedent exists for the reuse of primer molecules, it was concluded that plasmid primase must be transferred in a form that can initiate DNA synthesis on the transferred plasmid strands and can also substitute for host primase in the replication of the resident chromosome.

Since, in similar experiments, it was found that the truncated polypeptides encoded by pLG214 in donor cells could also stimulate DNA synthesis on the recipient DNA templates, it was suggested that only the N-terminal domain of the 240kD polypeptide, required for primase activity, may actually be transmitted (Boulnois *et al.* 1982; Chatfield and Wilkins, 1984). However, these experiments yielded no information about the sizes or amount of *sog* polypeptide transmitted during conjugation.

The results presented in this chapter elaborate further on these important aspects of the mode of action of plasmid primase by demonstrating physically that the *sog* polypeptides, synthesised in the donor cell, are amongst a limited group of proteins retained by the recipient cell following conjugation.

6.2. Experimental strategy and controls

The method used was adapted from the phage T6 lysis procedure used to measure DNA transfer (Wilkins et al., 1971). Total protein in T6-sensitive donor cells harbouring pLG221 (Sog^+) was labelled by adding a small amount of [^{35}S]methionine to bacteria growing in methionine-free medium. After 15 min at 37°C, the labelled cells were mated for 30 min with UV-irradiated, Rif-treated, T6-resistant recipient cells in an excess of unlabelled methionine. Rif was added to inhibit the transcription of incoming plasmid DNA, thus amplifying the plasmid transfer (Boulnois and Wilkins, 1978). Thus, under these conditions the only labelled protein present in the recipient cells following mating was of donor cell origin. The UV-irradiation allowed comparison with experiments previously performed to measure the transfer of plasmid DNA (Chatfield and Wilkins, 1984). After mating, the donor cells were selectively lysed using phage T6 and the lysate removed as described in section 2.11. The labelled polypeptides present in the recipient cells were then analysed by SDS-polyacrylamide gel electrophoresis and autoradiography.

One of the control experiments involved the use of a recipient strain harbouring a cloned $IncI_1$ exclusion gene. Exclusion acts to limit conjugation between cells carrying plasmids which specify similar transfer systems (Lederberg et al., 1952). Similarly, in R144, an $IncI_1$+B plasmid related to ColIb-P9 (Chabbert et al., 1978; Guerry and Falkow, 1971; Falkow et al., 1974), the products of a cloned entry exclusion gene (exc) act to limit DNA transfer between mating cells, but do not inhibit mating pair formation (Hartskeerl and Hoekstra, 1984; 1985). The entry exclusion system of F, encoded by traS, acts in a similar manner (Achtman et al., 1977).

Hartskeerl et al. (1985), showed that plasmids R144 and ColIb-P9 belong to a single exclusion group. The comparison of the restriction endonuclease cleavage sites in the region around the exclusion genes of R144 (exc; Hartskeerl et al., 1983) and ColIb-P9 (eex; Figure 6.1), showed that both plasmids have a 1.16kb EcoRI-HindIII fragment adjacent to a 0.46kb HindIII-SalI fragment, suggesting that this region is common to both plasmids. The presence of the cloned eex gene in the recipient cells reduced DNA transfer and the formation of pLG221 transconjugants by over 98% (Wilkins et al., 1985). Similar reductions in DNA transfer and transconjugant formation observed in equivalent experiments with R144

Figure 6.1 Comparison of restriction endonuclease cleavage sites in the regions of the R144 and ColIb-P9 entry exclusion genes

A. Restriction map of plasmid pLG252 (Chatfield, 1984) derived from results shown in C. The bold line indicates the extent of pBR325 vector DNA and the light line shows the ColIb-P9 *drd-1* *Eco-9* fragment. This cloned fragment encodes the entry exclusion system (Chatfield, 1984; Wilkins *et al.*, 1985). The origin of vegetative replication (*oriV*) is shown. Inner circle shows scale in kb and arrows show extent and direction of transcription of genes indicated.

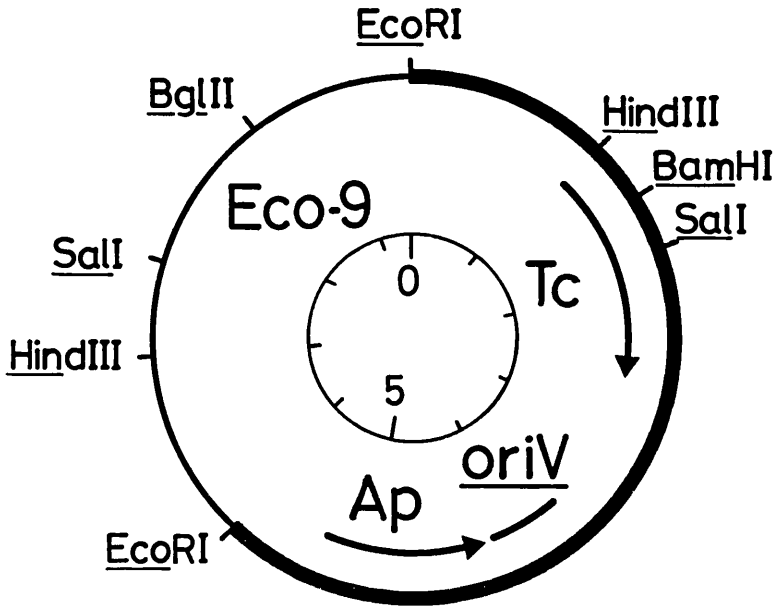
B. Restriction endonuclease cleavage sites in the region of the entry exclusion genes harboured by R144 (*exc*; Hartskeerl *et al.*, 1983) and ColIb-P9 (*eex*; A). Arrows represent the 13kD and 19kD polypeptides encoded by *exc* (Hartskeerl *et al.*, 1983; 1985b).

C. Molecular weights of DNA fragments obtained by restriction enzyme cleavage of pLG252.

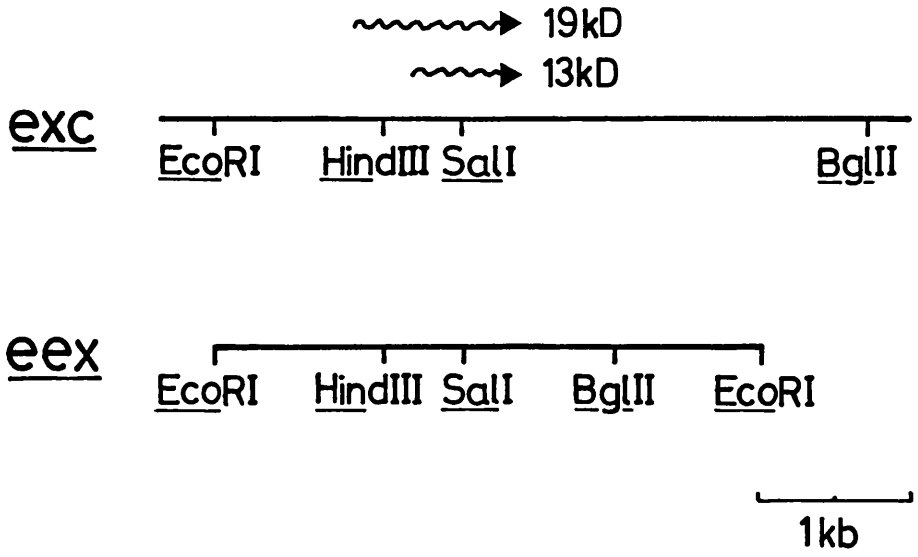
Enzyme	*Bgl*II	*Eco*RI	*Hind*III	*Pst*I	*Sal*I
	~9.3	5.9	5.8	~9.4	5.7
		3.4	3.55		3.65

Enzyme	*Hind*III *Bgl*II	*Hind*III *Eco*RI	*Hind*III *Pst*I	*Hind*III *Sal*I
	5.75	4.65	3.55	5.1
	2.2	2.35	3.5	3.05
	1.45	1.2	2.32	0.64
		1.16		0.46

A.


EcoRI
BglII
HindIII
BamHI
SalI
Eco-9
SalI
0
Tc
HindIII
5
oriV
Ap
EcoRI

B.


19kD
13kD
exc
EcoRI
HindIII
SalI
BglII
eex
EcoRI
HindIII
SalI
BglII
EcoRI
1kb

(Hartskeerl et al., 1985a; Hartskeerl and Hoestra, 1985), suggests that the two plasmid exclusion systems are also functionally similar. Presumably therefore, the observation that R144 entry exclusion acts after the formation of mating aggregates, but prior to DNA transfer (Hartskeerl and Hoekstra, 1984; 1985) can be extented to ColIb-P9.

Chatfield and Wilkins (1984) demonstrated that the transmission of donor-encoded primase activity was blocked by the presence of the $IncI_1$ exclusion gene in the recipient cells. Therefore, the analysis of polypeptides retained by recipient cells harbouring the ColIb-P9 eex gene should enable the proteins transferred as a result of cell-to-cell contact to be distinguished from those reliant on the formation of an effective conjugative system. As a second control, the transmission of polypeptides from labelled plasmid-free 'donor' cells would allow the quantification of any conjugation-independent protein exchange.

In order to label donor-encoded proteins efficiently, the cells must be grown in a methionine-free growth medium prior to the addition of [^{35}S]methionine. In addition, the transfer frequency of pLG221 was known to be greatly enhanced if the cells were grown in a rich medium prior to mating (B.M.Wilkins, personal communication). Difco Methionine Assay (DMA) medium was initially used in an attempt to satisfy these requirements, but for some unknown reason, the growth rate of strains in this medium was very much reduced when compared to that in SGC. Therefore, a methionine-free SGC medium was used, where the casamino acids were replaced by a mixture of all amino acids except methionine. The strains were found to grow equally well in this medium, named SGA, as in SGC.

6.3. Retention of donor-encoded polypeptides by recipient cells following ColIbP9-specified conjugation

Total protein was labelled in BW96(pLG221) cells prior to mating with BW40 recipient cells under the conditions described in section 2.11. The measurement of acid-precipitable radioactivity indicated that 0.91% of the label present in the mating mixture immediately after the addition of the donor cells, was retained by the washed recipient cells following donor cell lysis (Table 6.1). Similar measurements in the control experiments indicated that this value was reduced to 0.38% when recipient cells harboured the eex gene, showing that at least some of the label was

Table 6.1 Amount of [^{35}S]methionine-labelled donor-specified polypeptide retained by recipient cells following IncI$_1$ plasmid-mediated conjugation

Strains	Total acid-precipitable material (cpm)[1] Before mating	After mating	% retained by recipients[2]
BW96 + BW40	4.9×10^7	6.7×10^4	0.14
BW96(pLG221) x BW40	3.6×10^7	3.3×10^5	0.91
BW96(pLG221) x BW40(pLG252)	5.7×10^7	2.1×10^5	0.38

[1] Radioactivity in acid-precipitable material in samples taken from the mating mixture immediately after the addition of BW96 donor strains, and from washed BW40 recipient cells following mating and selective T6 lysis was determined as follows: 3 x 0.1ml samples were taken into 300mM TCA containing 0.6mM methionine. After 30 min on ice, samples were washed through Sartorius cellulose nitrate filter (diameter, 25mm; pore size, 0.45µm) with 15ml ice-cold TCA + methionine. Filters were dried and radioactivity determined as described in section 2.8.2.

[2] Total radioactivity retained by recipient cells following conjugation expressed as a percentage of radioactivity initially present in the mating mixture.

retained as a consequence of the formation of an active conjugative system. However, since this value was reduced still further in the absence of a conjugative plasmid, the formation of mating aggregrates must also account for the retention of some polypeptides (Table 6.1).

The analysis of the recipient cell protein by SDS-polyacrylamide gel electrophoresis and autoradiography (Figure 6.2), indicated that the retained radioactivity was concentrated mainly in a number of discrete polypeptide bands. Following matings involving a fully active conjugation system, about 20 major polypeptide bands were retained by recipient cells, amongst the most prominent of these being those of apparent molecular weight 205, 145, 37, 35 and 28kD. The largest two of this group were shown to comigrate with the sog polypeptides synthesised in minicells, suggesting that at least the 240kD sog protein is transmitted in an unprocessed form during conjugation. It should be noted that the molecular weight of the sog polypeptides has been known for some time to differ from 240kD and 180kD (B.M.Wilkins, personal communication), but the latter designations are used to avoid unnecessary confusion with the published literature.

Since the 180kD sog polypeptide comigrates with the β subunit of RNA polymerase (Figure 6.2), it was impossible to determine from this experiment whether the smaller sog polypeptide was also retained by recipient cells. However, the sample generated in this mating was used subsequently in Western blot analysis, performed by C.Rees and N.Smith, with an antibody raised against truncated sog primase (Wilkins et al., 1981). This demonstrated that the 205 and 145kD polypeptides were related to the sog gene products (Appendix I). The nature of the other polypeptides bound by the antiserum is unknown, but since the majority of these are present in the BW86 strain, it is suggested that they are host-encoded. As the antiserum raised against the sog polypeptides was polyclonal, it is suggested that these other polypeptides are bound by the antibody as a result of topological features shared with the sog polypeptides. Thus, it is clear that both sog polypeptides are retained by recipient cells following $IncI_1$-mediated conjugation. The nature of the other polypeptides retained by recipient cells will be discussed in a later section.

When labelled donors of pLG221 were mated with recipient cells harbouring the eex gene, a significant reduction in the amount of radioactivity

Figure 6.2 Analysis of donor-encoded polypeptides retained by recipient cells following ColIb-P9-mediated conjugation

Polypeptides retained by recipient cells after mating were obtained by labelling total protein in BW96 ($T6^S$) donor strains, at A_{600} = 0.3, with [^{35}S]methionine for 15 min at 37°C. 1.5ml labelled culture (~3 x 10^8 cells) was mixed with 1.5ml UV-irradiated BW40 ($T6^r$) recipient cells (at A_{600} = 0.35) and cells allowed to mate at 37°C for 30 min in presence of Rif (100µgml^{-1}) and methionine (350mM). Donor cells were lysed with T6 phage and the lysate treated with DNase, RNase A, protease K and Brij 58 as described in section 2.11. Intact recipient cells were pelleted and washed extensively before labelled proteins were released by boiling in 100µl SDS-PAGE sample buffer. 25µl samples were electrophoresed through a 10% SDS-polyacrylamide gel as described in section 2.10.6.

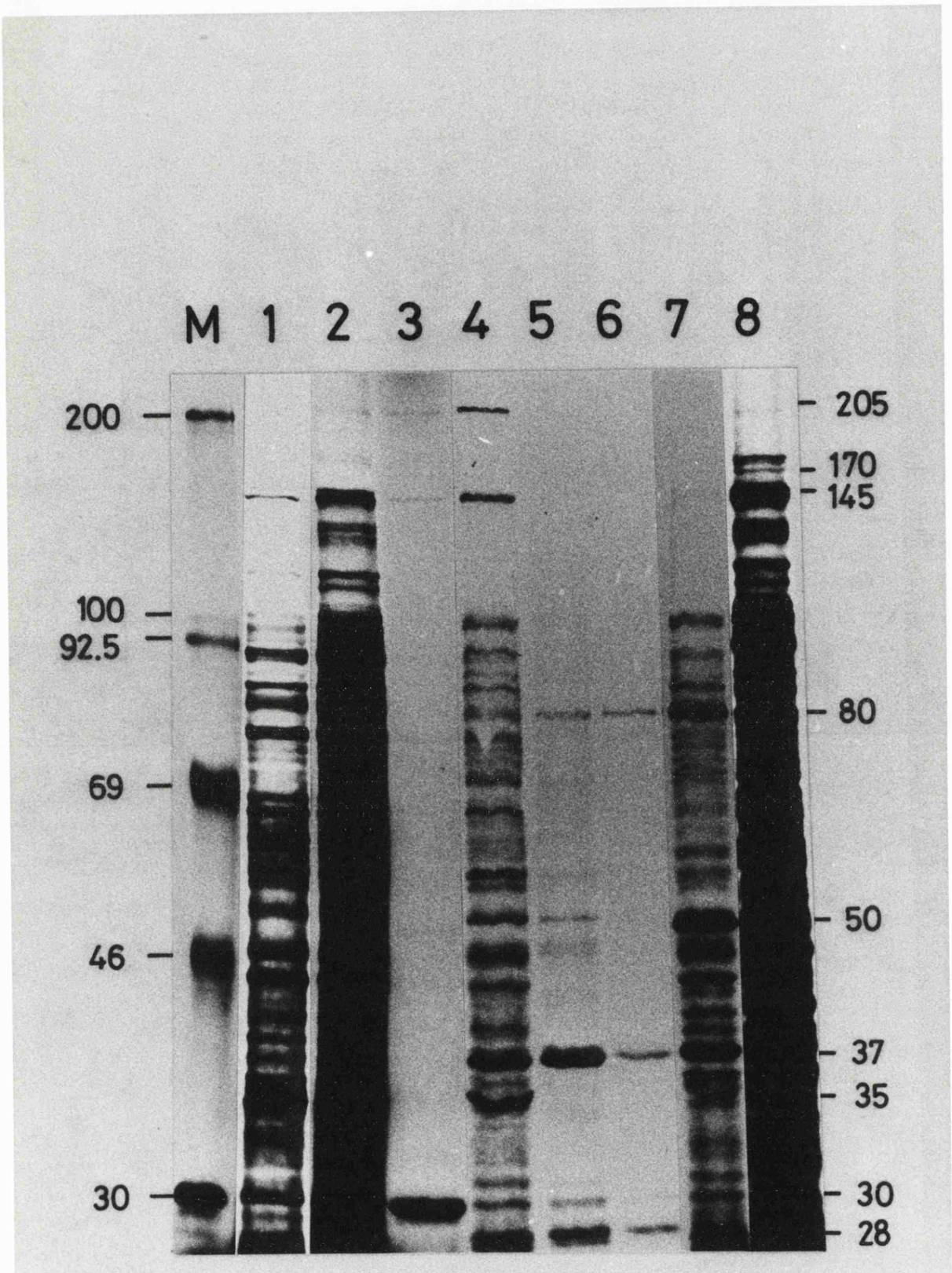
Total cell protein in BW86 strains harbouring pLG221 or pLG264 was labelled as described in section 2.10.5. Samples were diluted 20-fold before loading. Protein equivalent to ~1 x 10^6 cells was loaded (~40µl).

Minicells from strain DS410(pLG215) were prepared and polypeptides labelled as described in section 2.10.1. 30µl of labelled cells were loaded.

Molecular weights of radioactively labelled marker polypeptides (lane M) are shown to the left. Apparent molecular weights of pertinent proteins are shown to the right and are discussed in the text.

Samples were loaded as follows:

(1) Light exposure of Lane 2
(2) BW86(pLG221) - Total cell protein
(3) DS410(pLG215) - Plasmid-encoded polypeptides
(4) BW96(pLG221) x BW40 - Recipient retained polypeptides
(5) BW96(pLG221) x BW40(pLG252) - Recipient retained polypeptides
(6) BW96 + BW40 - BW40-retained polypeptides
(7) BW96(pLG264) x BW40 - Recipient-retained polypeptides } see Chapter 7
(8) BW86(pLG264) - Total cell protein


M 1 2 3 4 5 6 7 8
200
100
92.5
69
46
30
205
170
145
80
50
37
35
30
28

retained by the recipient cells was detected (Table 6.1). This was matched by a corresponding decrease in the number of polypeptide bands retained, such that only five major polypeptides of molecular weights 80, 50, 37, 30 and 28kD, along with a few more minor bands, were observed. No retention of the 205 and 145kD polypeptides was detected. Interestingly, three of the retained polypeptides (80, 37 and 28kD) were also transmitted between cells in the absence of any conjugative system (Figure 6.2). This suggests that small amounts of apparently specific polypeptides may be passed between cells by a conjugation-independent route. A mechanism for this process will be discussed later.

Therefore, in keeping with the results from functional tests (Chatfield and Wilkins, 1984), the transmission of donor-encoded primase polypeptides to recipient cells by a conjugation-specific process has been demonstrated. The physical evidence from these experiments shows that both sog polypeptides are retained by recipient cells in an apparently unprocessed form. The route by which this transmission occurs will be discussed.

6.4. Cellular location of sog polypeptides

In order to identify the cellular location of the sog polypeptides, protein encoded by the maxicell strain CSH26ΔF6 harbouring pLG215 were labelled and the cells fractionated by sonication. The inner and outer membrane proteins were then separated using the criterion of sarkosyl-solubility. The effectiveness of the separation procedure was illustrated by the absence of any of the periplasmic protein, β-lactamase, in the membrane fractions (Figure 6.3). This procedure showed that both sog polypeptides were located in the soluble fraction.

In order to determine whether the sog polypeptides were located in the periplasmic or cytoplasmic fractions, similarly labelled maxicells were subjected to the osmotic shock procedure of Nossal and Heppel (1960). This procedure indicated that none of the 240kD and only a little of the 180kD polypeptide was released by osmotic shock. A small proportion of the smaller polypeptide is therefore apparently located in the periplasmic fraction. Since no other polypeptides appear in this fraction, this cannot be attributed to cell lysis. The presence of almost all of the β-lactamase in the periplasmic fraction (Figure 6.3) indicated that spheroplasts were formed from the vast majority of cells by the osmotic shock.

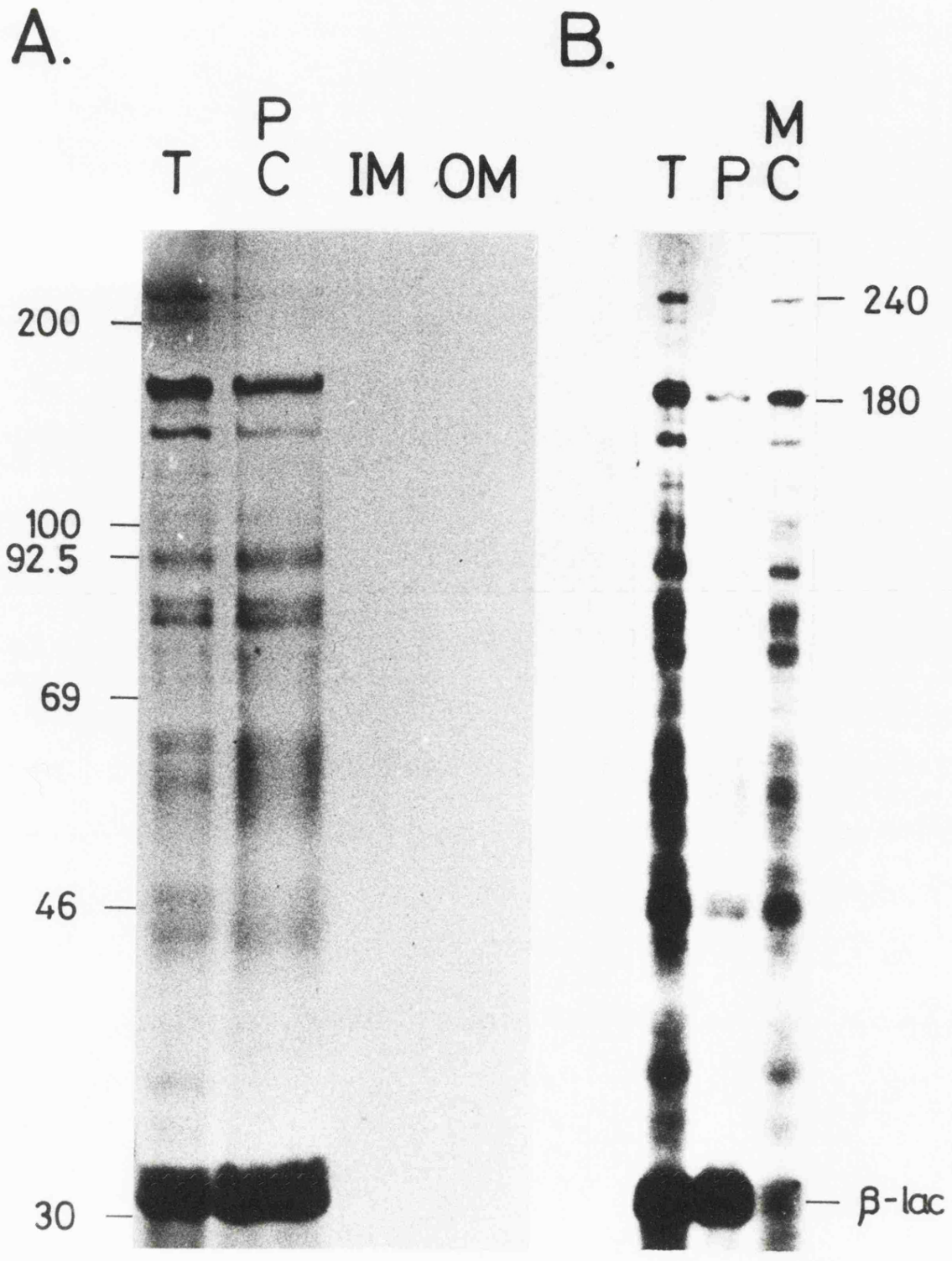
Figure 6.3 Determination of cellular location of sog polypeptides

A. Maxicells harbouring pLG215 were prepared and polypeptides labelled as described in section 2.10.2. A 50µl aliquot was retained as the 'Total' sample. The remainder of the cells were sonicated and the membrane and soluble fractions separated. The membrane proteins were further fractionated on the basis of sarkosyl-solubility (section 2.10.3.). The polypeptides in each fraction, derived from an approximately equivalent number of cells, were analysed by electrophoresis through a 13% SDS-polyacrylamide gel followed by fluorography and autoradiography (section 2.10.6). The autoradiograph shown is a 12 h exposure.

T - total cell sample 25µl
P C - periplasmic and cytoplasmic fractions 50µl
IM - inner membrane fraction 50µl
OM - outer membrane fraction 50µl

B. Maxicells harbouring pLG215 were prepared and polypeptides labelled as described in section 2.10.2. Half the cells were retained as the 'Total' sample. The remainder of the cells were osmotically shocked as described in section 2.10.4. The cytoplasmic and membrane fractions were separated from the periplasmic fraction by centrifugation. Samples were loaded such that the protein in the 'Total' sample was equivalent to the sum of that in the fractions. Samples were loaded onto a 13% SDS-polyacrylamide gel and electrophoresed before labelled protein was detected by fluorography and autoradiography (section 2.10.6.).

T - total cell sample 10µl
P - periplasmic fraction 10µl
M C - membrane and cytoplasmic fractions 10µl
βlac - β-lactamase (periplasmic protein)


A.
T
P C
IM
OM
200
100
92.5
69
46
30
B.
T P
M C
240
180
β-lac

These results indicate that the 240kD (Sog^+) polypeptide is located in the cytoplasmic fraction, in keeping with the suggested role for plasmid primase in conjugative DNA metabolism. The function of the 180kD polypeptide is unknown, but its presence in both soluble fractions may be significant. This will be discussed further in Chapter 7.

6.5. Discussion

In contrast to the extensive study of DNA transfer during conjugation, very little attention has been paid to the possible transmission of RNA or protein. This may reflect the paucity of knowledge relating to the contact formed between the mating cells. In the case of $IncI_1$ plasmids, this ignorance is further compounded by a poor understanding of the proteins involved in the conjugative transfer of DNA. However, the work presented here, involving one of the best characterised ColIb-P9 transfer genes, *sog*, may set a precedent which will allow the study of the interactions between host- and plasmid-encoded polypeptides during the conjugative process.

Previous work has established that, during ColIb-mediated conjugation, less than 0.3% of donor-encoded protein was transferred between mating cells, implying that a general mixing of the donor and recipient cytoplasm does not occur. However, this work was unable to rule out the possibility that small amounts of specific polypeptides could be transmitted (Silver and Ozeki, 1962; Silver *et al.*, 1965). This possibility was realised by the functional demonstration that $IncI_1$ primase, synthesised in the donor cell, could be transmitted to the recipient cell in order to initiate DNA synthesis (Chatfield *et al.*, 1982; Chatfield and Wilkins, 1984). Although these experiments predicted that the conjugative transfer of polypeptides would occur, neither the size of the transmitted products nor the nature of the mechanism employed could be determined.

The use of SDS-polyacrylamide gel electrophoresis has allowed the donor-encoded polypeptides retained by the recipient cell following conjugation to be analysed at the physical level. This approach revealed that a small group of about 20 polypeptides are apparently transmitted during conjugation and prominent amongst this group are the two *sog* polypeptides. This showed that both *sog* polypeptides are selectively transmitted as apparently unprocessed proteins, between donor and recipient cells during conjugation.

The nature or function of the other polypeptides retained by recipient cells is unclear, and some possibilities for this class of protein are considered later in this section. A comparison between the labelled polypeptides synthesised in the donor strain and those retained by the recipient cell, reveals that not all the major donor polypeptides are retained. Conversely, some minor bands in the donor strain, notably the sog polypeptides, appear to be enriched in the mated recipient. This indicates that the retention of polypeptides results from a selective process, and is not due to the random exchange of proteins. This conclusion is in keeping with the results of Silver et al. (1965).

Thus, although a discrete group of polypeptides are clearly transmitted during conjugation, the route between the mating cells remains uncertain. Since the retention of many polypeptides is substantially decreased by the presence of the entry exclusion gene in the recipient cells, it is tempting to suggest that the proteins are transferred with the plasmid DNA. However, the currently popular model of conjugation envisages a conjugation bridge involving the localised fusion of donor and recipient membranes at specific sites (Bayer, 1975; 1979; Willetts and Skurray, 1980), and the report that phage λ receptor sites can be exchanged bidirectionally during conjugation presumably confirms that at least the outer membranes are fused (Goldschmitt and Curtiss, unpublished result cited in Curtiss et al., 1977). This may therefore represent an alternative route of transmission for proteins located in the cell membranes during conjugation. Similarly, even in the absence of membrane fusion, the cell-cell contact established during conjugation could result in fragments of donor cell membrane in the vicinity of the conjugative bridge becoming attached to the recipient cell surface following donor cell lysis.

These models represent routes by which polypeptides could be retained by recipient cells following donor cell lysis and the distinction between them is difficult. The decrease in the retention of most polypeptides by the entry exclusion system may suggest that many proteins are transferred with the plasmid DNA. However, if entry exclusion acts to limit conjugation by preventing the formation of the conjugative bridge, the transmission of polypeptides by routes involving both membrane fusion and DNA transfer would be inhibited.

Therefore, other factors must be taken into consideration in order to

predict the route by which polypeptides are transmitted. In the case of sog primase, its ability to bind single-stranded DNA (Lanka et al., 1979), and its known role in DNA metabolism (Chatfield et al., 1982) suggest that the enzyme is transmitted in association with the transferred DNA strand. The demonstration that the 240kD polypeptide is located in the cytoplasm argues in favour of this model. Also, as revealed by examination of the sequence of the 5' end of the RP4 primase gene (Lanka et al., 1984), the RP4 primase polypeptides, which are functionally analogous to that of ColIb-P9, apparently lack an N-terminal signal sequence normally associated with the precursors of outer membrane proteins. This suggests, in keeping with the cytoplasmic location of the sog gene products, that primases are not be transferred directly as a result of membrane fusion. Since the two sog polypeptides are sequence-related (Boulnois et al., 1982), it is suggested that the 180kD polypeptide may also be transmitted in association with the transferred DNA.

It is noteable that the presence of the entry exclusion system does not block the retention of all polypeptides by the recipient cell. Interestingly, most of the prominent proteins in this group are also exchanged between cells in a conjugation-independent process. A possible explanation for the transmission of the latter group of proteins is suggested by the observation that growing E.coli cells release small blebs of envelope material during septum formation (Burdette and Murray, 1974). Loeb and Kilner (1978) showed that the material in such vesicles originated from the outer membrane, but that the polypeptide composition was atypical. Although some major outer membrane proteins, such as OmpF/C polypeptides (36-37kD) were present, others, such as the OmpA protein (35kD), were deficient. Since recipient cells in all experiments retained 80kD and 37kD polypeptides, it is possible that these proteins may be iron-uptake proteins and OmpF/C respectively (Lugtenberg and van Alphen, 1983), transferred in blebs of donor membrane. The prominent 35kD polypeptide transmitted only by the fully active conjugative system is suggested to be OmpA protein, which could be transmitted as the result of membrane fusion. Clearly immunopreciptation or Western blotting experiments would be required to confirm the identity of these polypeptides.

However, not all the polypeptides retained by recipient cells harbouring the eex gene were transmitted by the conjugation-independent process. The retention of the 50kD and 30kD polypeptides appears to be dependent on

the formation of mating aggregates, and is not significantly increased by the transfer of DNA. This suggests that these donor-specified polypeptides may be important in the formation of cell-cell contacts.

Clearly further work using this system would enable many of the proteins involved in mating pair formation and DNA transfer to be identified. Current knowledge of the process of conjugation, detailed in Chapter 1, allows the nature of some of the polypeptides retained by recipient cells to be predicted.

The popular model of conjugation envisages that, following the initiation and stabilisation of mating pair formation, a specific bridge is formed between the mating cells to allow the transfer of DNA. Therefore, following donor cell lysis, the recipient cell would be predicted to retain polypeptides involved in the formation of this bridge. This would include pilin subunits and the plasmid-encoded polypeptides involved in the stabilisation of mating pairs, such as the F *traG* and *traN* gene products. Many of the F *tra* gene products responsible for pilus assembly are located in the cell membrane (see Willetts and Skurray, 1980). Indeed the pilin subunit is synthesised as a precursor with an N-terminal signal sequence which presumably allows its passage through the inner membrane (Frost *et al.*, 1984). The signal sequence is cleaved by the F *traQ* gene product (Ippen-Ihler *et al.*, 1984) and presumably pilus assembly occurs within the cell membrane. Therefore, due to the intimate nature of the cell-to-cell contact during conjugation, the plasmid-encoded polypeptides associated with pilus synthesis would be expected to be retained on the surface of recipient cells following donor cell lysis. Furthermore, those proteins involved in the formation of the conjugation bridge should be retained, along with the host encoded outer membrane proteins in close proximity to this structure.

Those polypeptides so far mentioned would be predicted to be retained on the recipient cell surface. However, other donor-encoded polypeptides, mostly concerned with DNA metabolism, are predicted to function within the recipient cell, and as such would be transmitted during conjugation.

The DNA transferred by the F and $IncI_1$ plasmids is known to be single-stranded (Cohen *et al*, 1968; Vapnek and Rupp, 1971), as is the case for the mobilisable plasmid, ColEI (A.C.Boyd and D.J.Sherratt, personal communication). The transfer of DNA proceeds from the *oriT* site, which is

nicked by specific plasmid-encoded polypeptides. In ColEI transfer, this nicking reaction is catalysed by at least three polypeptides, one of which remains covalently bound to the 5' end of the induced nick (Blair and Helinski, 1975; Lovett and Helinski, 1975). The transfer of the ColEI DNA, therefore results in the transmission of the bound 60kD polypeptide to the recipient cell, where it is believed to have a role in the recircularisation of the transferred DNA (Warren et al., 1978). Similar polypeptides may also exist in other conjugative systems.

During the course of transfer, the single-stranded DNA is likely to be protected by a coating of single-stranded binding protein (SSB). F has been shown to encode an SSB (Kolodkin et al., 1983; Chase et al., 1983) and similarly, other plasmids, including ColIb-P9 (C.Howland, unpublished results), have been shown to possess regions homologous to the F ssb gene (Golub and Low, 1985). There is at present, some uncertainty concerning the roles of the host- and plasmid-encoded SSB in conjugation, but irrespective of their source, SSB proteins are hypothesised to be transmitted into the recipient cell.

In order to ensure the efficient formation of duplex DNA in the recipient cell, there may be a class of plasmid-encoded polypeptides which guide the transferred single-stranded DNA to the replicative machinery of the host. The prototype for such a protein is the M13 gene 3 product, which is thought to be responsible for directing the passage of the viral DNA through the cell membrane to the replisome on the inner membrane of the host cell (Marco et al., 1974; Kornberg, 1974). Hypothetical analogues of this polypeptide may also fulfil such a role in the transmission of plasmid DNA.

The retention of the 240kD sog polypeptide has been clearly shown. Since the efficient activity of primase has been demonstrated to require plasmid-encoded factors, at least in some systems (Chatfield, 1984; Chapter 5), the transmission of such cofactors would also be predicted to occur during conjugation.

Following the transfer of plasmid DNA, mating cells are known to disaggregate actively (Achtman, 1977). It is possible therefore, that a polypeptide bound to the end of the transferred strand could act to trigger this process, and clearly such a protein should be present in recipient cells.

Therefore, the work presented in this chapter has shown that a small group of donor-specified polypeptides, including both the sog proteins, can be retained by recipient cells following conjugation. The process of polypeptide transmission is clearly very selective and the specificity suggests a complex recognition system, possibly incorporated into the transmembrane pore thought to be involved in the transfer of DNA. Some of the retained polypeptides are suggested to be transmitted by a route involving the fusion of donor and recipient membranes, either during conjugation, or as a result of donor cell lysis. The study of these polypeptides should give an insight into the mechanism of cell-to-cell interactions. However, more relevant to this thesis, some polypeptides are thought to be transmitted in association with the transferred DNA, and the properties and role of plasmid primase predict that it should be transmitted in this manner. The sequence-related 180kD polypeptide has also been shown to be retained by the recipient cells, although the reason for the transmission of this protein is unclear since its role is unknown. Work presented in the next chapter clearly suggests that the transmission of this polypeptide is of great importance to the efficiency of the ColIb-P9 conjugative process.

CHAPTER SEVEN

ROLE OF THE 180kD *sog* POLYPEPTIDE IN IncI, PLASMID-MEDIATED CONJUGATION

7.1. Introduction

Previous work has shown that the *sog* gene encodes two antigenically related polypeptides, of 240kD and 180kD, which are the products of two overlapping, in-frame reading frames on a common transcript (Wilkins *et al.*, 1981; Boulnois *et al.*, 1982). Only the 240kD polypeptide specifies primase activity and the moiety responsible for this is confined to the N-terminal third of the polypeptide. Since truncated derivatives of the 240kD polypeptide, which have lost the C-terminal two-thirds of the protein, were still able to specify primase activity (Boulnois *et al.*, 1982), it was apparent that the *sog* gene products were multifunctional. The role of the non-primase section of the large polypeptide, and of the sequence-related 180kD protein is unknown. However, since the *sog* gene is coordinately expressed with the ColIb-P9 transfer genes, it is proposed that the polypeptides will have a role in the conjugative process (Wilkins, 1975; Wilkins *et al.*, 1981). Therefore, in order to ascertain the function of these proteins, the C-terminal region of the *sog* gene was inactivated by Tn*5* mutagenesis.

Initial attempts to isolate ColIb-P9 *sog*::Tn*5* derivatives failed due to the preferential insertion of Tn*5* into fragments *Sal*5 and *Sal*6, according to the nomenclature of Uemura and Mizobuchi (1982). The use of a different transposon, Tn*1723*, also failed to produce mutants of the *sog* gene (Cath Rees, unpublished results). This phenomenon of preferential transposon insertion has been reported in a wide variety of systems (see Berg *et al.*, 1983). To overcome this problem, the plasmid pLG215, which carries the *sog* gene, was used as the target for transposition events. This scheme facilatated the isolation and characterisation of transposon insertion mutants. The effect of these mutations on conjugation was studied by transferring them into ColIb-P9 by homologous recombination (Winans *et al.*, 1985).

7.2. Isolation of Tn*5* insertional mutants of the ColIb-P9 primase gene

The initial isolation of *sog*::Tn*5* mutations was performed by L.Capsey, a project student in the laboratory. The mutations were generated by

transposition of Tn5 from the lambda transducing phage λ::Tn5 (λ cI$_{857}$ b221 rex::Tn5 Oam; Table 2.3). Since the λ b221 and Oam mutations prevent the integration and replication of the phage respectively, the retention of the kanamycin resistance, specified by Tn5, could only be achieved by virtue of the transposition of Tn5 to the chromosomal or plasmid DNA. Hence, this phage was used to infect BW103 (recA1, Sup°) host cells harbouring pLG215, which carries the sog gene. Plasmid DNA prepared from pooled Tc^r Km^r colonies was used to transform BW86 to Km^r. Of the many pLG215::Tn5 plasmids isolated, two, namely pLG262 and pLG263 which harbour the sog-262 and sog-263 mutations respectively, were chosen for further analysis.

7.3. Characterisation of the sog::Tn5 mutations

The restriction endonuclease cleavage map of the two pLG215 sog::Tn5 plasmids was determined and showed that the Tn5 element in pLG262 and pLG263 was inserted at coordinate 8.90 and 6.75, respectively (Figure 7.1). The orientation of the element in the plasmids was different, the IS50R of Tn5 being to the right in pLG262, and to the left in pLG263. The ability of the plasmids to suppress the dnaG3 mutation carried by BW86, showed that the sog-262 gene specified active primase polypeptides, whereas the insertion in pLG263 disrupted primase activity (Table 7.1). These findings were in agreement with the results expected from the mapping data, and the deletion analysis of the sog gene (Boulnois et al., 1982).

The sizes of the polypeptides encoded by the mutant sog genes were determined using the minicell strain DS410. For a reason that remains unclear, the polypeptides encoded by pLG262 were poorly labelled in minicells, but the use of the maxicell strain CSH26ΔF6 overcame this problem (Figure 7.2). As expected, both plasmids expressed the prominent Tn5 polypeptides (58 and 54kD; Rothstein et al., 1980). The polypeptides encoded by the sog-262 gene were of molecular weight ~170 and 95kD, which presumably represent the truncated products of the 240 and 180kD proteins respectively. These truncated polypeptides were slightly smaller than those encoded by pLG228, in keeping with the position of the Tn5 insertion, relative to the pLG228 deletion end-point (Boulnois et al., 1982; Figure 7.4).

The plasmid pLG263 did not encode a 240kD polypeptide (Figure 7.2), as

Figure 7.1 Position of Tn5 insertions in pLG263 and pLG264

Map of pLG215 showing pBR325 vector DNA as a bold line and the approximate position and direction of transcription of the primase gene (sog) as an open arrow. Approximate regions encoding the 240 and 180kD sog polypeptides are shown above, and the positions of the Tn5 insertions in pLG262 and pLG263 represented by triangles below the line. The orientation of the Tn5 insert is shown by the position of IS50L (L) and IS50R (R). For clarity, the relevant features of transposon Tn5 are shown beneath and to the same scale (Auerswald et al., 1980; Beck et al., 1982).

Restriction enzyme cleavage sites are indicated as follows: BamHI (Bm), BglII (Bg), EcoRI (E), HindIII (H) and SalI (S).

Molecular weights of DNA fragments are shown below:

Enzyme	EcoRI	EcoRI BglII	EcoRI HindIII	EcoRI SalI	EcoRI BamHI
pLG262					
	~14.0	6.0	6.45	8.4	8.05
	6.0	4.15	4.8	5.7	6.1
		3.35	4.1	4.1	4.4
		3.35	3.45	1.85	1.6
		2.8	1.3		
pLG263					
	~14.0		9.4	~11.2	~12.0
	6.0		4.8	4.0	4.4
			3.4	3.7	3.4
			1.95	1.95	1.7
			1.3		

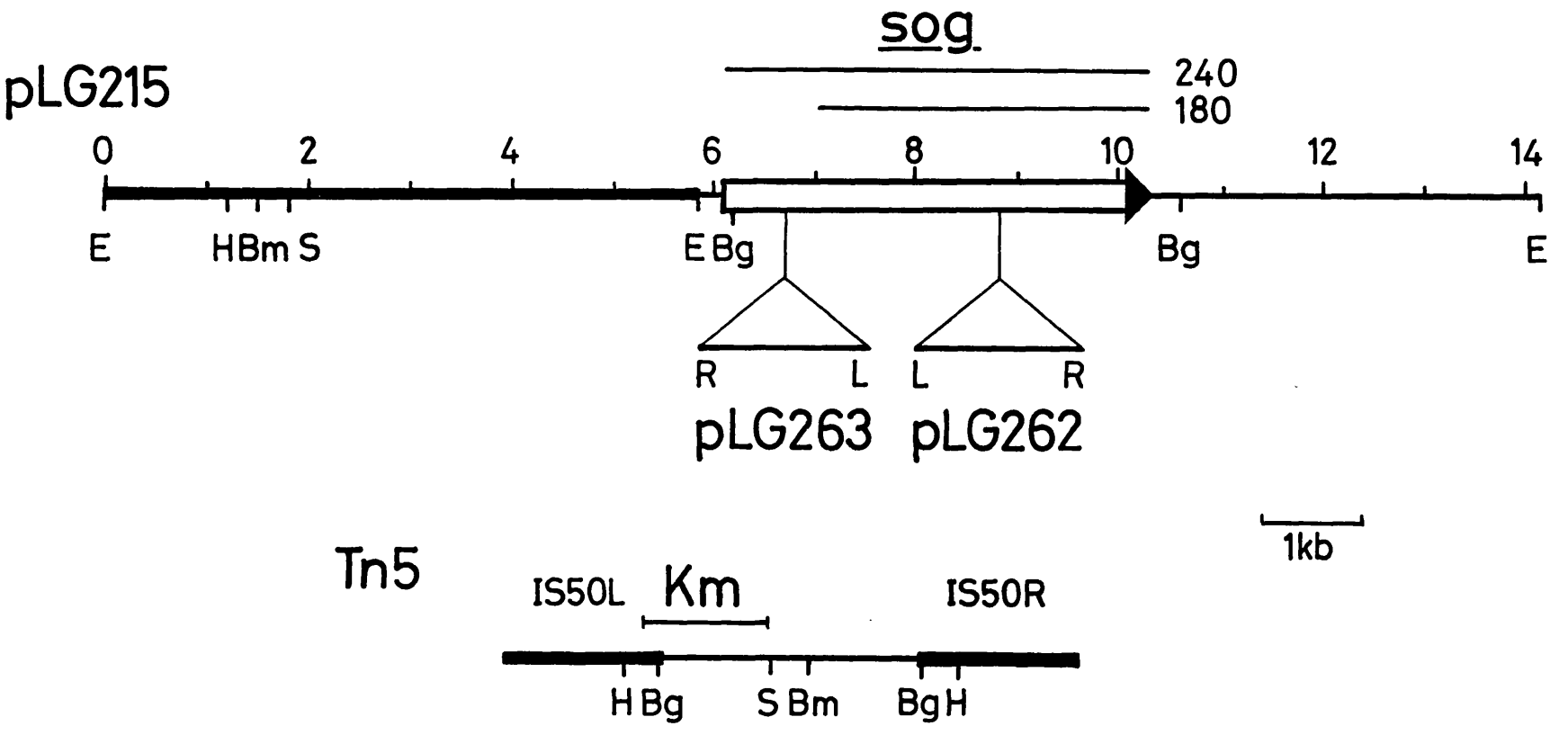

sog
240
180
pLG215
0
2
4
6
8
10
12
14
E
HBm S
E Bg
Bg
E
R
L
L
R
pLG263
pLG262
1kb
Tn5
IS50L
Km
IS50R
H Bg
S Bm
Bg H

Table 7.1 Plasmid primase-mediated suppression of the dnaG3 mutation

Plasmid	CFA[1]	DNA synthesis[2]
pLG215	1.5×10^{-4}	116.2
pLG262	1.1×10^{-5}	57.8
pLG263	2.8×10^{-7}	11.3
ColIb-P9drd-2	6.5×10^{-1}	248.0
pLG264	8.9×10^{-1}	273.6
pLG265	6.4×10^{-1}	ND

[1] Colony formation of BW86 strains at 40°C relative to 30°C (section 2.8.1.). BW86 = $<10^{-7}$.

[2] Radioactivity incorporated by DNA synthesis in BW86 strains. Measured in units of 10^3 cpm per ml per unit increase in absorbance at A_{450} (section 2.8.2.). BW86 = 9.8.

ND Not done.

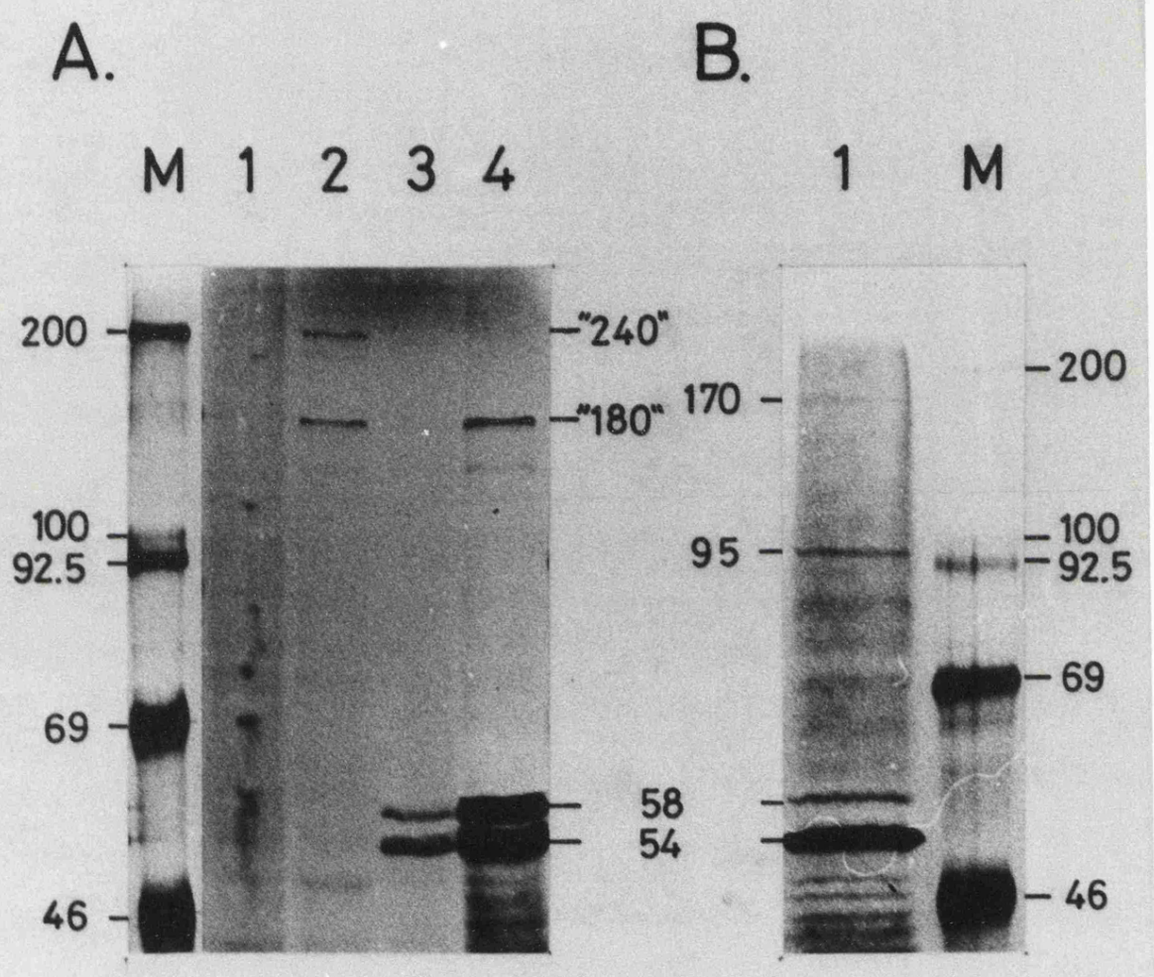
Figure 7.2 Polypeptides detected in DS410 minicell and CSH26ΔF6 maxicell strains

A. Labelled polypeptides in minicell strains were identified by fluorography and autoradiography following electrophoresis through a 13% SDS-polyacrylamide gel. The molecular weights, in kilodaltons (kD), of labelled polypeptides (lane M) are shown to the left and sizes (kD) of plasmid-encoded proteins are given to the right.

The plasmids harboured by DS410 are as follows:

(1) pBR328
(2) pLG215
(3) pLG262
(4) pLG263

B. Polypeptides encoded by maxicell strain CSH26ΔF6(pLG262) were labelled and analysed by fluorography and autoradiography after electrophoresis through a 10% SDS-polyacrylamide gel. The apparent molecular weight (kD) of the plasmid-encoded polypeptides are shown to the left. The sizes (kD) of the labelled polypeptides markers (lane M) are given to the right.


A.
M 1 2 3 4
200
100
92.5
69
46
"240"
"180"
58
54
B.
1 M
170
95
200
100
92.5
69
46

predicted from the absence of primase activity, but unexpectedly, apparently intact 180kD polypeptide was specified. Since both sog polypeptides are translated from a single transcript (Boulnois et al., 1982), it is suggested that the Tn5 insertion in pLG263 is translationally- but not transcriptionally-polar. Previous studies, involving the E.coli lac and Salmonella his operons, have also demonstrated the absence of transcriptional polarity in some Tn5 insertion mutants. This has been attributed to a promoter located within the terminal 186bp of the transposon (Berg et al., 1980). The ability of pLG263 to encode the 180kD polypeptide allows the translational start site for this protein to be positioned within a 750bp region, 850bp from the end of the cloned Eco-3 fragment (Wilkins et al., 1981; Figure 7.1).

7.4. Transfer of the sog::Tn5 mutations into ColIb-P9drd-2 by homologous recombination

To assess the effect of the sog::Tn5 mutations on the transfer efficiency of ColIb-P9, the sog locus of the conjugative plasmid was replaced by the mutant allele using the method of Winans et al. (1985). The recBC sbcB strain N1205 harbouring ColIb-P9drd-2 was transformed to Km^r using either pLG262 or pLG263 DNA linearised with EcoRI. This strain allows the efficient integration of the incoming linear DNA by homologous recombination (Clark 1973; Cosloy and Oishi, 1973). The transformation resulted in ~1250 and 338 Km^r transformants using pLG262 and pLG263, respectively. The difference probably results from the decreased length of homologous DNA flanking the transposon in pLG263 (Watt et al, 1985; Winans et al., 1985).

The transfer of the transposon to the conjugative plasmid was demonstrated by the loss of Tc^r and the replacement of the normal 8.0kb Eco-3 DNA fragment with the Eco-3::Tn5 fragment of ~14kb (Figure 7.3). Two conjugative plasmids, pLG264 and pLG265, which were derived from the transformations with pLG262 and pLG263 respectively, were chosen for further study.

As expected from the characterisation of pLG262, the sog polypeptides encoded by pLG264 were able to efficiently substitute for mutant host primase in BW86 (Table 7.1). The primase activity encoded by the sog-262 gene was enhanced following the transfer of the mutation into the conjugative plasmid. This phenomenom, also observed for the wild-type sog

Figure 7.3 Characteristion of plasmid pLG264

A. pLG264 DNA was cleaved in single and double digests with EcoRI and HindIII and ~0.5μg of cleaved DNA was electrophoresed through 0.7% agarose.

The lanes contain:

(1) ColIb-P9drd-2 x EcoRI
(2) ColIb-P9drd-2 x HindIII
(3) ColIb-P9drd-2 x EcoRI-HindIII
(4) pLG264 x EcoRI
(5) pLG264 x EcoRI-HindIII

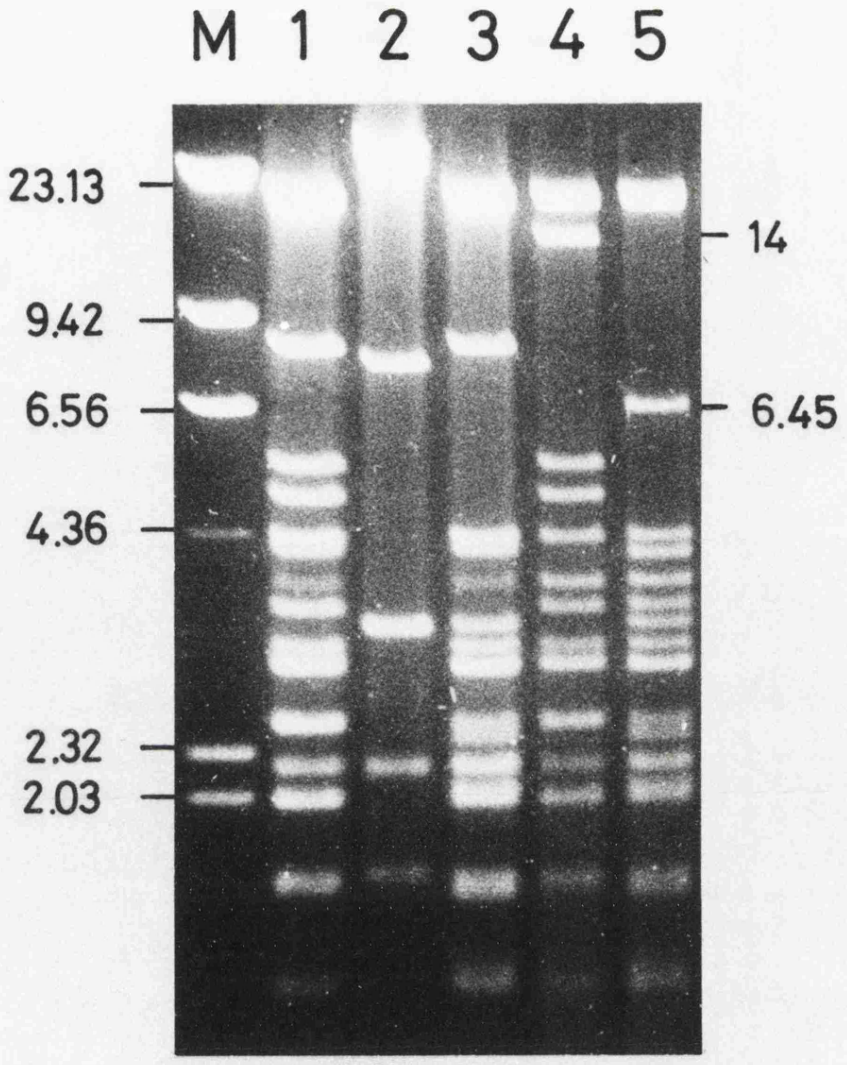
Molecular weights of λ x HindIII DNA markers (lane M) are given in kilobases (kb) to the left and apparent molecular weight (kb) of the distinguishable Eco-3::Tn5 fragments of pLG264 given to the right.

B. Total protein was labelled in BW86 strains and analysed by fluorography and autoradiography following electrophoresis through a 10% SDS-polyacrylamide gel. The lanes contain protein derived from strains:

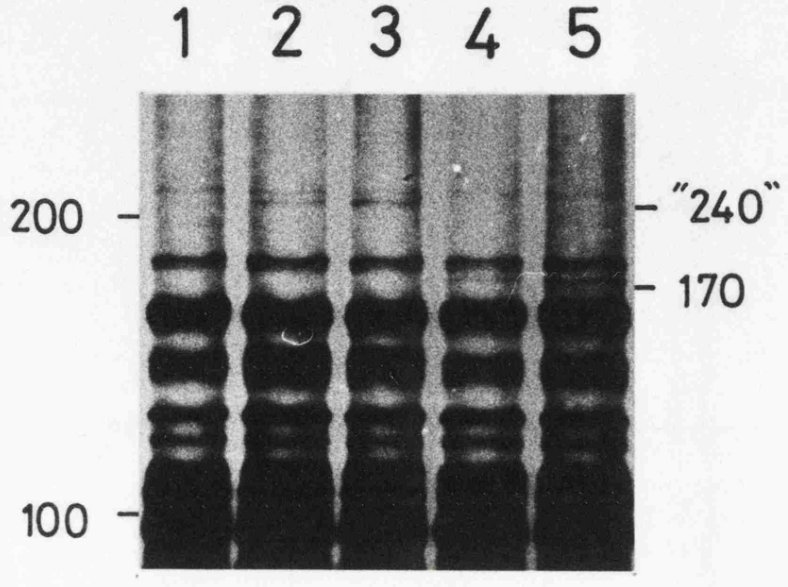
(1) BW86
(2) BW86(ColIb-P9drd-2)
(3) BW86(pLG215)
(4) BW86(pLG262)
(5) BW86(pLG264)

The molecular weights (kb) of the labelled polypeptides markers are shown to the left and the apparent molecular weights of the discernible sog polypeptides are given to the right.

A.



B.



gene, is thought to be due to the presence of ColIbP9-specified cofactors (Chatfield, 1984). The transfer of the sog-262 mutation to pLG264 was further confirmed, by demonstrating that EcoRI-HindIII digests of pLG264 yielded the 6.5kb fragment of Eco-3::Tn5 also seen in digests of pLG262. Also, both plasmids were shown to synthesise the ~170kD truncated sog gene product (Figure 7.3). The 95kD polypeptide encoded by the mutant sog gene in maxicells is presumed to be obscured by other host-encoded proteins.

The transfer of the sog-263 mutation to ColIbP9drd-2 was less successful. In contrast to the polypeptides specified by pLG263, those encoded by pLG265 were able to efficiently suppress the dnaG3 mutation in BW86 (Table 7.1). Therefore, clearly the precise transfer of the sog::Tn5 mutation from pLG263 to the conjugative plasmid had not occurred. In an attempt to isolate a Sog^{+} derivative, plasmid DNA prepared from N1205(ColIbP9drd-2) cells previously transformed to Km^{r} by pLG263, was used to transform BW86. Of the 642 Km^{r} Tc^{s} isolates screened, 5 were found to be unable to suppress the dnaG3 mutation. However, EcoRI-HindIII digestion of the conjugative plasmids harboured by these 5 isolates revealed that, although Tn5 was present in the Eco-3 fragment, two other fragments were absent and a new 6.6kb fragment had been formed, presumably due to the presence of a deletion. The reason for this effect is unclear, but since the transfer of the sog-263 mutation is apparently detrimental to the survival of the strain, further analysis of this mutation was not performed.

7.5. Transfer efficiency of plasmid pLG264

To assess the effect of the sog-262 mutation on conjugation, the efficiency with which pLG264 was transferred in matings between BW103 donors and BW97 recipient strains was tested. Relative to the pC2 control plasmid, pLG264 formed 150-fold fewer transconjugants (Table 7.2), indicating that either the sog polypeptides, or proteins encoded by a gene promoter-distal to sog, were important in the efficient transfer of ColIbP9.

In order to distinguish between these two possibilities, complementation tests were performed using pLG264 and various derivatives of the cloned Eco-3 fragment. The nature of the recombinants used is indicated in Figure 7.4. BW103 cells harbouring pLG264 and an appropriate recombinant plasmid were used as donor strains in matings with BW97 recipient cells. The recombinant plasmids could be divided into two distinct classes on the

Table 7.2 Complementation of transfer-defective plasmid pLG264 with recombinants carrying derivatives of the sog gene

Plasmids harboured in BW103 donors	BW97 recipients	Transconjugants per ml[1]
pC2[2]		6.7×10^7
pLG264		4.5×10^5
pC2, pLG215		2.4×10^8
pC2, pLG226		2.4×10^8
pC2, pLG228		1.0×10^8
pC2, pLG229		1.3×10^8
pC2, pLG214		1.2×10^8
pLG264, pLG215		8.5×10^7
pLG264, pLG226		1.2×10^8
pLG264, pLG228		7.5×10^5
pLG264, pLG229		3.8×10^5
pLG264, pLG214		2.3×10^6
pLG264, pLG218		9.2×10^7
pC2	pLG215	1.1×10^8
pLG264	pLG215	1.8×10^4

[1] Yield of Nal^r Km^r transconjugants derived from 1 h matings at 37°C as described in section 2.6.1.

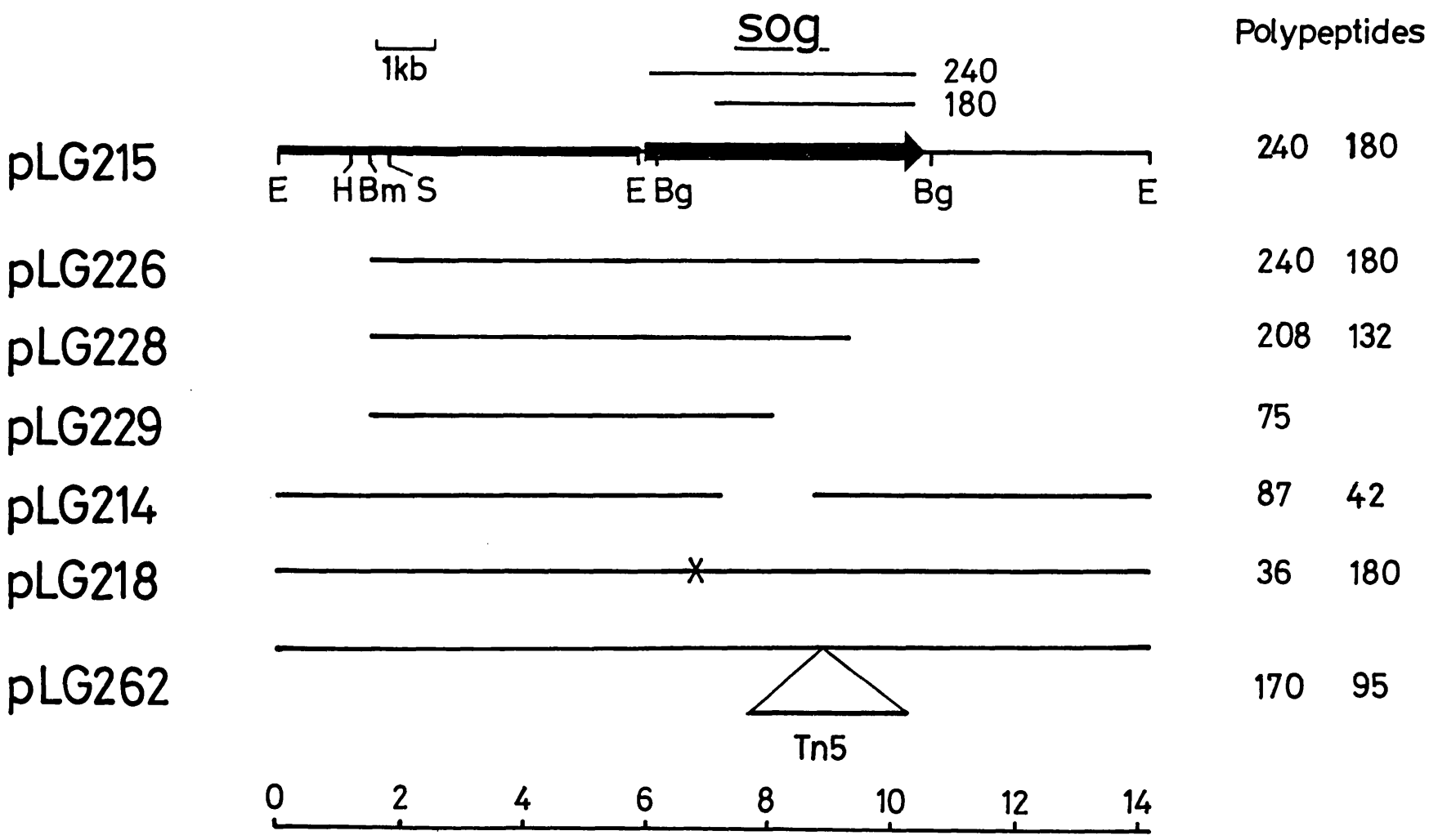
[2] pC2 is a ColIb-P9*drd*-2 derivative carrying Tn*5* in *Sal*-5 fragment in a region inessential for plasmid transfer or maintenance (C.Rees, unpublished results).

Figure 7.4 Map of plasmid pLG215 and various derivatives

Plasmid pLG215 consists of pBR325 vector DNA, shown as a bold line, and the Eco-3 fragment of ColIb-P9drd-1 (Wilkins et al., 1981). The approximate location and direction of transcription of the primase gene (sog) is represented by the arrow, and the approximate regions encoding the 240 and 180kD polypeptides are shown above. Restriction cleavage sites are indicated as follows: BglII (Bg), BamHI (Bm), EcoRI (E), HindIII (H) and SalI (S).

The lines below the map indicate the extent of remaining DNA in the deletion mutants pLG226, pLG228, pLG229 and pLG214, as described previously (Wilkins et al., 1981; Boulnois et al., 1982). Plasmid pLG218 contains an amber mutation at a position approximately indicated by the cross. The location of the Tn5 insertion in plasmid pLG262 is represented by the triangle (not to scale; see Figure 7.1).

The apparent molecular weights (kD) of the sog gene products specified by the plasmids are indicated (Wilkins et al., 1981; Boulnois et al., 1982).


sog
1kb
240
180
Polypeptides
pLG215
E H Bm S E Bg Bg E
240 180
pLG226
240 180
pLG228
208 132
pLG229
75
pLG214
87 42
pLG218
36 180
pLG262
170 95
Tn5
0 2 4 6 8 10 12 14

basis of their ability to complement to pLG264 transfer deficiency (Table 7.2). In the presence of the recombinants pLG215 and pLG226, the transfer efficiency of pLG264 was increased to a level approximately equal to that of the control plasmid pC2. However, recombinant plasmids pLG228 and pLG229, which carry deletions resulting in the loss of the C-terminal region of the sog polypeptides, were unable to significantly affect the pLG264 transfer deficiency.

Thus the complementing factor must be partially, or completely encoded by the 2.1kb region of DNA between the deletion end-points of pLG226 and pLG228. Since the end of the sog gene is thought to lie at coordinate 10.4kb, as predicted from polypeptide sizes, it is possible that a promoter-distal gene, in the region 10.4-11.3kb, may be responsible for the complementation (Figure 7.4). However, this region is present on pLG214, which carries a deletion internal to the sog gene (Wilkins et al., 1981). Although this deletion truncates both sog polypeptides, DNA located promoter-distal to sog will be transcribed, thus allowing the normal expression of any gene located in this region. Since pLG214 fails to complement the transfer deficiency of pLG264 (Table 7.2), it can be concluded that a domain at the shared C-terminal end of the two sog polypeptides is important in the efficient transfer of the conjugative plasmid.

To determine which of the sog polypeptides was important in this complementation, the recombinant plasmid pLG218 was used. This plasmid carries an amber mutant of the sog gene which allows the translation of the 180kD polypeptide, but causes the premature termination of the 240kD protein (Boulnois et al., 1982). When pLG218 was present in the BW103 (Sup°) donor strain, the pLG264 transconjugant yield was increased over 200-fold (Table 7.2). This showed that the 180kD polypeptide was able to efficently complement the transfer deficiency resulting from the sog-262 mutation, suggesting that this polypeptide has an important role in the $IncI_1$-mediated conjugative process.

7.6. Determination of the role of the 180kD polypeptide in ColIbP9-mediated conjugation

It was demonstrated in the previous chapter that 180kD polypeptide was retained by recipient cells following ColIb-P9-mediated conjugation. This implies that the 180kD protein may be involved in a process subsequent to

the transfer of plasmid DNA. In order to test this model, an active *sog* gene, carried by pLG215, was provided in BW97 recipient cells during a mating with BW103 donors harbouring either pC2 or pLG264. The results showed that the transfer deficiency of pLG264 could not be complemented by 180kD polypeptide synthesised in the recipient cell (Table 7.2). The same result was obtained using BW86 donor strains, thereby absolving the host recombination-deficency from any part in this lack of complementation. This suggests that the 180kD polypeptide may be involved in a process which occurs initially in the donor cell.

A series of experiments were therefore performed in order to establish which of the conjugative processes involved the 180kD polypeptide. The observation that cells harbouring pC2 and pLG264 exhibited similar sensitivity to the I_1-type pilus-specific phage Iα and PR64FS, indicated that flexible pilus formation was not significantly affected by the *sog-262* mutation. Similarly, the possibility that the 180kD protein may have a role in the formation of the conjugative bridge was dispelled by showing that pLG264 was able to mobilise the small non-conjugative IncQ plasmid, pGSS33 (Table 7.3). This recombinant carries the antibiotic resistance genes of pBR328 but has the vegetative origin and mobilisation functions of R300B (Sharpe, 1984). In broad agreement with previous estimates (Datta and Hedges, 1972; Guerry *et al*, 1974), the IncQ plasmid was mobilised with an efficiency of 3.8×10^{-4} by pC2. Interestingly, the frequency with which pGSS33 was transferred, as indicated by the yield of Cm^r transconjugants, was over 50-fold higher when stimulated by pLG264, relative to the control plasmid pC2 (Table 7.3). This suggests that the transfer of conjugative and mobilisable plasmids may be competitive at some stage in the conjugative process. This possibility is discussed in a later section.

Therefore, the role of the 180kD polypeptide appears to relate specifically to the transfer of ColIb-P9 DNA. To investigate the possibility that the protein may be required for the efficient nicking of the plasmid DNA at *oriT*, the ability of pC2 and pLG264 to mobilise pLG2009, a small recombinant plasmid carrying the *oriT* region of ColIb-P9 (Wilkins *et al*., 1985), was assessed. As expected, pLG2009 was mobilised very efficiently by pC2, at a frequency approximately equal to the transfer frequency of the conjugative plasmid (Table 7.3). Similarly, the presence of pLG264 stimulated a high frequency of transfer of the small plasmid. Indeed, as was the case in the mobilisation of pGSS33, the

Table 7.3 Mobilisation efficiency of an IncQ and an oriT recombinant plasmid

Donor	Recipient	Transconjugants/ml[1]		
		Km^r	Cm^r	Ap^r
BW103(pC2, pGSS33)	BW97	1.4×10^8	5.4×10^4	
BW103(pLG264, pGSS33)	BW97	9.4×10^3	3.1×10^6	
BW103(pC2, pLG2009)	BW97	1.3×10^7		1.2×10^7
BW103(pLG264, pLG2009)	BW97	2.2×10^5		1.1×10^8

[1] Matings were performed in liquid for 1 h at 37°C as described in section 2.6.1. Transconjugants were selected with Nal and an antibiotic appropriate to the plasmid.

transfer of pLG2009 was more efficient when mediated by pLG264 than by pC2.

7.7. Retention of donor-specified polypeptides by recipient cells following pLG264-mediated conjugation

In the previous chapter, it was shown that a specific group of polypeptides, including the *sog* proteins, were retained by recipient cells following conjugation. To assess the behaviour of the polypeptides encoded by the *sog*-262 in this regard, the retention of donor-encoded polypeptides by BW40 recipient cells following mating with BW96 donor cells harbouring pLG264 was analysed as described in the previous chapter. This showed that the ~170 and 95kD polypeptides encoded by the *sog*-262 gene were not retained by recipient cells (Figure 6.2). This observation was borne out by the inability of the retained polypeptides to bind antibody raised against *sog* primase in Western blotting experiments (Appendix 1). By comparison with the proteins retained following pLG221-mediated conjugation, the *sog*-262 mutation had little effect on the retention of the majority of polypeptides by the recipient cells. However, it is noted that pLG264 apparently enhances the transmission of two polypeptides of molecular weight 50 and 80kD, but retards the retention of the 35kD protein by recipient cells.

7.8. Discussion

As described in the introduction, the polypeptide products of the ColIb-P9 primase gene *sog*, are likely to be multifunctional. Although the role of the C-terminal region of the 240kD protein, and of the sequence-related 180kD polypeptide was uncertain, it is likely that these polypeptides will be involved in conjugation since *sog* is classified as a transfer gene (Wilkins, 1975).

To investigate the role of this C-terminal region common to both *sog* polypeptides, Tn*5* insertion mutants of the primase gene were isolated and characterised. One such *sog*::Tn*5* mutant, *sog*-262, which truncated both *sog* polypeptides so removing the C-terminal region without affecting the moiety required for primase activity, resulted in a 150-fold transfer deficiency when present in ColIb-P9*drd*-2. This demonstrated that the Tn*5* insertion inactivates a product important for the efficient conjugative transfer of ColIb-P9.

Complementation tests involving various derivatives of pLG215 in the donor cell, established that the products of the sog gene were responsible for the transfer-deficiency. Furthermore, the complementation of pLG264 by a recombinant plasmid able only to synthesise the smaller sog polypeptide, showed that the 180kD protein, rather than the C-terminal end of the 240kD protein, was important. By comparison, Chatfield et al. (1982) found that mutations which inactivate the primase moiety of the larger sog polypeptide without affecting the 180kD protein, only decreased the transconjugant yield by 3-fold. This clearly illustrates that the 180kD sog polypeptide is of greater importance than primase in the conjugative process.

To discover the role of the smaller sog polypeptide, the effect of the sog-262 mutation on various aspects of conjugation was investigated. It is worth stressing at this point that, although the yield of transconjugants is diminished in the presence of the sog-262 mutation, the transfer efficiency of pLG264 is still substantial. Thus, it is probable that the 180kD polypeptide will either have an optimising role in conjugation, or have a host-encoded analogue which can partially substitute for the mutant plasmid-specified protein.

The ability of pLG264 to stimulate the transfer of the mobilisable plasmid pGSS33, showed that the sog-262 mutation did not affect either the formation of stable mating aggregates, or the establishment of a functional conjugative bridge between mating cells. Since F derivatives mutant in various aspects of conjugative DNA synthesis are able to stimulate normal transfer of mobilisable plasmids (Alfaro and Willetts, 1972; Willetts and Maule, 1979; Everett and Willetts, 1980), it is proposed, by analogy with F, that the 180kD polypeptide may have a role in the conjugative DNA metabolism. Furthermore, since the recombinant pLG2009, which harbours the oriT sequence of ColIb-P9, was efficiently transferred by pLG264, the possible role of the 180kD protein can be narrowed down further, since clearly the site-specific nicking of ColIb-P9 DNA at oriT is unaffected.

In the previous chapter it was demonstrated that a relatively large amount of both the 180 and 240kD sog polypeptides were retained by recipient cells following conjugation. Since the sog polypeptides are principally located in the cytoplasm and are able to weakly bind single-stranded DNA, it was proposed that their transmission resulted from an association with

the transferred DNA. Following similar mating experiments using pLG264, the presence of mutant sog polypeptides in the recipient cell could not be detected. This could result either from a reduction in the transfer of plasmid DNA, or from the inability of the mutant sog polypeptides to bind single-stranded DNA. Since the provision of an active sog gene in the recipient cell was unable to rescue the transfer deficiency of pLG264, it seems most probable that the sog-262 mutation results in the inefficient transfer of pLG264 DNA.

This proposed reduction in DNA transfer may result from the reduced ability of the DNA to traverse the hypothetical transmembrane pore. If this is the case, then the 180kD protein may act to guide ColIb-P9 DNA through the putative transmembrane pore to the recipient cell in a manner analogous to that of the pilot protein encoded by phage M13 gene 3 (Marco et al., 1974; Kornberg, 1974). However, in contrast to the result observed, this model would also predict that the transfer of pLG2009 (ColIb-P9 oriT) would be reduced, since presumably both ColIb-P9 and this recombinant would be guided to the recipient cell in an similar manner. Alternatively, and by analogy with phage and bacterial DNA replication, the 180kD polypeptide may act to protect the transferred single-stranded DNA from nuclease attack, both in the donor and recipient cells. This role, which would involve the transmission of the protein with the DNA, would account for the relatively large amount of 180kD polypeptide retained by the recipient cells following effective conjugation. Also, this role for 180kD polypeptide may explain the location of a proportion of the protein in the periplasm, since the plasmid DNA would require protection from degradative enzymes present in this fraction. Thus, it is proposed that the 180kD protein is a conjugation-specific analogue of single-stranded binding protein which ensures the efficient transmission of DNA to the recipient cell in a form which can be recognised by the host replication system.

It is interesting to speculate that other conjugatively transmitted plasmids, including mobilisable plasmids, may also encode a specific SSB-type polypeptide. However, the significant number of pLG264 transconjugants formed in the absence of the 180kD polypeptide, suggests that a host factor, possibly host SSB, can substitute for the 180kD protein, albeit with a lower efficiency. Hence, it is possible that this host-encoded factor may be able to ensure the efficient transmission of small mobilisable plasmids, but is unable to mediate the transfer of the

larger conjugative plasmids with a high efficiency. Therefore, in the absence of 180kD polypeptide, the transfer efficiency may be dependent on plasmid size. The recent finding that a larger recombinant plasmid (26.2kb) carrying the ColIb-P9 oriT region is mobilised by pLG264 at the intermediate frequency of 7.3×10^5 transconjugants per ml (B.M.Wilkins, unpublished result), corroborates this hypothesis. Therefore, this model proposes that 180kD protein is required to ensure the efficient transfer of the large conjugative plasmid. Furthermore, these observations suggest that the transfer of mobilisable plasmids is not reliant on all aspects of the conjugative system.

This proposal is in keeping with observations which show that derivatives of F, mutant at traI, traM or traZ, are all able to efficiently mobilise ColEI (Alfaro and Willetts, 1972; Willetts and Maule, 1979; Everett and Willetts, 1980). These F transfer genes are all concerned with conjugative DNA metabolism, and, as yet, are the only transfer genes found to be inessential for ColEI mobilisation. This suggests that mobilisation only requires those functions of the conjugative plasmid concerned with the formation of the route between mating cells, and that aspects concerning conjugative DNA synthesis are provided either by the mobilisable plasmid or the bacterial host. This point is further emphasised by the demonstration that the polypeptides responsible for the recognition and nicking of the ColEI oriT sequence, and possibly the recircularistion of the plasmid in the recipient cell, are encoded by the ColEI mob region (Blair and Helinski, 1975; Lovett and Helinski, 1975; Warren et al., 1978; A.C.Boyd and D.J.Sherratt, personal communication).

The notion that mobilisable and conjugative plasmids share, and therefore presumably compete for, the conjugative bridge between mating cells, may offer an explanation for the apparent enhancement of mobilisable plasmid transfer when stimulated by pLG264, relative to pC2. It has been proposed that the absence of 180kD polypeptide reduces the efficiency of conjugative DNA transfer. Therefore, since mobilisable plasmids are not reliant on this protein, a decrease in conjugative plasmid transfer will reduce competition for the conjugative bridge, so enhancing the transfer of the mobilisable plasmids.

It was observed that the polypeptides retained by the recipient cell following conjugation were not generally affected by the presence of the sog-262 mutation. This is in agreement with the proposal that many of

these polypeptides are transmitted as a result of the establishment of a functional conjugative bridge (Chapter 6). Also, since it is proposed that pLG264 does not efficiently transfer DNA, it appears that only a minority of retained proteins are transmitted in association with the DNA. This minority however, clearly includes the sog polypeptides.

Interestingly, it appears that pLG264-mediated conjugation results in the enhanced retention of at least two polypeptides, of molecular weight 50 and 80kD. The nature of these two proteins is unclear, although Western blotting experiments have shown that they are not bound by antibody raised against truncated sog primase (Wilkins et al., 1981; Appendix 1). It is notable that the 50kD polypeptide is retained by recipient cells in a process reliant on the formation of cell-cell interaction, whereas the 80kD polypeptide is apparently transmitted in a conjugation-independent process. Therefore, the enhancement of transmission does not appear to relate to any particular process.

Thus it can be concluded that the 180kD protein plays an important role in ColIb-P9-mediated conjugation. It is proposed that this protein ensures the efficient transfer of ColIb-P9 DNA, possibly by protecting it from nuclease attack. Since the successful transfer of ColIb-P9 relies on the transmission of about 100kb of single-stranded DNA as an intact strand, the importance of protecting the DNA becomes apparent. Therefore, it is suggested that, like plasmid primase, the 180kD polypeptide is amongst a specific group of donor-encoded proteins which have a role in the recipient cell, and which are transmitted by association with the DNA. It may be hypothesised that, given the proposed roles of the 180kD polypeptide and plasmid primase during conjugation, that the fusion of the two genes may have occurred in order to ensure the efficient transmission of the primase moiety. It is therefore interesting to speculate that, since both RP4 and R16 primase genes also encode two sequence-related polypeptides, a similar gene fusion event may also have occurred during the history of these plasmids.

CHAPTER EIGHT

SUMMARY AND FUTURE PERSPECTIVES

Conjugation is a specific plasmid-encoded process which allows the transfer of DNA between bacterial cells. In those cases so far studied the DNA is transmitted as a single strand. Consequently one of the most important processes in the establishment of the plasmid concerns the synthesis of DNA on this transferred template to reform the duplex molecule. Previous work, using both ColIb-P9 and RP4 plasmids, has indicated that plasmid-encoded DNA primase has a central role in this process (Lanka and Barth, 1981; Chatfield et al., 1982).

Chatfield et al. (1982) showed that ColIb-P9 primase is required for efficient conjugative DNA synthesis in both donor and recipient cells. Similarly, Lanka and Barth (1981) hypothesised that RP4 primase may have an important role in the establishment of this broad host-range plasmid in the recipient cell. Work presented in this thesis, using a primase-defective derivative of RP4, confirmed this hypothesis by demonstrating that RP4 primase was required for efficient transconjugant formation in both E.coli and S.typhimurium hosts. Furthermore, complementation tests showed that, as is the case for ColIb-P9, the requirement for RP4 primase in the recipient cell could be satisfied by enzyme synthesised in the donor cell. Since the nature of the DNA transferred by RP4 is unknown, the role of RP4 primase will remain uncertain. However, by consideration of the reaction catalysed by this enzyme and by analogy with the role of ColIb-P9 primase, the RP4 primase is suggested to initiate complementary strand synthesis in the recipient cell, thus implying that RP4 transfers single-stranded DNA.

The strain and species variation observed in complementation experiments using the primase-defective RP4 derivative clearly demonstrates that the interactions between host and plasmid systems during conjugation are complex. However, it is notable that the transfer efficiency of primase-proficient RP4 derivatives varied very little, irrespective of the strains used. This suggests that plasmid primases act to optimise the efficiency with which immigrant plasmid DNA becomes established in the recipient cell, presumably by synthesising primers on the plasmid such that the DNA is efficiently synthesised by the host machinery.

A model proposed in Chapter 4, suggests that RP4 plasmid primase is required in conjugation due to the absence of sites recognised by the host initiation systems on the transferred RP4 strand. This implies that primases in general may be specific for certain templates *in vivo*. This is substantiated by the requirement for specific host priming systems during the replication of some phage.

In keeping with the concept of primase specificity, it was demonstrated in Chapter 5 that both ColIb-P9 and RP4 primases are unable to efficiently substitute for each other during conjugation. This finding is in contrast to previous observations that both plasmid primases were able to initiate DNA synthesis on a wide variety of single-stranded phage templates *in vitro* (Lanka *et al.*, 1979; Lanka and Barth, 1981). This apparent contradiction was explained by proposing that during conjugation, plasmid primases act within a complex of polypeptides which exhibits template specificity. Since purified primases can act to initiate phage replication, it was proposed that the primases acted without such a complex in this process, and consequently were not template specific.

The basis of the inability of ColIb-P9 and RP4 primases to cross-complement during conjugation is unclear. One explanation is that non-homologous primase is not recognised by the 'priming complex' and consequently cannot gain access to the transferred DNA. However, since non-homologous 'priming complexes' were also unable to efficiently act on the transferred DNA, it is apparent that specific recognition sequences must exist on the plasmid DNA.

The demonstration that ColIb-P9 plasmid primase is amongst a discrete group of polypeptides transmitted between mating cells during conjugation (Chapter 6) offers another explanation of primase specificity. Since it was demonstrated that the transmission of polypeptides to the recipient cell was specific, the inability of donor-encoded RP4 primase to complement the mutant *sog* primase may result from the failure of the non-homologous primase to be recognised by the process responsible for polypeptide transmission.

In order to further understand the nature of the plasmid primase specificity *in vivo*, the DNA-protein and protein-protein interactions involving primase must be investigated. However, before such models can be formulated for the RP4-mediated conjugation system, the nature of the

DNA transferred, and the transmission of the RP4 primase must be demonstrated.

One of the most exciting prospects arising from the work in this thesis relates to the physical demonstration of polypeptide transfer between mating cells. Results given in Chapter 6 showed that about 20 donor-specified polypeptides are prominently retained by the recipient cell following conjugation. Since the retained radioactivity only amounted to less than 1% of that present in the donor cells at the start of the mating, it was apparent that the process responsible for protein transmission was very specific.

The currently popular model for conjugation predicts that DNA transfer occurs via a transmembrane pore formed by the localised fusion of donor and recipient membranes, brought into close contact by the retraction of the pilus. From this model, two principle routes for polypeptide transmission can be envisaged: the transmission of proteins into the recipient cell by association with the transferred DNA, and the retention of proteins in the recipient envelope following membrane fusion. By confirming which, if either, of these routes is important, and the identification of the polypeptides involved, a clearer picture of the conjugative process can be obtained.

Some of the polypeptides currently hypothesised to be associated with the DNA transferred during conjugation have been mentioned in Chapter 6. Previous work determined that some, and possibly all, plasmids are transferred as single-stranded DNA molecules. Therefore, by analogy with bacterial and phage DNA metabolism, an obvious candidate for transmission to the recipient cell is single-stranded binding protein (SSB). Several conjugative plasmids, including ColIb-P9, have recently been demonstrated to encode their own SSB (Golub and Low, 1985; C.Howland, unpublished results). However the role of these proteins has yet to be determined and it is possible that the roles of E.coli and plasmid-encoded SSB may be specific. The relative importance of the two SSB proteins in conjugation could be determined by detecting the amount of each retained by the recipient cell.

It has been demonstrated that ColIb-P9 primase is retained by recipient cells in large amounts in an apparently unprocessed form (Chatfield and Wilkins, 1984; Chapter 6). The ability of primase to bind weakly

single-stranded DNA (Lanka et al., 1979) implies that it too may be transmitted in association with the DNA. This hypothesis is corroborated by the role of this enzyme and the cytoplasmic location of primase encoded by the cloned sog gene. However, it is possible that the presence of other ColIb-P9-specified cofactors may alter the cellular location of primase. This possibility could be investigated by fractionating whole cells harbouring ColIb-P9drd-1 and immunoprecipitating the sog polypeptides from each fraction. A similar procedure could also be used to determine the location of the primase retained by recipient cells. Information gained from such studies will relate to the route of primase transmission during conjugation.

Such experiments would also allow the role of the 180kD sog polypeptide to be further clarified. This protein is known to be retained by recipient cells following conjugation. Furthermore, genetic experiments have determined that this protein has an important role in the conjugative process, possibly by ensuring the efficient transfer of plasmid DNA. The determination of the cellular location of this protein, both in donor and recipient cells, and information concerning its ability to bind DNA, will allow the role of this polypeptide to be elucidated further.

Since sog primase can bind DNA, the regions of the protein responsible for the interaction with the DNA could be determined by investigating the ability with which various truncated sog polypeptides can bind DNA. Furthermore, by using a variety of cloned ColIb-P9 DNA fragments, it may be possible to identify any sequences that are preferentially bound by either sog polypeptide. This possibility is most exciting since it may also allow the identification of other plasmid-encoded polypeptides which are potentially of importance in aspects of replicative or conjugative DNA metabolism. Such proteins may include analogues of the 60kD ColEI-specified protein thought to be important in nicking and recircularisation (Lovett and Helinski, 1975), and possibly analogues of the M13 pilot protein (Marco et al., 1974; Kornberg, 1974) which may act to guide the DNA through the cell membranes during conjugation.

As well as identifying polypeptides transmitted in association with the DNA, the study of recipient-retained protein offers a method by which the nature of the conjugative bridge may be investigated. In Chapter 7 it was proposed that DNA transfer was reduced in the absence of 180kD sog polypeptides. However, it was demonstrated that this did not effect the

retention of the majority of polypeptides by the recipient cell during conjugation. This suggests that many of the proteins are retained as a result of localised membrane fusion, which is popularly envisaged to occur during the formation of the conjugative bridge. By evaluating the transmission of donor-encoded polypeptides normally located in the cytoplasm, periplasm or the inner or outer membrane, the extent of cellular fusion can be elucidated. Similarly, the identification of the recipient-encoded polypeptides retained by the donor cells following conjugation will provide information about the nature of any asymmetry in the exchange of polypeptides.

Experiments reported in Chapter 6 have shown that donor-encoded polypeptides of 35 and 38kD appear prominently in the recipient cells and it is possible that these polypeptides correspond to the major outer membrane proteins OmpA and OmpF/C, respectively. In order to verify this, and to identify other transmitted host-encoded proteins, an approach involving the fractionation of the recipient cells followed by immunoprecipitation with specific antisera could be exploited. Once the retained polypeptides have been identified, the use of appropriate mutant host strains will allow the involvement of these proteins in conjugation to be assessed.

Thus, the approaches described here will provide an insight into the role of both host and plasmid-encoded proteins of importance in conjugation. This includes not only those proteins concerned with conjugative DNA metabolism, but also those involved in the poorly understood cell-to-cell interactions which are fundamental to the process of conjugation.

Appendix 1 Identification of *sog* polypeptides using Western blotting analysis

This experiment was performed by C.Rees and N.Smith.

Whole cell protein was derived from 10 A_{450} units of cell culture by boiling in 300µl SDS-PAGE sample buffer for 10 min. The lysate was centrifuged at 81000g for 30 min to remove the chromosomal-membrane complex.

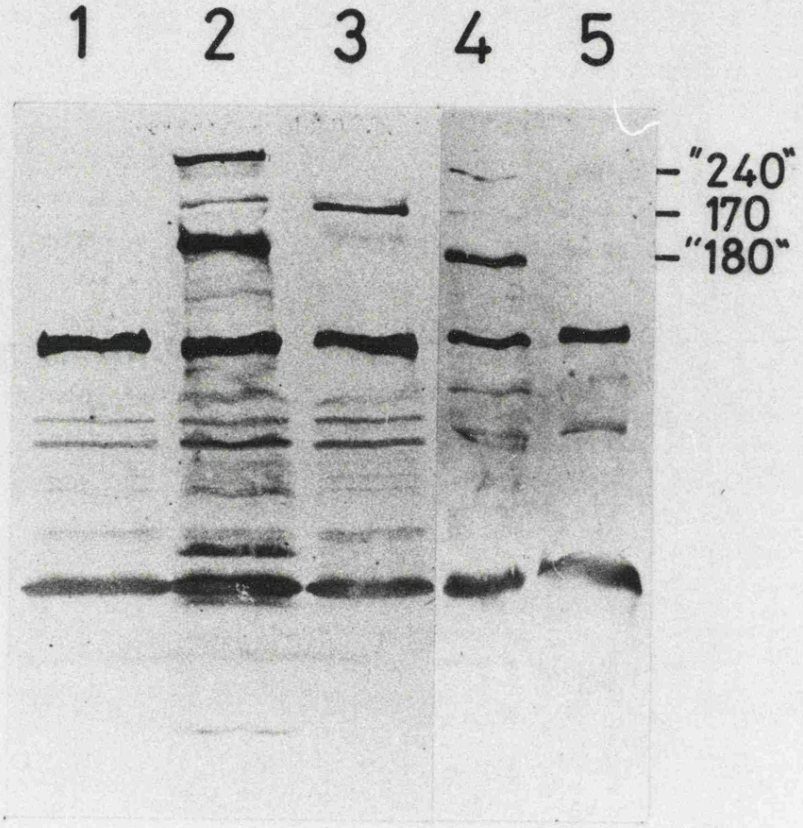
Samples of polypeptides retained by recipient cells following conjugation with labelled donor strains were from the same preparations as described in Figure 6.2.

Samples were electrophoresed through a 10% SDS-polyacrylamide gel before transferring the proteins onto a cellulose nitrate sheet as described by Towbin *et al.*, (1979). After washing in phosphate-buffered saline containing 0.05% (v/v) Tween, the cellulose nitrate sheet was gently shaken in the presence of (i) rabbit anti-87kD *sog* polypeptide (Wilkins *et al.*, 1981), (ii) swine anti-rabbit IgG (iii) rabbit anti-horse radish peroxidase. The bound antibody-complex was detected by soaking the filter in the presence of 8mM Tris-Hcl, 42mM NaCl, 16%(v/v) methanol, 0.05% (w/v) hydrogen peroxide and 0.5mgml^{-1} 4-chloro-1-naphthol for 1 min.

Samples were as follows:

(1) BW86 - whole cell protein 30µl
(2) BW86(pLG221) - whole cell protein 30µl
(3) BW86(pLG264) - whole cell protein 30µl
(4) BW96(pLG221) x BW40 - recipient-retained polypeptide 25µl
(5) BW96(pLG264) x BW40 - recipient-retained polypeptide 25µl

Molecular weights of *sog* polypeptides, shown to the right, were estimated from Coomassie blue-stained protein markers electrophoresed concurrently with the samples shown.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