ROLE OF PLASMID ColIb-P9 DNA PRIMASE

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by

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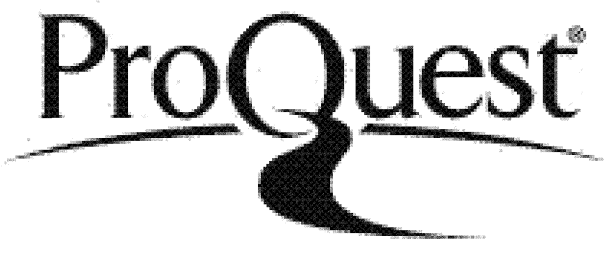

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This thesis is dedicated to my mother and to my father

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Most importantly of all, I wish to thank my parents, to whom this thesis is dedicated. Their helpful encouragement has made all this possible.

Abbreviations commonly used in the text

The International System (S.I.) of units for mass, length, time, temperature, etc. was used.

Miscellaneous abbreviations

A	absorbance
b.p.	base pair
dal	dalton
Inc	incompatibility
Kb	kilobase pair
kdal	kilodalton
m.o.i.	multiplicity of infection
MW	molecular weight
p.f.u.	plaque-forming unit
R.I.	refractive index
UV	ultraviolet light

Antibiotics

Ap	ampicillin
Cm	chloramphenicol
Kan	kanamycin
Nal	nalidixic acid
Rif	rifampicin
Sm	streptomycin
Tc	tetracycline

CONTENTS

CHAPTER 1

General Introduction

'I wrote and printed a book in which I discuss this doctrine ..., and adduce arguments of great cogency in its favour, without presenting any solution of these; and for this cause I have been pronounced by the Holy Office to be vehemently suspected of Heresy ...'

The Confession of Galileo Galilei to Cardinal Bellarmine.

1.1 Introduction

Bacterial conjugation is a process of plasmid-mediated DNA transfer requiring the formation of specific physical contact between the participating donor and recipient cells. In view of its early discovery (Lederberg and Tatum, 1946a,b) and the quantity of literature devoted to this subject (for recent review articles see Clark and Warren, 1979; Manning and Achtman, 1979; Willetts and Skurray, 1980) it is paradoxical that many aspects of conjugation remain obscure. This deficiency in our knowledge is attributable partly to the sheer number of different conjugation systems which appear to exist in nature. Conjugative plasmids, isolated from Gram-negative bacterial species alone, so far comprise more than 20 distinct incompatibility groups (Datta, 1979). Since conjugation requires the activities of many plasmid-encoded transfer gene products, and members of different plasmid incompatibility groups usually share little DNA homology, as evidenced by both DNA-DNA hybridisation experiments and heteroduplex analysis (Guerry and Falkow, 1971; Grindley *et al.*, 1973; Falkow *et al.*, 1974; Anderson *et al.*, 1975; Roussel and Chabbert, 1978; Gorai *et al.*, 1979), as many genetically distinct conjugation systems may

exist as there are incompatibility groups (Achtman and Skurray, 1977). In contrast to this diversity, however, most studies concerning conjugation have involved the archetypal plasmid F (IncFI; Datta, 1979). Other conjugative plasmids have yet to be investigated in such detail, and so our understanding of these transfer systems has proceeded largely by analogy with F. Obviously, this imbalance must be redressed before a truly generalised model of bacterial conjugation can be constructed.

This thesis involves studies on the role of a plasmid-encoded DNA primase enzyme during conjugative transfer of the archetypal IncIα (= $IncI_1$; Coetzee et al., 1982) plasmid ColIb-P9, and examines the implications of the mode of action of this protein on existing models of IncIα plasmid transfer. This introductory chapter will therefore describe what is known about the biology of this plasmid and its conjugation system before considering the metabolism of plasmid DNA during transfer. In order to place the physiological role of the plasmid-encoded DNA primase in its correct context, it will also be necessary to include a consideration of other primer-generating enzymes, and a review of prokaryotic DNA replication.

1.2 An overview of the archetypal IncIα plasmid ColIb-P9

Many strains of the Enterobacteriaceae support the production of bactericidal proteins called colicins, and a large range of biochemically distinct colicins has been identified (for a review see Hardy, 1975). The existence of the colicinogenic factor ColIb-P9 was first reported by Fredericq in 1954, but a decade passed before it was fully realised (Monk and Clowes, 1964b) that the genetic factors specifying these proteins were autonomous plasmid molecules, comprised of double-stranded covalently closed circular (CCC) DNA. Following its discovery in Shigella sonnei P9

(Fredericq, 1954), it was demonstrated that ColIb-P9 could promote its own transfer, and hence also ability to produce colicin, between strains of Escherichia coli K-12 (Clowes, 1961) and Salmonella typhimurium LT2 (Ozeki and Howarth, 1961). Taking advantage of this property, Silver and Ozeki (1962) estimated the size of this colicin factor to be about 60 kilobases (Kb) of DNA, by the measurement of the amount of [^{14}C]-thymidine-labelled DNA transferred from colicinogenic donor cells. However, isolation of purified ColIb-P9 DNA by sedimentation in neutral sucrose density gradients (Clewell and Helinski, 1970) led to the more accurate estimate of a molecular weight (MW) of about 61.5×10^6 (about 93 Kb). This value is in agreement with estimates drawn from the sizes of restriction endonuclease-generated fragments of ColIb-P9 DNA. Uremura and Mizobuchi (1982a) placed the molecular weight of ColIb-P9 at between 56.3×10^6 (from digestion with HindIII and SalI) and 58.7×10^6 (HindIII), while Walia et al., (1982) reported values between 60.1×10^6 (HindIII) and 63.8×10^6 (XhoI). Although Skorupska et al. (1979) described essentially identical MW values for ColIb-P9, also calculated from restriction enzyme analysis, their HindIII digest differed considerably from that reported by both Uremura and Mizobuchi (1982a) and Walia et al. (1982). As with most large bacterial plasmids, ColIb-P9 is present as one or at most a few copies per cell (Clewell and Helinski, 1970; Isaacson and Konisky, 1974a).

ColIb-P9 shares membership of the Iα incompatibility group with the closely related conjugative plasmids R64 and R144 (Hedges and Datta, 1973). Other members of the Iα incompatibility group have been identified, such as R483 and R648 (Hedges and Datta, 1973), but are not as well characterised as the afore-mentioned examples, and therefore will not be considered in detail here. Comparison of restriction endonuclease-

generated digests (Chabbert et al., 1978) and DNA-DNA reassociation experiments (Guerry and Falkow, 1971; Falkow et al., 1974) have revealed that Iα plasmids share a considerable degree of homology. Falkow et al. (1974) estimated that ColIb-P9 and R64 were 81.4 and 78% homologous respectively with R144, and ascribed this high level of homogeneity to phylogenetic relatedness between genes responsible for pilus biosynthesis and transfer functions. Furthermore, Lehrbach and Broda (1984) have recently examined the relationships between various colicin-producing plasmids and demonstrated that ColIb-P9 shares between 44 and 51 Kb of DNA in common with the Iα plasmid ColIa-CA53. These authors propose a common origin for these two plasmids, but the lack of common restriction endonuclease fragments points to extensive evolutionary divergence as well. Thus, it seems justified to allude to aspects of R144 and R64-mediated transfer throughout this thesis, since the conjugation systems of these plasmids are considered to be essentially similar to that of ColIb-P9. However, it must be noted that R144, and its derepressed derivative R144 drd-3, are incompatible with the Iγ plasmid R621a (Bird and Pittard, 1982). It may therefore be the case that R144 possesses two distinct incompatibility loci, and hence cannot be considered as a typical IncIα plasmid. This observation emphasises the potential shortcomings of classifying plasmids on the basis of incompatibility interactions alone.

The above members of the Iα group of plasmids appear to have a fairly restrictive host range, limited to species of Escherichia, Salmonella, Shigella and Klebsiella (Datta and Hedges, 1972; Jacob et al., 1977), and this property is apparently shared by naturally occurring plasmids which are incompatible with members of this group (Grant et al., 1980). Derivatives of ColIb-P9 carrying antibiotic resistance genes have also been isolated from pathogenic organisms (Anderson and Smith, 1972; S.K. Walia, in: Walia et al., 1982; Hughes and Datta, 1983).

As suggested by the ability to transfer colicin production between bacteria, ColIb-P9 is a conjugative plasmid, capable of promoting the transfer of its DNA to plasmid-free recipient cells. Although this property is considered in greater detail in section 1.7 of this chapter, it is important to note at this stage that much of the experimental work performed on conjugation mediated by ColIb-P9 has made use of derepressed (drd) mutants of this plasmid, which transfer at a higher frequency than the wild-type plasmid. The transfer system of ColIb-P9 is normally repressed, so that only a small proportion of a population of colicinogenic cells are initially competent donors (Ozeki and Howarth, 1971; Monk and Clowes, 1964a). The drd mutation removes this repression, apparently allowing a constitutive mode of transfer to operate (Meynell and Datta, 1967; Ohki and Ozeki, 1968; Edward and Meynell, 1968). The properties of drd mutants will be considered in greater detail in section 1.7.

Although bacterial plasmids exist as independent autonomous replicons, it appears to be a general property of these molecules to integrate into the host chromosome. In this integrated or Hfr (for high frequency of recombination) state the conjugative plasmids F, R100, ColV2, R6K and RP1 are able to transfer chromosomal DNA to recipient cells at a high frequency (Zinder, 1960a and b; Moody and Runge, 1972; Nishimura et al., 1973). In contrast however, the transfer of chromosomal markers during ColIb-P9-mediated conjugation was found to occur at only a low frequency of about 10^{-7} to 10^{-9} per donor (Ozeki and Stocker, 1961), and Clowes and Moody (1966) detected no such transfer using recombination-deficient donors. The integration of a transmissible plasmid into its host chromosome can also lead to suppression of temperature-sensitive dnaA mutations that affect initiation of chromosomal replication at the replication origin oriC (Marsh and Worcel, 1977), by initiation instead at the integrated plasmid

replication origin (Nishimura et al., 1971). Moody and Runge (1972) were unable to demonstrate the integration of any of the Iα plasmids ColIb-P9drd-1, R64drd-11 and R144drd-3 into the host chromosome by this criterion, but Datta and Barth (1976) were able to demonstrate that R144 and the atypical Iα plasmid R483 could suppress the DnaA phenotype by an unstable recombinational event apparently requiring the recA$^+$ gene product. Thus it appears that ColIb-P9 can form only an unstable cointegrate with the chromosome in contrast to the behaviour of most other conjugative plasmids.

ColIb-P9 has a number of other interesting features besides its conjugative ability and the production of, and immunity to, colicin Ib-P9. The plasmid is also able to confer a limited resistance to ultraviolet (UV) light and to inhibit the growth of the bacteriophages BF23 and T5 (Howarth, 1965; Strobel and Nomura, 1966; Nisioka and Ozeki, 1968). Since these phenomena are not central to this thesis, consideration of these aspects will be kept brief.

The colicin Ib-P9 protein is a bacteriocidal molecule of about 80,000 MW, and shows antigenic relatedness to another colicin of apparently identical size, Ia-CA53 (Konisky and Cowell, 1972), although the immunity systems of the respective plasmids ColIb-P9 and ColIa-CA53 are able to distinguish between the two proteins (Stocker, 1966). Both of these colicins can cause impairment of the energised state of cellular membranes, possibly by acting on the potassium ion transport system of the cell (McCorquodale et al., 1979), and colicin Ib-P9 has also been shown to inhibit the synthesis of cellular macromolecules (Levisohn et al., 1967). Only about 0.1% of a population of ColIb-P9-containing bacteria produce colicin at any one time (Ozaki et al., 1959), although exposure to UV light can induce colicin synthesis in a greater proportion of these cells

(Ozeki et al., 1959; Monk and Clowes, 1964b). Colicin synthesis is thus normally repressed; it is presumed that cleavage of a repressor molecule by UV results in the expression of the colicin Ib-P9 gene, cib (Isaacson and Konisky, 1974a,b). This repressor is thought to be the lexA gene product in E.coli, from studies on both ColIb-P9 and the related I_1 plasmid TP110, which also specifies colicin Ib-P9 production (Kenyon and Walker, 1980; Pugsley, 1981; Glazebrook et al., 1983). The colicin Ia-CA53 structural gene (cia) has been cloned by Weaver et al. (1981), while the cib gene of ColIb-P9 has been cloned independently by Boulnois (1981), Uemura and Mizobuchi (1982b) and Pinkerton et al. (1981). The location of cib has been mapped by hybridisation of plasmid restriction fragments to cloned DNA and Tn5 mutagenesis (Walia et al., 1982) and also by analysis of Cib^- ColIb-P9-R222 recombinant plasmids (Uemura and Mizobuchi, 1982a,b).

The mechanism by which ColIb-P9 confers immunity to the effects of colicin upon its host cell is unknown, but the system is highly specific, and is thought to function at the level of the cytoplasmic membrane (Hardy, 1975; Weaver et al., 1981). Presumably the gene for immunity (imm) is expressed constitutively, since all ColIb-P9-containing cells are resistant to this colicin. Levisohn et al. (1968) have shown that immunity is not absolute, since it can be overcome by a 50-fold increase in colicin concentration. The immunity genes of ColIa-CA53 (Weaver et al., 1981) and ColIb-P9 (Uemura and Mizobuchi, 1982a,b) have been cloned, and in each case lie closely linked to the structural colicin genes of each plasmid.

Infection of cells harbouring ColIb-P9 by either of the closely-related bacteriophages BF23 (Strobel and Nomura, 1966; Nisioka and Ozeki, 1968) or T5 (Moyer et al., 1972) results in the inhibition of phage protein synthesis and infection fails. This abortion of phage infection is

associated with extensive membrane damage and depolarisation (Glenn and Duckworth, 1980) leading to an efflux of K^+ and ATP and leakage of β-galactosidase (Cheung and Duckworth, 1979), eventually resulting in cell lysis. Since this form of membrane damage is similar to that caused by colicin Ib-P9, McCorquodale et al. (1979) suggested that this protein was the agent for cell lysis during abortive infection. However, the genetic information for abortive phage infection was found to be encoded by a 2.2 Kb EcoR1 fragment of ColIb-P9 DNA, and cib to be located on a separate 2.8 Kb fragment (Boulnois, 1981). Similar results were obtained by Pinkerton et al. (1981) and Uemura and Mizobuchi (1982a,b). The latter authors also report that two genes are involved in this process, and that mutation of only one of these, ibfA, was required for loss of the Ibf phenotype. The product of the ibfB gene apparently acts in trans to promote ibfA activity. These proteins have yet to be identified, however, and so the mechanism of this phenomenon remains unknown, although it appears to be complex and involves host, plasmid and bacteriophage gene products (McCorquodale et al., 1979).

Like many other bacterial plasmids, ColIb-P9 also apprently encodes or stimulates a DNA repair mechanism, capable of increasing the resistance of its host cell to UV light (Howarth, 1965; Khmel et al., 1979). This process, being error-prone, also results in a greater rate of mutagenesis in such cells following exposure to UV or other mutagenic agents causing repairable single-strand gaps in DNA (Howarth, 1966; Hanawalt et al., 1979; Khmel et al., 1980). This mutagenic, UV-protective phenotype (Mut, Uv; Novick et al., 1976) has also been found to be associated with members of the incompatibility groups B, FIV, Iγ, J, L, M and N as well as Iα (Molina et al., 1979; Pinney, 1980), but this range is thought to reflect

a number of different mechanisms (Chernin and Mikoyan, 1981). In the case of ColIb-P9, involvement of the plasmid is thought to be indirect, involving a stimulation of a host repair system, rather than the direct involvement of a plasmid-encoded product (Khmel et al., 1979; Chernin and Mikoyan, 1981). One further ColIb-P9-encoded product of interest to this thesis, namely an EDTA-resistant endonuclease (Winans and Walker, 1983) which could be involved in this DNA repair activity, will be considered further in Chapter 5. Firstly, however, I shall turn to the subject of bacterial DNA replication.

1.3 Bacterial DNA replication

The synthesis of bacterial DNA is a complex process involving the sequential actions of a large number of DNA replication enzymes. Many recent reviews already deal with this subject (Wickner, 1978; Ogawa and Okazaki, 1980; Kornberg, 1980, 1982; Nossal, 1983), and since this thesis is concerned with the stages in this process by which new DNA chains are initiated, this section will attempt to place these reactions in context.

DNA synthesis is carried out by polymerase enzymes, but no known DNA polymerase is able to initiate DNA synthesis de novo on an exposed template strand. Instead, the presence of the 3'-hydroxyl terminus of a polynucleotide primer molecule base-paired to the template strand is required (Ogawa and Okazaki, 1980). There are two requirements for primer synthesis during replication of the bacterial chromosome; firstly in the initiation of replication of the genome at the single vegetative origin, oriC (Marsh and Worcel, 1977), and subsequently in the discontinuous synthesis of DNA fragments during elongation. Initiation at oriC is a rifampicin-sensitive process, requiring RNA polymerase and the activities of the dnaA, dnaC,

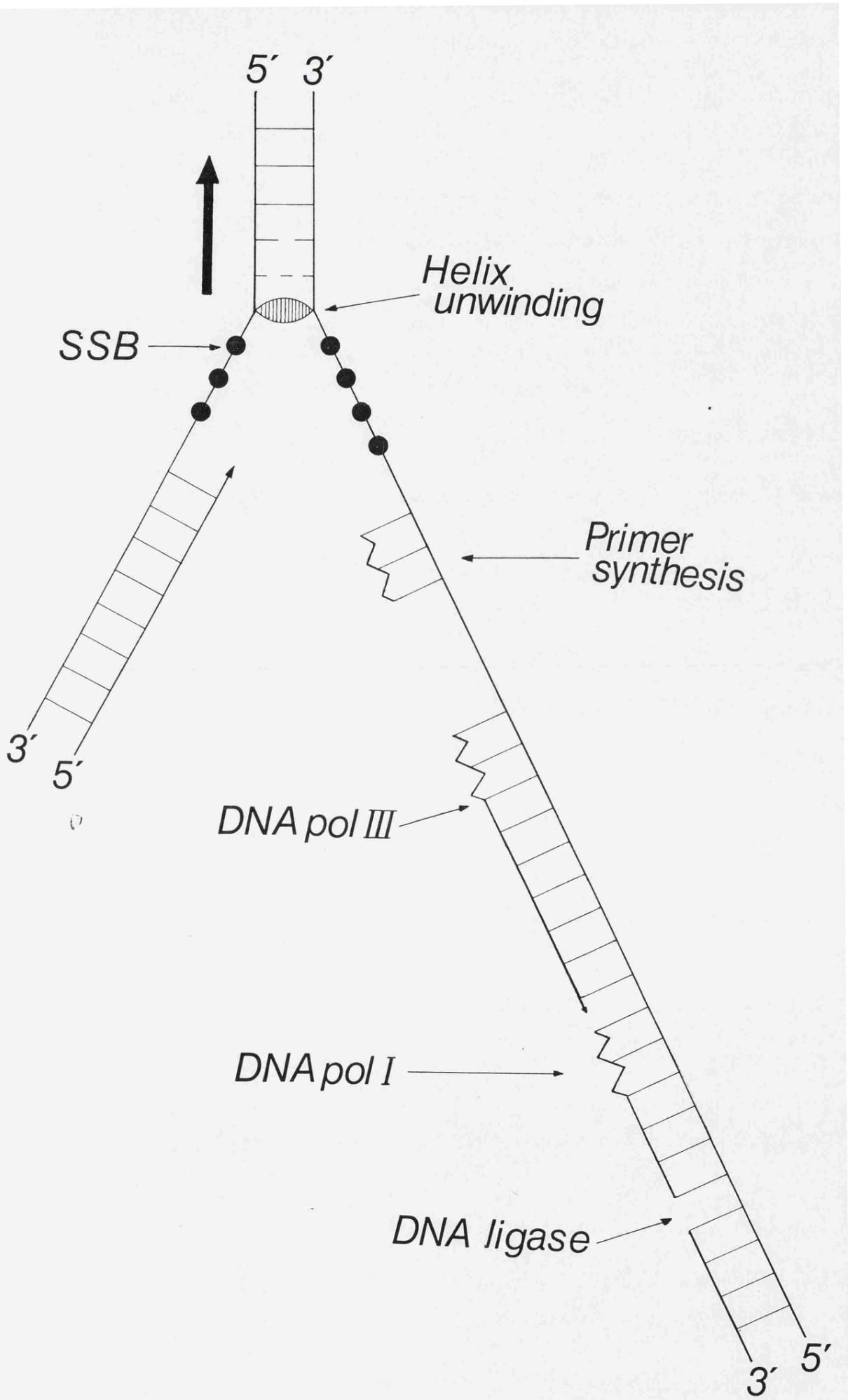
dnaI and *dnaP* gene products (Beyersmann *et al.*, 1974; Wada and Yura, 1974; Nusslein-Crystalla *et al.*, 1982; Walker *et al.*, 1982), and it also appears that the origin is associated with the cell membrane at this stage (see Hendrickson *et al.*, 1982, for a recent discussion). Semiconservative replication (Meselson and Stahl, 1958) proceeds bidirectionally from this point to the terminus (Prescott and Kuempel, 1972) by the progress of mobile replication forks and their associated enzymic activities. A current scheme for the structure and composition of such a replication fork is depicted in Figure 1-1.

Separation of the double strands of DNA in order to provide single-stranded template for DNA synthesis is generated by the action of DNA unwinding enzymes (Geider and Hoffmann-Berling, 1981), and single-strand binding protein (SSB) stabilises the separated strands (Molineux *et al.*, 1974; Meyer *et al.*, 1979). The *ssb* gene has been mapped at about 90.8 min on the *E.coli* chromosome (Glassberg *et al.*, 1979) and its product acts as a tetramer present at about 270 copies per replication fork (Kornberg, 1980). Since DNA polymerase may only utilise the 3'-OH terminus of primer molecules, polymerisation can proceed only in a 5'-3' direction (Ogawa and Okazaki, 1980). As a consequence of both this and the antiparallel nature of DNA, synthesis of the lagging strand must be discontinuous (Okazaki *et al.*, 1968), and it seems likely that the leading strand may also be replicated in a similar fashion. Thus, elongation requires multiple priming events on at least one, and possibly both, strands.

Primers are extended by the major replicative enzyme of *E.coli*, DNA polymerase III holoenzyme, which catalyses the addition of deoxyribonucleotides to the 3'-OH terminus of the primer molecule. This complex holoenzyme consists of at least seven subunits (Nossal, 1983), of which

Figure 1-1 Bacterial DNA replication fork

The figure shows a schematic representation of a replication fork, progressing in the direction indicated by the heavy arrow. A more detailed discussion of the enzymology associated with this structure is provided in sections 1.3 and 1.4. The double helix ahead of the fork is unwound by the action of DNA unwinding enzymes, and the single-stranded regions stabilised by single-strand binding protein (SSB). Primers for lagging strand synthesis are extended by DNA polymerase III holoenzyme and removed and replaced by the action of DNA polymerase I. Discontinuous DNA fragments are joined by DNA ligase. Leading strand synthesis is depicted as a continuous process in the 5' to 3' direction.


5′ 3′
Helix unwinding
SSB
Primer synthesis
3′ 5′
DNA pol III
DNA pol I
DNA ligase
3′ 5′

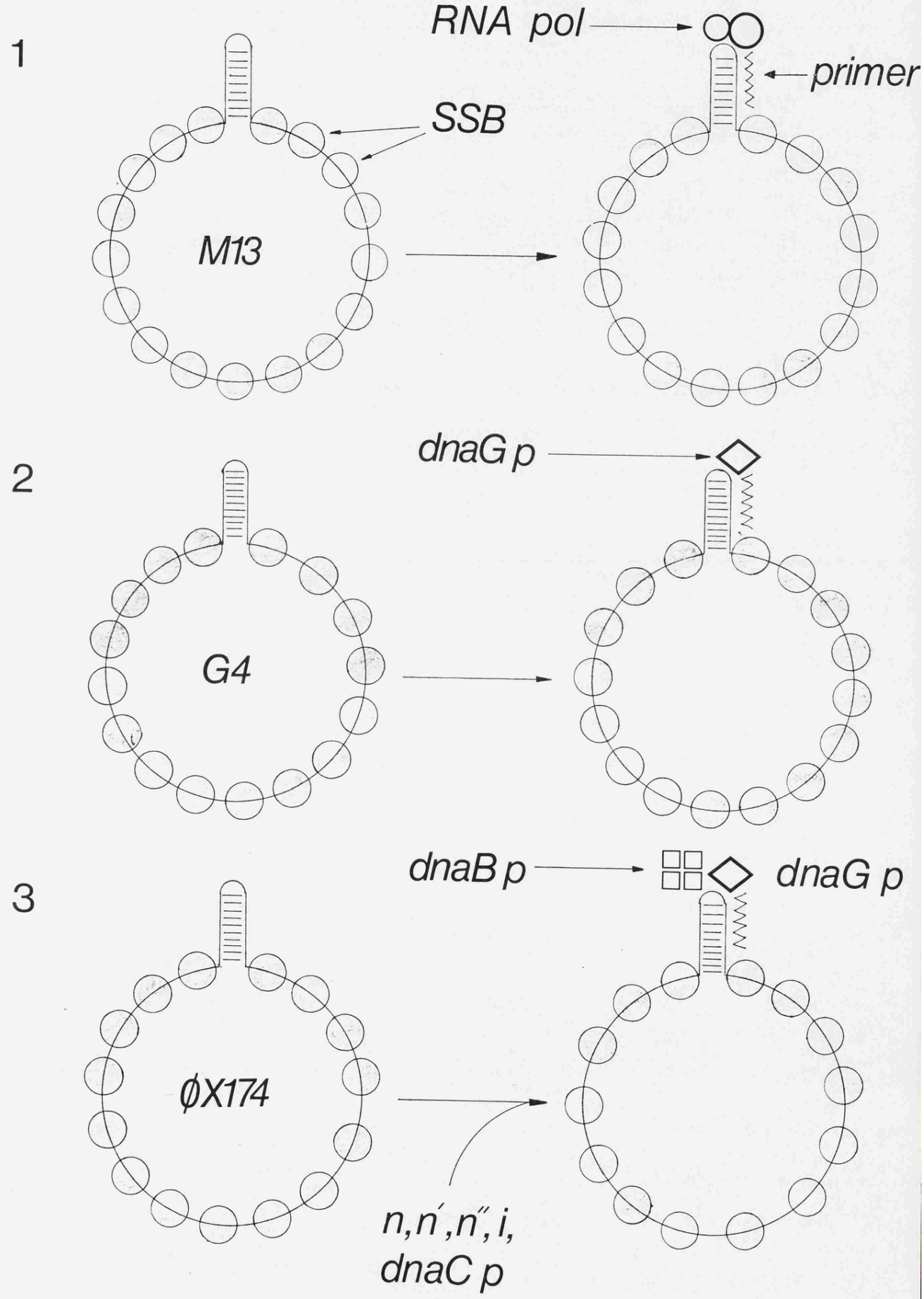
three (the α, ε, and θ subunits) form the core enzyme. The 140 kilodalton (kdal) α-subunit, the product of the dnaE gene (Welch and McHenry, 1982) has been shown to possess polymerase activity (Spanos et al., 1981), but the roles of the ε and θ subunits remain unknown. Addition of the ζ-subunit to the core constitutes polIII enzyme, which allows synthesis of DNA on single-stranded fd template (McHenry, 1982), implying that this subunit allo the core enzyme to synthesise long stretches of DNA. The further interacti of the δ-(dnaX product; Hübscher and Kornberg, 1979) and γ-subunits (dnaZ protein; Wickner and Hurwitz, 1976) forms the polIII* complex, and the final association of the β-subunit (dnaN protein; Burgers et al., 1981) completes the holoenzyme complex. In this form, DNA polymerase III is able to carry out the processive synthesis of DNA on primed single-stranded template strands (McHenry and Kornberg, 1977), and presumably the holoenzyme extends primer molecules on bacterial DNA until it reaches the 5' terminus of an adjacent primer. Primers are then envisaged to be excised and replaced with DNA by the 5'-3' exonuclease and polymerase activities of DNA polymerase I, and gaps are sealed by DNA ligase (Kornberg, 1980).

1.4 Bacterial primer-generating enzymes

Two primer-generating enzymes have been identified for DNA replication in E.coli. These enzymes, DNA-dependent RNA polymerase and primase, will be described with reference to the single-stranded bacteriophage replication systems which exploit the specificities of these enzymes towards different bacteriophage DNA templates (see Figure 1-2). All of the systems involve the conversion of the viral single-stranded DNA to the double-stranded replicative form. In vitro reconstruction of these replication systems, using purified phage DNA and cell extracts from define

Figure 1-2 Action of bacterial primer-generating enzymes on single-stranded bacteriophage DNA templates in E.coli

The scheme depicts the formation of primers for viral (+) strand synthesis of (1) M13, (2) G4 and (3) ØX174 DNA. In each case, the template (-) strand is coated with E.coli single-strand binding protein (SSB) except for a unique region of potential secondary structure indicated here as a single hairpin. This region serves as a site for the recognition and initiation of primer synthesis by the bacterial enzymes supplied by the host cell. As indicated in the figure, primer synthesis is carried out by a different enzymatic mechanism in each case. Details of these, and of the enzymes involved, are given in section 1.4 of the text.


1
RNA pol
primer
SSB
M13
2
dnaG p
G4
3
dnaB p
dnaG p
ØX174
n,n´,n˝, i,
dnaC p

mutant strains of E.coli has enabled the biochemistry of the priming reactions of these enzymes to be understood, and in the case of the ØX174 system, provides an analogy for the priming of chromosomal DNA synthesis.

In the case of M13 (or the closely related bacteriophages fd and f1), the single-stranded circular DNA molecule is coated with E.coli single-strand binding protein (SSB) except for a unique region of potential secondary structure as shown in Figure 1-2, which is transcribed by RNA polymerase to generate a 30 ribonucleotide primer (Brutlag et al., 1971; Geider and Kornberg, 1974; Geider et al., 1978) which initiates DNA synthesis by DNA polymerase III holoenzyme (Geider and Kornberg, 1974). Three other factors have been implicated in the specificity of this priming reaction, but their role in this process is obscure, and only one of these factors (RNase H) has been identified (Vicuna et al., 1977a; Vicuna et al., 1977b). It also appears that if the initiation site specifying the primer synthesised by RNA polymerase is made unavailable, due to deletion of this region of M13 DNA, then the bacteriophage replicates by means of priming reactions at secondary sites (Ray et al., 1981).

RNA polymerase holoenzyme is composed of at least two α-subunits (gene = rpoA), and one each of the subunits β (rpoB), β' (rpoC) and σ (rpoD), while the role of an additional subunit, ω, has yet to be determined (Williamson and Doi, 1979). Detachment of the σ subunit converts RNA polymerase to the core polymerase, which cannot utilize an intact duplex template, but is still able to function on single-stranded DNA (Harris et al., 1978). The enzyme is inhibited by rifampicin, an antibiotic which binds to the β subunit and prevents the initiation of transcription; rifampicin-resistant mutations map within the rpoB gene (Wehrli and Staehelin, 1971).

In contrast to the apparent simplicity of the RNA polymerase priming reaction, E.coli primase, the product of the dnaG gene (Rowen and Kornberg, 1978) can participate in at least three different priming systems. The simplest reaction is observed on viral strands of G4 DNA coated with SSB (Bouché et al., 1975; Bouché et al., 1978; Rowen and Kornberg, 1978), in which primase synthesises a 26 to 29-ribonucleotide primer at a unique origin sequence. Primase binds specifically to the DNA at or near this site prior to primer synthesis (Sims and Benz, 1980) by the recognition of two hairpin structures in this region. The enzyme cannot be classified as an RNA polymerase, since it is able to incorporate deoxyribonucleotides into its primer molecules (Wickner, 1977). Hybrid primers containing both deoxy-ribo- and ribonucleotides are shorter than corresponding ribonucleotide molecules (Rowen and Kornberg, 1978).

The dnaG gene has been cloned and sequenced (Lupski et al., 1982; Smiley et al., 1982) and has been located adjacent to the rpoD gene (Gross et al., 1978) with which it is thought to be co-ordinately expressed (Smiley et al., 1982). The dnaG gene product is a rifampicin resistant monomeric protein with a MW of about 60,000 (Rowen and Kornberg, 1978) and is maintained at a low copy number apparently since large amounts of primase can kill host cells (Wold and McMacken, 1982). Primase can therefore be distinguished from RNA polymerase by the action of rifampicin, the different primer compositions, and the specificity of each enzyme for DNA templates.

Primase is also able to synthesise primers on single-stranded DNA in the absence of SSB, but this activity requires instead the presence of the dnaB gene product (Bouche et al., 1978; Arai and Kornberg, 1981b), a 300 kdal hexamer (Reha-Krantz and Hurwitz, 1978). Primer molecules formed are composed of between 10 and 60 ribonucleotides (Arai and Kornberg, 1981c),

and a wide range of single-stranded DNA templates can be utilised in this way. This generalised priming system appears to proceed by the ATP-dependent binding of dnaB protein to the DNA, which enables the dnaG protein to synthesise primers on the uncoated template strand (Arai and Kornberg, 1981c). However, the cooperation between the dnaB and dnaG proteins is not yet fully understood, and it remains possible that the dnaB product may act in another capacity than that of a mobile promoter in this system.

The dnaG and dnaB proteins also participate in the synthesis of primers on viral strands of SSB-coated ØX174 DNA (Figure 1-2). This priming system is more complex than those previously described, and serves as a current model for the priming of discontinuous bacterial DNA replication. The process requires a prepriming reaction involving at least six proteins in addition to primase and SSB (Weiner et al., 1976); dnaB and dnaC products and the proteins n, n', n'' and i (Wickner, 1978; Meyer et al., 1978; Shlomai and Kornberg, 1980; Arai et al., 1981; Low et al., 1982). This complex of proteins is termed the primosome, and is envisaged to be assembled on SSB-coated ØX174 DNA at the protein n' recognition site, after which it migrates along the DNA in the 5' to 3' direction priming discontinuous DNA synthesis at subsequent sites (Arai and Kornberg, 1981a; Arai et al., 1981). Protein n' is thought to recognise a unique region of secondary structure on ØX174 DNA (Zipursky and Marians, 1980), and stimulate the binding of protein n (Low et al., 1982). Next, dnaB and dnaC proteins form a protein complex, which also associates with the proteins n, n' and n'' in a reaction requiring protein i (Kobori and Kornberg, 1982) before this structure can be utilised by primase. The precise roles of the subunits of the primosome are largely obscure, but it is proposed that dnaB protein recognises, and perhaps modifies the secondary

structure of, specific but as yet undefined DNA sequences as the primosome migrates along the template strand (McMacken et al., 1977; Arai and Kornberg, 1981b). Protein n', besides being involved in the initial site-recognition reaction, may also be involved in the movement of the primosome (Arai et al., 1981). It should be pointed out, however, that movement of the primosome has not yet been demonstrated directly, but is instead inferred from the ability of the ØX174 system to synthesise primers at locations other than at the protein n' recognition site. This assembly of proteins is assumed to act in a similar fashion during discontinuous chromosomal DNA replication.

1.5 Bacteriophage-encoded priming enzymes

E.coli also supports the replication of certain bacteriophages, which, unlike the single-stranded phages described in the previous section, supply their own priming enzymes rather than relying on host gene products. The bacteriophage T7 genome is a double-stranded linear DNA molecule of some 40 Kb in length, which replicates bidirectionally primarily from a unique internal origin (Wolfson et al., 1972a; 1972b), although secondary origins have also been identified (Tamanoi et al., 1980). The gene 4 product of T7 is a multifunctional enzyme, possessing both DNA primase and ATP-dependent helicase properties (Scherzinger et al., 1977; Romano and Richardson, 1979). This 58 kdal protein functions during T7 DNA replication to separate the two DNA strands and to then synthesise short oligoribonucleotide primers on the single-stranded template; this latter reaction is stimulated approximately 10-fold by the presence of either E.coli- or T7-specified SSB (Romano and Richardson, 1979a). The primase recognises a 5 bp template sequence, yielding characteristic tetranucleotide primers

(Tabor and Richardson, 1981; Fujiyama et al., 1981) which are elongated by T7 DNA polymerase (Romano and Richardson, 1979b) to yield discontinuous fragments up to 6 Kb in length.

The representative T-even phage T4 possesses a 165 Kb linear duplex DNA molecule, which is replicated by a more complex series of events. Following infection of E.coli, this linear molecule is apparently replicated bidirectionally from a unique origin by the host RNA polymerase (Luder and Mosig, 1982). Secondary initiations lead to the formation of a highly complex and branched intermediate structure, with up to sixty replication forks, before the inhibition of RNA polymerase activity by T4-encoded proteins (Luder and Mosig, 1982). Formation of these intermediates apparently requires recombination between replicating molecules (Broker and Doermann, 1975).

Priming of discontinuous T4 DNA synthesis is carried out by the products of the phage genes 41 and 61; these proteins, acting in concert on single-stranded DNA, synthesise pentanucleotide primers composed only of ribonucleotides (Liu and Alberts, 1980). Gene 41 protein possesses both nucleotidase and helicase properties (Liu and Alberts, 1981; Venkatesan et al., 1982), and is thought to act as an oligomer in association with a monomer of gene 61 protein in order to synthesise primers (Silver and Nossal, 1982). This complex is postulated to migrate along the template DNA strand in a 5' to 3' direction, synthesising primers, with the helicase activity destabilising the helix ahead of the replication fork (Alberts et al., 1980).

1.6 Plasmid DNA replication

Although bacterial plasmids are generally regarded as autonomous replicons, all examples so far studied rely on host gene products at some stage of their replication cycle. The 6.4 Kb multicopy non-conjugative plasmid ColE1 (Clewell and Helinski, 1972) is perhaps unusual in this respect, in that it is entirely reliant on existing host replication enzymes for this purpose (Donoghue and Sharp, 1978). Replication in *E.coli* requires at least RNA polymerase, *dnaB*, *dnaC* and *dnaG* gene products, gyrase, DNA polymerases I and III and RNase H (reviewed in Tomizawa and Selzer, 1979). Initiation at the ColE1 origin involves the synthesis of a long RNA primer molecule (termed RNAII) at a region upstream from the origin on the H (heavy; more dense) strand by RNA polymerase (Itoh and Tomizawa, 1980). This RNA is cut by RNase H to yield a 555-bp primer which is elongated by DNA polymerase I, and is subsequently removed by RNase H action (Staudenbauer *et al.*, 1979; Hillenbrand and Staudenbauer, 1982). DNA synthesis from this point proceeds unidirectionally (Staudenbauer, 1979). A second, labile, RNA molecule of 108 bp (RNAI) is synthesised from the L strand, and acts to prevent initiation by preventing the processing of the RNAII primer molecule, perhaps by modifying the secondary structure of this primer transcript (Tomizawa and Itoh, 1981). The DNA sequence for RNAI is located within the RNAII coding region; thus these two molecules are presumed to hybridise with each other to achieve this effect (Conrad and Campbell, 1979; Tomizawa and Itoh, 1981). After the initial extension of RNAII primer by DNA polymerase I, priming and elongation reactions are assumed to be analogous to discontinuous chromosomal DNA replication. The plasmids ClodF13, RSF1010 and pBR322 are also thought to replicate by a similar process to that of ColE1 (Nossal, 1983).

Less is known of the replication systems of larger, conjugative plasmids, but it is apparent that a number of different mechanisms exist. R6K (IncX) is a 38 Kb antibiotic resistance plasmid with a copy number of between 10 and 15 per cell (Kontomichalou et al., 1970), for which three origins of replication have been identified (Shafferman et al., 1981). Replication is sequentially bidirectional, occurring predominantly in one direction at first, and then in the opposite direction from the same origin (Crosa et al., 1976). A 1565 bp fragment of R6K has been cloned which is capable of autonomous replication, containing both a replication origin and the gene for a protein termed π that is required for this process (Stalker et al., 1982; Kolter and Helinski, 1982).

The closely-related plasmids R1, R12 and R100 (IncFII) (Diaz et al., 1981; Miki et al., 1980) replicate unidirectionally from a single origin, which is essentially identical in all three cases (Armstrong et al., 1981). Synthesis of repA protein, which is required for initiation of replication from oriV, is controlled by the action of the copA and copB gene products (Light and Molin, 1981). The copA transcript (RNA I) hybridises to the leader sequence of the RNA II transcript, which encodes the repA protein, to prevent translation of RNA II, while copB protein represses RNA II synthesis by binding to the RNA II promotor region (see Scott, 1984 for a recent review).

Following initiation of replication of such large plasmids, primers for discontinuous synthesis of the lagging strand are presumably made by the activity of the primosome, as suggested by the requirement of dnaB protein for replication of F and IncIα plasmids (Marinus and Adelberg, 1970; Vapnek and Rupp, 1971; Fenwick and Curtiss, 1973a; Wilkins and Hollom, 1974) and identification of a primosome assembly site in the region of the ori2 replication origin of the mini-F plasmid (Imber et al., 1983).

1.7 Bacterial conjugation

Transfer of the colicin Ib-P9 gene to both *S.typhimurium* LT2 and *E.coli* K-12 strains was found to be accompanied, at a much lower frequency, by transmission of chromosomal markers (Ozeki and Howarth, 1961; Clowes, 1961) and also by mobilisation of the non-conjugative plasmids ColE1 and ColE2 (Smith *et al.*, 1963). It was therefore realised that ColIb-P9 transfer proceeds by a process analogous to that reported for F (Zinder, 1960). However, since the transfer systems of these two plasmids are genetically distinct, and this chapter is intended as a review of ColIb-P9 transfer, I shall refer to the F system only by way of analogy where details of IncIα plasmid transfer are obscure.

The transfer system of ColIb-P9 is normally repressed, so that only about 0.02% of *S.typhimurium* LT2 (Ozeki and Howarth, 1961) and 0.1% of *E.coli* K-12 (Monk and Clowes, 1964a) colicinogenic cells are initially competent donors. However, if such a ColIb$^+$ culture of *S.typhimurium* LT2 was mixed with a vast excess of plasmid-free recipient cells, then after 18h between 30 and 70% of this culture acquired the ability to synthesise colicin Ib-P9, and these newly-infected cells were in turn shown to be capable of transferring the plasmid to fresh recipients with a frequency of between 50 and 90% in subsequent 2h matings (Stocker *et al.*, 1963). This HFCT (high frequency colicinogeny transfer) state can also be attained with *E.coli* strains (Monk and Clowes, 1964a). The HFCT state is a reflection of the physiological escape of plasmid transfer genes from self-repression, since those few plasmids transferred initially are able to transfer copies to fresh recipients before the host can synthesise those proteins responsible for repression of transfer (Willetts, 1974; Cullum *et al.*, 1978). This model explains why the epidemiological spread can be maintained for only 3

to 7 generation times in such cultures (Stocker *et al.*, 1963; Monk and Clowes, 1964a), since presumably the repression system is eventually re-established as fewer recipient cells are left in the culture.

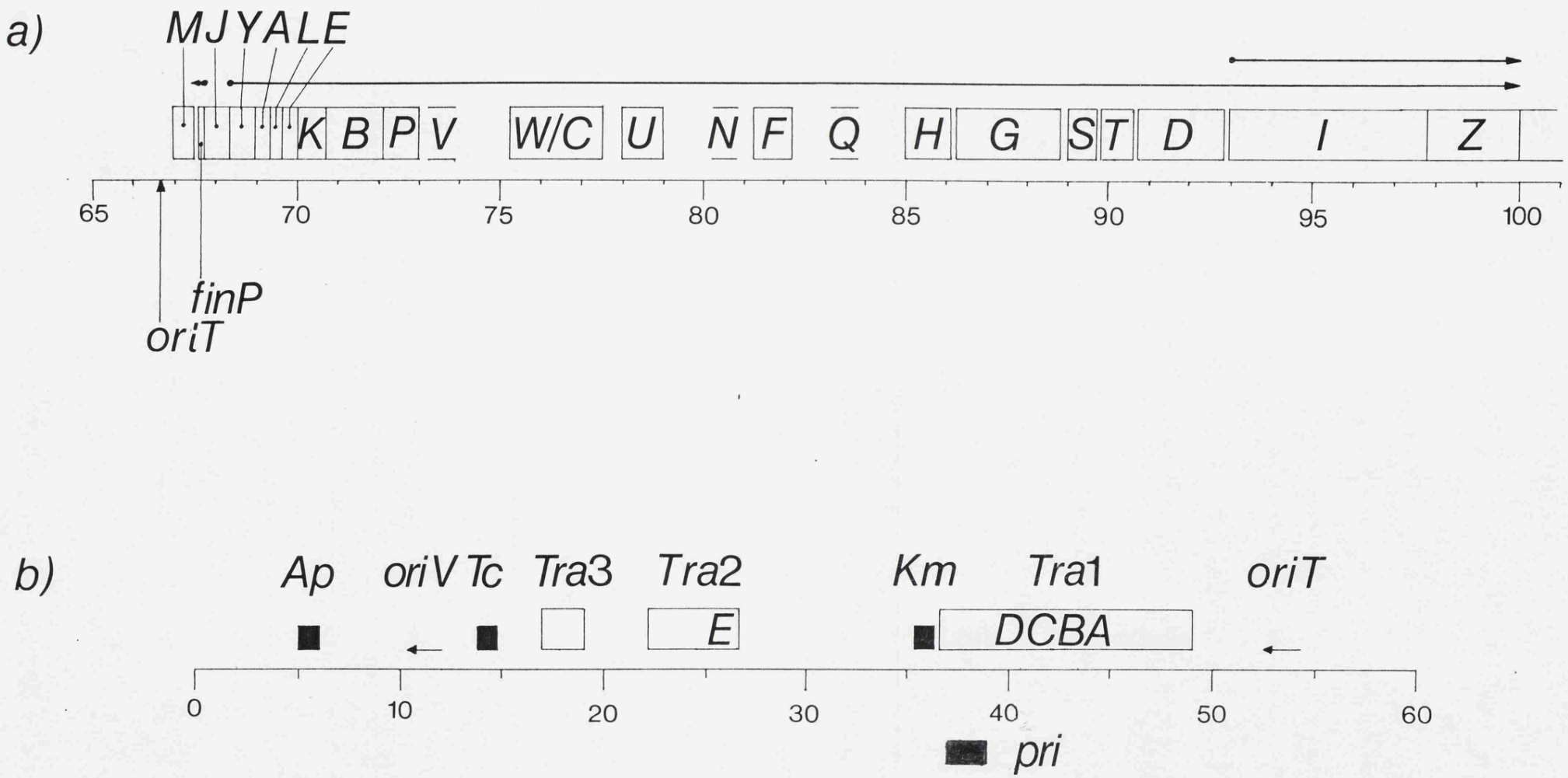
Derepressed (*drd*) mutants of ColIb-P9 and of the other IncIα plasmids R64 and R144 can be obtained which mimic the HFCT state and transfer at a high frequency (Meynell and Datta, 1967, Edward and Meynell, 1968). Such "authentic" *drd* mutations, which result in constitutive expression of transfer genes, can be accompanied by a further mutation on the plasmid leading to an even higher extent of transfer proficiency, at least for the IncIγ plasmid R621a (Sasakawa and Yoshikawa, 1978). The *drd* mutation of Iα plasmids may reflect a two-protein repression system similar to the *finO*-*finP* system of F-like plasmids (Willetts and Finnegan, 1972). The three Iα plasmids, ColIb-P9 *drd-1*, R144*drd-3* and R64*drd-11* have all proved useful in the elucidation of Iα plasmid transfer, and will be referred to throughout this chapter.

Little is known about the organisation of the transfer (*tra*) genes of Iα plasmids, so reference will be made here to the F and RP4 transfer systems, which have been examined in much greater detail. So far 23 *tra* genes of F have been mapped, and are located within a single region comprising approximately one third of the plasmid DNA (Willetts and Skurray, 1980). The products of the *finO* and *finP* genes negatively regulate the transcription of *traJ*, which in turn positively controls transcription of the other transfer genes (Finnegan and Willetts, 1973; Willetts, 1977). F itself is *finO* *finP*$^{+}$, and so expresses transfer functions at a high level, but closely related plasmids such as R100 can complement this deficiency since their repression and transfer systems are very similar and, in contrast to *finP* product, the *finO* protein is relatively nonspecific (Willetts and Skurray, 1980). The organisation of F transfer genes is depicted in Figure 1-3a,

Figure 1-3 Organisation of the transfer region of F and RP4

a) The figure depicts the transfer region of the F plasmid. Transfer (tra) genes, described in section 1.7, are denoted by their assigned letter and their positions within the operon indicated by the closed boxes above the scale for kilobase coordinates (65 to 100 Kb). Transcription of the entire traY to traZ operon, indicated by the larger horizontal arrow, is positively regulated by the product of traJ, which is in turn negatively controlled by the finO and finP products. Since F is finO$^-$, the traY to traZ operon is expressed constitutively. The last two genes in the operon are also expressed constitutively from a second, weaker, promotor (smaller horizontal arrow). See Willets and Wilkins (1984) for further details.

b) The map represents RP4 DNA linearised by cutting at the unique EcoRI site of this plasmid. Numbers denote kilobase co-ordinates proceeding clockwise from this site according to the map of Lanka et al. (1983), and the positions of the genes for resistance to ampicillin (Ap), tetracycline (Tc) and kanamycin (Km), and the orientations of the origins of DNA replication (oriV) and transfer (oriT) derived from maps in Lanka and Barth (1981) and Lanka et al. (1983). The three groups of transfer genes in RP4 are denoted by closed boxes and the letters A to E represent the five complementation groups described by Barth et al. (1976) and Lanka and Barth (1981). The position of the primase gene (pri) is indicated below the kilobase scale. DNA replication from oriV proceeds from right to left and DNA transfer from oriT runs from left to right with Tra1 being transferred last.


a)
MJYALE
K B P V W/C U N F Q H G S T D I Z
65 70 75 80 85 90 95 100
finP
oriT
b)
Ap oriV Tc Tra3 Tra2 Km Tra1 oriT
E
DCBA
0 10 20 30 40 50 60
pri

from which it can be seen that traJ regulates transcription of the long traY to Z operon and traM. The traI and Z genes are also expressed constitutively at a low level independently of traJ action (Achtman et al., 1978; Willetts and Wilkins, 1984). The products of traA, L, E, K, B, V, W/C, U, F, Q, H and G are involved in formation of the F pilus, while traM, Y, D, I, and Z are needed for conjugative DNA metabolism. Transfer genes N and G are required for the stabilisation of mating pair formation, and traS and T for surface exclusion (reviewed in Willetts and Skurray, 1980 and Willetts and Wilkins, 1984).

The IncP plasmid, RP4, has also been analysed genetically and three transfer regions identified (Barth et al., 1978; Lanka and Barth, 1981; Lanka et al., 1983; see also Figure 1-3b) through studies of deletion and Tn7 mutants. Although five complementation groups have thus been identified, the polar effects of Tn7 insertion have resulted in some of these groups containing more than one phenotypic class. The transfer regions of the closely related IncP plasmids RK2 and R751 appear to be organised in a similar fashion (Meyer and Shapiro, 1980), and two transfer regions have been proposed for the IncP-10 plasmid R91-5, each containing the respective genes for conjugative DNA metabolism and pilus synthesis (Corrigan et al., 1978; Moore and Krisnapillai, 1982a,b).

Bacteria containing ColIb-P9drd-1 constitutively produce I pili attached to the outside of the cell. These filamentous structures are about 2 μm in length with a diameter of between 6 and 12 nm (Lawn, 1966; Tomoeda et al., 1975) and are distinct from F-specified sex pili by their morphology, antigenic specificity (Lawn and Meynell, 1970; Bradley, 1980) and sensitivity to F-specific and I-specific bacteriophages (Willetts, 1977). The I pilus is composed of subunits of I pilin, distinguishable

from F pili by amino-acid composition (Brinton, 1965) and susceptibility to proteolytic cleavage by trypsin (Brinton and Bear, 1967). The bacteriophages If1 (Meynell and Lawn, 1968), PR64FS (Coetzee et al., 1980) and Iα (Coetzee et al., 1982) all infect Iα plasmid-containing bacteria by attachment to the I pilus. The pili of ColIb-P9, R144 and R64 are all similar, although R64-specified pili are distinguishable serologically from the former (Lawn and Meynell, 1970). Bradley (1983) has also reported that R144drd-3 and R64drd-11 determine a second, thicker, type of pilus with a diameter of about 10.5 nm. These thick pili have knobs at one end and a point at the other, distal, terminus. I-specific bacteriophages do not adsorb to these pili, and their role remains uncertain at present.

Although sex pili are essential for conjugative transfer of these plasmids, the precise role of this structure is still in doubt. It is known, however, that the F pilus is responsible for the formation of specific contacts between the donor bacterium and recipient cells. This shear-sensitive union is subsequently stabilised by a process involving the traG and N gene products (Manning et al., 1981) and the pili are then thought to retract, bringing the cells into wall-to-wall contact (Curtiss, 1969; Clark and Warren, 1979). In the case of I sex pili, the receptor for pilus recognition is a lipopolysaccharide present in the recipient cell wall, and conjugation-deficient (Con^-) cells with altered lipopolysaccharide component can be obtained (Havekes et al., 1977a). There is also evidence that I pili can contract (Lawn and Meynell, 1972).

It has been proposed, by analogy with infection by small filamentous bacteriophages, that the pilus might conduct DNA between mating cells along its axial channel, although there is no direct evidence for this suggestion (Brinton, 1965; Ou and Anderson, 1970). Alternatively, since wall-to-wall

contact appears essential for transfer (Achtman *et al.*, 1978), the DNA may travel via a channel formed either by fusion of the outer membrane of mating cells and holes in the peptidoglycan layers of the cell envelopes or by some other specialised structure bridging the cells (Clark and Warren, 1979). In the case of ColIb-P9, the amount of plasmid DNA transferred to recipients is limited by the action of a protein or proteins synthesised from the incoming DNA (Boulnois and Wilkins, 1978). ColIb-P9 DNA transfer can be amplified approximately 4-fold by mating rifampicin-resistant (*rpoB*) donors and sensitive recipients in the presence of the drug, presumably due to the prevention of transcription of plasmid gene(s) normally responsible for the limitation of DNA transfer (Boulnois and Wilkins, 1978). This appropriate gene product(s) appears to destroy the competence of newly formed transconjugants to act further as recipients (Boulnois and Wilkins, 1978), but the precise mechanism is unknown. F plasmid transfer is affected by two gene products; *traS*, which limits DNA transfer by affecting conjugative DNA metabolism, and *traT*, which inhibits the formation of mating pairs to limit DNA transfer, and also to prevent F^+ cells acting as recipients (Achtman *et al.*, 1980). The effect of such proteins during a normal mating is to halt DNA transfer and destabilise cell to cell contacts so that the bacteria separate.

In contrast to DNA transfer, there is no evidence that RNA or protein molecules are transferred during conjugation. Previous attempts to quantitate protein and RNA transfer by selectively labelling these components in donor cells have failed to detect such material in unlabelled recipients after matings mediated by either ColIb-P9 or F (Silver and Ozeki, 1963; Silver, 1963; Silver *et al.*, 1965). Thus, if conjugal transfer of protein or RNA occurs, it involves less than 0.3% of the total cellular protein or RNA content of mating cells, which was the limit of resolution

of these experiments (Silver and Ozeki, 1963). Such a result, however, does not preclude the selective transfer of specific molecules associated with the conjugative process, and this point will be reconsidered later in this thesis.

1.8 Conjugative DNA metabolism

Having summarised relevant aspects of both DNA replication and conjugation, a consideration will be given of the metabolism of plasmid DNA during its transfer; a series of events central to this thesis. Transfer of plasmid DNA apparently requires that the supercoiled plasmid molecule is converted to a 'relaxed' open circular form by the introduction of a nick into one of DNA strands. In the cases of ColE1 (Warren *et al.*, 1978), R12 (Morris *et al.*, 1973) and RK2 (Guiney *et al.*, 1979), this reaction has been shown to be highly specific, with the nick being introduced into a specific strand of the plasmid DNA at or near a unique site termed the origin of transfer (*oriT*). The transfer origins of ColIb-P9 (this thesis; C. Wymbs and B. Wilkins, personal communication), F (Everett and Willetts, 1982), R46, R1, R100 (see Willetts and Wilkins, 1984), RK2 (Guiney and Yakobson, 1983) and RP1 (Watson *et al.*, 1980) have been cloned, as well as the origins of transfer of other, smaller, non-conjugative plasmids. In all of the above instances, a short sequence, often containing regions of dyad symmetry, is required for DNA transfer. The nicking reaction implicated with F plasmid transfer is postulated to occur at one of two discrete sites contained within the inverted repeats found within the cloned transfer origin (see Willetts and Wilkins, 1984), and requires the activities of both the *traY* and *traZ* gene products (Everett and Willetts, 1980). A complex of the two proteins is thought to act as a site-specific endonuclease, recognising the transfer origin and introducing a single-strand nick into the DNA at this point.

Although both ColIb-P9 (Clewell and Helinski, 1970) and R64 (Kupersztoch-Portnoy et al., 1974) have been reported to exist as relaxation complexes, no strand- or site-specific nicking event has yet been demonstrated. The possibility remains that relaxation may be attributable to the effects of a plasmid-encoded endonuclease, nicking the plasmid DNA at random sites. Such endonucleases have been reported for ColIb-P9, R64 and R144drd-3 by Winans and Walker (1983); see also Chapter 5 of this thesis.

It remains uncertain whether nicking at oriT is the trigger for DNA transfer. Everett and Willetts (1980) have proposed that the oriT site of F is repeatedly nicked and religated in cells not involved in conjugation, so that an equilibrium between these two states is reached. It may therefore be mating pair formation that initiates DNA transfer and conjugative DNA metabolism, rather than the nicking event. Once this event has occurred, the nicked strand is transferred to the recipient. Transfer of a specific single-strand of DNA has been demonstrated for R64drd-11 (Vapnek, Lipman and Rupp, 1971) as well as for F (Cohen et al., 1968; Vapnek and Rupp, 1970, 1971). The gradient of recombinants generated in matings with R144-mediated Hfr donor strains indicates unidirectional transfer of the IncIα plasmid strand, but it is not known whether it is transferred with a leading 5' terminus, as in the case of F (Rupp and Ihler, 1968; Ohki and Tomi zawa, 1968).

Conjugative transfer of Iα plasmids appears to involve genomic lengths of DNA. Fenwick and Curtiss (1973a,b) observed only monomeric lengths of single-stranded R64drd-11 DNA transferred to minicell recipients, with a delay of several minutes between successive rounds of transfer. Similar results have been reported by Boulnois and Wilkins (1978) using rifampicin-treated recipients of ColIb-P9, and the concept is further supported by the requirement of an RNA primer for synthesis of DNA to replace the strand transferred to the recipient cell (see later chapters).

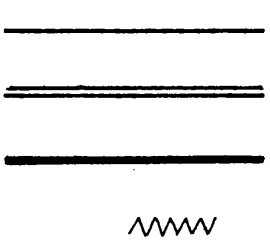
Jacob et al. (1963) proposed that DNA synthesis in the donor provided the force driving DNA transfer to recipients, an hypothesis elaborated by Gilbert and Dressler (1968) in their rolling circle model of transfer, in which extension of the 3'-OH terminus at a nicked origin of transfer displaces the 5' terminus of this strand into the recipient cell. However, studies using thymine starvation (Pritchard, 1965; Ishibashi, 1966; Maturin and Curtiss, 1981) refute this conclusion. In the case of F, the 180,000 MW product of traI (Willetts and Maule, 1979), DNA helicase I, is postulated to bind to a single-stranded region of plasmid DNA and migrate in a 5' to 3' direction to displace the transferred strand in an ATP-dependent process (Kingsman and Willetts, 1978; Abdel-Monem et al., 1983). Roles may also exist for DNA gyrase (Gellert, 1981) and single-strand binding protein (SSB) (Geider and Hoffmann-Berling, 1981). Evidence for the involvement of DNA gyrase (Fenwick and Curtiss, 1973c), a host enzyme which introduces negative supercoils into duplex DNA (Gellert et al., 1976), is dependent on studies involving the inhibitor nalidixic acid (Sugino et al., 1977), and they are open to criticism since it appears that the drug may cause gyrase-DNA complexes that result in breakage of DNA as well as disruption of DNA metabolism (Gellert, 1981). Until the involvement of gyrase in DNA transfer is tested by a different means, perhaps by utilizing the temperature-sensitive gyr mutants reported (Kreuzer and Cozzarelli, 1979; Orr et al., 1979), the involvement of DNA gyrase in both F and I plasmid transfer must remain uncertain.

Conjugative DNA transfer of ColIb-P9 is a replicative event accompanied by synthesis of a replacement DNA strand in the donor and of a complementary strand in the recipient, (Wilkins and Hollom, 1974), as shown in Figure 1-4. These separate processes can be measured by the specific

Figure 1-4 Conjugative synthesis of ColIb-P9 DNA

The figure depicts a simplified scheme for the conjugative synthesis of ColIb-P9 DNA, with events occurring in the donor and recipient bacteria displayed respectively on the left and right of the vertical line. Following the nicking of the unique plasmid strand destined for transfer at the origin of transfer, oriT (1), this strand is displaced into the recipient cell with its 5' terminus leading (2). The circular strand retained in the donor serves as template for the synthesis of a replacement strand (RSS), while the transferred strand is converted to duplex material in the recipient cell by complementary strand synthesis (CSS; 3). RSS may proceed continuously, but CSS is thought to be a discontinuous process and hence to require multiple priming events. (4) Second rounds of transfer may result from transmission of the replacement strand generated during the previous round of transfer.

Key:

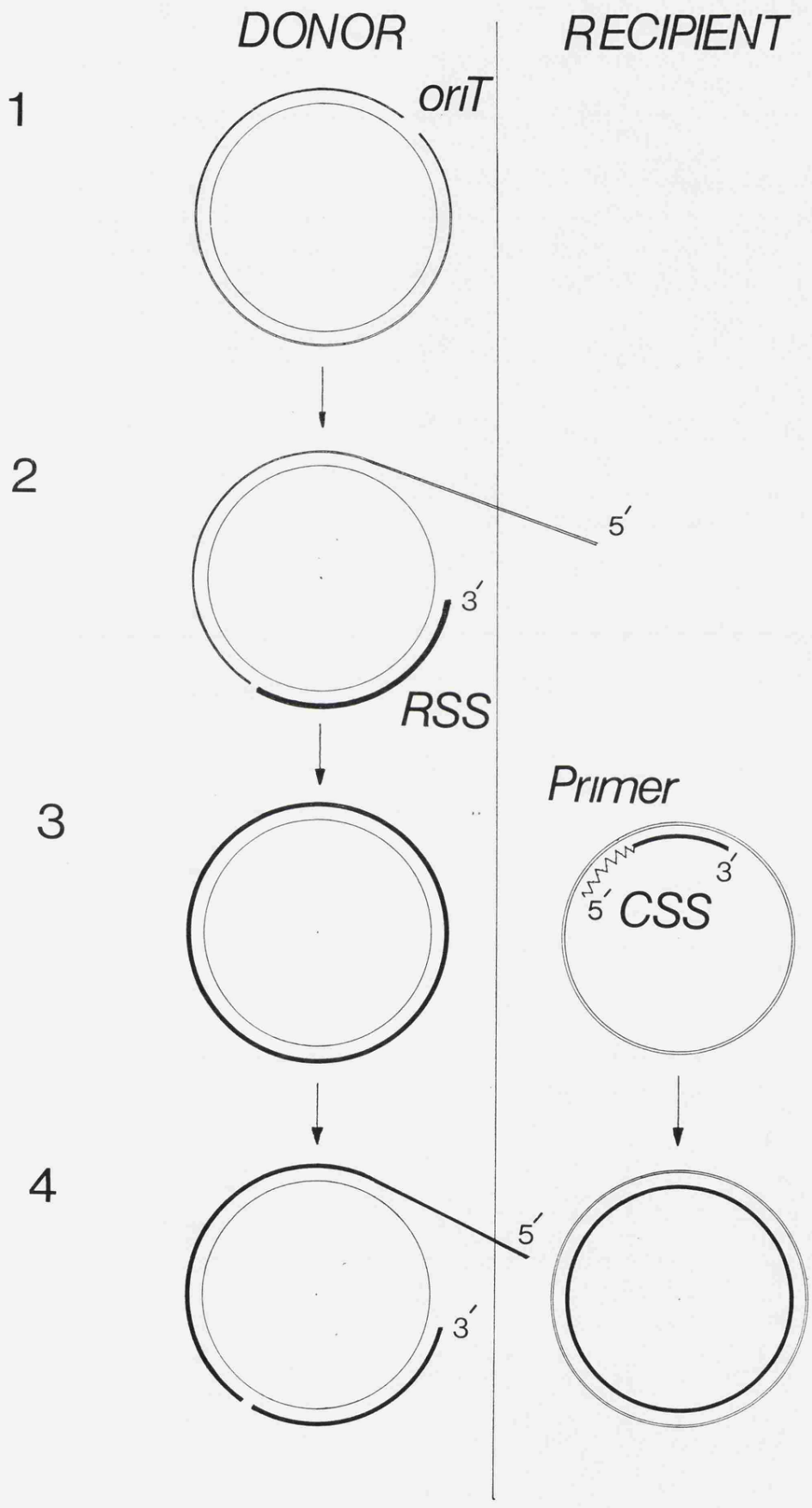


Plasmid DNA strand retained in donor

Transferred plasmid strand

Conjugatively synthesised plasmid DNA

Primer


DONOR
RECIPIENT
1
oriT
2
5´
3´
RSS
Primer
3
3´
5´
CSS
4
5´
3´

incorporation of radioactively-labelled thymine into donor or recipient strains (Vapnek and Rupp, 1971; Wilkins and Hollom, 1974). Incorporation of labelled thymine by either parental strain can be prevented by using tdk bacteria deficient in thymidine kinase activity (Freifelder and Freifelder, 1968) and hence unable to incorporate exogeneous thymine or thymidine into DNA (Higara et al., 1967). The use of temperature-sensitive dnaB mutants as donors of ColIb-P9 has allowed detection of replacement strand synthesis since vegetative replication of an IncIα plasmid, but not conjugative DNA synthesis, is dependent on dnaB protein (Fenwick and Curtiss, 1973a; Wilkins and Hollom, 1974). Complementary strand synthesis in recipients can in turn be detected either by using a dnaB(ts) strain at restrictive temperature (Bresler et al., 1973; Wilkins and Hollom, 1974) or by ultraviolet (UV) irradiation of recipient cells prior to mating to render the resident DNA unsuitable as template for DNA synthesis (Freifelder and Freifelder, 1968).

Synthesis of the complementary strand in recipients is carried out by DNA polymerase III holoenzyme (Wilkins and Hollom, 1974), necessitating a requirement for primer synthesis during conjugative DNA metabolism. In the case of IncIα plasmids, RNA primers are generated by a plasmid-encoded DNA primase, which will be considered in more detail in the next section. This accounts for the reported independence for conjugative DNA synthesis of the host priming enzymes RNA polymerase and dnaB and dnaG proteins (Wilkins and Hollom, 1974; Boulnois and Wilkins, 1979) and presumably explains the decrease in transconjugant frequency when RNA synthesis in dnaB donors of R64drd-11 was inhibited by the prevention of cytidine utilisation (Maturin and Curtiss, 1981), since primers synthesised by plasmid primase possess a characteristic cytidine residue at their 5' terminus (E. Lanka unpublished data in Willetts and Wilkins, 1984).

Presumably the post-priming stages of conjugative DNA synthesis, such as the removal of primers, their replacement with DNA, and the ligation of discontinuous fragments is analogous with the corresponding steps in discontinuous chromosomal DNA replication (Section 1.3). There may also be a requirement for a plasmid-specified single-strand binding protein in this process, since these proteins are associated with both phage and bacterial DNA replication. Both F and R64drd-11 (but not the repressed plasmid R64) have been shown partially to suppress temperature-sensitive ssb-1 mutations in E.coli (Kolodkin et al., 1983; Willetts and Wilkins, 1984). The F gene responsible for this suppression has been designated ssf and its product has extensive homology with the host SSB, implying a similarity of roles in DNA synthesis (Chase et al., 1983).

Circularisation of the transferred strand in the recipient is necessary for the stable inheritance of the plasmid. This process does not require transcription of plasmid DNA in recipients, thus implying that it involves donor-supplied products in the cases of ColIb-P9 (Boulnois and Wilkins, 1978) and F (Hiraga and Saitoh, 1975). It has been proposed, by analogy with circularisation of ØX174 (Eisenberg et al., 1976), that a protein with both endonuclease and ligase activities covalently attaches to the 5' terminus created as it nicks at oriT and after each round of DNA transfer, recognises the 3'-OH terminus of the same strand and religates the two ends (Warren et al., 1978). Upon completion of the synthesis of the replacement strand, a topoisomerase such as DNA gyrase converts the relaxed duplex DNA molecule to a negatively supercoiled form.

1.9 Plasmid-encoded DNA primases

The first evidence for the existence of plasmid-encoded priming enzymes stemmed from the observation that the IncIα plasmids ColIb-P9, R64 and R144 partially suppressed temperature-sensitive *dnaG3* mutations, allowing chromosomal DNA synthesis to proceed at restrictive temperature (Wilkins, 1975). Suppression was increased if the plasmids carried an authentic *drd* mutation derepressing transfer genes (Wilkins, 1975) and was enhanced further by a second plasmid-located mutation causing an even higher level of transfer gene expression (Sasakawa and Yoshikawa, 1978).

IncIα plasmid DNA primase was initially purified from cells harbouring R64*drd-11* and shown to be capable of initiating DNA synthesis on a wide range of DNA templates (Lanka *et al.*, 1979). This plasmid primase can prime DNA synthesis on single-stranded phage templates of fd (M13), G4 and ØX174 *in vitro*, thus substituting for the host priming enzymes RNA polymerase and *dnaB*, *C* and *G* proteins (Lanka *et al.*, 1979). The enzyme can also substitute for *dnaG* protein during discontinuous replication of bacteriophage λ DNA, Iα and ColE1-like plasmids and the *E.coli* chromosome (Wilkins, 1975; Lanka *et al.*, 1979; Wilkins *et al.*, 1981) although it cannot bypass the requirement for *dnaB* protein in this latter process (Wilkins and Hollom, 1974; Boulnois and Wilkin, 1978).

Plasmid DNA primase is distinguishable antigenically from *dnaG* protein and, unlike RNA polymerase, it synthesises primers in a rifampicin-resistant process (Lanka *et al.*, 1979; Wilkins *et al.*, 1981). Functional primers made *in vitro* consist of between only 2 and 10 nucleotides, with a characteristic cytidine or cytidine-5'-monophosphate at the 5' terminus followed by an adenosine-5'-monophosphate nucleotide (E. Lanka, unpublished data in Willetts and Wilkins, 1983). This contrasts with primers

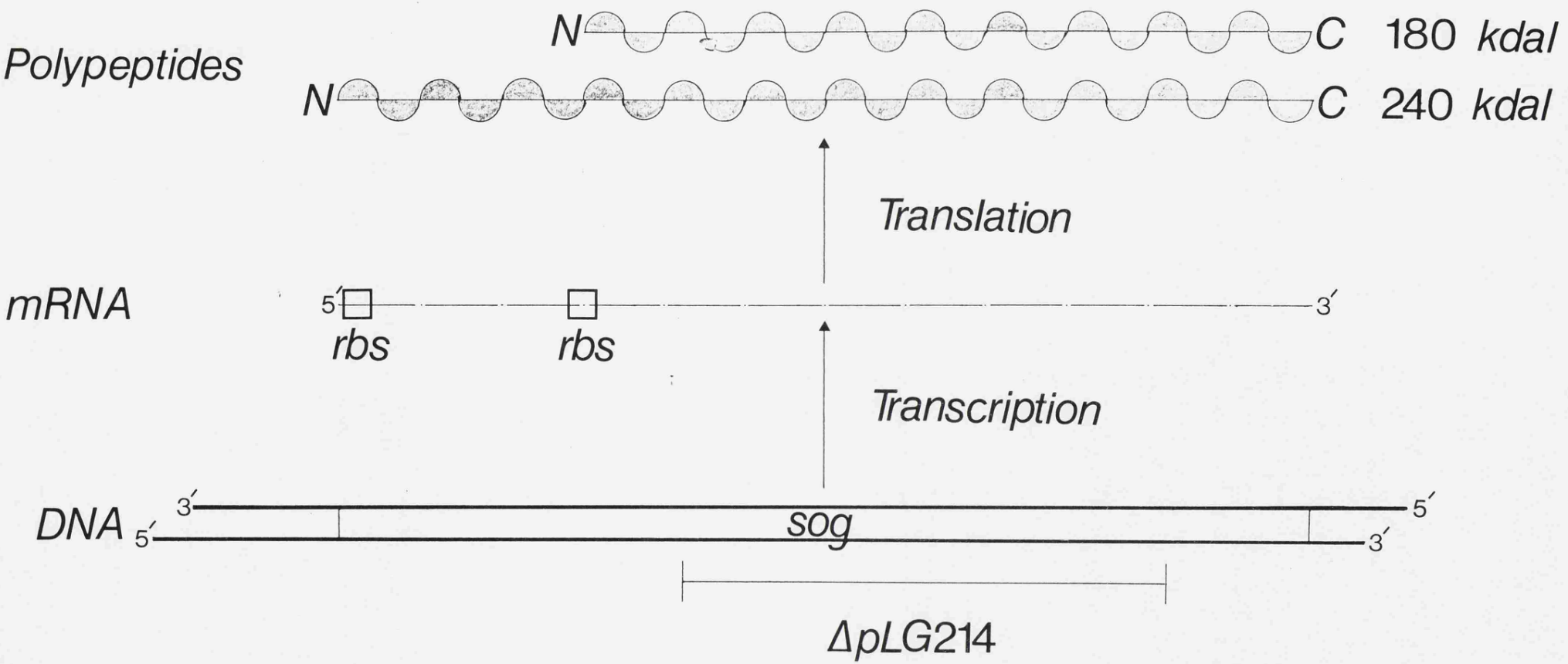
synthesised by dnaG primase, which are initiated by a 5' purine nucleotide (Rowen and Kornberg, 1978; Arai and Kornberg, 1981c). The plasmid enzyme thus recognises at least a 3'-dG-dT-5' sequence on single-stranded DNA for primer synthesis, and interestingly, it can use the 3' terminal nucleotide of a linear template to synthesise a complementary primer-DNA complex with a flush end (Willetts and Wilkins, 1984). Primers are extended by E.coli DNA polymerase III holoenzyme (Lanka et al., 1979; Wilkins et al., 1981).

The ColIb-P9 gene for plasmid DNA primase has been designated sog (for suppression of dnaG; Boulnois and Wilkins, 1979) and shown to be homologous with the primase genes of R64, R144, (IncIα), R621a (IncIγ), R864a and RIP72 (IncB). These plasmids can be considered as producing the same priming enzymes as judged by measurements of apparent MW and immunological cross-reaction (Wilkins et al., 1981; Dalrymple et al., 1982; Dalrymple and Williams, 1982). The sog gene of ColIb-P9 has been cloned on a 7.9 Kb EcoR1 restriction fragment into the multicopy vector plasmid pBR325 (Bolivar, 1978), and the resultant recombinant designated pLG215 (Wilkins et al., 1981). The sog locus, (see Figure 1-5) is thought to specify two antigenically related polypeptides of apparent molecular weight 240,000 and 180,000 (Wilkins et al., 1981; Boulnois et al., 1982). These proteins are apparently translated in the same reading frame from separate initiation sites on a single mRNA transcript, so that the two polypeptides consequently share a common amino acid sequence such that the smaller protein corresponds to the C-terminal portion of the larger polypeptide (Boulnois et al., 1982). DNA primase activity has been associated with the unique N-terminal portion of the larger protein, requiring a domain of between 36 and 75 kdal, as judged by analysis of Sog$^-$ amber and deletion mutants (Boulnois et al., 1982). The 240 kdal sog protein is therefore likely to be multifunctional, but the role of the C-terminal region of this polypeptide, and of the entire 180 kdal protein has yet to be determined.

Figure 1-5 Schematic representation of the sog locus of ColIb-P9

The diagram, which is purely schematic and not drawn to scale, depicts current ideas on the organisation and products of the sog locus of ColIb-P9. The sog gene is transcribed to yield a single messenger RNA species, which in turn is translated from two separate ribosome binding sites (rbs) to generate two polypeptides of about 240 and 180 kdal. The initiation codons are thought to be in the same reading frame, giving two sequence-related sog proteins with the entire amino acid sequence of the smaller polypeptide comprising the C-terminal region of the larger. Primase activity requires at least part of the unique N-terminal region of the 240 kdal polypeptide.

The deletion in the sog gene present in recombinant plasmid pLG214, is indicated in the figure. It does not eliminate the primase domain but apparently removes a considerable portion of the region common to both sog polypeptides.


Polypeptides
N
C
180 kdal
N
C
240 kdal
Translation
mRNA
5´
3´
rbs
rbs
Transcription
DNA
3´
5´
5´
sog
3´
ΔpLG214

Plasmid-encoded DNA primases appear to be common amongst conjugative plasmids (see Table 8-1), and have been associated with members of 11 incompatibility groups so far (Lanka et al., 1979; Lanka and Barth, 1981; Dalrymple et al., 1982; Dalrymple and Williams, 1982). Although some other plasmids, most notably F, have been designated as primase-negative (Lanka and Barth, 1981), they might encode primases that are unable to function on the viral strands of fd or M13 in the in vitro assay used to determine primase activity, and so the range of such enzymes may be greater than assumed. Three distinct plasmid DNA primase genes have been identified so far, encoded by the prototype plasmids ColIb-P9 (IncIα), RP4 (IncP) and R16 (IncB) (Wilkins et al., 1981; Lanka and Barth, 1981; Dalrymple et al., 1982). The respective genes are distinguishable by DNA hybridisation techniques, and their products are antigenically distinct (Lanka and Barth, 1981; Dalrymple et al., 1982).

The primase gene (pri) of RP4 has been assigned to the Tra1 region of the plasmid (Barth et al., 1976; Lanka and Barth, 1981; Lanka et al., 1983) and shown to produce two antigenically related polypeptides of 118 kdal and 80 kdal as separate translation products from overlapping genes (Lanka et al., 1984). These two polypeptides are coordinately regulated with other Tra1 gene products, and are apparently translated from a polycistronic messenger RNA containing the information for at least three other RP4 gene products (Lanka et al., 1984). Both proteins possess DNA primase activity (Lanka and Barth, 1981; Lanka et al., 1984) and can operate on fd, G4 and ØX174 viral templates (Lanka and Barth, 1981). A recombinant plasmid carrying the pri gene of RP4 and the related IncP plasmid R68.45 (Ludwig and Johanson, 1980), are capable of dnaG suppression, apparently as a result of a mutational change in the primase gene (Lanka et al., 1984). In this

regard the gene also appears similar to sog, since spontaneous deletion of 1.5 kb from the cloned sog gene on pLG215 results in the subsequent plasmid (pLG214) specifying a truncated DNA primase with an increased efficiency of dnaG suppression (Wilkins et al., 1981; Boulnois et al., 1982; see also Fig. 3-1 and Chapter 3).

The R16 (IncB) pri determinant has also been cloned and shown to encode two proteins which can be altered by deletions in a similar fashion to that reported for Iα and RP4 DNA primases, and still retain priming ability (Dalrymple, 1982). Furthermore, it has been suggested that this locus also contains two overlapping genes, but deletion analysis suggests that, unlike sog (Boulnois et al., 1982), the proteins specified by the R16 pri locus share a common N-terminal sequence (Dalrymple, 1982). This DNA primase is also active on M13 viral DNA templates in vitro, and in dnaG suppression (Dalrymple et al., 1982).

As shown in Table 8-1, there is no simple correlation between groups of plasmids sharing homologous plasmid DNA primase genes and classification by incompatibility grouping. Members of the IncIα (R64, R144), Iγ (= IncB; R621a) and B (R864a, RIP72) groups share nucleotide sequences homologous to the sog locus of ColIb-P9, while homology with the prototype R16 pri gene has been demonstrated for plasmids of the Iζ (R805a), K (R387) and B (TP125, pLG101) incompatibility groups (Dalrymple et al., 1982; Dalrymple, 1982). The existence of two such non-homologous DNA primase genes among a group of related conjugative plasmids as typified by the B group plasmids presumably reflects previous evolutionary gene exchange between diverse groups of plasmids.

The biological role of plasmid DNA primase forms the central theme of this thesis. Conjugative DNA synthesis of the IncIα plasmid R144drd-3 in recipient cells can proceed in the absence of active host priming enzymes RNA polymerase and dnaG protein (Boulnois and Wilkins, 1979), and so it was proposed that plasmid primase carried out the initiation of complementary strand synthesis on the transferred plasmid DNA. Using the analogous RP4 system, it was subsequently demonstrated that E.coli donors of RP4::Tn7 Pri$^-$ plasmids were deficient in generating transconjugants when mated with certain other bacterial species including S.typhimurium, Proteus mirabilis and Klebsiella aerogenes (Lanka and Barth, 1981). Back transfer experiments with these strains revealed that this deficiency was recipient specific, suggesting that RP4 primase was involved in the priming of plasmid DNA synthesis in recipient cells whose own primer-generating enzymes were unable to function efficiently on single-stranded RP4 DNA (Lanka and Barth, 1981). Since loss of RP4 primase activity also resulted in a detectable level of plasmid instability, these authors conferred two physiological roles for this enzyme(s), the priming of the transferred strand of plasmid DNA during conjugation and also of discontinuous plasmid DNA replication following transfer. These two functions may contribute to the broad host range of RP4 (Lanka and Barth, 1981).

The physiological role and mode of action of the plasmid DNA primase specified by ColIb-P9 will be discussed in the remaining sections of this thesis.

1.10 Introduction to the results

This thesis is a continuation of an analysis of the conjugation system of IncIα plasmids using ColIb-P9 as a representative of this group. My studies have concentrated on the involvement of the DNA primase encoded by the *sog* gene of this plasmid in the conjugative process. As described in the previous section, Boulnois and Wilkins (1979) proposed a role for the *sog* gene product in the conjugative synthesis of plasmid DNA in recipient cells, based on the observation that the priming of complementary strand synthesis of R144*drd-3* in recipients was independent of host-encoded enzymes. The candidature of *sog* product in this respect was supported by its associated DNA primase activity (Lanka *et al.*, 1979) and the coordinate expression of *sog* with transfer functions (Wilkins, 1975). Subsequent genetic analysis of *sog* has required this hypothesis to be modified. The *sog* locus is comprised of two overlapping genes, and specifies two sequence-related polypeptides of which only the larger possesses DNA primase activity, localised in the N-terminal portion unique to this larger molecule (Wilkins *et al.*, 1981; Boulnois *et al.*, 1982). Thus the *sog* locus may encode gene products with multiple functions only one of which is involved with priming activity. Clearly, therefore, the mode of action of plasmid primase may be more complex than previously realised.

The first part of this thesis is concerned with the experimental verification of the original hypothesis that Iα plasmid primase is involved in the initiation of complementary plasmid strand synthesis during conjugation. The approach taken involved the construction of Sog$^-$ derivatives of ColIb-P9*drd-1* by the *in vivo* recombination of defined mutations from a cloned *sog* gene into the parental Iα plasmid. This procedure, and the subsequent characterisation of these plasmids is described in Chapter 3. Using these

mutants (designated pLG250 and pLG251) in conjunction with Sog^+ recombinant plasmids, the involvement of plasmid primase in conjugative and replicative synthesis of ColIb-P9 is examined in Chapter 4. Both Sog^- Iα plasmids were deficient in supporting complementary strand synthesis in recipient cells, and a role is also suggested for plasmid primase in donor bacteria. The most important conclusion drawn from these results is that the enzyme achieves initiation of complementary strand synthesis following its transmission from the donor. This hypothesis is supported by the ability of rifampicin-treated dnaG recipients to synthesise chromosomal DNA in matings with Sog^+ donors. As detailed in Chapter 5, this conclusion is drawn from measurements of conjugative DNA synthesis in UV treated and untreated dnaG recipient cells, and analysis of conjugatively synthesised DNA by DNA-DNA reassociation experiments. Requirements for this recovery process are examined in Chapter 6, and models for the action of plasmid primase discussed in Chapter 8.

CHAPTER 2

Materials and Methods

2.1 Bacterial strains and plasmids

Descriptions and sources of bacterial strains and plasmids used in this thesis are given in Tables 2-1 and 2-2. BW92 was derived from BW82 by selection for chlorate-resistant mutants under anaerobic conditions by the method of Adhya *et al.* (1968) and screening of chlorate-resistant colonies for sensitivity to UV light. BW92, which also has a requirement for biotin, is therefore considered to specify a deletion covering the *chlA-uvrB-bio* region. Selection for T6-resistant mutants of BW92 gave rise to BW93. BW98 and BW101 were each isolated by plating BW61 or BW82 on nutrient agar containing naladixic acid ($50\mu g ml^{-1}$) or rifampicin ($100\mu g\ ml^{-1}$) as appropriate. Plasmid containing strains are denoted by the convention : strain number (plasmid).

2.2 Media and chemicals

Liquid growth media for bacterial strains was either nutrient broth 'E' (London Analytical and Bacteriological Media Ltd., 13g per litre) or Luria broth (L broth contains 10g tryptone (Oxoid), 5g yeast extract (Oxoid) and 5g NaCl (Fisons) per litre at a pH of 7.0). SGC, a salts-glucose-casamino acids medium, contained, per litre : Na_2HPO_4, 6g; KH_2PO_4, 3g; NH_4Cl, 1g; NaCl, 0.5g; $MgSO_4$, 0.12g; $CaCl_2$, 11mg; glucose, 4g; Casamino acids (Difco), 2g; thiamine HCl (Sigma), 1mg. Where required by auxotrophic strains, both the above liquid media , and nutrient agar (Oxoid No.2) plates were supplemented with either 0.2mg of biotin per litre

Table 2-1 Bacterial strains

Strain	Genotype or description	Source
E.coli K-12		
BW40	*dna*$^+$ *tdk-1 uvrB5 rpsL31 tsx-33 proA2 leu-8 thr-4 argE3 his-4 thi ara-14 mtl-1 xyl-5 gal-2 lac-1*	Wilkins *et al.* (1971)
BW61	*dnaG3 polA1 leu metE thyA drm* ColI^R	Wilkins (1974)
BW68	*dnaB70 thyA deoB deoC rpsL tsx lacY14 rha* ColI^R	Boulnois and Wilkins (1978)
BW69	*dnaB70 thyA deoB deoC* Δ(*chlA-uvrB-bio*) *tsx rpsL* ColI^R	Boulnois and Wilkins (1978)
BW82	*dnaG3 leu deoA deoC tdk rpsL* ColI^R	Boulnois *et al.* (1979)
BW84	*dnaG3 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)
BW88	*dna*$^+$ *leu thyA deoB rpsL* ColI^R *rpoB*	Boulnois *et al.* (1979)
BW89	*dna*$^+$ *leu thyA deoB rpsL* ColI^R Δ(*chlA-uvrB*)	Boulnois and Wilkins (1979)
BW92	*dnaG3 leu deoA deoC tdk rpsL* ColI^R Δ(*chlA-uvrB-bio*)	from BW82
BW93	*dnaG3 leu deoA deoC tdk rpsL* ColI^R Δ(*chlA-uvrB*) *tsx*	from BW92
BW96	*dna*$^+$ *leu deoA deoC tdk rpsL* ColI^R *rpoB*	Boulnois and Wilkins (1979)

Table 2-1 Continued

Strain	Genotype or description	Source
E.coli K-12		
BW97	*dna*$^+$ *leu thyA deoB rpsL* ColI^R Δ(*chlA-uvrB*) *nal*	Boulnois and Wilkins (1979)
BW98	*dnaG3 polA1 leu metE thyA drm* ColI^R *nal*	from BW61
BW101	*dnaG3 leu deoA deoC tdk rpsL* ColI^R *rpoB*	from BW82
J08	*dna*$^+$ *leu deoB rpsL* ColI^R *recA1*	Laboratory stock
DB56	*dna*$^+$ *metB trpR supE44 supF* $hsdM_K^+$	D. Burt
AB259	HfrH *thi*	Laboratory stock
BC1304	*dnaB1304 dnaC201 polA1 thyA rpsL end arg*	Shuster *et al.* (1977)
W3110	prototrophic	
S.typhimurium LT2		
ST1	wild type	Laboratory stock
ST203	Δ*his*	Laboratory stock

Genotypic symbols are defined in Bachmann (1983). ColI^R is the phenotypic symbol for resistance to colicin Ib-P9.

Table 2-2 Plasmids

Plasmid	Genotype or description	Source or reference
Vector plasmid		
pBR322	$Ap^R Tc^R$	Bolivar *et al.* (1977)
pBR325	$Ap^R Tc^R Cm^R$	Bolivar (1978)
IncIα plasmids		
pLG221	ColIb-P9 *drd-1 cib*::Tn*5* *sog*$^+$	Boulnois (1981)
pLG250	pLG221 *sog-217*	This work
pLG251	pLG221 *sog-220*	This work
Recombinant plasmids		
pLG211	pBR325Ω[*sog*$^+$, *Eco*RI 7.9Kb, 0.27Kb]	Wilkins *et al.* (1981)
pLG214	pBR325Ω[*sog*$^+$, *Eco*RI 7.9Kb Δ1.5Kb]	Wilkins *et al.* (1981)
pLG215	pBR325Ω[*sog*$^+$, *Eco*RI 7.9Kb]	Wilkins *et al.* (1981)
pLG217	pBR325Ω[*sog-217*, *Eco*RI 7.9Kb,] 0.27Kb	Boulnois *et al.* (1982)
pLG220	pBR325Ω[*sog-220*, *Eco*RI 7.9Kb,] 0.27Kb	Boulnois *et al.* (1982)
pLG2000	pBR325Ω[*oriT*, *Eco*RI], 3 fragments	This work

Table 2-2 Continued

Plasmid	Genotype or description	Source or reference
Recombinant plasmids		
pLG252	pBR325Ω[*eex*$^+$, *Eco*RI 3.5Kb]	This work
pKC3	pBR325Ω[*eex*$^+$, *Eco*RI 3.5Kb]	This work
pKC4	pBR325Ω[*eex*$^+$, *Eco*RI 3.5Kb, and second small fragment]	This work

Plasmid nomenclature follows the guidelines in Novick *et al.* (1976).

or 20µg of thymine (Sigma) per ml as appropriate. In the case of strains harbouring pBR325 or recombinant plasmids derived from this vector, tetracycline (Tc) was added to $7.5\mu gml^{-1}$ to select for the presence of the plasmid. Other antibiotics were used at the following concentrations; ampicillin (Ap) $25\mu gml^{-1}$; kanamycin (Km) $50\mu gml^{-1}$; nalidixic (Nal) 50 μgml^{-1}; rifampicin (Rif) $100\mu gml^{-1}$ and streptomycin sulphate (Sm) 100 μgml^{-1}. All antibiotics were purchased from Sigma chemical company with the exception of Sm (Glaxo), and solutions of these sterilised prior to use either by filtration or, in the case of Tc and Rif, by making the solution in 50% ethanol. For Rif, the stock solution also contained 0.05M K_2CO_3 to aid dissolution.

The radiochemicals [2-^{14}C]thymine, [methyl-^{3}H]thymine, [methyl-^{3}H] thymidine, and [methyl-^{3}H] deoxythymidine 5'-triphosphate, were purchased from Amersham International.

2.3 Storage and identification of strains

Bacterial strains were maintained on nutrient agar plates supplemented with thymine or Tc as appropriate and stored at 4°C. For long-term storage, 5 mls of 25% glycerol solution in nutrient broth was inoculated with several loopfuls of cells grown previously on nutrient agar plates and vortexed briefly. After 30 min at room temperature, samples were frozen at -20°C. Bacteria were recovered from storage by thawing vials until just the top layer of the sample was liquid and innoculation of a 5 ml overnight culture with 0.1 ml of the cell suspension.

The phenotype of recovered strains was confirmed prior to use in experiments in order to prevent cross-contamination or mis-identification by applying the strain to the following tests as indicated by the genotype of the appropriate strain. Auxotrophic mutants were streaked to single

colonies across minimal medium (made up as SGC except for the omission of Casamino acids and addition of 15gl^{-1} Davis agar) containing, as appropriate, thymine (Thy, 20μgml^{-1}), leucine (Leu, 40μgml^{-1}), methionine (Met, 20μgml^{-1}) or histidine (His, 20μgml^{-1}) and strains carrying antibiotic resistance markers tested on agar plates containing the relevant antibiotic. Amino acids were purchased from Sigma. The sensitivity of strains to ultraviolet light was determined by exposure of freshly-streaked cells on nutrient plates to a range of doses (0 to 60Jm^{-2}) of UV light from a Hanovia germicidal lamp. The dose rate was set using a Latarjet dosimeter to 2Jm^{-2} per sec. output and the streaked cells shielded partially with a cardboard screen for various intervals, (0, 8, 10, 15 and 30 sec) to achieve a range of exposures. Plates were incubated overnight to determine the viability of exposed cells. Susceptibility of strains to bacteriophages was tested by cross-streaking cells with a suspension of either T6 or PR64FS. Temperature-sensitive *dnaG3* and *dnaB70* mutants were tested by their ability to form colonies at restrictive temperature, as described fully in section 2.10.1. These strains were routinely grown at 31°C and *dna*$^{+}$ strains at the optimum temperature of 37°C, except where indicated.

The *tdk* and *thyA* genotypes of strains intended for use in *in vivo* DNA labelling and DNA synthesis experiments was verified by detection of the ability to incorporate exogenous [*methyl*-^{3}H] thymine into trichloroacetic acid (TCA)-precipitable material. Overnight cultures of strains to be tested were diluted 0.25ml into 4.75 ml of pre-warmed SGC medium supplemented with 2μg thymine and 200μg deoxyguanosine (Sigma) per ml, and grown by shaking for 90 min at 31°C in an orbital shaker water-bath. 1.5 ml of this exponentially growing culture was then added to 1.5 ml of pre-warmed SGC medium containing 2μg thymine, 200μg deoxyguanosine and

15 μCi [methyl-^{3}H]thymine ml^{-1}. After a further 90 min incubation, 1 ml aliquots were sampled by addition to 2 ml of ice-cold TCA stock solution (6.7% trichloroacetic acid and 134μg thymine per ml) to give a final concentration of 4.5% TCA and 100μgml^{-1} thymine. Samples were vortexed briefly and kept on ice for at least one hour and TCA-precipitable radioactivity determined by filtering the contents of each tube through a 27 mm Sartorius membrane filter (pore size 0.45 μm) pre-soaked in a 0.2% thymine solution. Each filter was washed through ten times with boiling water and thoroughly air-dried. 5 ml of scintillant (5g 2,5-diphenyloxazole; Fisons, and 0.3g of 1,4-bis-2(4-methyl-phenyloxazolyl)benzene; Fisons, per litre of toluene; Cambrian Chemicals) was added to each filter in a scintillation vial, and the amount of radioactivity present in each vial measured in a Packard liquid scintillation spectrophotometer. The tdk-1 mutant BW40 and the thyA strain BW86 were routinely used as negative and positive controls for this procedure. The retention of background radioactivity by filters was estimated by processing four aliquots of 1 ml cell-free culture medium and 2 ml TCA solution in the same way. The average value was subtracted from those obtained for samples as a correction for non-specific retention of label.

2.4 Bacterial matings

Bacteria were grown before use in mating experiments in 10 ml broth cultures by aeration at 37°C or, in the case of temperature-sensitive strains, at the permissive temperature of 31°C. Usually, 0.2 ml of an overnight culture was added to 10 ml of broth and cells grown for at least 3 to 4 mass-doublings to a density of 1-2x10^8 cells per ml. Growth was monitored by the absorbance of cultures at 600mm (A_{600}) with a Bausch and

Lomb spectrophotometer. Donor and recipient cultures were mixed so that the recipient was present in an excess (usually 1:10) and gently swirled in conical flasks at the required temperature for 1 hour. Transconjugants were assayed, either at timed intervals during the mating, or at the end of this time, by plating serial dilutions by the mating mixture in sodium phosphate buffer (PB; 60.9g NaH_2PO_4 and 208.5g Na_2HPO_4 per litre at pH7.5) on selective agar plates. When required for accurate calculation of transconjugant frequency, donor cells in the mating mixture were also assayed in this way, and in interrupted mating experiments samples (0.35 ml) were added to 3.15 ml PB and vortexed vigorously in a mechanical agitator to separate mating cells. Undiluted 0.1 ml samples of donor and recipient cultures were always plated to the selective medium used for transconjugants as controls.

2.5 Bacteriophage production

Bacteriophage PR64FS (Coetzee et al., 1980) was produced by a large scale method essentially similar to that described by Yamamoto et al. (1970). A 40 ml culture of W3110 (pLG221) in LB was grown for 3-4 mass-doublings at 37°C to a density of about $2x10^8$ cells ml^{-1} at 37°C. 20 ml of this culture was added to 20 ml of pre-warmed LB at 37°C to give approximately 10^8 cells ml^{-1} and PR64FS added at a multiplicity of infection (m.o.i) of around 0.05. This culture was shaken at 37°C for 5h and 1 ml samples extracted at 30 min intervals. These samples were treated with 0.1 ml of ether, and held at 37°C for 10 min before titration on nutrient agar plates with a lawn of W3110 (pLG221) as indicator bacteria. Controls plated on nutrient agar plates showed that the ether treatment was completely effective in the killing of bacteria from the culture. After 5h, the increase in A_{600} had ceased and the culture contained approximately

$5x10^9$ plaque-forming units (p.f.u.) per ml. Bacteria were sedimented by centrifugation in a Sorvall SS34 rotor at 10,000 rpm and 4°C for 10 min. Polyethylene glycol (PEG_{6000}; BDH Chemicals Ltd.) and NaCl were added to the supernatant to achieve 6% (w/v) and 0.5M respectively. Bacteriophage were sedimented at 4°C overnight, and pelleted by centrifugation as above. The pellet was resuspended in 1 ml of phosphate buffer, and contained approximately $5x10^{11}$ p.f.u.. Phage were stored over 0.1 ml of ether at 4°C.

Bacteriophages M13, fd and T6 were obtained from Dr B.M. Wilkins and titred on AB259 (Hfr) and W3110 (pLG221) as appropriate.

2.6 DNA preparation

2.6.1 Isolation of pBR325-based recombinant plasmid DNA

Large amounts of recombinant plasmid DNA were isolated by the cleared lysate method described below. A 20 ml NB culture of the plasmid-containing strain was grown overnight in the presence of Tc to select against the loss of plasmids, and used to inoculate a 1 l flask of NB also containing Tc. The culture was grown to an A_{600} of 0.6 and plasmid DNA amplified overnight with either chloramphenicol (Cm, 200μgml^{-1}; Sigma) for recombinant plasmids or spectinomycin (300μgml^{-1}; Sigma) in the case of the Cm^R vector plasmid pBR325. The culture was decanted into 250 ml polypropylene tubes and cells collected by centrifugation in an ice-cold Sorvall GS3 head at 5,000 rpm for 3 min. The pellets were resuspended in a total of 16 ml 25% sucrose in 50mM Tris (tris(hydroxymethyl)aminomethane; Trizma) solution, pH8.0, and divided into four equal aliquots in silicone-coated Sorvall SS34 tubes, in an ice-bath. To each was added 0.3 ml of a freshly-prepared 10$mgml^{-1}$ lysozyme (Sigma) solution, and samples held on ice for 5 min before the addition of 0.2 ml 0.25M EDTA

(ethylenediaminetetraacetic acid) pH8.0 to achieve formation of spheroplasts. Cleared lysates were prepared from this solution by the addition of 4 ml lysis mixture (50mM Tris, 62.5mM EDTA, 0.1% Triton X-100; pH8.0) to each tube, and left at room temperature until viscous (about 5 min). Cellular debris was sedimented at 18,000 rpm for 40 min and the supernatant centrifuged to equilibrium in a caesium chloride (CsCl) - ethidium bromide gradient. Gradients contained 7.7 ml supernatant, 8.15g CsCl (Fisons) and 0.6 ml 5mgml^{-1} ethidium bromide (Sigma) solution, and the refractive index (R.I) measured using a Bellingham and Stanley refractometer. The R.I. was adjusted to 1.394 with saturated CsCl solution and spun at 38,000 rpm for 40h at 15^{o}C in a fixed angle Beckman 50Ti rotor, which was allowed to coast to a standstill at the end of the run. DNA bands were visualised under UV light (365 nm wavelength) from a UV transilluminator, and the lower, more dense, band containing plasmid DNA collected by puncturing the nitrocellulose tube with a 0.7 mm diameter stainless steel needle and withdrawing the DNA into a disposable syringe. Samples were treated four times with equal volumes of propan-2-ol saturated with an aqueous solution of CsCl, with the upper phase containing extracted ethidium bromide being discarded each time. The resultant solution was dialysed extensively against four changes of 1 l 10mM Tris pH8.0 at 4^{o}C over 48 hours.

Rapid, small-scale plasmid isolation was performed by the method of Klein et al. (1980), using either a 5 or 10 ml culture of cells which had been treated with amplifying antibiotics overnight. Cells were spun down and resuspended in 0.5 ml 50 mM Tris pH8.0 in 1.5 ml conical polypropylene tubes (Eppendorf). 50µl of a 10mgml^{-1} solution of freshly-prepared lysozyme was added and samples held at room temperature for 15 min, before the addition of 0.5 ml of phenol mixture (100g phenol (BDH Chemicals Ltd),

0.1g 8-hydroxyquinoline (BDH Chemicals Ltd), 4 ml iso-amyl alcohol (Fisons) in 100 ml chloroform (Fisons); stored at 4°C under 10mM Tris buffer pH7.5). Tubes were capped and inverted three times, and centrifuged for 15 min in a bench-top Eppendorf microfuge at maximum speed. The upper, aqueous layer was transferred to a new tube using a Gilson hand pipette and disposable tips, avoiding the flocculent material at the interface. This solution was extracted with 0.3 ml of phenol mixture in the same way, and again transferred to a new Eppendorf tube. Residual phenol was removed by addition of an equal volume of a chloroform : iso-amyl alcohol mixture (24:1 ratio by volume) and the aqueous layer again transferred to a new tube. DNA was precipitated with 3 volumes of 100% ethanol in a bath of dry ice and methanol for 5 min, and pelleted in an Eppendorf microfuge. The pellet was washed with 70% ethanol and dried under vacuum in a desicator, before a final resuspension in 100 µl of autoclaved distilled water. 20 µl of this solution was normally sufficient for a single digest with restriction endonucleases.

2.6.2 Isolation of the DNA of large conjugative plasmids

In this section, only the protocols for the different methods of plasmid isolation used in the course of this work are described; comparison of the efficiencies and properties of the DNA samples obtained by each method is given in Chapter 5.

a) The procedure of Hansen and Olsen (1978)

Cultures of plasmid-containing strains were grown by shaking either overnight to late log-phase or to A_{600} of 0.6 following the inoculation of 1 l volumes of NB in 2 l flasks with a 10 ml pre-culture. After growth, the culture was divided into 250 ml aliquots, and the steps

described below refer to the manipulations of each aliquot. Cells were pelleted (Sorvall GS3 rotor, 7,000 rpm for 10 min), washed and resuspended in 6 ml 25% sucrose in 50mM Tris pH8.0 in plastic Nalgene tubes with sealing caps. Lysis was achieved by addition of 1 ml of 5mgml^{-1} lysozyme in 0.25M Tris pH8.0 and tubes inverted four times to mix the contents thoroughly. All inversion steps in this procedure were performed gently to reduce mechanical breakage of plasmid DNA, at a rate of one inversion every 3 seconds. After 5 min at room temperature, 2.5 ml of 0.25M EDTA pH8.0 was added, and mixed by a further 15 sec of inversion. After a further 5 min at room temperature, 2.5 ml of 20% sodium dodecyl sulphate (SDS) in 50mM Tris/20mM EDTA pH8.0 was added, and samples treated by 8 cycles of heat pulse and mixing. One cycle was 15 sec at 55°C in a water bath, followed by 15 sec of inversion out of the water bath. This was successful in producing a clear, viscous lysate, which was next subjected to an alkaline denaturation step designed to improve the yield of plasmid DNA and remove residual broken chromosomal DNA (Hansen and Olsen, 1978). At room temperature, 0.75 ml of 3M NaOH was added to each tube, and mixed by 20 inversions. Next, 6 ml 2M Tris pH8.0 was added, followed immediately with 3 ml 20% SDS and 6 ml 5M NaCl, and again mixed by 20 inversions. A white flocculant precipitate indicating the salt-precipitation of chromosome-membrane complexes was apparent at this stage, and tubes refrigerated at 4°C overnight. This floc-like material was pelleted by centrifugation at 18,000 rpm in an ice-cold SS34 head for 30 min, and the supernatants pooled in a chilled plastic measuring cylinder. The volume (normally about 85 ml per litre of input culture) was noted, and $\frac{1}{3}$ volume of a 42% PEG 6000 solution added. The contents were mixed by pouring into chilled polypropylene GSA bottles, and stored at 4°C overnight to encourage the precipitation of plasmid DNA. Precipitated DNA was collected

by centrifugation at 7,000 rpm for 5 min at 4°C, and the pellet dissolved in TE buffer (50mM Tris, 5mM EDTA pH8.0). The volume was made up to 8 ml per 250 ml input culture with TE, and 0.5 ml ethidium bromide solution ($510 mg ml^{-1}$) and 8g CsCl (also per 250 ml input) added to give a solution with R.I. 1.387. Caesium chloride-ethidium bromide gradients were spun to equilibrium in a Beckman 50 Ti rotor and DNA samples collected as described previously in this section.

Modification of the above method to avoid the use of PEG 6000 was achieved by precipitating plasmid DNA at this stage with ethanol. The supernatant obtained after SDS-NaCl precipitation of membrane-bound chromosomal DNA was extracted with butan-2-ol to a volume of about 10 ml, to which 2 volumes of ethanol and 1/10 volume of 2M sodium acetate pH5.6 were added. Samples were left at either 4°C overnight or at -80°C for 2h, before sedimentation of DNA at 7,000 rpm for 5 min at 4°C in a GSA head. Subsequent CsCl-ethidium bromide density gradient centrifugation was carried out as before.

b) The procedure of Barth and Grinter (1975)

Cells were grown as described above in 1 l cultures of NB and sedimented using a GS3 rotor at 7,000 rpm for 10 min, washed with 100 ml TES buffer (50mM Tris, 5mM EDTA, 50mM NaCl, pH8.0) and finally resuspended in 30 ml ice-cold TES containing $100 mg ml^{-1}$ sucrose and $1 mg ml^{-1}$ lysozyme. After 10 min on ice, cells were lysed in plastic Corex tubes by the addition of 7.5 ml 5M NaCl and 1.5 ml 20% SDS. In later experiments EDTA was also added to a final concentration of 60mM. Samples were stored at 4°C overnight and centrifuged in a SS34 head at 17,000 rpm and 4°C for 30 min to clear the lysate. Supernatants were decanted to fresh Corex tubes and extracted with a $\frac{1}{2}$ volume of phenol mixture. After centrifugation, the

aqueous layer was extracted with 24:1 chloroform : iso-amyl alcohol and DNA precipitated with 2 volumes of ethanol and 1/10 volume of sodium acetate pH5.6 at -80°C for 2h. DNA was sedimented in a GS3 rotor, re-suspended in 10 ml TES buffer and incubated for 1 hour in the presence of 100μgml^{-1} RNase A (heat-treated at 100°C for 15 min prior to use). The solution was again extracted with phenol mixture and dialysed extensively against 10mM Tris pH8.0 at 4°C.

c) The method of Uemura and Mizobuchi (1982)

A 500 ml culture of NB was inoculated with a pre-grown 10 ml overnight of W3110 (pLG221) and grown to an absorbance at 600 nm of between 0.8 and 1.2 at 37°C. Cells were sedimented (GS3 rotor, 7,000 rpm for 10 min at 4°C), washed in 125 ml of 50mM Tris pH8.0 and finally resuspended in 12.5 ml 25% sucrose in 50mM Tris pH8.0. This suspension was transferred to two plastic Nalgene tubes, and to each was added 1.25 ml 5 mgml^{-1} lysozyme in 250mM Tris pH8.0. Samples were held at 0°C in iced water for 5 min and 2.8 ml 0.25M EDTA pH8.0 added before a further 5 min on ice to produce spheroplasts. Lysis involved the addition of 10 ml of detergent mixture (1% Brij 58, 0.4% sodium deoxycholate, 62.5mM EDTA and 50mM Tris, pH8.0) to each tube and a further 10 min incubation at 0°C. Samples were centrifuged in a SS34 head 20,000 rpm and at 4°C for 25 min and the resultant cleared lysates decanted into fresh tubes.

Sarkosyl (N-lauroyl sarcosine sodium salt) (Geigy) was added to a final concentration of 1% and the solution incubated at 30°C for 10 min, before being put in an ice bath to chill. NaCl and PEG 6000 were added to 0.5M and 10% respectively and allowed to stand at 4°C overnight. PEG-precipitated DNA was sedimented by centrifugation at top speed in a bench-top centrifuge, and the pellets resuspended in 2 ml TES buffer. CsCl-

ethidium bromide gradients were made by the further addition of 0.2 ml 0.25M EDTA, 0.2 ml 250mgml^{-1} sarkosyl solution in TES buffer and 0.84 ml 5mgml^{-1} ethidium bromide. After gentle mixing of solutions, a further 3.24 ml TES buffer and 6.5g CsCl were added, and the R.I. adjusted to 1.392. Gradients were spun at 33,000 rpm in a Beckman 50 Ti rotor for 40h at 20°C and plasmid DNA collected, treated with CsCl-saturated propan-2-ol and dialysed as described in Section 2.6.1.

2.6.3 Preparation of chromosomal DNA

Bacterial DNA from BW86 was prepared by a method essentially similar to that described by Jeffreys and Flavell (1977). Two flasks, each containing 1 l NB supplemented with thymine were inoculated with 2 ml from an overnight culture of BW86 and grown at 31°C in an orbital shaker to late log phase or stationary phase overnight. Cells were sedimented and resuspended in 30 ml 10mM Tris pH8.0 containing 1% SDS per litre of original culture. After phenol extraction and ethanol precipitation as described previously, the resultant pellet was resuspended in 30 ml of 0.1 x TNE (20 x TNE contains 121g Tris, 117g NaCl, 37.2g EDTA per litre at pH7.5). RNase A (boiled for 15 min prior to use to remove residual contaminating nuclease activity) was added to 100μgml^{-1} and the sample incubated at 37°C for 15 min before the further addition of 3 ml 10% SDS, 1.5 ml 20 x TNE and 0.3 ml 10mgml^{-1} pronase (predigested for 2h at 37°C). Incubation was continued for another 15 min, and the sample then mixed with a $\frac{1}{2}$ volume of phenol mixture. Following a 2 min centrifugation at 10,000 rpm , the aqueous phase was precipitated at -80°C for 2h with 2 volumes of ethanol, and DNA pelleted by a further centrifugation. The precipitate was next resuspended in 10 ml 10mM Tris pH8.0, and equal volumes of 2.5M potassium phosphate pH8.0 and methoxyethanol added and mixed. After centrifugation

at 10,000 rpm for 2 min to separate the two phases of this mixture, the upper, aqueous phase was collected and precipitated with ethanol. The DNA pellet was washed with 70% ethanol and resuspended in 1 ml 10mM Tris pH8.0 before extensive dialysis against the same buffer at 4°C. Yields of chromosomal DNA obtained per litre of input cells ranged between 2 to 6 mg.

High molecular weight bacterial DNA from Proteus mirabilis 13 was prepared exactly as described by Chow et al. (1977).

2.6.4 Preparation of bacteriophage DNA

Single-stranded phage fd DNA was prepared by growth of the bacteriophage on the Hfr strain AB259. A 10 ml overnight culture of AB259 in NB was used to inoculate 500 ml NB in a 1 l flask. Cells were grown at 37°C with shaking to an absorbance at 600 nm of 0.6, and bacteriophage added at a m.o.i. of 0.1. Incubation was continued overnight, and the culture centrifuged the next day to pellet cells (GSA rotor, 15 min, 5,000 rpm, 4°C). The resultant supernatant was decanted into a polypropylene bottle, and PEG 6,000 and NaCl added to give final concentrations of 4% and 0.5M respectively. The mixture was shaken overnight and centrifuged at 3,000 rpm for 5 min. After resuspension in 10mM Tris pH8.0 and extraction with phenol, bacteriophage DNA was purified by centrifugation in a discontinuous CsCl step gradient in a swinging bucket Spinco SW27 rotor for 20h at 23,000 rpm. Gradients consisted of three layers of CsCl solution (1.2, 1.3 and 1.4 gml^{-1}) and bacteriophage was added by layering on the top of the gradient. Phage fd bands at a density of 1.29 (Yamamoto et al., 1970) and was collected by aspiration. CsCl was removed by dialysis against 10mM Tris pH8.0.

DNA was purified further from this sample by phenol extraction and ethanol precipitation, as described below.

2.6.5 Further purification and measurement of DNA concentration

DNA concentration and purity of all samples obtained by the above methods was determined by measurement of the absorbance of the solution between 230 and 300 nm using a Unicam SP1800 spectrophotometer. A_{260} allows estimation of the DNA concentration (one A_{260} unit corresponds to a nucleic acid concentration of 50μgml^{-1}) and proteins absorb maximally at 280 nm. Measured values were converted to absolute amounts using a nomograph. Since RNA also absorbs at 260 nm, this method may overestimate the concentration of DNA in the sample, and so contaminating RNA molecules were detected by agarose gel electrophoresis (Section 2.8) as a diffuse band running well ahead of DNA.

Residual amounts of RNA and protein were removed by digestion with RNase A (pre-boiled for 15 min at 100°C) and proteinase K (pre-digested for 2h at 37°C) at concentration of 50 to 150μgml^{-1} and 150μgml^{-1} respectively for 1 to 2h at 37°C. EDTA was then added to 20mM and samples extracted with a half volume of phenol mixture in 1.5 ml Eppendorf tubes. The aqueous phase was removed after a 2 min centrifugation in an Eppendorf microfuge and extracted with an equal volume of 24:1 chloroform : iso-amyl alcohol mixture to remove residual phenol. The aqueous phase was again collected and DNA precipitated with 2 volumes of ethanol and 1/10 volume of 2M sodium acetate pH5.6 in a dry ice/ethanol bath for 5 min. Precipitated DNA was collected by sedimentation in an Eppendorf microfuge and washed with 70% ethanol. Samples were dried under a vacuum and re-suspended in 10mM Tris pH8.0. DNA was stored at 4°C.

2.7 Preparation of [^{3}H] labelled DNA

2.7.1 Isolation of [^{3}H] labelled BW86 DNA

A 5 ml overnight culture of BW86 in SGC medium supplemented with 20 μgml^{-1} thymine was spun down and cells resuspended in 1 ml SGC containing 200μgml^{-1} deoxyguanosine. This suspension was used to inoculate two 10 ml volumes of pre-warmed SGC and deoxyguanosine (200μgml^{-1}) in 250 ml conical flasks. One of these flasks contained [methyl-^{3}H] thymine at a specific activity of 40 $\mu Ci\ \mu g^{-1}$ (5 $Ci\ mmol^{-1}$) and a concentration of 2μgml^{-1}. The second flask contained only 'cold' thymine at the same concentration. Both flasks were inoculated with resuspended cells to A_{600} of 0.03, and incubated at 31°C with shaking. The A_{600} of the unlabelled culture was followed until it reached 0.5 (4 mass-doublings), when the [^{3}H] labelled cells were collected by centrifugation in a Sorvall SS34 rotor for 5 min at 10,000 rpm and 4°C. Unincorporated label was removed by three cycles of sedimentation and resuspension in fresh ice-cold TES buffer and cells finally resuspended in 1 ml 10% sucrose solution made up in 0.1M Tris pH8.0, and chilled for 5 min on ice. Lysozyme was added to 600μgml^{-1} from a freshly-made stock solution of 3$mgml^{-1}$ in 0.1M Tris pH8.0 and the sample held on ice for a further 10 min. EDTA (0.25 ml of 300mM stock, pH8.0) was added and the mixture kept on ice for another 5 min to allow formation of spheroplasts, and a cleared lysate achieved by the addition of 0.25 ml of a 2.8% solution of sarkosyl. The mixture was held at room temperature until clear (about 3 to 5 min). RNase A, prepared as described in Section 2.6.5 was added to give a final concentration of 150μgml^{-1} and the mixture incubated at 37°C for 1h. The lysate was vortexed twice for 7 sec each time

and proteinase K (section 2.6.5) added to 150μgml^{-1} and returned to 37°C for a further 2h. The lysate (2 ml) was added to 8 ml TE buffer and 12.9g CsCl and the mixture allowed to dissolve slowly, with gentle occasional mixing, at room temperature. Refractive index was adjusted to 1.4015 and the gradient centrifuged to equilibrium at 35,000 rpm for 40h in a Beckman 50Ti rotor at 15°C.

Gradients were punctured at their base using a fractionator, and 25 drop fractions collected in plastic Eppendorf tubes. 5 μl of each fraction was spotted onto 2.5 cm Whatman No.1 filters and dried under a 250W lamp. Filters were washed three times in 200 mls of an ice-cold 5% TCA solution containing thymine at 100μgml^{-1} for successive periods of 15, 10 and 10 min, and finally for 10 min in 200 mls of methanol. Filters were air-dried, and radioactivity bound to each measured as described above (Section 2.3). The major fractions containing chromosomal DNA were pooled and CsCl removed by dialysis against 10mM Tris pH8.0. Typical preparations yielded about 12 μg of DNA at a specific activity of 226 cpm per ng.

2.7.2 Isolation of [^{3}H] labelled pLG221 DNA

A 7.5 ml culture of BW86 (pLG221) in SGC medium containing 200μgml^{-1} deoxyguanosine and [methyl-^{3}H] thymine (2μgml^{-1}; 50 μCi μg^{-1}; 6.3 Ci mmol^{-1}) was set up as described in Section 2.7.1 and grown from an absorbance of 0.03 to 0.5 at 600 nm by aeration at 31°C. Cells were washed and cleaved lysates formed by the procedure described above. The sample was centrifuged to equilibrium in a CsCl-ethidium bromide gradient consisting of 7.4 ml lysate (made up to volume with TE buffer), 250 μl 10mgml^{-1} ethidium bromide solution and 7.1g CsCl with a R.I. of 1.3910. Two- or three-drop fractions were collected from the gradient as before and the three fractions containing the plasmid DNA pooled. Fractions containing chromosomal DNA

were used to determine the specific activity of labelled plasmid DNA. Plasmid DNA was purified further by a second CsCl ethidium bromide gradient centrifugation (R.I. = 1.3920), and two-drop fractions collected. Plasmid fractions were again pooled, and dialysed extensively after extraction with CsCl-saturated propan-2-ol.

This approach yielded approximately 40 ng of labelled plasmid DNA with a specific activity of 1,680 cpm ng^{-1}.

2.7.3 Isolation of [^{3}H]labelled DNA from recipient cells

Conjugatively-synthesised DNA in unirradiated rifampicin-treated BW86 recipients was labelled with [methyl-^{3}H] thymine (1$\mu g ml^{-1}$; 33.3 μCi μg^{-1}; 4.2 Ci $mmol^{-1}$) in the presence of rifampicin and deoxyguanosine as described in Section 2.11, except that the volume of mating mixture was 10 ml. Controls for the mating, consisting of separately-incubated BW96 (pLG221) and BW86 strains, are described in the text of Chapter 5. After 60 min of mating, 1 ml of 100mM Tris pH containing 1 mg thymine, 1 mg thymidine and 6.5 mg KCN were added, followed by 0.2 ml 1M NaOH to adjust the pH to 7.6. Cells were then treated as described in the preceding sub-sections to give a cleared lysate, and spun to equilibrium in a CsCl density gradient (R.I. = 1.4015). Fractions containing DNA (33.5 cpm ng^{-1}) were pooled and dialysed against four 1 l changes of 10mM Tris pH8.0 at $4^{o}C$.

2.8 Restriction endonuclease digestion of DNA and electrophoresis of DNA fragments

Digestion of DNA samples with the restriction endonucleases EcoRI (Boehringer Mann heim GMBH) and HindIII (BRL Inc.) was carried out according

to the manufacturer's instructions. Digests contained, as well as DNA, 1/5 volume of a X 5 stock of salt mix; 500mM Tris pH7.5, 50mM $MgCl_2$, 250mM NaCl (EcoRI buffer) or 100mM Tris pH8.0, 35mM $MgCl_2$, 2.5mM DTT, 300mM NaCl (HindIII buffer), and 1 unit of the appropriate enzyme per µg of DNA. Samples were incubated at 37°C for 1-2h and loaded immediately onto agarose gels for electrophoresis. Molecular weight markers included phage λ DNA digested either singly or doubly with EcoRI or HindIII (Daniels et al., 1980) and pBR322 DNA digested with TaqY1 (BRL Inc.) (Sutcliffe, 1978). Digestion of pBR322 was performed at 65°C in the appropriate buffer (50mM Tris pH8.4, 30mM $MgCl_2$, 500mM NaCl, 30mM 2-mercaptoethanol) for 2h.

Agarose was obtained from Sigma, and gels made up as an appropriate percentage of agarose in running buffer (25mM Tris-acetate pH7.7 and 1mM EDTA) disolved by boiling. Agarose solutions were cooled to around 60°C before pouring into a horizontal tray and allowed to set with a well mould in place. The gel was freed from its mould and transferred on its glass base plate to an electrophoresis tank containing running buffer. 2µl of tracking dye (80mM Tris pH6.8 containing 10% glycerol and 0.001% Bromophenol blue) was added to each digest and mixed. Samples were loaded onto the submerged gel and electrophoresed at either 20V (overnight) or 100V (2-3h) until the lower dye band was about 2/3 to 3/4 down the gel. DNA fragments were stained by placing the gel in a bath of 0.5µgml^{-1} ethidium bromide for 30 min, or run in the presence of the stain. The gels were then photographed on top of a long wavelength UV transilluminator using a Polaroid land camera with type 57 or 55 Polaroid film or a Nikon 135mm SLR camera loaded with Kodak HS23 film.

Table 2-3 Molecular weight markers for agarose gel electrophoresis

	Molecular weight standard		
Fragment	λHindIII	λEcoR1	λHindIII EcoR1
A	23.13	21.23	21.22
B	9.42	7.42	5.15
C	6.56	5.80	4.97
D	4.36	5.65	4.27
E	2.32	4.88	3.52
F	2.02	3.52	2.02
G	0.56		1.91
H			1.71
I			1.38
J			0.95
K			0.83
L			0.56

Numbers indicate the size of the restriction fragments in kilobases.

Data are drawn from Sanger et al., 1982.

2.9 Transformation of bacteria

Recombinant plasmid DNA was transformed into bacterial cells by the method described below. A 10 ml culture of NB or LB was inoculated from an overnight culture of the plasmid-free strain and grown by aeration at the appropriate temperature from A_{600} of 0.05 to 0.4 in one step. Cells were sedimented in a pre-cooled Sorvall SS34 rotor at 7,000 rpm and 0°C for 3 min, and resuspended in 10 ml ice-cold 0.1M $MgCl_2$. The cell suspension was divided between 2 chilled SS34 tubes and sedimented as before. Care was taken throughout these manipulations to keep bacteria at 4°C as much as possible, and to use pre-cooled solutions, tubes and rotors. Cells were resuspended in 5 ml volumes of 0.1M $CaCl_2$ and held on ice for 20 min. Following another sedimentation, bacteria were resuspended in 0.75 ml 0.1M $CaCl_2$, and decanted in 250 µl volumes to sterile 1.5 ml Eppendorf tubes. 1µg of plasmid DNA was added to the competent cells for transformation; controls included the addition of an equal volume of phosphate buffer instead of DNA. Samples were held on ice for at least 1h, and cells kept in suspension by occasional finger-flicking before a 3 min heat pulse at 42°C with very gentle shaking. The transformed cells were returned to ice for 2 min and then used to inoculate a 3 ml shake culture of LB. Cultures were shaken for $1\frac{1}{2}$ to 2h, and finally the cells were collected and resuspended in 0.4 ml phosphate buffer. Undiluted samples from this final suspension and 10^{-1} and 10^{-2} dilutions were plated to selective media to isolate transformants. 1 µg of DNA usually yielded a total of about 2-3,000 transformants using this procedure.

2.10 Measurement of *dnaG3* suppression and DNA primase activity

The ability of various plasmids to suppress the temperature-sensitivity of the host *dnaG3* lesion was determined by the methods detailed below.

2.10.1 Colony formation

Overnight cultures of plasmid-containing BW86 strains (5 ml NB supplemented as necessary with thymine or Tc) were grown at 30°C and diluted to give an absorbance of 0.1 at 600 nm (about 5×10^7 cells ml^{-1}) in fresh NB, warmed to 30°C prior to use. Cells were plated at appropriate dilutions onto pre-warmed selective agar plates at 30 and 40°C and incubated overnight. Colony formation is the ratio of yields of colonies formed at 40°C compared to 30°C.

2.10.2 DNA synthesis at 41°C

A 5 ml culture of the strain under test was set up in SGC medium supplemented with deoxyguanosine and thymine (and Tc if required) as described in Section 2.3, and grown in a 50 ml flask at 30°C for 90 min in an orbital shaker. Two 1.5 ml aliquots were drawn from this culture, and used to inoculate two 1.5 ml shaking solutions of SGC and deoxyguanosine ($200 \mu g ml^{-1}$) and thymine ($2 \mu g ml^{-1}$). One of these contained [methyl-^{3}H] thymine at a specific activity of 320 mCi $mmol^{-1}$ and was shaken at 41°C for 90 min. Two 1 ml aliquots of the culture containing [methyl-^{3}H]thymine were taken into 2 ml of TCA stock solution and stored on ice for at least 1h. Acid-precipitable radioactivity was determined as in Section 2.3. The A_{450} of the other culture was measured using a Gilford 300-N spectrophotometer, and DNA synthesis in cultures at 41°C expressed as cpm ml^{-1} per A_{450} to standardise results.

2.10.3 Plasmid DNA primase activity

DNA primase activity in crude cell extracts (CCEs) was determined by the ability to prime DNA synthesis on single-stranded phage fd or M13 DNA. CCEs were prepared from plasmid-containing derivatives of BW82. 5 ml overnight cultures in NB, supplemented with Tc in the case of pBR325-based plasmids, were diluted 0.5 ml into 10 ml fresh NB and grown at 31°C in an orbital shaker to late exponential-phase. Cells were chilled and collected by centrifugation, washed in 1 ml N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid (HEPES) buffer (Sigma) pH8.0 containing 50mM KCl and 1mM dithiothreitol (DTT) and resuspended in 200 µl of ice-cold spheroplast buffer (5% sucrose, 10mM KCl, 4mM spermidine-3HCl, 1mM DTT, 1mM EDTA and 0.2 mg of lysozyme in 25mM HEPES buffer, pH8.0 in 1.5 ml Eppendorf tubes). After 30 min on ice, cells were lysed with Brij 58 (final concentration 0.25%) and left on ice for a further 40 min. Samples were centrifuged to pellet debris in an Eppendorf microfuge at 4°C and the supernatant decanted and stored at -80°C. Protein content of CCE s was determined by the method of Bradford (1976) as modified by Spector (1978).

Receptor extract for the *in vitro* primase assay was prepared from the *dnaB dnaC* strain BC1304 by a modification of the method of Lanka *et al.* (1979). A 50 ml overnight culture was grown at 31°C and used to inoculate 1 l of TY broth (8g tryptone (Difco), 5g yeast extract (Difco) and 5g NaCl made up to volume with distilled water). After shaking overnight at 31°C, cells were collected and washed in 30 ml 50mM Tris buffer pH7.5. The final pellet was weighed (wet weight was about 2g) and resuspended in 1 ml 50mM Tris pH7.5 containing 10% sucrose per gram wet paste. The cells were frozen in liquid nitrogen and stored at -80°C overnight. 1 ml aliquots were thawed below 10°C in polycarbonate tubes and, following the addition of DTT (10mM), EDTA (1mM), spermidine-3HCl (10mM) and lysozyme (200µgml^{-1}), pH

was adjusted to 7.5 with 1M Tris buffer. Samples were left on ice for 30 to 45 min to allow the formation of spheroplasts and then shifted to 37°C to allow the temperature to rise to 20°C. Once this temperature was reached, samples were again cooled to 4°C on ice with gentle mixing using a glass rod. Following centrifugation for 45 min at 30,000 rpm in a Beckman 45 Ti rotor. The supernatant was decanted and fresh streptomycin sulphate added to 4%. Samples were stirred at 4°C and centrifuged for 30 min at 30,000 rpm in a Beckman 60 Ti head. Supernatant was decanted, and the pH adjusted to 7.5 with 1M NaOH. Receptor extract was frozen and stored under liquid nitrogen.

Plasmid-specified priming activity in CCE s was assayed in mixtures (25 µl) containing 25mM HEPES buffer pH8.0, 25mM KCl, 5mM magnesium acetate, 0.2mM EDTA, 2.5mM spermidine 3HCl, 1mM DTT, 20µM nicotinamide adenine dinucleotide (NAD) 4mM creatine phosphate (Sigma), 3µg creatine kinase (Sigma), 20µM cyclic adenosine 5'-monophosphate (cAMP) (Sigma), 20mM adenosine triphosphate (ATP) (Sigma), 25µgml^{-1} rifampicin, 125µM each of rCTP, rGTP and rUTP (Sigma), 12.5µM each of dATP, dCTP and dGTP (Sigma), 650 pmol of [methyl-^{3}H]deoxythymidine 5'-triphosphate (650 or 1000 cpm per pmol), 0.5µg of single-stranded fd or M13 DNA, 5 µl receptor extract and an appropriate amount of CCE. A range of volumes of undiluted and diluted CCE s were assayed in pilot experiments to ensure that the reaction did not proceed to completion before the end of the incubation period, and 2.5 µl of a ten-fold dilution of CCE was usually found to be optimal in this respect. Reactions were carried out in triplicate in sterilised 1.5ml Eppendorf tubes at 30°C for 60 min. Mixtures lacking CCE were used as controls. The reaction was arrested by the addition of 250 µl of stop mix (0.5M NaOH, 0.5% SDS, 10% sodium pyrophosphate, 0.5mgml^{-1} calf thymus DNA; Sigma) and mixtures shifted to an ice bath. After 5 min, 500 µl 2M

TCA was added to each tube and held on ice for a further 30 min at least. TCA-precipitated radioactivity was determined as described in Section 2.3. One unit of primase activity is defined as the incorporation of 1 μmol of dTMP into TCA-precipitable material under the above conditions.

2.11 Conjugative DNA synthesis

Measurement of conjugative DNA synthesis in mating bacteria was performed by the method of Boulnois and Wilkins (1979). Bacteria were grown by aeration at 31°C (*dnaG* strains), 33°C (*dnaB*) or 37°C (*dna*$^+$) for 3 to 4 mass doublings to 2×10^8 cells per ml in SGC medium. Cultures of thymine-requiring strains were supplemented with 2 μg thymine and 200μg deoxyguanosine per ml, and strains harbouring recombinant plasmids grown in the presence of Tc. The above supplements were removed prior to mating by sedimentation and resuspension in pre-warmed SGC (containing deoxyguanosine in the case of thymine-requiring strains). [2-^{14}C]thymine was preconditioned by the method of Boulnois and Wilkins (1978). This procedure removes trace amounts of undefined labelled compounds present in the solution which are incorporated into the TCA-precipitable material of cells. The radiochemicals were added to a small culture (less than 2 ml) of exponentially growing BW40 bacteria at about 10^8 cells ml^{-1} and incubated at 37°C for 30 min. The culture was filtered to remove cells and the filtrate stored on ice until used in matings. Controls for experiments consisted of parental cultures incubated separately.

2.11.1 Conjugative DNA synthesis in recipient cells

UV irradiated recipients in mating mixtures were grown as described previously and pelleted by centrifugation. Following resuspension in 20 ml phosphate buffer, cells were irradiated with $400Jm^{-2}$ of UV light and then resuspended in prewarmed SGC medium containing deoxyguanosine to A_{600} of 0.65 (about $5x10^8$ cells ml^{-1}). Recipients were prewarmed at 41°C for 10 min and in the presence of rifampicin for 5 min prior to mating. Mating mixtures (4.7 ml) were set up by adding 1 ml of recipient culture (at 10 min before the start of mating), 1 ml of prewarmed SGC containing $200\mu gml^{-1}$ deoxyguanosine and $400\mu gml^{-1}$ rifampicin (at t = -5 min), 0.7 ml of prewarmed label medium (preconditioned [2-^{14}C]thymine in SGC containing 100 μgml^{-1} deoxyguanosine and $200\mu gml^{-1}$ rifampicin; at t = -2 min) and 2 ml of donor cells (at the start of mating) to a 250 ml conical flask at 41°C (43°C for *dnaB* matings) in an orbital shaking water bath. Mixtures therefore contained about 10^8 bacteria of each parental strain per ml, $100\mu gml^{-1}$ rifampicin and $2\mu gml^{-1}$ [2-^{14}C]thymine (63 mCi $mmol^{-1}$). Mating was continued for 1h, and 0.5 ml samples extracted from the mixture into 2 ml ice-cold TCA stock solution at 10 min intervals. Determination of TCA-precipitable radioactivity was carried out as detailed in Section 2.3.

2.11.2 Conjugative DNA synthesis in donor cells

Experiments were carried out essentially as described above, as detailed in the text of Chapter 4. Preconditioned [*methyl*-^{3}H]thymidine (2.42 Ci $mmol^{-1}$) was used as label, and matings were performed at 43°C. The *dnaB* donor cells were preincubated for 15 min at this temperature prior to addition to mating mixtures.

2.12 Conjugative DNA transfer

Experiments were essentially similar to those described in Section 2.11, except for the following points. Donor strains were grown in 10 ml SGC medium containing 2μgml^{-1} [2-^{14}C]thymine (63 mCi mmol^{-1}) and deoxyguanosine. Label was included as before during mating, so that donors were labelled prior to and during mating. The volume of mating mixtures was 3.5 ml. After 60 min of mating, 2.5 ml was extracted and added to 0.6 ml of 10mM Tris pH8.0 containing 10mM KCN, 40μgml^{-1} tryptophan and 100 μgml^{-1} thymine. Samples were blended for 5 sec and 0.5 ml of T2 adsorption buffer (4 gl^{-1} NaCl, 5 gl^{-1} K_2SO_4, 1.5 gl^{-1} K_2SO_4, 3 gl^{-1} Na_2HPO_4, 10mM $MgSO_4$, 0.5mM $CaCl_2$ and 10 mg per l gelatin, pH7.1) containing 5x10^{11} phage T6, irradiated with 110Jm^{-2} of UV light prior to use. After a 5 min incubation at 37°C, deoxyribonuclease (Sigma) and RNase A (Sigma) were added to 150 and 100μgml^{-1} respectively and incubation continued for a further 15 min, with a gentle swirl given to the solution every 5 min. Pronase (Sigma) was next added to 375μgml^{-1}, and samples incubated for a further 15 min before being transferred to a repel-coated Sorvall SS34 tube on ice. Next, Brij 58 was added to 0.5% and samples vortexed briefly to mix contents. Finally, 1 ml of packing cells (BW40 bacteria treated with 500Jm^{-2} of UV light at a density of 2x10^{8} cells per ml in phosphate buffer containing 100μgml^{-1} of thymidine) was added to encourage pellet formation, and samples washed by four cycles of centrifugation (Sorvall SS34 rotor, 10,000 rpm, 3 min, 0°C) and resuspension in phosphate buffer containing 100 μgml^{-1} thymidine. Each final sample (2.5m) was divided into 0.5 ml aliquots and TCA-precipitable radioactive material, representing DNA transferred to T6^{R} recipients, determined as in Section 2.3.

2.13 DNA-DNA reassociation

DNA-DNA reassociation experiments were performed by a method essentially similar to that described by Barth and Grinter (1975). The preparation of DNA species for use in these experiments is described in Sections 2.6 and 2.7, and details of individual experiments in Chapter 5. Salmon sperm DNA was purchased from Sigma Chemical Co.

2.13.1 Preparation of hybridisation mixtures

DNA was sonicated using an MSE 150W ultrasonic disintegrator operating at maximum output (setting 1; amplitude 18 to 20 μm) with a 3 mm diameter probe to generate fragments of between 0.3 and 0.8 Kb. Samples were kept on ice throughout the procedure in 1.5 ml Eppendorf tubes and given twelve 15 sec bursts of sonication with 15 sec intervals for cooling. The amounts and ratios of DNA species included in hybridisation mixtures for each experiment varied (see Chapter 5), but normally either 2.5 or 20 mg of radioactivity-labelled probe DNA was mixed with a thousand-fold excess of unlabelled DNA. Mixtures (0.1 ml) contained NaCl at a final concentration of 0.42M, and were overlaid with 3 drops of paraffin oil in 1.5 ml Eppendorf tubes.

Hybridisation mixtures were heated to 105°C for 10 min in a bath of PEG 400 to denature DNA, and then shifted to another PEG 400 bath at 75°C for 17h to allow reassociation of samples. Cot values for reassociation reactions are explained and defined in Chapter 5. Assays were performed in triplicate.

2.13.2 Analysis of reassociated DNA

Following reassociation of DNA samples, 0.9 ml prewarmed reaction mixture was added to samples at 75°C to give final concentrations of 0.168M NaCl, 30mM sodium acetate pH4.8, 1mM $ZnSO_4$ and 20$\mu g ml^{-1}$ sheared denatured calf thymus DNA (Sigma). S1 nuclease (Bethesda Research Labs.) was diluted as required in storage buffer (20mM Tris pH7.5, 50mM NaCl, 0.1mM $ZnCl_2$ and 50% glycerol) and 150 units (5 µl) added to reaction mixtures. Incubation at 75°C was continued for 20 min, and reactions halted by addition of 0.2 ml 2M TCA and immersion in an iced water bath. After 1h, samples were washed through filters with boiling water. Filters were dried and transferred to scintillation vials containing 5 ml of Fiso-fluor 1 non-aqueous scintillation fluid (Fisons). Radioactivity was determined as in previous sections.

Controls for the S1 assay, identical in composition to hybridisation mixtures, were either denatured or left as double-stranded material, and similarly assayed by the S1 nuclease method. The number of input counts was determined by processing duplex DNA samples to which S1 nuclease was not added.

CHAPTER 3

Construction and characterisation of ColIb-P9drd-1 plasmids mutant in sog.

3.1 Introduction

As stated in the introductory chapter, synthesis of the complementary strand of ColIb-P9 DNA in recipient cells is carried out by host-specified DNA polymerase III holoenzyme (Wilkins and Hollom, 1974), creating a requirement for a primer-generating enzyme in this process. Boulnois and Wilkins (1979) demonstrated that conjugative DNA synthesis of the closely-related Iα plasmid R144drd-3 was independent of the two defined E.coli priming enzymes, dnaG protein and RNA polymerase, leading to the proposal that a plasmid-specified product is responsible for the priming of complementary strand synthesis in recipients. The obvious candidate for this role is the product of the Iα plasmid gene sog, which has been shown to possess DNA primase activity in vitro (Lanka et al., 1979) and to participate in chromosomal DNA replication in dnaG mutants (Wilkins, 1975; Wilkins et al., 1981). Co-ordinate expression of sog with transfer genes further implied a role for this protein in conjugation (Wilkins, 1975).

In order to subject this hypothesis to genetic investigation, it was necessary to obtain Sog^- derivatives of a de-repressed (drd) Iα plasmid, defective in ability to specify DNA primase activity. The Iα plasmid pLG221 (Boulnois, 1981) was chosen for this purpose, and is a derivative of ColIb-P9drd-1 containing Tn5 in the structural gene for colicin Ib-P9 (cib). The advantage of pLG221 is that it does not specify synthesis of colicin, thus avoiding the need to work with colicin-resistant bacteria, and

the Tn*5*-specified kanamycin resistance provides a convenient genetic marker.

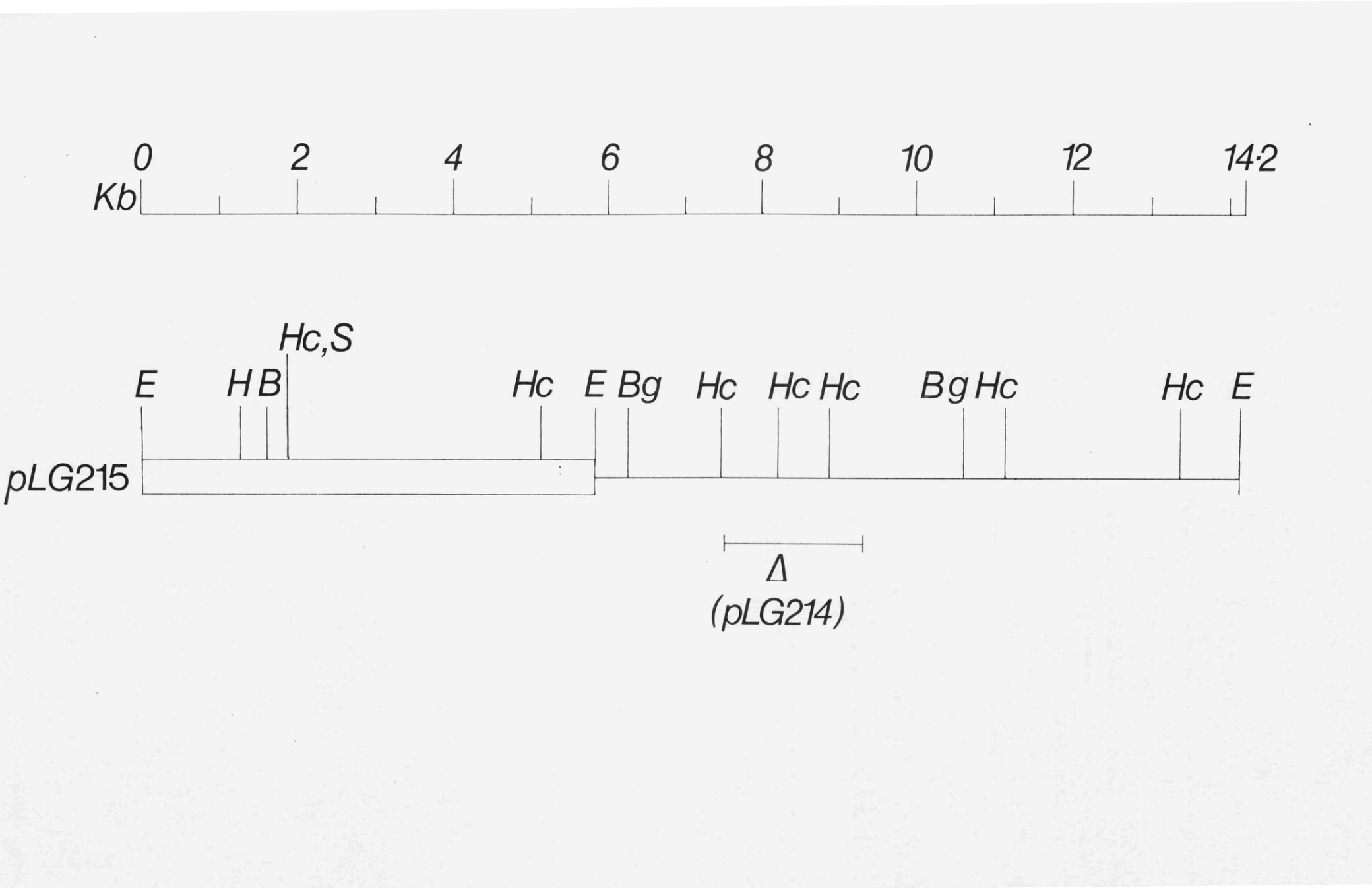
This study has also made use of a number of recombinant plasmids carrying *sog* and various derivatives of these. A 7.9 kb *Eco*RI fragment of ColIb-P9*drd-1* DNA had previously been ligated (Wilkins *et al.*, 1981) into the unique *Eco*RI site in the chloramphenicol acetyltransferase gene of the vector plasmid pBR325 (Bolivar, 1978) and shown partially to suppress the temperature-sensitive phenotype of *dnaG3* mutants and to specify DNA primase activity (Wilkins *et al.*, 1981). Ligation of *Eco*RI-generated fragments of ColIb-P9*drd-1* DNA into pBR325 yielded the Sog^+ recombinant plasmid pLG211, which was found to contain two *Eco*RI fragments of ColIb-P9*drd-1* DNA; one minor fragment of 0.27 Kb and a 7.9 Kb fragment specifying DNA primase activity. Removal of the smaller fragment by *Eco*RI cleavage and religation generated the Sog^+ recombinant plasmid pLG215 (Wilkins *et al.*, 1981). This plasmid is described in Figure 3-1.

Boulnois *et al.* (1982) had also isolated a number of hydroxylamine-induced Sog^- derivatives of pLG211 defective in the gene for plasmid DNA primase. Two of these plasmids, pLG217 and pLG220 each apparently contain at least a single point mutation in their cloned *sog* genes. SDS polyacrylamide gel electrophoresis of the products synthesised from these plasmids in sup^o minicells, coupled with a screen for amber mutations by lysogenisation with a *supF* lambdoid recombinant phage, showed that these lesions are neither extensive deletions nor amber mutations (Boulnois *et al.*, 1982). Thus pLG217 and pLG220 apparently possess amino acid-substituting mutations in *sog*.

Given the availability of these plasmids and the absence of either a detailed physical map of ColIb-P9 or a library of characterised transfer

Figure 3-1 Restriction endonuclease map of pLG215

The figure describes the positions of sites for the restriction enzymes BamHI (B), BglII (Bg), EcoRI (E), HindIII (H), HincII (Hc) and SalI (S) on the Sog^+ recombinant plasmid pLG215, based on the data of Boulnois et al. (1982). The plasmid was generated by the ligation of a cloned EcoRI fragment of ColIb-P9 DNA specifying sog into the vector plasmid pBR325 (Wilkins et al., 1981). pBR325 DNA is indicated by the double lines at the left hand side of the map. The extent of the deletion in sog specified by pLG214 is denoted below the restriction map.


0
2
4
6
8
10
12
14·2
Kb
Hc,S
E
H B
Hc
E Bg
Hc
Hc Hc
Bg Hc
Hc
E
pLG215
Δ
(pLG214)

mutants, it was decided to obtain Sog$^-$ derivatives of pLG221 by *in vivo* recombination with the mutant *sog* genes of pLG217 and pLG220, rather than by direct mutagenesis of pLG221 itself. Such a procedure not only offers the advantage of using well-characterised mutations, but it avoids both the possibilities of polar effects on neighbouring genes and of mutagenesis of other plasmid genes that could be incurred by the use of transposon insertions or chemical mutagens such as nitrosoguanidine. As will be described in the remainder of this chapter, this approach yielded two derivatives of pLG221 carrying the respective mutations *sog-217* and *sog-220*, each defective in specifying an active plasmid DNA primase.

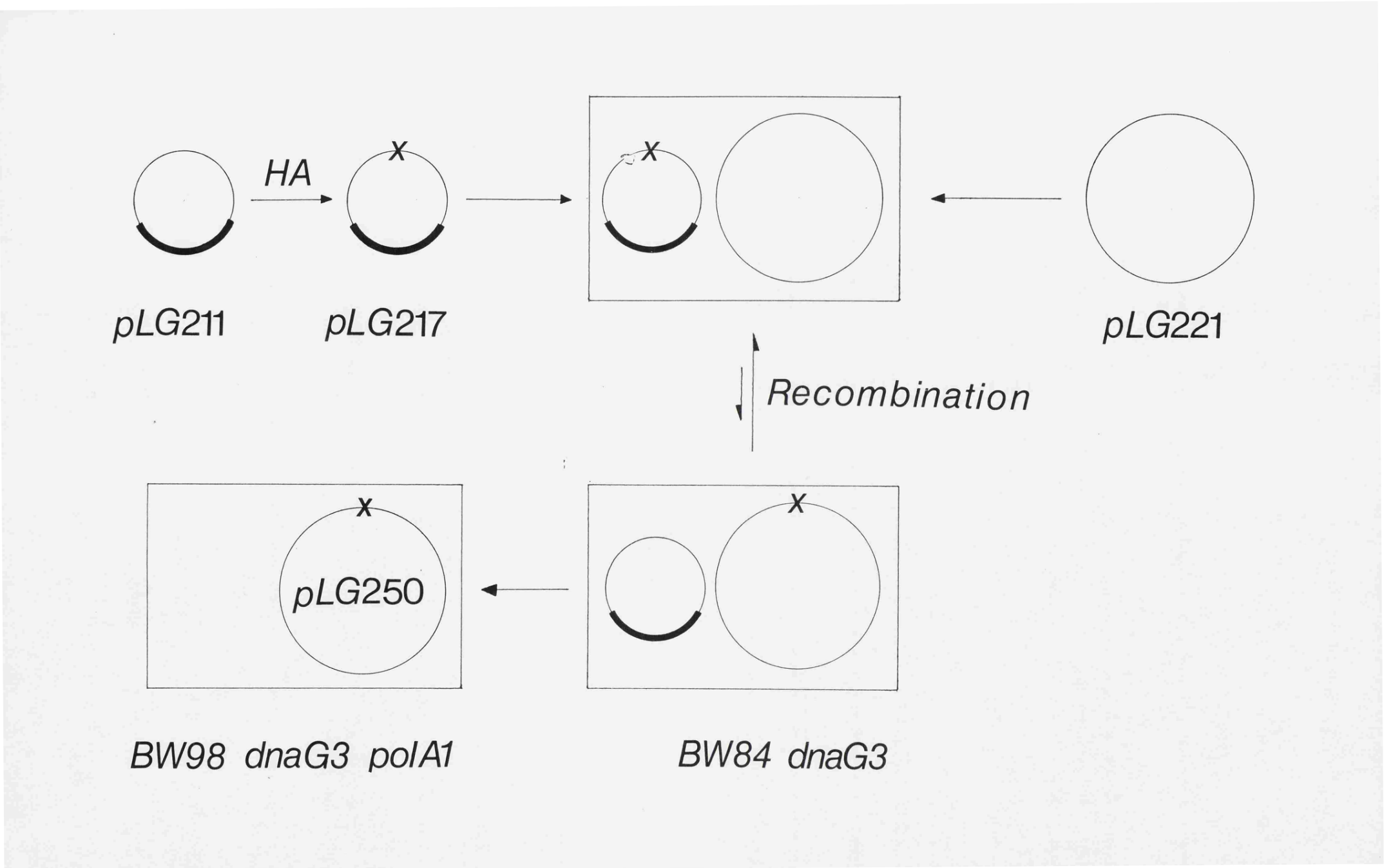
3.2 Isolation of pLG221 derivatives mutant at sog

The strategy followed for recombining the *sog-217* and *sog-220* mutations into the Iα plasmid (pLG221) from the pBR325-derivatives (pLG217 and pLG220) is explained diagramatically in Figure 3-2. BW84 was used as the host strain for the *in vivo* recombination since it is *rec*$^+$ and derivatives harbouring pLG217 and pLG220 were available. Thus, pLG221 was transferred by conjugation from the donor strain W3110(pLG221) into the two recipient strains BW84(pLG217) and BW84(pLG220) generating transconjugants at the high frequency of approximately 5×10^7 per ml of mating mixture. Since the 7.9 Kb *Eco*RI fragments of pLG217 and pLG220 containing the *sog* locus will share almost complete homology with the corresponding region of pLG221, recombination events can be expected to occur between the respective pairs of *sog* alleles present in BW84 (pLG221, pLG217) and BW84 (pLG221, pLG220) such that the lesions *sog-217* and *sog-220* are introduced into the *sog* gene of pLG221.

The detection of such Sog$^-$ recombinants is made simpler by the inability of *sog-217* and *sog-220* to suppress the temperature-sensitivity of *dnaG3*

Figure 3-2 Construction of the Sog$^-$ Iα plasmid pLG250

The rationale behind the scheme presented opposite is described in detail in section 3.2. DNA of the Sog$^+$ recombinant plasmid pLG211 was mutagenised *in vitro* with hydroxylamine (HA) to generate the Sog$^-$ plasmid pLG217. BW84 was transformed with pLG217 and the Iα plasmid pLG221 was introduced into the resultant strain by conjugation. Recombination between the homologous regions of these two plasmids resulted in the formation of Sog$^-$ derivatives of pLG221. Mating BW86 (pLG221, pLG217) bacteria with the *dnaG3*, *polA1* strain BW98, and replica plating of transconjugants to high temperature resulted in the isolation of the Sog$^-$ Iα plasmid pLG250. An identical scheme, using the Sog$^-$ recombinant plasmid pLG220 in place of pLG217, yielded pLG251.


HA
pLG211
pLG217
pLG221
Recombination
pLG250
BW98 dnaG3 polA1
BW84 dnaG3

bacteria (Boulnois *et al.*, 1982; see also Table 3-1), so that *sog* mutants of pLG221 should fail to allow growth of such a host at 40°C. This deficiency can be screened for by replica plating colonies and incubating replicas at high temperature, but in order to perform this test, it was first necessary to obtain segregation of pLG221 from the pBR325-derived plasmids, pLG217 and pLG220. This was achieved by mating BW84 (pLG221, pLG217) and BW84 (pLG221, pLG220) strains with the *nal dnaG3 polA1* recipient strain, BW98, at 30°C and selecting for Kan^R Nal^R transconjugants. The strain BW98 was obtained from BW61 (*dnaG3 polA1*; Wilkins, 1975) by selection for resistance to naladixic acid (*nal*). Since pBR325 is a non-mobilisable vector plasmid (Bolivar, 1978), pLG217 and pLG220 should not be inherited by recipients unless integration into pLG221 has occurred to create a co-integrate molecule. This rare event was detected in equivalent matings involving pLG221 and other pBR325-based recombinant plasmids carrying *EcoR1*-generated fragments of ColIb-P9*drd-1* DNA from rec^+ donors (see Chapter 7) at the low frequency of approximately $1x10^{-4}$ per pLG221 transconjugant. This event is *recA*-dependent, since matings using the same plasmids in a *recA* donor strain yielded no transconjugants containing the pBR325-based plasmid (see Chapter 7). However, should such a recombination event result in the transfer of either pLG217 or pLG220 to BW98, these plasmids should not be maintained in this *polA1* host, since the vector pBR325 is derived from a ColE1-derived plasmid (Bolivar, 1978) and so requires the *polA* gene product, DNA polymerase I, for replication (Staudenbauer, 1978).

This approach was validated by assaying transconjugants from matings of BW84 donor cells containing pLG221 and a pBR325-based recombinant plasmid with either BW98 (*nal polA1*) or BW97 (*nal* $polA^+$) recipients. In the matings with BW97, Tc^R transconjugants harbouring the pBR325-based recombinant

were identified at a frequency of about $4x10^{-6}$ per Kan^R transconjugant. Despite the reported 1% residual DNA polymerase I activity of polA1 mutants (DeLucia and Cairns, 1969; Lehman and Chien, 1973), not a single Tc^R transconjugant of BW98 was obtained in these matings, so that it appears that the combined effects of the recipients polA1 mutation and the selection for segregation of pLG221 by conjugation are sufficient to isolate pLG217- or pLG220-free cells.

Kan^R Nal^R transconjugants, isolated from matings at a frequency of about $5x10^6$ cells per ml of mating mixture, were grown at 30°C to yield small colonies and then replica plated to 40°C. Most of these replicas grew at the higher temperature due to suppression of the dnaG3 mutation of BW98 by pLG221, but temperature-sensitive clones were detected at a frequency of about $2x10^{-4}$. A number of such clones, from separate matings involving either pLG217 or pLG220, were isolated and purified. Two of these clones were chosen as representative of Sog^- derivatives of pLG221 as a result of genetic exchange with either pLG217 or pLG220, and respectively designated as pLG250 (sog-217) and pLG251 (sog-220).

3.3 Characterisation of pLG250 and pLG251

The ability of dnaG3 strains harbouring various plasmids to form colonies and make DNA at restrictive temperature were determined as described in Chapter 2, and values are given in Table 3-1. Plasmid DNA primase activity in crude cell extracts of these strains was also determined (Table 3-1) using an in vitro system to assay the ability of these extracts to initiate DNA synthesis on single-stranded phage M13 DNA. It is important to note that the vector plasmid pBR325 does not specify DNA primase activity. By all three criteria, the Iα plasmids pLG250 and pLG251 are judged to be

Table 3-1 DNA primase properties of plasmids

Host strains and definition of units are given in section 2.10. Further details of data and of plasmids used in this table are to be found in sections 3.2 and 3.3 of this chapter.

Table 3-1 Properties of plasmids

PLASMID IN HOST	DESCRIPTION OF PLASMID	SUPPRESSION OF *dnaG3* MUTATION		
		COLONY FORMATION	DNA SYNTHESIS (41°C)	PLASMID PRIMASE
		40°/30°C	cpm ml^{-1}/A_{450}	mU mg^{-1} CCE
pLG214	pBR325 Ω [EcoR1 7.9 kb, Δ1.5 kb, *sog*$^+$]	1.0	363	234
pLG215	pBR325 Ω [EcoR1 7.9 kb, *sog*$^+$]	5.8×10^{-4}	220	109
pLG217	pBR325 Ω [EcoR1 7.9 kb, 0.27 kb, *sog-217*]	$< 10^{-7}$	11	1
pLG220	pBR325 Ω [EcoR1 7.9 kb, 0.27 kb, *sog-220*]	$< 10^{-7}$	11	1
pLG221	ColIb-P9*drd1* *cib*::Tn5 *sog*$^+$	5.0×10^{-1}	176	117
pLG250	pLG221 *sog-217*	$< 10^{-7}$	10	1
pLG251	pLG221 *sog-220*	$< 10^{-7}$	9	1
pLG250, pLG214		1.1	392	256
pLG250, pLG215		3.3×10^{-2}	194	109
pLG250, pLG217		$< 10^{-7}$	8	ND
pLG251, pLG220		$< 10^{-7}$	12	ND
pLG250, pLG220		1.2×10^{-4}	11	ND
pLG251, pLG217		1.6×10^{-3}	11	ND
pBR325	Ap^r Tc^r Cm^r	< 10-7	9	1

defective in sog. The validity of these measurements is supported by the equivalent values obtained in these experiments with the defined Sog^- plasmids pLG217 and pLG220 and by the positive values obtained with pLG221 and pLG215. It should be noted that the Sog^+ recombinant plasmids pLG211 and pLG215 gave approximately equal values for both colony formation and DNA synthesis measurements; thus these two plasmids can be regarded as equivalent in Table 3-1.

The Sog^+ recombinant plasmid pLG214, which will be referred to throughout the remainder of this thesis, is introduced at this point. This plasmid (Wilkins et al., 1981) was derived from the recloning experiment which generated pLG215 from pLG211. The Sog^+ EcoR1-generated fragment of ColIb-P9drd-1 carried by pLG214 has undergone an internal deletion of about 1.5 Kb, mapping within the coding region of sog (Figure 3-1), and results in the production of truncated sog polypeptides of MWs 87,000 and 42,000 (Wilkins et al., 1981). This deletion, rather than impairing primase activity, results in a more efficient rescue of chromosomal DNA synthesis in dnaG cells.

Complementation studies involving the pairing of pLG250 or pLG251 with various recombinant plasmids in the same, dnaG3 strain (Table 3-1) confirm that pLG250 and pLG251 respectively possess the sog-217 and sog-220 lesions carried by the pBR325-derived plasmids used in their genesis. Either of the Sog^+ recombinant plasmids, pLG215 or pLG214, are capable of complementing the Sog^- phenotype of these plasmids, whereas pairing of the homologous mutants pLG217 and pLG250 or pLG220 and pLG251 failed to result in any detectable suppression of the host dnaG3 mutation. However, formation of colonies at 40°C at an intermediate frequency was observed when either pLG250 was partnered with pLG220, or pLG251 with pLG217. This observation implies the formation of Sog^+ recombinants as a result of intragenic recombination

and indicate that the sog-217 and sog-220 lesions map at different positions within the gene. As the frequency of such recombinants did not exceed 10^{-3}, their presence was not sufficient to be detected by measurement of DNA synthesis at high temperature.

In order to characterise the lesions determined by pLG250 and pLG251, it was decided to screen cells containing these plasmids for spontaneous reversion to Sog^+. However, the only practical way of screening large numbers of bacteria for this event is by selection for suppression of the temperature-sensitivity of a dnaG3 host, and this may be occluded by reversion of the dnaG mutation. It was decided to make use of the transfer properties of pLG250 and pLG251 to distinguish between these two classes of revertant. Strains of BW98 (dnaG3) containing either pLG250 or pLG251 were plated out at 40°C and temperature-resistant colonies, arising at a frequency of about 10^{-8}, were subsequently mated at 30°C with the dnaG3 strain BW82. Transconjugants were tested for ability to form colonies at 40°C and where this occurred with high efficiency, it was judged that reversion of the sog mutation in the plasmid had occurred. Sog^+ revertants of pLG251 were detected at a frequency of about 10^{-9}, so that this plasmid probably has a single point mutation, but the procedure failed to discern similar revertants of pLG250. It therefore appears that the sog-217 lesion may affect more than one base pair.

3.4 Discussion

The in vivo recombination of the defined mutations sog-217 and sog-220 from a cloned sog gene into the Iα plasmid pLG221 represents a potentially valuable general strategy for the isolation of plasmids mutant at specific loci, since the approach avoids any requirement for genetic mapping information. The method successfully yielded the primase-defective Iα

plasmids pLG250 and pLG251. Each was shown to carry the lesion specified by the appropriate pBR325-*sog* recombinant plasmid used in their construction. Since *sog* is common to each of the Iα plasmids ColIb-P9, R144 and R64 (Dalrymple *et al.*, 1982), the consistent results obtained with pLG250 and pLG251 in subsequent chapters indicate that the behaviour of these may be taken as representative of a primase-defective Iα plasmid.

The data expressed in Table 3-1 is in agreement with previous results reported by Wilkins *et al.* (1981), and both show that pLG215, although encoding an intact *sog* gene, is less efficient than pLG221 in promoting colony formation of BW86 at 40°C. Heterogeneous colonies are formed by BW86 (pLG215) at a lower frequency than the homogeneous colonies of BW86 (pLG221), suggesting some deficiency in pLG215-mediated *dnaG* suppression. Wilkins *et al.* (1981) have demonstrated that BW86(pLG215) is deficient in accumulating DNA at high restrictive temperature, and proposed that an undefined mutation was required for colony formation by this strain. Analysis of plasmids extracted from independently isolated colonies of BW86 (pLG215) showed that the mutation was not plasmid-located, and since plasmid-free segregants of these strains obtained a fully thermo-resistant phenotype upon transformation with pLG215, the mutation was deduced to be chromosomal (Wilkins *et al.*, 1981). This mutation does not revert the *dnaG3* lesion (Wilkins *et al.*, 1981).

One insight into this deficiency, and perhaps into the mechanism by which plasmid primase acts in chromosomal DNA replication, may be offered by the observation that the complementation of pLG215 by either pLG250 (Table 3-1) or pLG251 (data not shown) result in the formation of colonies similar in heterogeneity to those formed by BW86(pLG215), but at the increased frequency of 3×10^{-2}. This intermediate frequency, 50-fold higher than that of BW86(pLG215), may imply the existence of a co-factor

for plasmid primase functioning in dnaG suppression. The gene for this protein would presumably be located on pLG250, outside the 7.9 Kb EcoR1 cleavage fragment encoding sog, and be a functional analogue of that mutated host gene necessary for efficient colony formation by BW86(pLG215).

The existence of this accessory protein could be investigated once the chromosomal mutation specified by thermo-resistant colonies of BW86(pLG215) is identified. Plasmid-free segregants of these strains might be suitable hosts for the cloning of the iso-functional gene carried by ColIb-P9.

CHAPTER 4

Plasmid DNA primase is involved in conjugative DNA synthesis in donor and recipient bacteria

4.1 Introduction

It was demonstrated by Boulnois and Wilkins (1979) that synthesis of the complementary strand of an IncIα plasmid in recipients was independent of host-specified priming enzymes, a discovery which led these authors to propose that the product of the plasmid-located gene *sog* was the enzyme responsible for this process. The purpose of this chapter is to describe experiments which test this hypothesis by the use of the Sog$^-$ derivatives of the conjugative Iα plasmid pLG221 (pLG250 and pLG251) described in the previous chapter. These plasmids were also used to examine the involvement of plasmid primase in other forms of DNA synthesis.

Measurements of transconjugant formation in matings mediated by pLG221 *sog*$^+$ and pLG250 *sog* between *E.coli* and *S.typhimurium* strains show that pLG250 is only partially defective in conjugation, indicating an involvement, but not an absolute requirement, for plasmid primase in the conjugation process between *dna*$^+$ bacteria. However, using an experimental system designed to measure complementary strand synthesis of plasmid DNA in rifampicin-treated *E.coli dnaG* recipients (Boulnois and Wilkins, 1979), plasmid primase activity is shown to be required for efficient initiation of this conjugative DNA synthesis. The limited transfer proficiency of the Sog$^-$ Iα plasmids is explained by the ability of host-specified *dnaG* protein to substitute partially for defective *sog* product during conjugative DNA metabolism, as shown by experiments using *dna*$^+$ recipients. This

finding is compatible with the notion that DNA primases from different sources are, at least to some extent, interchangeable between different templates for DNA replication (Wilkins et al., 1981) and supports the idea of fundamental similarities between prokaryotic priming mechanisms.

The deficiency of conjugative DNA synthesis of pLG250 can be rectified by the inclusion of a Sog^+ recombinant plasmid, either pLG215 or pLG214, in the donor or recipient cells. Such studies revealed more efficient complementation when the donors contained active plasmid primase, implying roles for this enzyme in both parents during conjugation. Measurements of both transconjugant formation and conjugative DNA synthesis in mated dnaB donors of pLG221 and pLG250 indicate that the active enzyme is required for efficient transfer of pre-existing plasmid strands and for the initiation of plasmid DNA synthesis in the donor to replace the transferred material. Implications of these findings on models for plasmid DNA transfer and its metabolism during conjugation are discussed.

Plasmid primase is not apparently required for vegetative replication of either ColIb-P9 or the DNA of the filamentous bacteriophage PR64FS (Coetzee et al., 1980), although results may also be interpreted as reflecting the lack of an absolute requirement for the enzyme in these forms of DNA replication, by analogy with the interchangeability of plasmid primase with dnaG protein. Thus, the biological significance of plasmid primase activity appears to be limited to conjugative events, in keeping with the classification of this enzyme as a transfer gene product (Wilkins, 1975).

4.2 Plasmid primase and conjugation

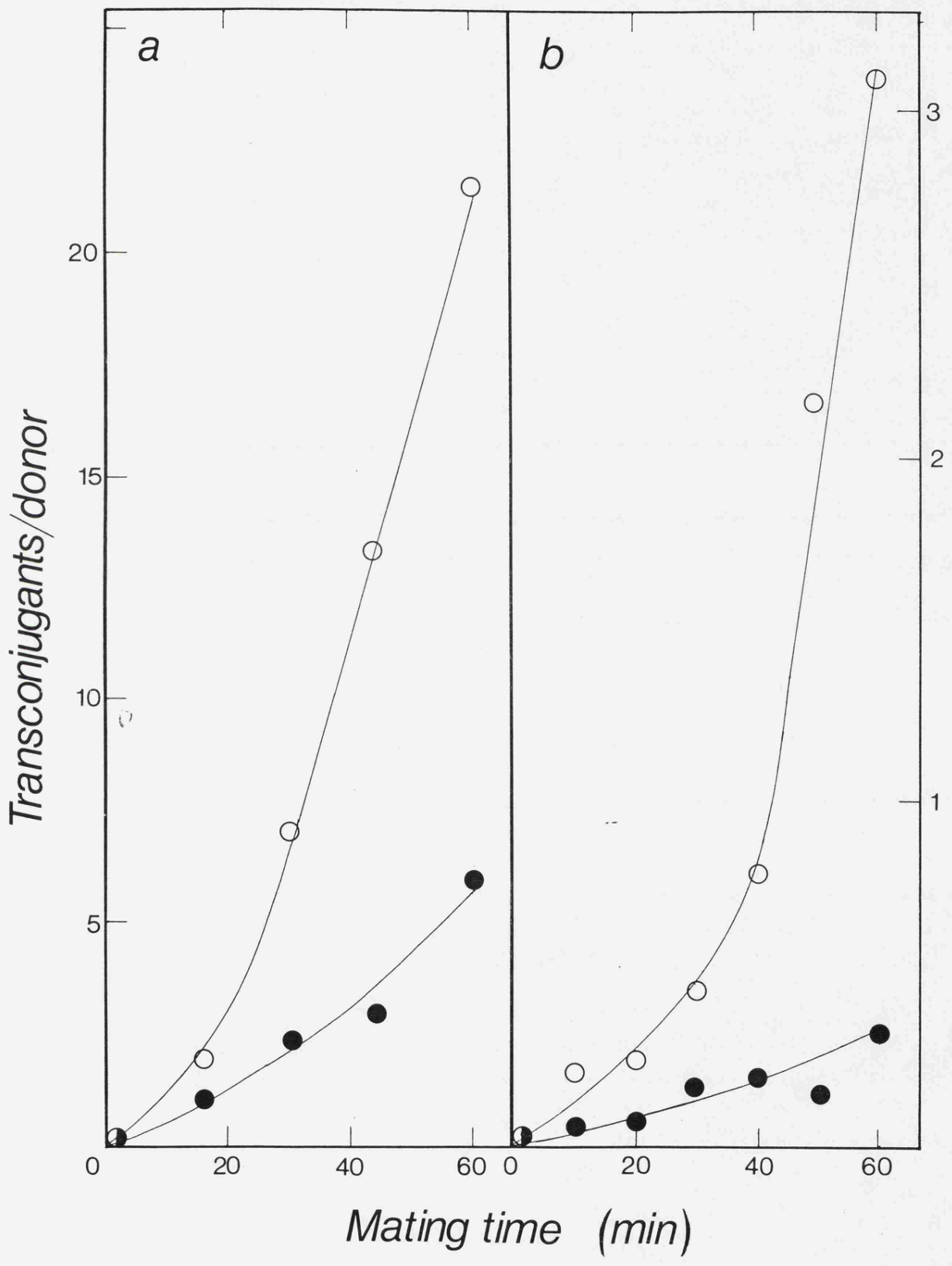
The involvement of plasmid primase in bacterial conjugation was initially investigated by comparison of the numbers of transconjugants formed in 1h broth matings mediated respectively by the Iα plasmids pLG221 and pLG250. Matings were performed using *E.coli* K-12 and *S.typhimurium* LT2 strains, since IncIα group plasmids apparently have a narrow host range, with maintenance only in *Escherichia*, *Salmonella*, *Klebsiella* and *Shigella* hosts (Jacob *et al.*, 1977). For *E.coli* matings, BW96 donors of either pLG221 or pLG250 were mixed with a ten-fold excess of BW97 (*nal*) recipient cells in nutrient broth at 37°C and mated with gentle shaking. Interrupted samples from the mixture were assayed over a 1h period by selection for Kan^r Nal^r transconjugants (Figure 4-1a). Comparison of the two matings indicates that, by this criterion, pLG250 is partially transfer defective, generating only 29% of the number of transconjugants formed in the equivalent Sog^+ mating after one hour. A similar pattern was observed in *S.typhimurium* matings involving ST1 (wild-type) and ST203 (*Δhis*) strains, with selection for Kan^rHis^+ transconjugants carrying pLG221 or pLG250. After 1h, mating cultures containing pLG250 yielded 87% less transconjugants than Sog^+ cultures (Figure 4-1b). The lower overall efficiency of transconjugant formation by *Salmonella* as opposed to *E.coli* cultures is interpreted to reflect the less stable mating aggregates formed between cells of the former species (Sanderson *et al.*, 1981).

Lanka and Barth (1981), using primase-defective *pri* mutants of the IncP group plasmid RP4 generated by Tn*7* insertion, also demonstrated that loss of RP4 DNA primase activity affected the efficiency of conjugation in crosses between *E.coli* donors and certain other bacterial species, including

Figure 4-1 Transconjugant formation in matings mediated by pLG221 and pLG250

The figure shows the number of transconjugants formed per input donor cell during matings using the conjugative plasmids pLG221 (Sog^+; o) and pLG250 (Sog^-; •). Bacterial strains used as donors and recipients of these plasmids were a) Escherichia coli K12 : BW96 and BW97, and b) Salmonella typhimurium : ST203 and ST1. The input numbers of E.coli and S.typhimurium donors were assayed on nutrient agar or minimal media as appropriate containing kanamycin ($50\mu g ml^{-1}$). Matings were performed at $37^{\circ}C$ with gentle agitation in a total volume of 10 ml nutrient broth with a donor:recipient ratio of 1:10. Transconjugants were assayed by the removal of 0.35 ml of mating mixture into 3.15 ml of cold phosphate buffer, quickly followed by mechanical shearing to separate mating cells. Serial dilutions were assayed for a) Kan^R Nal^R or b) Kan^R His^+ transconjugants.

Data are the average of a) 2 and b) 3 experiments.


a
b
Transconjugants/donor
Mating time (min)
0
20
40
60
5
10
15
20
1
2
3

S.typhimurium and Klebsiella aerogenes. These authors demonstrated that this reduction in transconjugant frequency was dependent on the recipient strain used in crosses, and for S.typhimurium recipients the deficiency was up to 95%. This is similar to the 87% reduction observed with pLG250 matings (Figure 4-1b), but while transfer of RP4 Pri^- plasmids between E.coli bacteria is apparently normal (Lanka and Barth, 1981), the yield of E.coli transconjugants in matings with either BW96 (Figure 4-1a) and ST203 donors (data not shown) was reduced by 71% and 88%, respectively, when the IncIα plasmid was primase-negative. The uniformity of the pLG250-associated deficiency in these experiments and the restrictions imposed by the narrow host range of Iα plasmids prevented further genetic attempts to investigate the role of plasmid primase in conjugation, and it was instead decided to concentrate on the physiological events occurring in genetically amenable E.coli recipient cells.

4.3 Plasmid primase and conjugative DNA synthesis in recipient bacteria

The mating system used to examine the effect of sog protein on complementary strand synthesis of plasmid DNA in recipient bacteria during conjugation has been described previously (Boulnois and Wilkins, 1978, 1979). BW96 (tdk, rpoB) donors of pLG221 or pLG250 were mated with BW86 (thyA, dnaG3, Δ(chlA-uvrB)) recipient cells in the presence of rifampicin ($100\mu g ml^{-1}$) and [^{14}C]thymine ($0.5\mu Ci\ \mu g^{-1}$, $2\mu g\ ml^{-1}$) at 41°C. Incorporation of exogeneous label by donor cells is prevented by the absence of thymidine kinase activity. Chromosomal DNA synthesis, which would mask plasmid DNA synthesis in thymine-requiring recipients, is prevented by two separate mechanisms; the high mating temperature of 41°C inactivates temperature-sensitive dnaG protein in recipients, thus preventing discontinuous DNA

replication, and irradiation of cells prior to mating with UV light prevents the usage of native DNA as template. The $400Jm^{-2}$ dose of UV light used in these experiments is calculated to induce approximately 24,000 pyrimidine dimers per E.coli chromosome (data of Rupp and Howard-Flanders, 1968) and is sufficient to prevent the incorporation of exogenous label into the TCA-precipitable material of BW86 cells under these conditions, as shown by the control values for Figure 4-2. Thus, incorporation of thymine by mating cultures predominantly reflects synthesis of plasmid DNA complementary to the transferred strand in recipients.

Rifampicin was added to mating cultures in order to maximise conjugative DNA synthesis, since it has been shown that the antibiotic prevents development of a transfer limitation system, presumably by inhibiting transcription of plasmid genes in the drug-sensitive recipients (Boulnois and Wilkins, 1978). Use of rifampcin -resistant donors therefore results in an amplification of the number of plasmid strands transmitted under these conditions (Boulnois and Wilkins, 1978). It should also be noted that the rifampicin-treated dnaG recipients are deficient in the priming activities of the two defined priming enzymes, RNA polymerase and bacterial primase.

The results depicted in Figure 4-2a show that conjugative DNA synthesis in rifampicin-treated dnaG recipient cells was virtually abolished during matings with the Sog^- Iα plasmid pLG250. It was shown in Chapter 3 that the Sog^+ recombinant plasmids pLG215 and pLG214 were able to complement partially the Sog^- phenotype of pLG250 in temperature-sensitive BW86 bacteria, and the results of Figures 4-2b and c (line c in both diagrams) demonstrate that the presence of either of these recombinants in the parental strains was also able to resolve the synthesis of complementary

Figure 4-2 Conjugative DNA synthesis in UV irradiated, rifampicin-treated recipient cells at 41°C

Growth of bacteria and measurement of conjugative DNA synthesis have been described in section 2.11. Recipient strains were irradiated with $400 Jm^{-2}$ of UV light immediately prior to mating. Matings were carried out at 41°C in the presence of [^{14}C]thymine ($0.5 \mu Ci \mu g^{-1}$, $2 \mu g\ ml^{-1}$) and rifampicin ($100 \mu g ml^{-1}$) for 60 min. Samples (0.5 ml) from mating mixtures were taken into TCA and insoluable radioactivity determined as described in section 2.11. Control mixtures, consisting of parental strains, were incubated separately and treated as for mating cultures. Control values for donor and recipient bacteria incubated separately did not exceed a maximum of about 10^3 cpm ml^{-1} in total, and results are presented after subtraction of these amounts. Data presented is the average of at least three separate experiments.

Strains used (donor x recipient) were:

a) ○ BW96 (pLG221*sog*$^+$) x BW86; line a

● BW96 (pLG250*sog*) x BW86; line b

b) △ BW96 (pLG250*sog*,pLG215*sog*$^+$) x BW86 (pLG215*sog*$^+$); line c

▲ BW96 (pLG250*sog*,pLG215*sog*$^+$) x BW86; line d

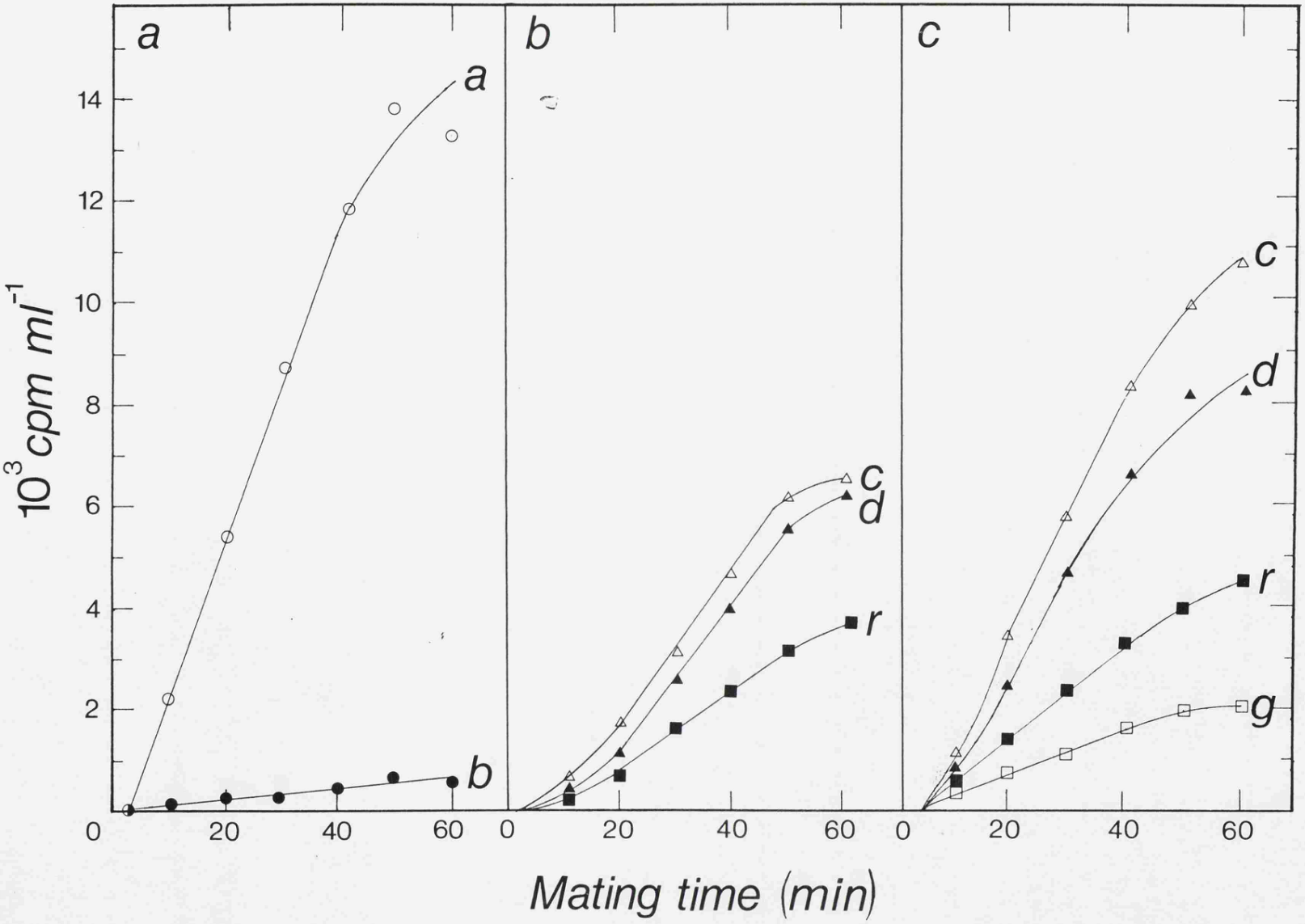
■ BW96 (pLG250*sog*) x BW86 (pLG215); line r

c) △ BW96 (pLG250*sog*,pLG214*sog*$^+$) x BW86 (pLG214*sog*$^+$); line c

▲ BW96 (pLG250*sog*,pLG214*sog*$^+$) x BW86; line d

■ BW96 (pLG250*sog*) x BW86 (pLG214*sog*$^+$); line r

□ BW96 (pLG250*sog*) x BW89 *dna*$^+$; line g


a
b
c
a
b
c
d
r
c
d
r
g
14
12
10
8
6
4
2
0
20
40
60
10^3 cpm ml^{-1}
Mating time (min)

plasmid DNA in the recipient. Complementation apparently relies to a greater extent on the presence of active plasmid primase in the donor cell, since experiments limiting the non-mobilisable Sog^+ recombinant to only one parent of each mating (lines d and r) demonstrate that rescue of conjugative DNA synthesis was most efficient when the plasmid was present in the donor. These results are consistent with the enzyme being supplied by the donor cell in order to promote conjugative DNA synthesis in recipient cells, and also imply another role for plasmid primase in the donor, such as in the synthesis of replacement plasmid strands.

Matings using rifampicin-treated BW89 (dna^+) recipients (Figure 4-2c, line g) indicate that active dnaG primase of recipient cells can initiate weak complementary strand synthesis of pLG250. Since sog can partially complement the dnaG3 lesion carried by BW86, as shown in Chapter 3, it appears that the converse can occur during conjugation. This finding prompts an explanation for the less pronounced effect of sog mutations on transconjugant formation (Figure 4-1) in dna^+ strains compared with measurements of conjugative DNA synthesis in dnaG recipients, since the host-specified DNA primase could substitute for mutant sog product in transconjugants.

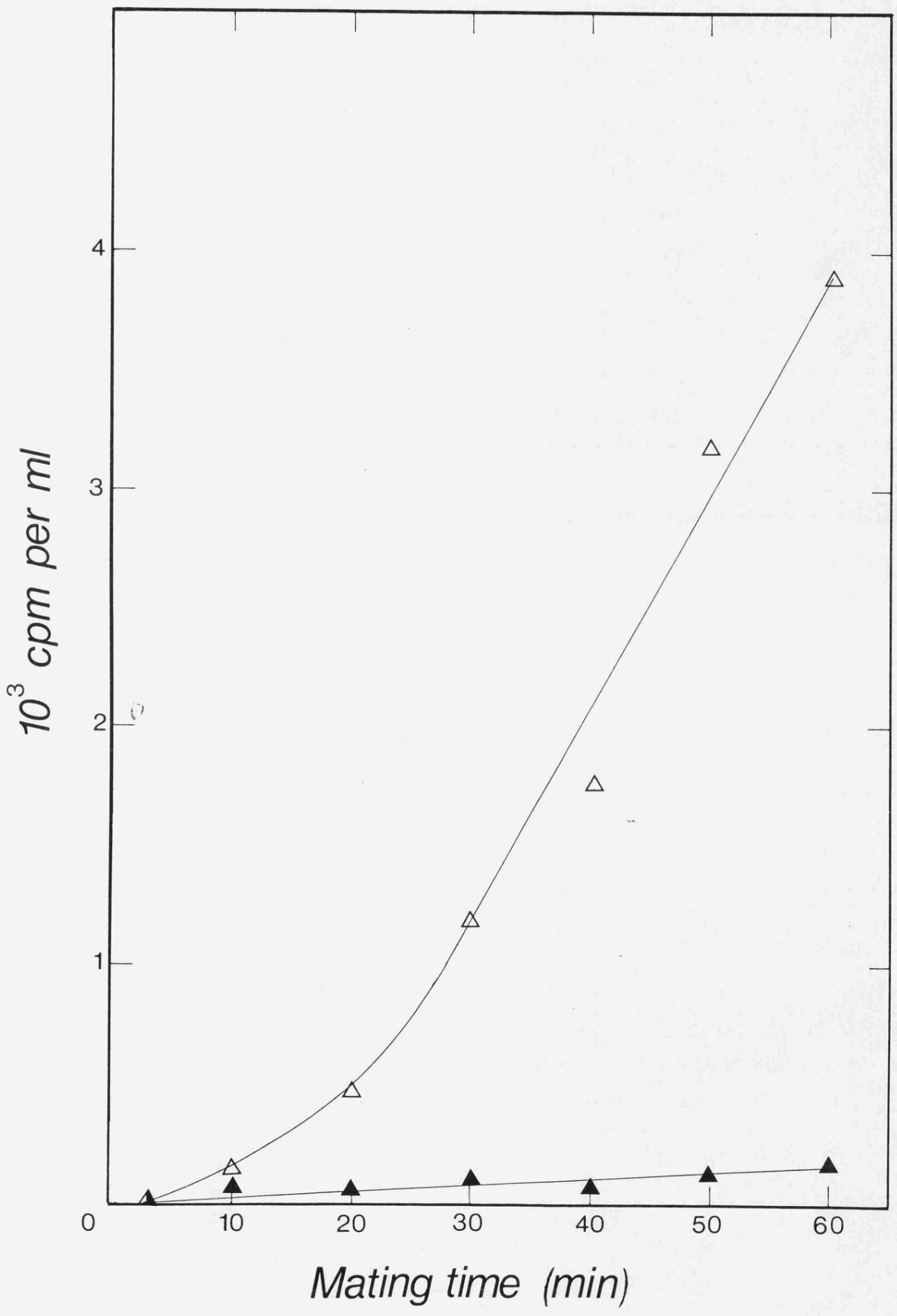
When BW101 (dnaG3, rpoB, tdk) donors were mated with dnaG recipients (Figure 4-3), conjugative DNA synthesis in the recipients was detected provided that the plasmid was Sog^+. This demonstrates that donor specified primase is not required for conjugative DNA metabolism. The lower levels of conjugative DNA synthesis supported by matings using BW101 donors as opposed to BW96 (dna^+) cells are attributed to decreased fertility of rifampicin-resistant dnaG3 donor cells. BW101 was derived from BW82 (dnaG3, tdk) by selection on nutrient agar plates containing $100\mu gml^{-1}$ of

Figure 4-3 Conjugative DNA synthesis in Sog^+ and Sog^- matings between bacteria mutant at *dnaG*

Experimental procedure and mating conditions were as described for Figure 4-2. Parental control values for separately incubated strains did not exceed 200 cpm ml^{-1} each, and results are presented after subtraction of appropriate control values. Mating cultures contained (donor x recipient):

Δ BW101 *dnaG3* (pLG221*sog*$^+$) x BW86 *dnaG3*

▲ BW101 *dnaG3* (pLG250*sog*) x BW86 *dnaG3*


10^3 cpm per ml
0
1
2
3
4
10
20
30
40
50
60
Mating time (min)

rifampicin, and all four clones isolated showed longer mass-doubling times than homogenic strains mutant at only rpoB or dnaG3 (Chapter 6). This impairment of growth rate could account for the low fertility of BW101 donor cells.

Experiments described in this section using pLG250 were repeated with the separately isolated Sog^- plasmid pLG251, and identical results obtained. Thus the data are regarded as representative of the behaviour of a primase-defective Iα conjugative plasmid.

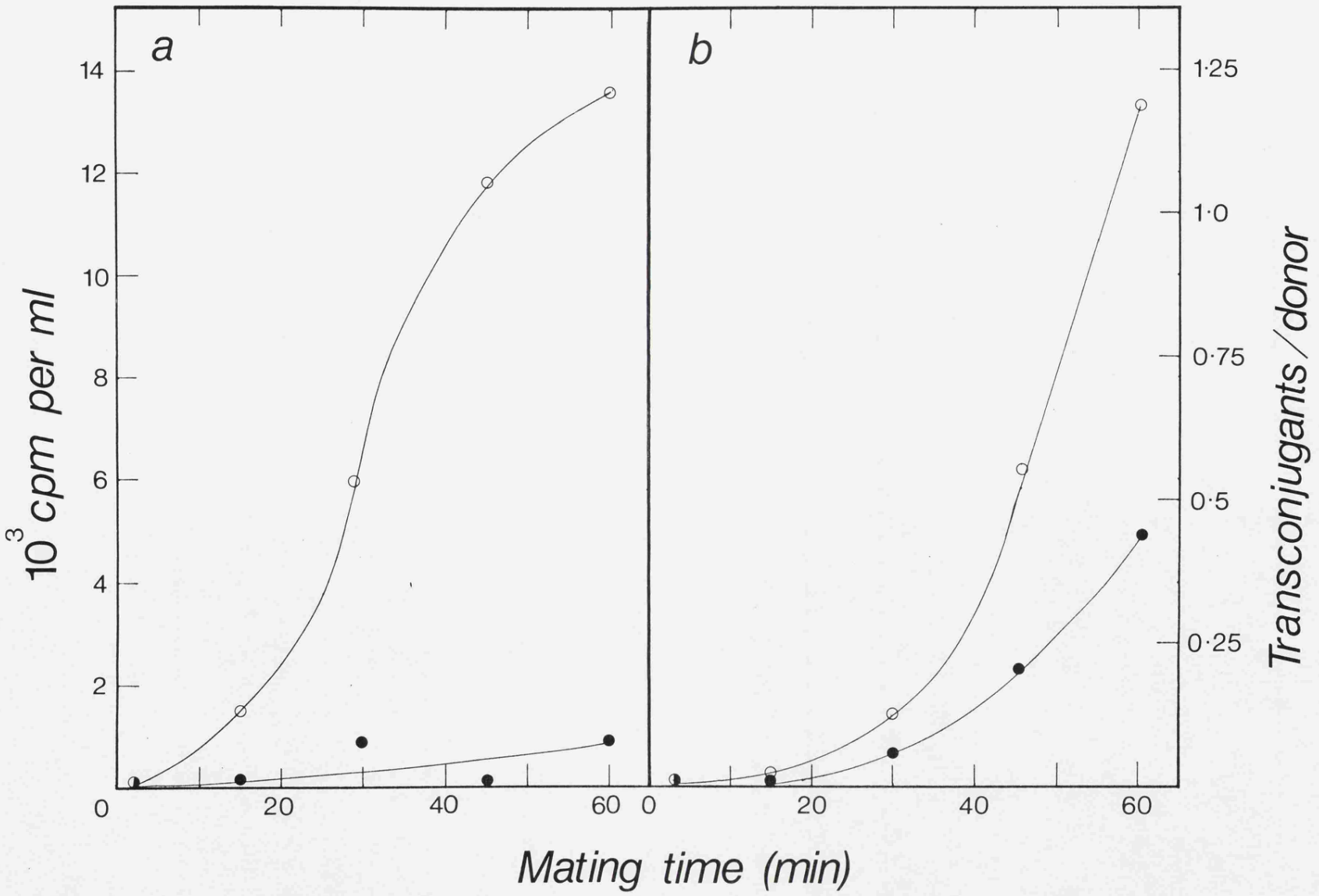
4.4 Plasmid primase and conjugative DNA synthesis in donor bacteria

Measurement of conjugative DNA synthesis in donor, rather than recipient bacteria, is less amenable to investigation since donor cells cannot be irradiated with UV light to prevent chromosomal DNA replication without damaging plasmid DNA. Instead, temperature-sensitive dnaB70 mutants were used as donors to exploit the requirement for dnaB protein in discontinuous bacterial DNA replication. This enzyme is not needed for the conjugative synthesis of plasmid DNA during Iα transfer (Fenwick and Curtiss, 1973a; Wilkins and Hollom, 1974), and so incorporation of [^{3}H]thymidine by BW68 (dnaB70, thyA) donors of pLG221 (Figure 4-4a) is taken to reflect synthesis of DNA to replace the transferred strand. In contrast, a clear deficiency was observed in homogenic matings with pLG250 (Figure 4-4a), and similar results were obtained with BW68 (pLG251) donor cells in equivalent matings. Recipients (BW96) were dna^+ and contained the Sog^+ plasmid pLG214 in order to maximise the initiation of the complementary strand on transferred plasmid. Thus, events in recipients were kept as near to normal as possible.

Figure 4-4 Conjugative DNA synthesis and transconjugant formation during matings with Sog^- (pLG250) donor and Sog^+ (pLG214) recipient strains

a) BW68 *dnaB70* donor cells of pLG221 sog^+ (o) or pLG250 *sog* (●) were mated with BW96 dna^+ (pLG214 sog^+) recipients at 43°C in the presence of $[^3H]$thymidine (2.42 Ci $mmol^{-1}$) and conjugative DNA synthesis in donor cells determined as described in section 2.11.2. Results are presented after correction for the control values derived from the identical incubation and treatment of individual parental strains. Control values increased progressively to about 5,750 cpm ml^{-1} at 60 min. Data are the average of three experiments.

b) Strains and mating conditions were identical to those described above. Transconjugants were assayed by platingserial dilutions of mating mixtures following mechanical interruption of mating cells, and selection for growth at 37°C on nutrient agar plates containing kanamycin (50μgml^{-1}) and tetracycline (7.5μgml^{-1}). The input number of donor cells was determined by assaying samples of the donor culture at the start of mating on nutrient agar plates containing kanamycin.


a
b
14
12
10
8
6
4
2
0
20
40
60
1·25
1·0
0·75
0·5
0·25
10^3 cpm per ml
Transconjugants / donor
Mating time (min)

Matings were repeated under identical conditions (with the single omission of labelled thymidine) in order to evaluate the frequency with which transconjugants were formed in Sog^+ and Sog^- cultures (Figure 4-4b). This was necessary since the deficiency of conjugative DNA synthesis in BW68 (pLG250) donor cells may reflect some involvement of plasmid primase in the transfer of pre-existing strands of plasmid DNA, as well as in the priming of replacement strand synthesis. Since transconjugants were formed in pLG250-mediated matings under these conditions, it appears that plasmid primase is not essential for the transfer of pre-existing DNA. However, an involvement in this process cannot be ruled out, since the yield of transconjugants at one hour was reduced by about 64% when the plasmid was Sog^-. In this regard, it should be pointed out that measurement of transconjugant formation in the Sog^+ mating may overestimate first-round DNA transfer, due to the transmission of replacement plasmid strands synthesised during mating. Taking these points together, it is concluded that the results are consistent with a role for the enzyme in replacement strand synthesis, but no firm conclusion may be drawn concerning its involvement in the transfer of pre-existing DNA strands.

4.5 Plasmid primase and vegetative DNA replication

So far, roles have been assigned to plasmid primase in both conjugative DNA synthesis and in *dnaG*-deficient DNA replication (Wilkins *et al.*, 1981). These findings, together with the wide range of DNA templates which can be used by the enzyme *in vitro* (Lanka *et al.*, 1979), prompted investigation of a role in the vegetative replication of ColIb-P9. Cultures of BW97 containing either pLG221, pLG250 or pLG251 were grown for 20 generations, after which 200 colonies isolated from each culture were tested for the

plasmid-associated kanamycin resistance. Since these plasmids are conjugative, it was necessary to prevent reinfection of possible plasmid-free segregants by conjugation. The pBR325-based recombinant plasmid pLG252 (Tc^R Ap^R Eex^+), which contains a 3.5 Kb EcoR1 fragment of ColIb-P9 drd-1 specifying an active entry exclusion (Eex) system, was introduced into the above strains and maintained by the presence of tetracycline ($7.5\mu gml^{-1}$). This plasmid is described in greater detail in Chapter 7, but it is enough at this stage to know that the plasmid effectively prevents DNA transfer to its host cell. A reconstruction experiment, involving the incubation of a mixed culture of BW97(pLG221, pLG252) and BW97(pLG252), showed that the ratio of Kan^R Tc^R to Kan^S Tc^R bacteria was virtually unchanged after 20 generations.

Analysis of colonies obtained from culturesof BW97(pLG252) also harbouring either pLG221, pLG250 or pLG251 (Table 4-1a) showed that all colonies tested were resistant to kanamycin, implying that the Iα plasmids were stably maintained over 20 generations of growth despite the Sog^- phenotype of pLG250 and pLG251. Thus, no requirement was demonstrated for plasmid primase in the maintenance of Iα plasmids, and vegetative replication of ColIb-P9 presumably relies on host priming enzymes.

The efficiency of plaque formation on BW86(pLG250) and BW86(pLG251) by the I-specific bacteriophage PR64FS (Coetzee et al., 1980) at 30°C did not differ significantly from that obtained with BW86(pLG221), as shown in Table 4-1b. Plaques formed on the Sog^- strains were slightly smaller than those generated on the control strain, so that although an absolute requirement for plasmid primase in PR64FS replication can be ruled out by these results, a minor involvement in this process remains a formal possibility. Since PR64FS adsorbs to the tips of pili specified by Iα plasmids

Table 4-1 Plasmid primase and vegetative DNA replication

a) Plasmid maintenance

Plasmid-containing strains were grown for 20 generations following the addition of 0.1 ml of a 10^{-4} dilution from a pre-culture (A_{600}=0.35) to 10 ml of nutrient broth containing thymine (2µg ml^{-1}) and Tc (7.5µg ml^{-1}), and incubation for 17h in an orbital shaker at 29°C. The input numbers of cells were assayed on nutrient agar plates containing thymine and Tc. After growth, single colonies were isolated by plating serial dilutions on nutrient agar, and 200 colonies of each strain scored for resistance to Km by toothpicking to appropriate media containing or lacking the antibiotic.

b) Propagation of PR64FS

Bacteriophage PR64FS, from a stock containing about 10^{11} p.f.u. ml^{-1}, was titred on the strains indicated in the table. 0.5 ml of an overnight culture was added to 2 ml of nutrient broth and shaken for 2½h at 30°C. 0.1 ml aliquots were mixed with 3.5 ml of soft nutrient agar and poured onto nutrient agar plates to give a bacterial lawn. When dry, 20 µl aliquots of diluted phage suspension were spotted onto the lawn, and allowed to dry before incubation at 30°C overnight.

Table 4-1

a)

Strain	Phenotype of colonies	
	Kan^R	Kan^S
BW97(pLG221*sog*$^+$,pLG252*eex*$^+$)	200	0
BW97(pLG250*sog*,pLG252*eex*$^+$)	200	0
BW97(pLG251*sog*,pLG252*eex*$^+$)	200	0

b)

Strain	Titre of PR64FS (p.f.u. ml^{-1})
BW86(pLG221)	1.45×10^{11}
BW86(pLG250)	1.50×10^{11}
BW86(pLG251)	0.83×10^{11}

(Coetzee et al., 1980; Coetzee et al., 1982), it appears that the mutations in sog carried by pLG250 and pLG251 do not affect the expression of transfer genes required for pilus synthesis.

4.6 Discussion

Use of the Sog$^-$ Iα plasmids pLG250 and pLG251 clearly confirm a role for plasmid primase in the synthesis of ColIb-P9 DNA during conjugation, and as no such requirement was detected in vegetative replication of the plasmid, the enzyme is deduced normally to function only in conjugative events. This conclusion is in keeping with the classification of sog as a transfer gene (Wilkins, 1975). Presumably, therefore, ColIb-P9 replication is initiated by a host-supplied priming enzyme.

Plasmid primase initiates conjugative DNA synthesis in both donor and recipient bacteria. Measurements of complementary strand synthesis in rifampicin-treated dnaG recipient cells (Figure 4-2) directly implicate the enzyme in this process, but the behaviour of dna$^+$ recipients (Figure 4-2c, line (g)) also shows that the dnaG protein of these cells can substitute weakly in this regard. This observation would explain the partial transfer proficiency of pLG250 and pLG251, since both synthesis of the complementary Iα strand and subsequent replication of the plasmid would be carried out by the host dnaG protein.

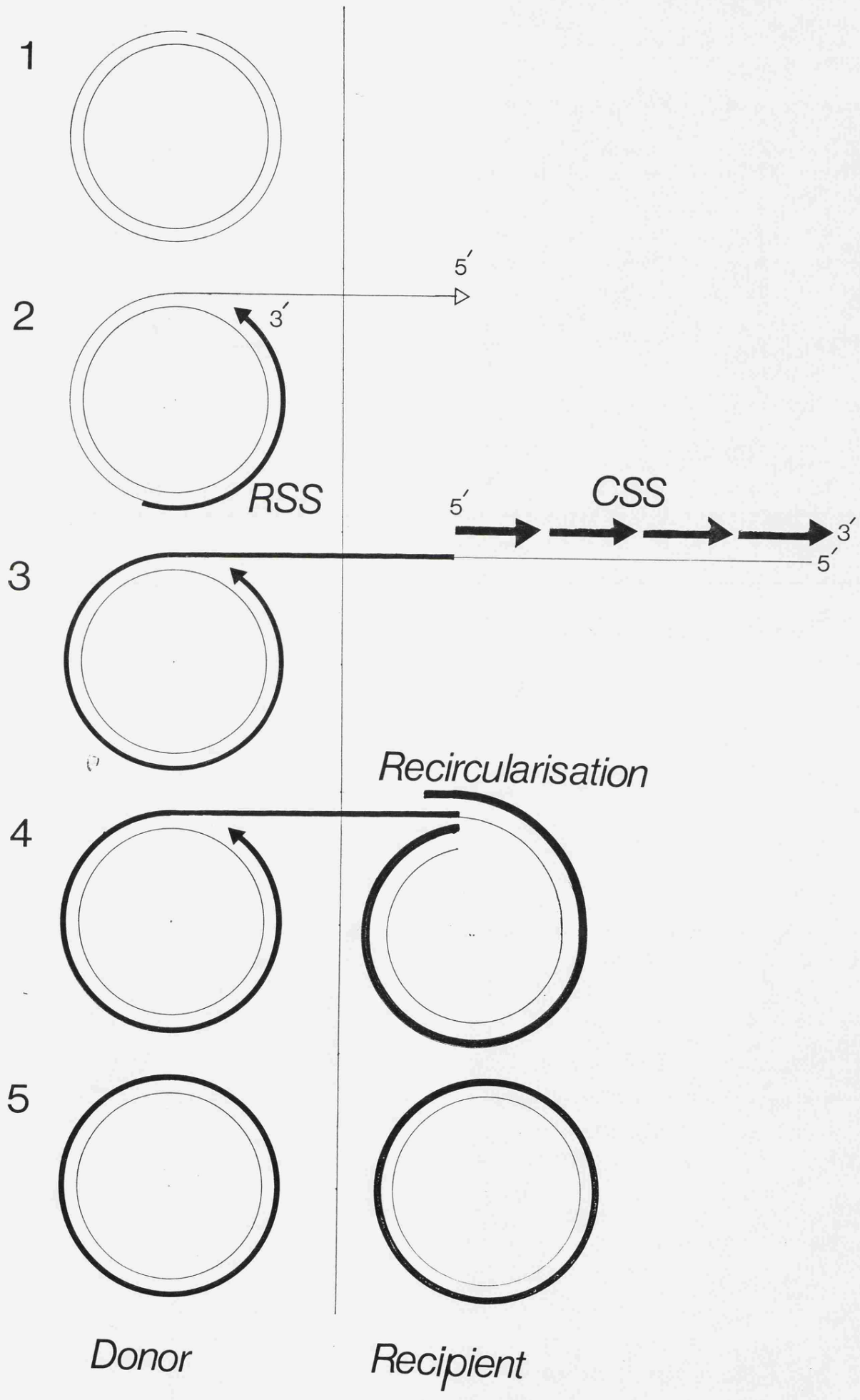
The action of plasmid primase in the donor bacterium is less clear than the corresponding events in the recipient. A role in the donor is implied by the ability of the Sog$^+$ recombinant plasmids pLG214 and pLG215 to complement pLG250 more effectively when present in the donor rather than recipient cell. One likely reason for this is that the enzyme is required in the donor to initiate synthesis of replacement strands of pLG250, so

that only pre-existing DNA strands were transmitted to recipients from Sog^- donors, thus limiting the amount of template for replacement strand synthesis in recipients (Figure 4-2b and c, line (r)). The use of *dnaB* donors of pLG221 and pLG250 (Figure 4-4) clearly shows that the enzyme is not required for the transfer of pre-existing strands of pLG250, since Sog^+ recipients were able to form about 36% of the normal yield of transconjugants. In contrast, conjugative DNA synthesis in the donor cells was reduced by over 95%, so that it appears that plasmid primase is essential for the synthesis of DNA to replace the transferred material. Furthermore, since rifampicin-resistant (*rpoB*) $dnaG^+$ donors were used in these experiments, it appears that, unlike events in the recipient cell, the host priming enzymes cannot substitute for defective *sog* product. Thus, since a unique strand of Iα plasmid DNA is transmitted to recipients (Vapnek *et al.*, 1971), plasmid primase can apparently initiate DNA synthesis on both strands of ColIb-P9 during conjugation.

A requirement for a primer-generating enzyme in replacement strand synthesis contrasts with a central feature of the rolling circle model for DNA transfer proposed by Gilbert and Dressler (1968). This model is depicted and explained in Figure 4-5, and it can be seen that it requires extension of the 3'-OH terminus formed by nicking the strand destined for transfer at *oriT* to initiate replacement strand synthesis. The replacement strand is produced as a multimeric molecule which is displaced from its template strand and transmitted with the 5' terminus leading to recipient cells, where it is resolved into monomeric lengths, presumably by internal recombination after its conversion to double-stranded material. This model is contradicted not only by the requirement for plasmid primase, but also by the requirement for *de novo* protein synthesis for each round of DNA

Figure 4-5 The original rolling circle model

The scheme presented is discussed in section 4.6, and in a modified form of that originally proposed by Gilbert and Dressler (1968). Events occurring in the donor and recipient cells are depicted on the left and right of the vertical line respectively, and the stages in the process of conjugative DNA metabolism predicted by the rolling circle model are as follows: 1) The plasmid strand destined for transfer is nicked at oriT, creating a free 3'-OH terminus. 2) The nicked strand is transferred to the recipient cell with its 5' terminus leading, while the 3'-OH end is elongated by DNA polymerase III holoenzyme to synthesise a replacement strand. 3) Continued replacement strand synthesis (RSS) and DNA transfer result in the transmission of a multimeric strand of DNA which acts as template for complementary strand synthesis (CSS). 4) The multimeric strands are resolved into circularised monomeric molecules by recombination leading to the stable inheritance of the plasmid by both mating cells (5).


1
2
5′
3′
RSS
CSS
5′
3′
5′
3
Recircularisation
4
5
Donor
Recipient

transfer (Fenwick and Curtiss, 1973b), the reported delay of several minutes between successive rounds of transfer (Fenwick and Curtiss, 1973a) and the accumulation of monomeric lengths of plasmid DNA in minicells (Fenwick and Curtiss, 1973a) and in recipients treated to enhance DNA transfer (Boulnois and Wilkins, 1978). Thus, transfer of ColIb-P9 DNA appears to be a discontinuous process, whereby successive monomeric strands are initiated individually by the action of plasmid primase in the donor cell, and transmitted separately to recipients.

The conclusion that plasmid primase is not required for first round transfer of pre-existing Iα plasmid DNA also contradicts models that require that transfer of R64*drd-11* be initiated by RNA synthesis carried out by RNA polymerase (Curtiss and Fenwick, 1975). These models rest on the evidence that DNA transfer from *dnaB* donors was more sensitive to rifampicin than to chloramphenicol at 42°C (Fenwick and Curtiss, 1973a). However, rifampicin did not block first round transfer of ColIb-P9*drd-1* from *dna*$^+$ donors at 37°C (Boulnois *et al.*, 1979) and it was also shown that the drug caused a general disruption of DNA metabolism in cells containing an Iα plasmid which was more pronounced at elevated temperature. Thus, it was proposed that the results of Fenwick and Curtiss reflected disruption of R64 metabolism rather than a specific requirement of RNA synthesis for transfer (Boulnois *et al.*, 1979). The reduction in transconjugant frequency observed when RNA synthesis in *dnaB* donor cells of R64*drd-11* was blocked by cytidine starvation (Maturin and Curtiss, 1981) may be interpreted as evidence for involvement of plasmid primase in replacement strand synthesis, since primers synthesised by this enzyme are initiated with cytidine or cytidine 5'-monophosphate (Lanka and Fürste, 1984). The reported requirement of RNA synthesis for the initiation of first round

transfer of R64drd-11 DNA (Maturin and Curtiss, 1981) contrasts with the results of this chapter using ColIb-P9 drd-1. Clarification of the role (if any) of plasmid primase in this respect requires further understanding of the relationship between DNA transfer and priming events in the donor cell.

In conclusion, roles are assigned to plasmid primase in conjugation for initiating synthesis of replacement strands in the donor bacterium and of complementary plasmid DNA in the recipient. The mechanism by which the enzyme carries out this latter function is the subject of the next two chapters.

Chapter 5

Plasmid DNA primase supplied by donor cells can promote chromosomal DNA synthesis in mated dnaG recipient bacteria

"In order to ascertain the cost of any one luncheon, it must come to the same amount upon two different assumptions'.

Charles Lutwidge Dodgson (Lewis Carroll)

5.1 Introduction

In the previous chapter I defined a physiological role for the DNA primase of ColIb-P9 in recipient bacteria during conjugation. Under the routine conditions of rifampicin treatment used to enhance plasmid transfer, drug-sensitive recipients are presumably unable to transcribe immigrant plasmid DNA, and hence cannot support synthesis of the plasmid-specified enzyme. Furthermore, it was shown that the presence of a non-mobilisable Sog^+ recombinant plasmid in the donor could rectify the deficiency in conjugative DNA synthesis in recipients during matings with the Sog^- plasmids pLG250 and pLG251. One corollary of these observations is that the enzyme must be synthesised and supplied by the donor cell, even though its product of reaction is utilised in the recipient. This property of plasmid primase could be explained if the enzyme were transmitted to recipient cells during conjugation, and it is this possibility which is examined in this and the following chapter.

The approach taken exploited the ability of dnaG recipients to regain the capacity to synthesise chromosomal DNA under conditions that prevent the expression of sog on incoming plasmid DNA. Using this system,

conjugative DNA synthesis in recipient bacteria is significantly greater when the dnaG recipients were left untreated, rather than irradiated with UV light prior to mating. Since UV treatment renders the resident chromosome unavailable as template, DNA synthesis in UV irradiated recipients reflects conjugative synthesis of plasmid DNA (Chapter 4). Additional synthesis detected in equivalent matings involving unirradiated cells is taken to represent use of undamaged chromosomal DNA as template. This is confirmed by analysis of the DNA synthesised in rifampicin-treated dnaG recipients of pLG221 by DNA-DNA reassociation experiments, which demonstrate extensive homology between this conjugatively synthesised DNA and chromosomal DNA of recipient cells.

Recovery of chromosomal DNA synthesis is attributed to the suppression of the host dnaG phenotype by the action of plasmid primase, transferred from donor cells. The possibility that rescue is mediated by mobile, trans-acting primer molecules supplied by the donor cell, is open to criticism. There are two ways in which such primers could operate; they could either be conducted to recipients in the form of RNA-DNA duplexes, presumably at priming sites on the transferred strand of plasmid DNA, or as free RNA molecules. The action of free RNA molecules acting in trans is considered unlikely, since short primers are generally accepted to be generated at their site of origin, and degraded following their extension. Furthermore, the primers synthesised by plasmid primase in vitro are no more than 10 ribonucleotides in length (Lanka and Fürste, 1984) and so would presumably require stabilisation during transfer. Although it is attractive to speculate that the primer-generating enzyme itself participates in this respect, this possibility still requires transfer of the plasmid primase enzyme, which remains a more probable candidate than trans-acting primer molecules for the initiation of chromosomal DNA synthesis in recipients.

5.2 Conjugative DNA synthesis in UV irradiated and unirradiated dnaG recipients of pLG221

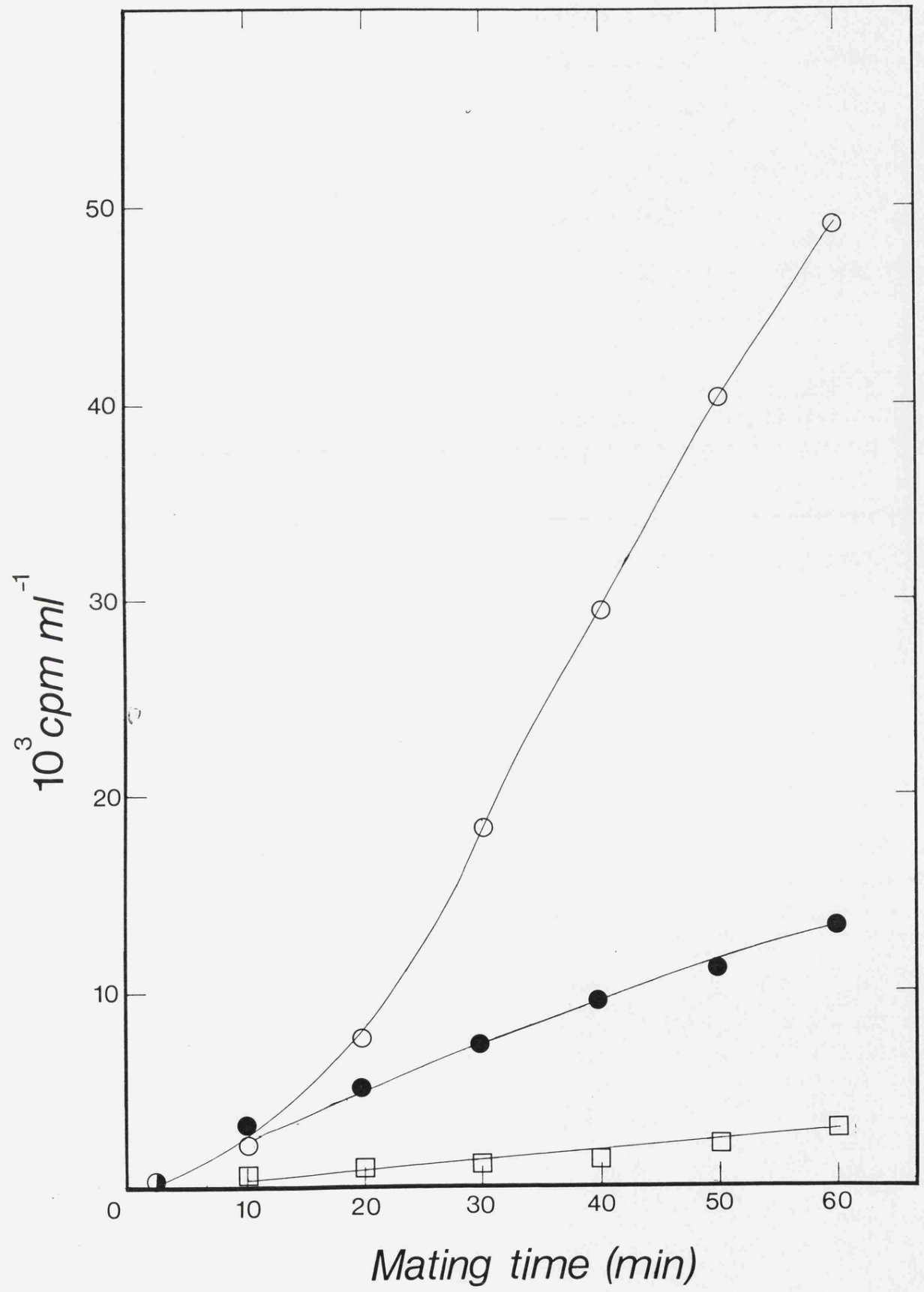
The effect of UV irradiation of *dnaG* recipient cells on conjugative DNA synthesis was determined by mating irradiated and unirradiated bacteria with rifampicin-resistant (*rpoB*), *tdk* donors of the ColIb-P9*drd-1* derivative pLG221. The rifampicin-sensitive, UV sensitive (*Δ(chlA-uvrB)*), *dnaG3* recipient bacteria were either irradiated with UV light prior to the start of mating, or left untreated, and then mated at 41°C in the presence of rifampicin to maximise plasmid DNA transfer (Boulnois and Wilkins, 1978). Results obtained are depicted in Figure 5-1.

Controls of unmated bacteria, incubated separately under the same conditions, gave no more than 400 cpm ml^{-1}, with the exception of the unirradiated *dnaG* recipient (Fig. 5-1) which progressively incorporated up to approximately 1500 cpm ml^{-1} after 1 hour of incubation. Since both bacterial primase and RNA polymerase were inactivated in these cells under the conditions used (Boulnois and Wilkins, 1979), this residual DNA synthesis presumably reflects weak leading strand synthesis at pre-existing replication forks on the host chromosome, which is prevented in the corresponding irradiated control by the presence of UV-induced pyrimidine dimers. The dose of UV light given to these cells is sufficient to render the chromosomal DNA unsuitable as a template for DNA synthesis. However, since the residual DNA synthesis detected in unirradiated BW86 amounts to only some 3% of the radioactivity incorporated in its mating with BW96 (pLG221), the effect of this DNA synthesis is insignificant to the conclusions drawn from these experiments.

It is clear from Figure 5-1 that, after 60 min of mating, approximately 3.7 times more [^{14}C]thymine was incorporated into TCA-precipitable

Figure 5-1 : Effect of UV irradiation on conjugation-dependent DNA synthesis in dnaG3 recipient bacteria

BW96 (pLG221) was mated with rifampicin-sensitive BW86 recipients at 41°C in the presence of [^{14}C]thymine (0.5 μCi μg^{-1}) and rifampicin (100 $\mu g ml^{-1}$). Recipient bacteria were either irradiated with 400Jm^{-2} of UV light immediately prior to mating (•) or left untreated (o). Samples (0.5 ml) were taken into TCA, and insoluble radioactivity determined. Control samples of donors and irradiated recipients incubated separately each incorporated about 250 and 400 cpm ml^{-1} respectively after 1h of mating. Counts incorporated by the unirradiated control culture of BW86 are shown (□). Data presented for the mating mixtures have been corrected by subtraction of the control values.


50
40
30
20
10
0
10
20
30
40
50
60
10^3 cpm ml^{-1}
Mating time (min)

material in matings involving unirradiated recipients (48,000 cpm ml^{-1}) than UV irradiated cells (13,000 cpm ml^{-1}). This latter amount of conjugative DNA synthesis is in accordance with previous results using UV treated cells (Chapter 4; Figure 4-2), and reflects complementary strand synthesis of pLG221 DNA in the recipients of the matings. It is reasonable, therefore, to assume that at least one fourth of the [^{14}C]thymine incorporated into the unirradiated recipient cells is due to complementary strand synthesis of plasmid DNA, and the obvious candidate as template for the remainder of this DNA synthesis is the undamaged recipient chromosome.

However, when comparing amounts of conjugative synthesis of plasmid DNA in UV irradiated and unirradiated recipient bacteria, it is necessary to allow for any reduction in the efficiency of DNA transfer to recipients as a result of UV induced damage. Therefore, before an accurate estimation of the relative amounts of plasmid and chromosomal DNA synthesis in unirradiated recipients could be derived using the data in Figure 5-1, the effect of UV irradiation on the competence of bacteria to act as recipients in such matings was investigated.

5.3 Effect of UV irradiation of recipient bacteria on plasmid DNA transfer

Although UV irradiation of bacterial cells renders the resident DNA unsuitable as a template for either DNA or RNA synthesis, it has been shown that the cells retain the abilities to act as recipients in conjugation (Freifelder and Freifelder, 1968; Falkow et al., 1971; Wilkins and Hollom, 1974) and subsequently as donors (Falkow et al., 1971), and also to synthesise RNA and proteins from undamaged DNA templates introduced after irradiation (Hendrix, 1971). However, it has also been reported (Boulnois, 1980) that a dose of 400 Jm^{-2} of UV reduced by about 50% the

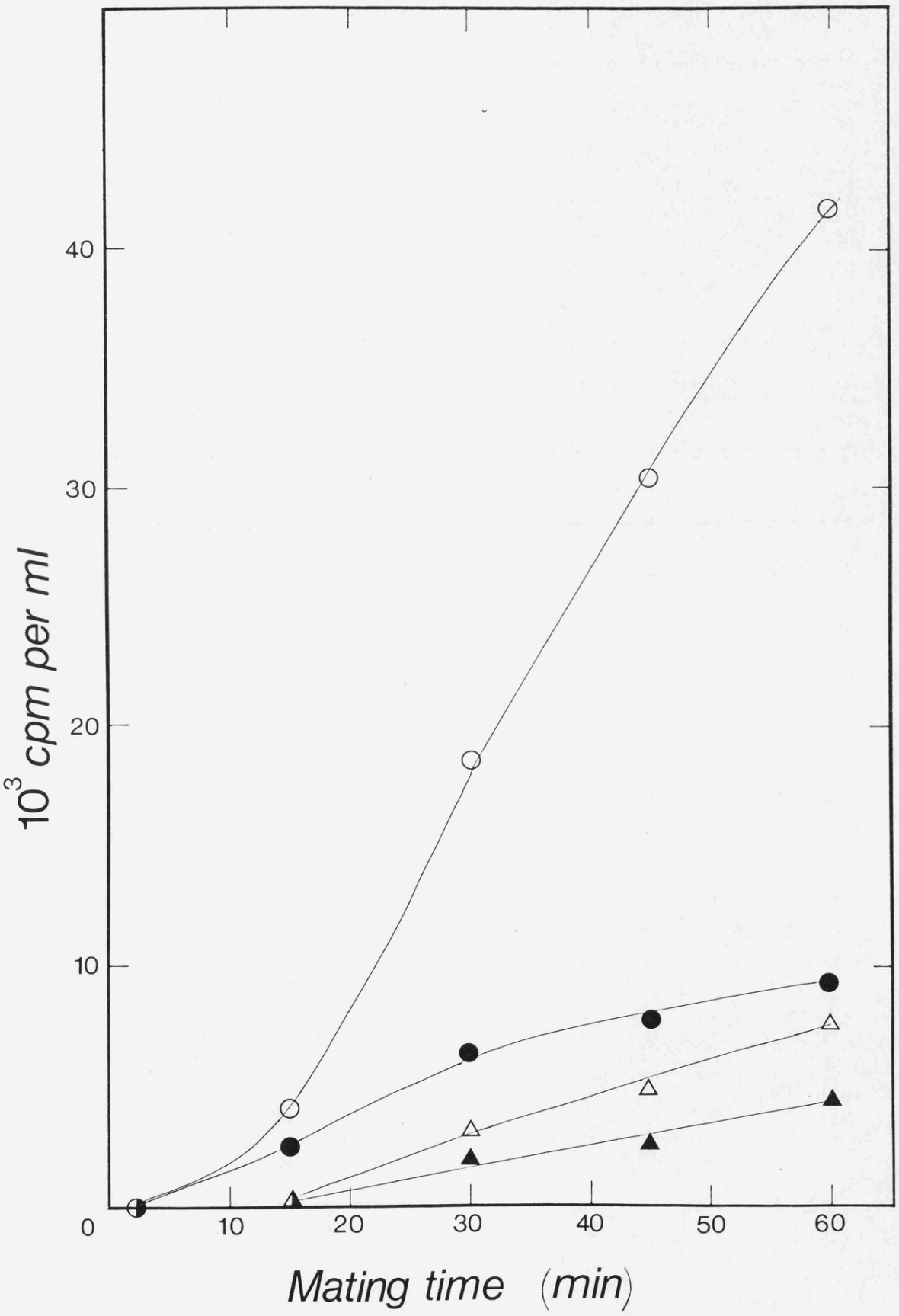
amount of Flac DNA transferred to recipient bacteria in a 60 min mating. Thus it is possible that the results in Figure 5-1 might reflect differing efficiencies of DNA transfer to unirradiated and irradiated recipients. However, recipient ability would have to be reduced by about 75% in order to account for these results.

Two methods for estimating DNA transfer were used to investigate this possibility. The first involved measurement of conjugative DNA synthesis in rifampicin-treated dnaB recipients, since it has previously been shown that this synthesis accurately reflects complementary strand synthesis (Boulnois and Wilkins, 1978). BW96 donors of pLG221 were therefore mated with irradiated or unirradiated dnaB70 recipient bacteria to estimate the relative amounts of plasmid DNA transferred in these matings. Results obtained are presented in Figure 5-2. The conditions of mating, including the donor strain, rifampicin treatment and UV dose were identical to those described for Figure 5-1, except that mating temperature was increased from 41°C to 43°C, since this higher temperature is necessary for efficient inactivation of mutant dnaB70 protein (B.M. Wilkins, personal communication). As a control, therefore, the experiments described in Figure 5-1 were performed at 43°C. Comparison of Figures 5-1 and 5-2 reveals very little difference in levels of conjugative DNA synthesis in dnaG recipients at 41°C and 43°C.

If conjugative DNA synthesis in unirradiated dnaG3 recipient bacteria occurs on both plasmid and chromosomal templates, then levels of DNA synthesis in UV treated and untreated dnaB recipients of pLG221 should be approximately equal, since pLG221 cannot bypass the requirement for dnaB protein in chromosomal DNA replication. Conversely, if UV seriously affects the competence of potential recipient cells, then substantially more DNA synthesis should be observed in untreated rather than irradiated

Figure 5-2 : Effect of UV-irradiation on conjugative DNA synthesis in dnaB recipients

BW96 (pLG221) was mated with appropriate recipients in the presence of rifampicin ($100 \mu g ml^{-1}$) and $[^{14}C]$thymine ($0.5\ \mu Ci\ \mu g^{-1}$) at $43^{\circ}C$. Recipients in each mating were : (o) unirradiated BW86 (dnaG), (•) irradiated BW86, (Δ) unirradiated BW69 (dnaB) and (▲) irradiated BW69. All four matings were performed in each single experiment, and data shown are the averages of 2 experiments.


40
30
20
10
0
10
20
30
40
50
60
10^3 cpm per ml
Mating time (min)

dnaB recipients. The results (Figure 5-2) clearly show that the stimulation observed in unirradiated *dnaG* recipients is not detected if cells are defective in *dnaB* product. A decrease in conjugative DNA synthesis of approximately 26% was observed after irradiation of *dnaB* recipients, insufficient to account for the results obtained with *dnaG* recipients.

These conclusions are supported by the direct measurements of DNA transfer to irradiated and unirradiated BW40 recipients described in Table 5-1. BW40 (*dna*$^+$) was selected as recipient in these experiments after it was found that a *tdk tsx* of BW86 (*dnaG3*) was unsuitable for this purpose. BW93 (*dnaG3 tdk tsx* Δ(*chlA-uvrB*)) was constructed in two stages from BW82 (*dnaG3 tdk*), firstly by selection for spontaneous chlorate-resistance under anaerobic conditions (Adhya *et al.*, 1968) and screening of mutants for sensitivity to UV light, and then by selection for resistance to phage T6. All four clones constructed showed mass-doubling times of more than 3h, and so were considered unusable for reliable measurements of DNA transfer. BW88 donors of pLG221 were labelled prior to and during mating with *tdk tsx* BW40 recipient cells for 1h at 41°C in the presence of rifampicin to stimulate DNA transfer. Plasmid DNA transferred to either irradiated or unirradiated recipients by this time was determined by TCA-precipitation, after selective lysis of the donor cells with phage T6 and DNase and RNase digestion of exposed nucleic acids. As controls, identical matings were performed with the presence of pLG252 in the recipient bacterium. pLG252 is a pBR325-based recombinant plasmid carrying the entry exclusion gene(s) from ColIb-P9 (see Chapter 7), and it can reduce conjugation by more than 50-fold between these strains under identical conditions as measured by transconjugant frequency.

Table 5-1 : Effect of UV-irradiation of recipient bacteria on pLG221 transfer

	Mating (M)[a]	Eex$^+$ Control[b] (C)	Transfer (M-C)
+ UV	5948	112	5836
- UV	6644	88	6556
Ratio-UV/+ UV			1.12

BW88 (pLG221) was labelled with [^{14}C]thymine (0.25 µCi µg^{-1}) both before and during mating with tdk BW40 recipient cells. Mating was performed for 1h at 41°C in the presence of rifampicin (200µgml^{-1}). Irradiated recipients were treated with 400Jm^{-2} of UV light immediately prior to mating. TCA-precipitable radioactivity transferred to recipient cells was determined after selective lysis of donors with irradiated T6.

a. Figures represent cpm per ml of mating mixture.

b. Control matings used BW40 (pLG252, Eex$^+$) as recipient to inhibit plasmid DNA transfer. These bacteria were grown in the presence of tetracycline (7.5µgml^{-1}), which was removed by sedimentation prior to mating, to select for the presence of pLG252.

Table 5-1 therefore shows that DNA transfer to UV irradiated bacteria was reduced by about 11%. Correction of the data displayed in Figure 5-1 for this reduction in the efficiency of DNA transfer to UV treated recipients predicts that 69% of the DNA synthesised in unirradiated dnaG recipients was bacterial, and 31% was plasmid-specific.

Use of chromosomal DNA as template in the absence of functional recipient-specified priming enzymes forms the core of the experimental evidence presented in support of the notion of transfer of a primer-generating enzyme from the donor cell, and so the proportions of plasmid and chromosomal DNA labelled in such a mating were also determined using DNA-DNA reassociation experiments involving the single-strand specific nuclease S1 from Aspergillus oryzae. A description of this method forms the next section of these results.

5.4 Parameters for S1 endonuclease assay and DNA-DNA reassociation experiments

The enzyme S1 from Aspergillus oryzae, first characterised by Ando (1966) and more extensively by Vogt (1973; 1980), is a single-strand specific nuclease, hydrolysing both RNA and single-stranded DNA in the presence of Zn^{2+} ions to yield 5'-mononucleotides (Ando, 1966). Under appropriate conditions S1 shows a very high specificity for single-stranded nucleic acid substrate, although the enzyme can introduce breaks in duplex DNA at high concentrations (Vogt, 1980). Sutton (1971) was the first to exploit these properties in a method for assaying reassociated eucaryotic DNA molecules in DNA hybridisation experiments, using the nuclease to degrade the single-stranded non-reassociated molecules and leave predominantly double-stranded material. Subsequent refinement of

this procedure for use with bacterial and plasmid DNA-DNA hybridisations (Crosa et al, 1973) and at the elevated temperature of 75°C (Barth and Grinter, 1975), has lead to an efficient and accurate quantitative assay for double-stranded DNA. Since the S1 assay obviated the need for immobilising DNA on nitrocellulose filters (Denhardt, 1966) and had advantages in terms of equipment and reported accuracy (Crosa et al., 1973; Barth and Grinter, 1975) over the technique using hydroxyapatite (HA) (Brenner et al., 1969), this method was selected to determine re-associated DNA in hybridisation samples.

In order to ensure that this method was reproducibly accurate in my hands, initial experiments were performed using [^{3}H]thymine-labelled chromosomal DNA from BW86 (thyA, dnaG) as radioactive probe in assays with unlabelled DNA from Proteus mirabilis 13, BW86, salmon sperm and calf thymus. Preparation of [^{3}H]labelled DNA by the lysozyme-SDS lysis method of Willetts et al. (1981) was rejected in favour of the lysozyme-sarkosyl lysis method described in detail in Chapter 2, since samples of TCA-precipitable material taken during an experiment with the former method showed that a substantial proportion of incorporated label was lost during phenol extraction of the lysate. This problem was easily circumvented by the use of caesium chloride gradients to purify and concentrate the labelled DNA in the final method adopted. Isolation of unlabelled BW86 DNA was performed routinely by the method of Jeffreys and Flavell (1977), and salmon sperm and calf thymus DNAs were purchased from Sigma Chemical Company. As Barth and Grinter (1975) reported 0% homology between the chromosomes of Proteus mirabilis 13 and E.coli strain J62, it was originally decided to use P.mirabilis DNA as a negative control in hybridisations with [^{3}H] labelled BW86 probe. However, difficulties in isolation of this DNA by the method of Chow et al. (1977) and the better performance of

salmon sperm in this respect lead to the routine use of salmon sperm DNA as the non-homologous control for this system.

Although the S1 assay was essentially as described by Barth and Grinter (1974; 1975) (see Chapter 2 forthe final procedure adopted), small modifications in the methodology were introduced. An appraisal of certain steps in the process are therefore appropriate at this point.

All DNA samples for hybridisation and S1 assay were sheared by ultrasonic treatment with an MSE ultrasonic disintegrator at 100W energy output. A total of twelve 15-sec bursts with 15-sec intervals for cooling were found to be sufficient to shear the majority of DNA molecules to between 1000 and 300 bp in size , near to the 2.5×10^5 molecular weight of fragments used by Barth and Grinter (1975). Denaturation of DNA samples (0.1 ml) was by heat at 105°C for 10 min in a PEG600 bath. Samples were then shifted directly to a 75°C bath for either reassociation or assay by S1, rather than fast-coded in ice water first, since this was found to cause some immediate reannealing (data not shown).

Hybridisation or assay samples comprised a final DNA concentration of $200 \mu g ml^{-1}$ in 0.1 ml of 0.42M NaCl. To these samples at 75°C was added 0.9 mls of reaction mixture to give final concentrations of 0.168M NaCl, 1mM $ZnSO_4$, 30mM sodium acetate buffer (pH4.6) and $20 \mu g ml^{-1}$ sheared denatured calf thymus DNA in a 1.0 ml final volume. Denatured calf thymus DNA was added since it has been reported that this practice overcomes the problem imposed by the inability of S1 nuclease completely to degrade low concentrations of single-stranded substrate (Sutton, 1971; Barth and Grinter, 1975). Hybridisation samples routinely contained 20µg of unlabelled DNA and 20ng of [^{3}H] labelled probe DNA. Different ratios of probe and excess unlabelled DNA were tried over the range of 1:500 to 1:3000,

and a thousand-fold excess finally selected for standard use. 150 U of S1 were found to be optimal for each assay (data not shown), and the reaction allowed 20 min at 75°C for completion. Sterilised eppendorf tubes (1.5 ml) were used as receptacles for both DNA samples and reaction samples, since these proved easily manipulable and disposible. Performance was not improved by repel-coating tubes, but overlay of the sample with 3 or 4 drops of parrafin oil improved reproducibility of results by preventing evaporation during incubation at 75°C and denaturation.

Table 5-2 demonstrates the activity of S1 nuclease on single-stranded and double-stranded BW86 chromosomal DNA, under the above conditions. The 96% and 3% activities on denatured and native DNA described in Table 5-2 are in accordance with the previously reported data of Barth and Grinter (1975), and so confirm the validity of this assay for our purposes.

Since pLG221 is much smaller than the E.coli chromosome, and rifampicin treatment of recipients causes transfer of multiple strands of pLG221 to each cell in a 60 min mating (Boulnois and Wilkins, 1979), the plasmid component in labelled DNA extracted from mating cells would be expected to reassociated at a faster rate than the chromosomal DNA fraction (Britten and Kohne, 1968). It was therefore decided to set the Cot value (C_o = initial concentration of DNA : t = time allowed for hybridisation, Britten and Kohne, 1968) for reassociation experiments to 10 times the $Cot_{\frac{1}{2}}$ value derived for BW86 DNA in 0.42M NaCl at 75°C. $Cot_{\frac{1}{2}}$ is the Cot value at which reassociation is 50% completed, and is a specific value for different species of DNA molecules, dependant on the size and complexity of each genome. This value was determined empirically by measuring the proportion of reassociated DNA in a number of separate samples containing

Table 5-2 : Action of S1 nuclease on single-stranded and double-stranded DNA at 75°C

Trichloroacetic acid-precipitable material (%)[a]		
Denatured DNA + S1	Native DNA + S1	Native DNA - S1
3 ± 2	96 ± 5	100[a]

DNA samples (0.1 ml) contained 20µg sheared BW86 DNA and 20 ng of sheared [^{3}H]labelled BW86 DNA (226 Cpm ng^{-1}) in 0.42M NaCl. Samples were either heat-denatured as described or left as native form, and then assayed in duplicate by addition of pre-warmed reaction mixture and 150 U of S1 endonuclease at 75°C for 20 min. Data are the average results of three separate experiments.

a. Results are expressed relative to the input of label, as determined by cpm recovered from native DNA without addition of S1.

denatured BW86 DNA incubated over a wide range of Cot values. Results are expressed in Figure 5-3 as a plot of percentage reassociation against the logarithmic values of Cot. The logarithm of $Cot_{\frac{1}{2}}$ is measured as the value on the x axis of this plot when 50% reassociation is reached, and conversion gives a $Cot_{\frac{1}{2}}$ value of 2.14×10^{4} μgml^{-1} x min in 0.42M NaCl at 75°C. Units of μgml^{-1} x min will be used in preference to the more usual Cot units of moles of nucleotides per litre x sec; conversion of the former to the latter requires division by a factor of 5,542 (Barth and Grinter, 1975).

Denatured DNA samples were therefore incubated to a Cot value of 10x $Cot_{\frac{1}{2}}$, equal to approximately 2.14×10^{5} μgml^{-1} x min (Britten and Kohne, 1968; Barth and Grinter, 1974; 1975). Since C_o = 200 μgml^{-1}, the incubation time for samples was 17h 45 min. This compares favourably with conditions for hybridisations involving bacterial DNA (Britten and Kohne, 1968; Crosa et al., 1973; Barth and Grinter, 1975). Table 5-3 confirms that hybridisations using these conditions allow the homologous reassociation reaction to proceed to virtual completion. The selection of salmon sperm DNA as a DNA species without significant homology with bacterial DNA is also validated by the low recovery of TCA-precipitable radioactivity from the non-homologous reassociation reaction.

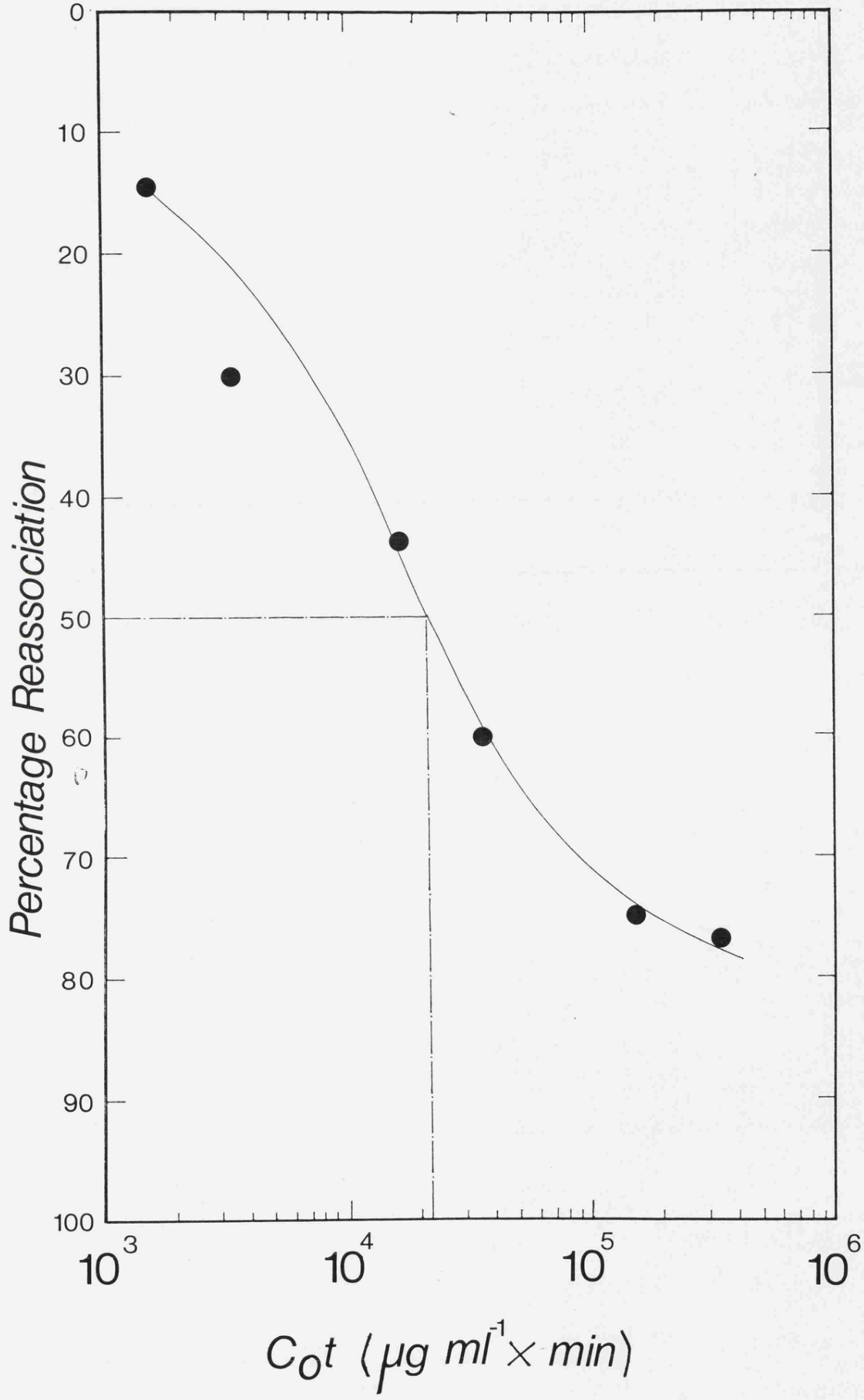
5.5 Preparation of [^{3}H] labelled probe DNA from unirradiated, rifampicin-treated thyA, dnaG3 recipients of pLG221

To ensure fidelity with the mating conditions and bacterial strains described in Figure 5-1, BW96 (tdk, rpoB) donors of pLG221 were mated with unirradiated BW86 (thyA, dnaG3) recipients in the presence of rifampicin ($100 \mu gml^{-1}$) and deoxyguanosine ($200 \mu gml^{-1}$) at 41°C in a total volume of

Figure 5-3 : The kinetics of BW86 DNA reassociation

0.1 ml samples of $[^3H]$ labelled and excess unlabelled BW86 DNA in 0.42M NaCl solution were denatured and incubated at 75°C over a range of $C_o t$ values from 1.5×10^3 to 3×10^5 $\mu g ml^{-1}$ x min. This distribution was achieved by setting the initial concentration of DNA in samples (C_o) at either 200, 500 or 1000 $\mu g ml^{-1}$ and varying incubation time up to 4h 45 min. All hybridisations were performed in duplicate and samples assayed by S1 endonuclease for reassociated DNA. Denatured and native DNA controls showed 95 and 3% activity of the enzyme on the respective substrates. Data are expressed as a percentage of the input counts, and have been corrected by subtraction of the denatured DNA control values.

Results are expressed as a plot of percentage reassociation against $C_o t$ value on a logarithmic scale to allow the determination of $C_o t_{\frac{1}{2}}$ for BW86 DNA under these conditions. Calculation of this value is explained in the text.


0
10
20
30
40
50
60
70
80
90
100
Percentage Reassociation
10^3
10^4
10^5
10^6
$C_o t$ ($\mu g\ ml^{-1} \times min$)

10 ml [^{3}H]thymine (33.3 μCi μg^{-1}) was added to the mating mixture at a concentration of 1μgml^{-1}. After 1h of mating, 2x0.5 ml samples were extracted for analysis by TCA-precipitation, and a mixture of KCN and excess thymine and thymidine added to the remaining 9 mls to halt conjugative DNA synthesis. Parental donor and recipient controls, incubated separately under identical conditions, incorporated approximately 4,600 and 12,000 cpm ml^{-1} respectively. These values amount to only 0.6% and 1.4% of the radioactivity incorporated in the mating mixture ($8.3x10^5$ cpm ml^{-1}), and so at least 98% of the radioactivity incorporated into the TCA-precipitable material of the mating mixture is attributable to conjugative DNA synthesis. Extraction of DNA from the mating culture was described in Chapter 2, and mating probe DNA was sonicated (section 5.4) before use in DNA-DNA reassociation experiments.

5.6 Analysis of DNA synthesised in unirradiated *dnaG* recipient using DNA-DNA reassociation experiments

The isolation of ColIb-P9 DNA of sufficient quantity and quality for use in DNA-DNA reassociation experiments, although technically arduous, provided further insights into the properties of this plasmid, and so will be discussed in the first part of this section in some depth. Precise details of the different methods used in the isolation of pLG221 DNA have been described in Chapter 2.

The method of Hansen and Olsen (1978) affords a reliable procedure for the isolation of large bacterial plasmids of comparable size to pLG221. The protocol makes use of gentle manipulations to reduce mechanical breakage of circular plasmid DNA by pipetting (Currier and Nester, 1976), and involves a lysozyme-sodium dodecyl sulphate (SDS) lysis

Table 5-3 : Reassociation of homologous and non-homologous DNA

	TCA-precipitable material (%)		
Unlabelled DNA	Denatured DNA control	Native DNA control	Hybridisation
BW86	2 ± 1	99 ± 4	95 ± 2
Salmon Sperm	3 ± 2	102 ± 3	2 ± 1

Samples (0.1 ml) each contained 20 ng of sheared [^{3}H]labelled BW86 DNA (226 cpm ng^{-1}) and 20µg of sheared unlabelled BW86 or salmon sperm DNA in 0.42M NaCl, and were denatured before incubation at 75°C for 17h 45 min to allow reassociation. Duplex DNA in the samples at the end of this period was then assayed by the S1 endonuclease method. Single-stranded and double-stranded DNA control mixtures, identical to hybridisation samples, were either denatured or left as native substrate, and immediately assayed by S1 nuclease. Values are expressed as the percentage of the counts recovered from identical control mixtures which were not treated with S1, and are the average of four separate experiments.

essentially similar to that of Guerry et al. (1973), which, due to a high salt concentration, allows sedimentation of membrane-bound chromosomal DNA by centrifugation. Alkali denaturation removes residual broken chromosomal DNA from the supernatant, and plasmid DNA was concentrated by the addition of PEG 6000. To remove any remaining chromosomal DNA contamination, lysates were centrifuged to equilibrium in CsCl-EtBr density gradients as described in Chapter 2, and both chromosomal and plasmid bands isolated separately for analysis.

Although it proved possible to isolate sufficient quantities of pLG221 DNA for my purposes by this method, with an initial 1 l culture of W3110 (pLG221) routinely yielding at least 150µg of plasmid DNA, this DNA could only rarely be restricted by digestion with EcoR1. Out of eight separate experiments, only two yielded DNA samples which were susceptible to restriction. Restriction analysis of the chromosomal and plasmid fractions from a successful preparation is depicted in Figure 5-4. The presence of plasmid EcoR1 fragments in the chromosomal fraction from CsCl density gradients confirms the existence of pLG221 in this sample, presumably in an open circular form, so that this sample may be regarded as plasmid-enriched chromosomal DNA for the purposes of reassociation experiments. Plasmid DNA samples from three separate preparations were mixed with λ HindIII marker fragments, and subjected to digestion by EcoR1 (Figure 5.5, tracks d, e and f). In only one of these three mixtures were λ HindIII EcoR1 double digest bands discernable after agarose gel electrophoresis, and the plasmid DNA of this mixture was subsequently shown to be the only sample of the three capable of yielding pLG221 EcoR1 fragments. Resistance to EcoR1 digestion precluded the use of two of the three DNA samples in reassociation experiments since it was not confirmed that they

Figure 5-4 Restriction endonuclease analysis of DNA samples prepared by the method of Hansen and Olsen (1978)

Digestion with restriction endonuclease EcoR1 and preparation of DNA samples have been described previously. Tracks contain 1 to 2μg of DNA as follows:

a) λHindIII EcoR1 DNA

b) Uncleaved DNA from chromosomal fractions

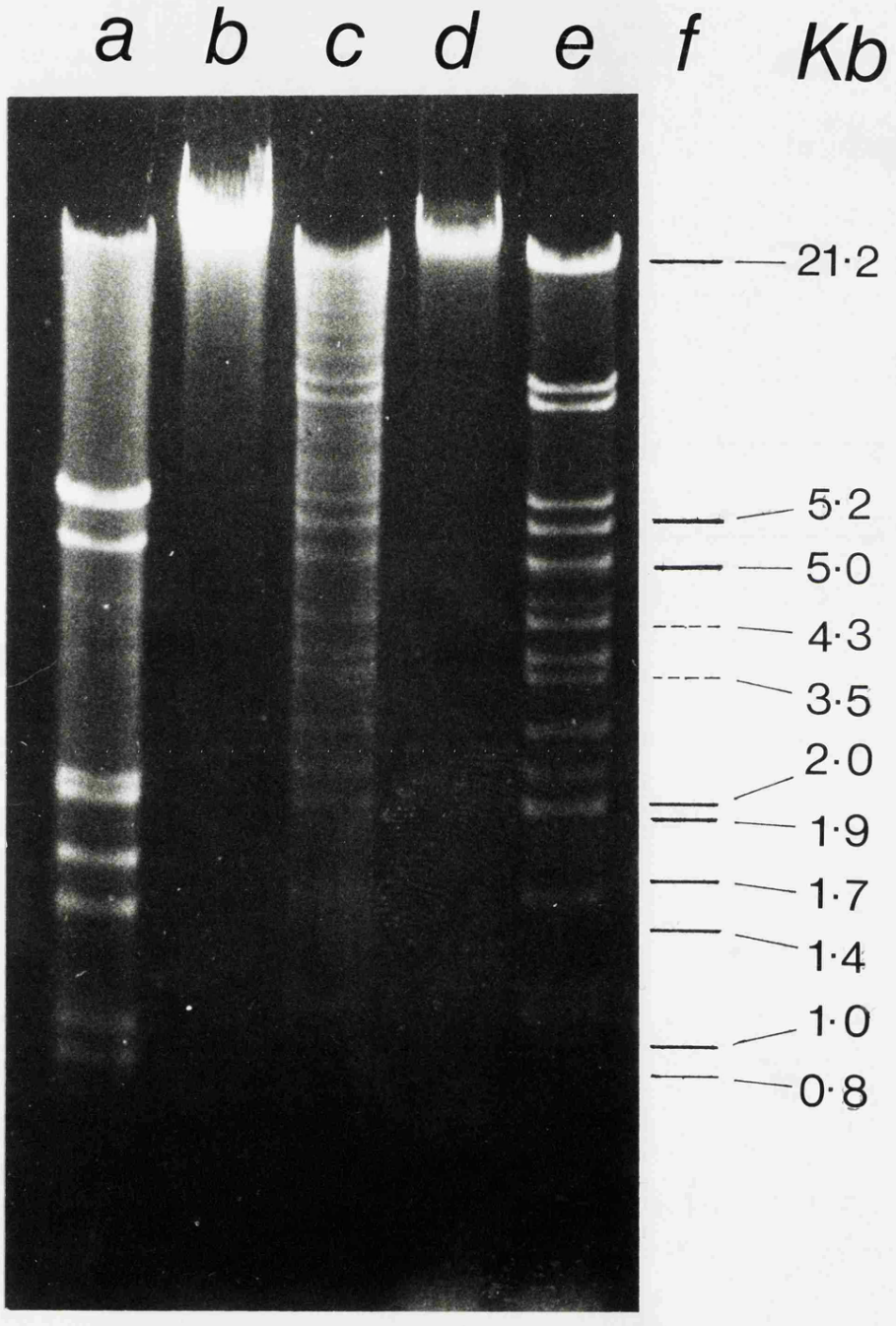
c) Chromosomal fraction DNA cut with EcoR1

d) Uncleaved pLG221 DNA

e) pLG221 EcoR1 DNA

f) λHindIII EcoR1 marker bands

Numbers indicate the molecular sizes of λHindIII EcoR1 in kilobases.


a
b
c
d
e
f
Kb
21·2
5·2
5·0
4·3
3·5
2·0
1·9
1·7
1·4
1·0
0·8

contained pLG221 DNA, and so the third preparation of DNA (Figure 5-5) was selected for this purpose. Pilot experiments (data not shown) revealed that DNA mixtures containing pLG221 DNA would not reassociate under the standard hybridisation conditions described earlier, and small amounts of this DNA sample were sufficient to prevent the reassociation of homologous DNA mixtures under identical conditions. Since the single- and double-stranded controls from these experiments show that the pLG221 DNA can be digested by S1 endonuclease after heat treatment at 105°C, it appears that this solution of DNA can be denatured, but is resistant to reassociation. As the pLG221 DNA samples prepared by the method of Hansen and Olsen (1978) can affect the reassociation and restriction properties of other DNA species, the causative agent is unlikely to be the plasmid DNA itself, but rather a contaminating chemical used in the method of preparation. The mechanism by which PEG 6000 can concentrate DNA is undetermined as yet, although it seems more likely to act by a process of phase partition, rather than a simple precipitation reaction (Yamamoto et al., 1970; Chun et al., 1967). Since PEG was used at a high concentration prior to density gradient centrifugation, it was felt that perhaps residual amounts of PEG, escaping removal by CsCl centrifugation (Chun et al., 1967), might be responsible for contamination of DNA samples. Attempts to purify these samples by phenol extraction and passage through DEAE-Sephacel were unsuccessful due to loss of DNA during these manipulations, and so the procedure of Hansen and Olsen (1978) was modified to circumvent the use of PEG. Concentration of plasmid DNA in the supernatant obtained after sedimentation of membrane-bound chromosomal DNA was achieved by ethanol precipitation in the presence of 200mM sodium acetate pH5.6.

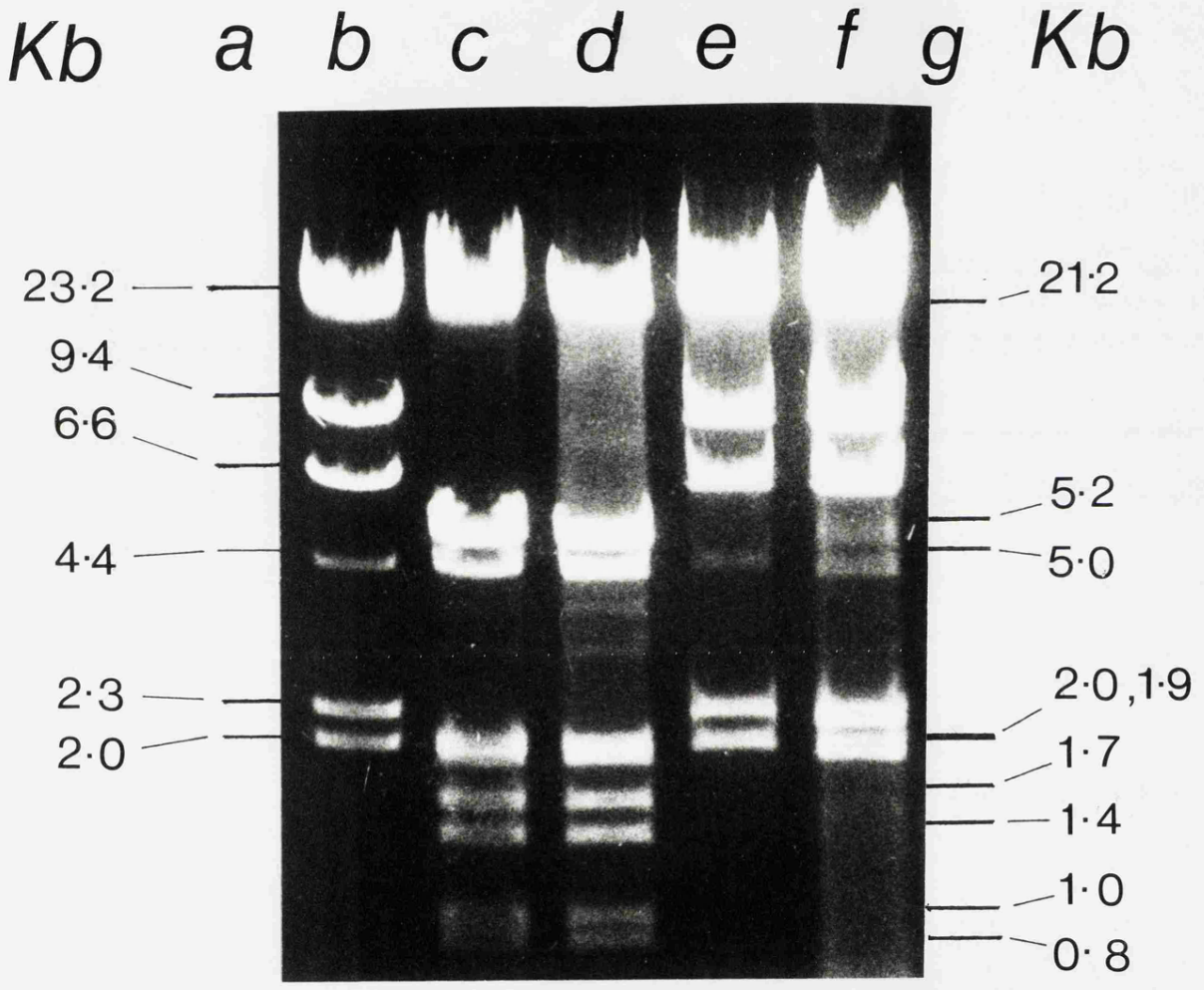
Figure 5-5 Endonuclease restriction of DNA samples prepared by the method of Hansen and Olsen (1978)

Procedure has been described previously (sections 2.8 and 5.6). Tracks contain about 1µg of DNA prepared by the method of Hansen and Olsen (1978) and 2µg of λHindIII DNA as appropriate. Tracks are:

a) and b) λHindIII

c) and g) λHindIII EcoR1

d) to f) pLG221 DNA samples mixed with λHindIII DNA and digested with EcoR1


Kb
a
b
c
d
e
f
g
Kb
23·2
9·4
6·6
4·4
2·3
2·0
21·2
5·2
5·0
2·0,1·9
1·7
1·4
1·0
0·8

Visualisation of the CsCl/EtBr density gradients produced using this modification under UV light revealed that the plasmid band was much fainter than previously attained by the standard method. The yield of pLG221 DNA was confirmed spectrophotometrically to be too low for practical use, but restriction analysis of the chromosomal DNA band taken from gradients revealed the presence of pLG221 EcoR1 fragments, and pilot reassociation experiments showed that, unlike the DNA isolated using PEG, this DNA sample did not affect the hybridisation properties of other DNA solutions. Presumably the plasmid-enriched chromosomal DNA band is a consequence of mechanical breakage of circular plasmid DNA during the ethanol precipitation stage. Essentially identical results were obtained for the isolation of ColIb-P9.

W3110, the host strain used in these preparations was selected since W3110T$^-$ has been used successfully in the past for isolation of the related plasmid R144 (Barth and Grinter, 1975). This E.coli K-12 strain can be expected to be reasonably homologous with the K-12 strain BW86 (Bachmann, 1972; Crosa et al., 1973), and ColIb-P9 and pLG221 differ in sequence only by the drd mutation and the presence of Tn5 (5.3 Kb; Auerswald et al., 1980) in the latter, so that these plasmids share at least 95% homology. Thus, the pLG221 and ColIb-P9-enriched W3110 chromosomal DNA from gradients could be expected to show almost complete homology with the [^{3}H]labelled probe DNA synthesised during conjugation in unirradiated, rifampicin-treated BW86 recipients of pLG221. As shown in Table 5-4, both ColIb-P9 and pLG221-enriched W3110 DNA allowed approximately 98% recovery of denatured mating probe DNA in reassociation experiments. The result for this near-homologous reaction confirms both the suitability of this DNA for reassociation experiments, and the conditions for hybridisation. Unlabelled plasmid-free BW86 DNA showed about 69% homology with conjugatively-

Table 5-4 : Reassociation of DNA labelled in unirradiated rifampicin-treated recipient cells with unlabelled DNAs

Unlabelled DNA	% Homology with mating probe
BW86 + pLG221	98.0 ± 18.5
BW86 + ColIb-P9	98.3 ± 5.4
BW86	69.1 ± 15.0
pLG221	24.3 ± 4.0
salmon sperm	0.0 - 1.4

Sheared [^{3}H] labelled probe DNA (20 ng), isolated from unirradiated rifampicin-treated BW86 recipients of pLG221, was mixed with 20µg of sheared unlabelled DNA as appropriate in 0.1 ml of 0.42M NaCl. Plasmid DNA for use in the homologous reactions (lines 1 and 2) was prepared by a modification of the method of Hansen and Olsen (1978), and purified pLG221 DNA (line 4) was isolated using the procedure of Uemura and Mizobuchi (1982), as described in the text. After denaturation and incubation at 75°C to allow DNA-DNA reassociation, samples were assayed by the S1 endonuclease method (Barth and Grinter, 1975), for renatured DNA. Assays were performed in triplicate, and results have been corrected by subtraction of the amount of denatured DNA resistant to S1 nuclease (5.5% of the input counts) and are expressed as a percentage of S1 nuclease-resistant DNA recovered from native DNA controls (98% of the input counts).

synthesised probe DNA, in close agreement with the value predicted by the hypothesis under test.

Although this experiment determines the proportion of conjugatively-synthesised chromosomal DNA present in the labelled probe, hybridisation against purified pLG221 is still required for conf rmation of this result. The method for preparing the unlabelled plasmid DNA described by Barth and Grinter (1975), is similar to the SDS-NaCl procedure of Vapnek and Rupp (1971), and both avoids the use of PEG and has been successfully used in the isolation of R144. However, although it proved possible to isolate both R144 and F DNA by this method, agarose gel electrophoresis of samples from ColIb-P9 and pLG221 DNA preparations, estimated spectrophotometrically to contain up to 50μg of nucleic acid, did not reveal the presence of high molecular weight plasmid DNA. Instead illumination by UV light revealed a very intense smear, presumably oligodeoxyribonucleotides, running ahead of a faint characteristic RNA band.

It is proposed that this result is due to degradation of plasmid DNA by a ColIb-P9-encoded or -activated nuclease, as a consequence of bacterial lysis. Precedents for this hypothesis, together with experimental support, will be considered in the discussion to this chapter, but since the method of Hansen and Olsen (1978) maintains a high concentration of EDTA (60-100mM) throughout the protocol, this would explain why degradation of DNA was not observed previously. However, although the EDTA concentration was raised to 100mM in subsequent preparations, and was successful in reducing degradation of DNA, it proved unfeasable to use this method for the generation of large stocks of pLG221 since, as with the modified Hansen and Olsen method, mechanical or perhaps nuclease-mediated breakage of plasmid DNA reduced drastically the size of the plasmid band in CsCl density gradients.

Unlike the more general methods of large plasmid isolation described previously, the method of Uemura and Mizobuchi (1982) is designed for purification of large recombinant derivatives of ColIb-P9, and was found to be the most effective method for preparation of pLG221 DNA. W3110 (pLG221) was lysed by a Brij 58-sodium deoxycholate method similar to that of Clewell and Helinski (1969) in the presence of 100mM EDTA, and chromosomal DNA was pelleted by centrifugation. Plasmid DNA in the supernatant was concentrated by addition of PEG, but unlike the method of Hansen and Olsen (1978), this did not affect restriction or hybridisation of the DNA, perhaps due to the presence of 1% sarkosyl. CsCl/EtBr gradients also contained sarkosyl, and both resultant plasmid and chromosomal bands were pronounced and well separated. Agarose gel electrophoresis of EcoR1-digested pLG221 and chromosomal fractions from gradients is depicted in Figure 5-6. The EcoR1 digest pattern of pLG221 is detectable in track (c) of Figure 5-6, confirming the presence of linearised or open circular forms of the plasmid in the chromosomal fraction.

In reassociation experiments (Table 5-4) pLG221 DNA, prepared by the method of Uemura and Mizobuchi (1982), showed a homology of about 24% with denatured mating probe DNA. A homologous control mixture of BW86 chromosomal DNA and pLG221 incubated with mating probe gave a 95% recovery of input counts after treatment with S1 nuclease, confirming the suitability of pLG221 DNA in these experiments.

Figure 5-6 Restriction endonuclease analysis of DNA prepared by the method of Uemura and Mizobuchi (1982)

Procedures have been described in section 2.8 and 5.6, and tracks contain between 1 and 2μg of DNA. Tracks are:

a) λHindIII

b) Uncleaved pLG221 DNA

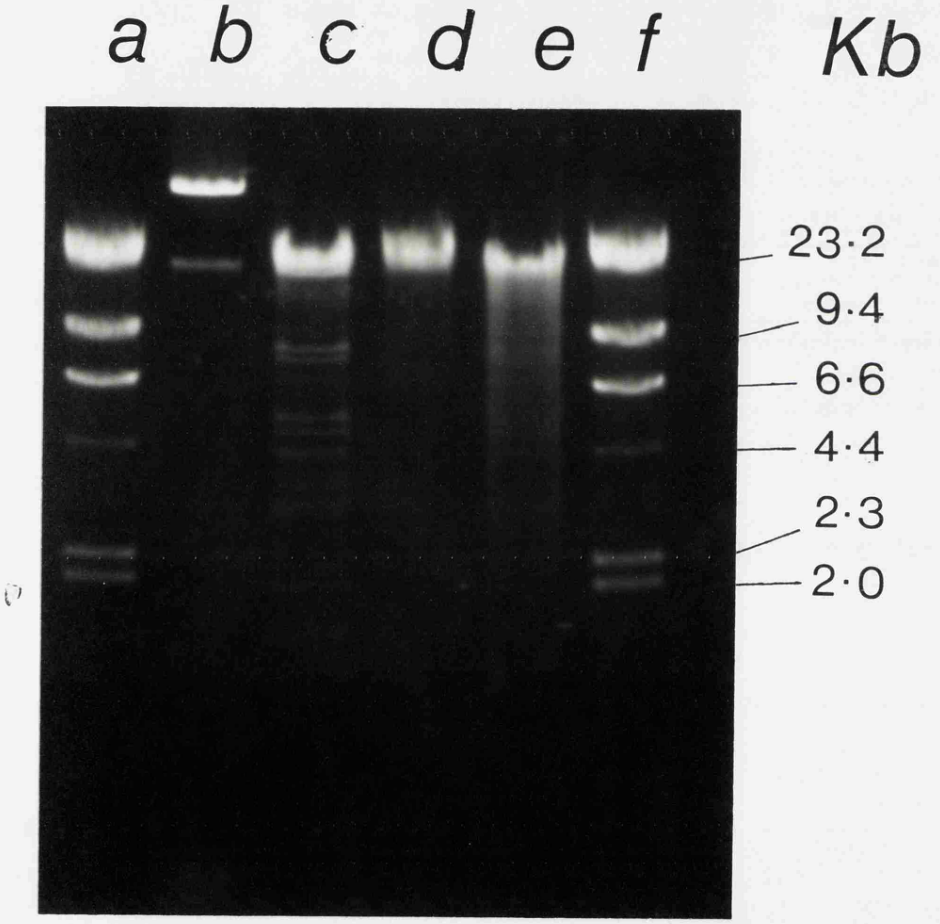
c) pLG221 EcoR1

d) Uncleaved chromosomal fraction DNA

e) Chromosomal fraction DNA cut with EcoR1

f) λHindIII

Numbers indicate the sizes of λHindIII fragments


a b c d e f
Kb
23·2
9·4
6·6
4·4
2·3
2·0

5.7 Determination of the extent of homology between pLG221 and BW86 DNA

$[^3H]$ labelled pLG221 DNA was prepared from a culture of BW86 (pLG221) containing $[^3H]$thymine (50 $\mu Ci\ \mu g^{-1}$, $2\mu g ml^{-1}$) by the production of a sheared lysate as described in Chapter 2. TCA-precipitable samples taken throughout this procedure showed significant incorporation of $[^3H]$ thymine by the culture, and an equivalent number of counts were detected after each successive manipulation of the cleared lysate obtained from this culture. Plasmid DNA was purified by two successive centrifugations in CsCl/EtBr density gradients as described in Chapter 2. The specific activity of the final pLG221 DNA isolate was 435 cpm ng^{-1}, and from the specific activity of DNA extracted from the chromosomal band of the first gradient, it is calculated that about 35 ng of pLG221 was recovered by this procedure. This compares with almost 100ng of plasmid DNA collected from the first gradient, and the loss is attributed to breakage of CCC DNA as a result of mechanical forces entailed by the second centrifugation procedure.

The labelled plasmid DNA was sheared, and used in reassociation experiments to determine the homology of pLG221 with BW86 chromosomal DNA (Table 5-5). In the homologous reaction, 88% reassociation was achieved against unlabelled pLG221 DNA prepared by the method of Uemura and Mizobuchi (1982), whilst only 1.2% homology was detected with BW86 chromosomal DNA. This low extent of homology was confirmed independently by the reassociation of $[^3H]$thymine-labelled BW86 DNA and unlabelled pLG221 DNA (Table 5-5), which estimated the homology between these two DNA species at only 2.7%. These values are in agreement with the 2.0% homology between ColIb-P9 and the E.coli chromosome reported by Isaacson and Konisky (1974a) using a filter hybridisation method, and with the 0.7%

Table 5-5 : Reassociation of labelled pLG221 and BW86 bacterial DNA with unlabelled DNAs

[^{3}H] labelled DNA	Unlabelled DNA		
	pLG221	BW86	salmon sperm
pLG221	87.8 ± 29.0	1.2 ± 0.7	0.0 ± 0.6
BW86	2.7 ± 2.6	91.9 ± 2.5	0.0 ± 1.1

Hybridisation mixtures consisted of 2.5 ng of sheared denatured [^{3}H]thymine labelled DNA and 2.5µg of sheared denatured unlabelled DNA in 0.1 ml of 0.42M NaCl. Following incubation at 75°C, the amount of TCA-precipitable labelled DNA remaining in mixtures after treatment with S1 nuclease was determined. Assays were performed in triplicate, and data have been corrected for the single- and double-stranded control values of 4% and 95% respectively, as described for table 5-4.

reassociation between the closely-related plasmid R144 (which shares 85% homology with ColIb-P9; Crosa et al., 1973) and E.coli DNA measured by the S1 nuclease method by Barth and Grinter (1975).

5.8 Discussion

It was demonstrated in the previous chapter that the DNA primase specified by ColIb-P9 in donor cells was responsible for the initiation of complementary strand synthesis of the plasmid in rifampicin-treated dnaG recipients. Presumably, either oligoribonucleotide primers or the primer-generating enzyme itself is transmitted to the recipient bacterium to promote this DNA synthesis. The results in this chapter support the notion of the transfer of the enzyme, rather than its product of reaction, although concomitant RNA transfer cannot be discounted. Previous attempts to examine the possibility of protein transfer during conjugation (Silver and Ozeki, 1962; Silver, 1963; Silver et al., 1965) involved the specific labelling of donor-specified proteins and lysis by phage T6 to isolate donor or recipient cells after mating. This method proved inconclusive due to the limit of resolution of these types of experiments, but it was established that not more than 0.3% of total cellular protein or RNA was transferred from donor cells of ColIb-P9 (Silver and Ozeki, 1962). This corresponds to an amount of protein roughly equivalent to 60% of the DNA transferred to recipients in these experiments, and so does not rule out a selective transfer of proteins directly involved in the conjugative process.

In keeping with this hypothesis, the additional DNA synthesis observed in unirradiated, compared to UV irradiated, dnaG recipients of pLG221 (Figure 5-1) is attributed to the rescue of dnaG-deficient chromosomal DNA synthesis by a transferred primer-generating enzyme from

the donor cell. The significance of this recovery can be assessed more easily if we consider that 1,200 cpm per ml of mating mixture corresponds to the synthesis of about 31×10^6 daltons of single-stranded DNA in each recipient cell under these conditions (Boulnois and Wilkins, 1979). Since almost 48,000 cpm per ml was detected in the unirradiated mating described in Figure 5-1, the average rifampicin-treated, dnaG recipient synthesised about 1.24×10^9 dal of single-stranded DNA in 1 hour of mating. This figure is equivalent to about 3,800 Kb of single-stranded DNA, enough to synthesise 35 complementary strands of pLG221 or to replicate about 48% of an E.coli chromosome.

Since conjugative DNA synthesis in UV irradiated recipients represents the synthesis of complementary strands of plasmid DNA (Chapter 4), the additional DNA synthesis detected in equivalent matings using unirradiated recipients provides an estimate of the amount of chromosomal replication occurring in these cells. However, for an accurate estimation the effect of UV on the recipient strains ability to receive plasmid DNA must be taken into account. Although UV treated bacteria can act as recipients of F (Friefelder and Friefelder, 1968; Falkow et al., 1971; Wilkins and Hollom, 1974), R1-drd-19 (Herschfield, 1973) R144drd-3 (Boulnois and Wilkins, 1979) and ColIb-P9drd-1 (Boulnois et al., 1979; this thesis), and no change in cell wall or outer membrane composition is detectable until several hours after irradiation (Herschfield, 1973), it was also shown that UV treatment equivalent to that used in my experiments halved the amount of Flac DNA transferred to recipients (Boulnois, 1980).

The measurements of DNA transfer given in Table 5-1 demonstrate that irradiated recipients received 11% less plasmid DNA than the correspondingly unirradiated cells. Together with the data of Figure 5-1,

these results predict that the proportions of plasmid and chromosomal DNA synthesised in unirradiated, rifampicin-treated dnaG recipients of pLG221 are 31% and 69% respectively after 1 hour of mating. Thus, the average unirradiated recipient synthesised about eleven complementary strands of pLG221 and replicated over 1.3×10^3 Kb (~ 33%) of the duplex bacterial chromosome in this time. The proportions of conjugatively-synthesised plasmid and chromosomal DNA are supported by the independent results obtained using DNA-DNA reassociation experiments (Table 5-4). DNA isolated from these recipients was shown to possess homologies of 24% and 69% with pLG221 and BW86 DNAs, respectively. This measurement is not complicated by significant homology between pLG221 and the E.coli chromosome (Table 5-5). Similar experiments were not performed using DNA from UV treated dnaG recipients as hybridisation probe, since it was found in initial experiments that the specific activity of this DNA was reduced, presumably due to the lower levels of DNA synthesis in these cells, thus increasing the significance of the background parental control values.

The major technical problem encountered in the course of these experiments was the isolation of sufficient quantities of chromosome-free pLG221 DNA for use as the unlabelled component in reassociation mixtures. Preparations of pLG221 and ColIb-P9, but not F or R144 showed evidence of degradation of DNA when isolated using methods involving low concentrations of EDTA. The methods of Hansen and Olsen (1978) and Uemura and Mizobuchi (1982) were successful in yielding stable plasmid DNA, presumably since they use 100mM EDTA or the presence of sarkosyl to inhibit nuclease-mediated degradation of samples. It therefore appeared that ColIb-P9 might encode a plasmid-specified endonuclease. Support for this suggestion was derived from the analysis of a bank of Tn5 insertions in

ColIb-P9 created for another project (C. Corker). Screening by agarose gel electrophoresis of small-scale DNA preparations made in the presence of low EDTA concentration revealed that many of the Tn*5* insertions created a phenotype that does not promote the degradation of plasmid DNA in samples. This high proportion may indicate that the gene responsible for DNA breakdown is part of a large operon, perhaps analogous to the large transfer operons of F (Manning and Achtman, 1979) or RP4 (Barth *et al.*, 1978). It was subsequently reported by Winans and Walker (1983) that some bacterial plasmids encode endonucleases which are resistant to low concentrations of EDTA. Out of 27 plasmids examined by these authors, twelve, including ColIb-P9, R64 and R144*drd-3*, were shown to be associated with such an enzyme.

The close agreement between DNA-DNA hybridisation experiments and the comparison of levels of DNA synthesis in UV treated and untreated recipients in elucidating the proportions of plasmid and chromosomal DNA synthesised in *dnaG* recipients validates the use of the latter method as a rapid and accurate system to investigate the requirements for the recovery of chromosomal replication. This approach is used in the next chapter to investigate the nature of this process, and to resolve the primer-generating enzyme that is supplied by the donor cell to mediate this recovery.

CHAPTER 6

Requirements for the recovery of chromosomal DNA synthesis in mated rifampicin-treated dnaG recipient cells

'I ask the reader to remember that what is most obvious may be most worth of analysis. Fertile vistas may open out when commonplace facts are examined from a fresh point of view.'

L.L. Whyte.

6.1 Introduction

It was demonstrated in the previous chapter that *dnaG* bacteria were able to recover limited ability to synthesise chromosomal DNA when used as recipients during matings with *dna*$^+$ donors of the IncIα plasmid pLG221. This conclusion, drawn from measurements of conjugative DNA synthesis in rifampicin-treated *dnaG3* recipient cells, was confirmed by DNA-DNA re-association analysis of [^{3}H]thymine-labelled DNA isolated from BW86 (*thyA*, *dnaG3*, *uvrB*) bacteria mated with *tdk dna*$^+$ *rpoB* donors of pLG221. In order to account for this observation, it was proposed that a donor-specified primer-generating enzyme, able to substitute for defective *dnaG3* protein in the initiation of discontinuous bacterial DNA replication, was transferred to recipient cells.

In this chapter I shall examine the requirements for recovery of chromosomal DNA synthesis in mated *dnaG* recipients. The approach taken made use of measurements of conjugative DNA synthesis in UV irradiated and unirradiated *dnaG* recipients, since it was demonstrated in chapter 5 that subtraction of the amounts of conjugative DNA synthesis detected in UV

treated recipients from that occurring in unirradiated cells is a valid method of estimating the proportion of chromosomal DNA labelled in the latter recipients. Allowance must be made for the 11% reduction in DNA transfer to irradiated bacteria (Table 5-1), but as the same recipient strain (BW86) is used throughout the experiments described in this chapter, this factor may be expected to remain a constant for this set of results.

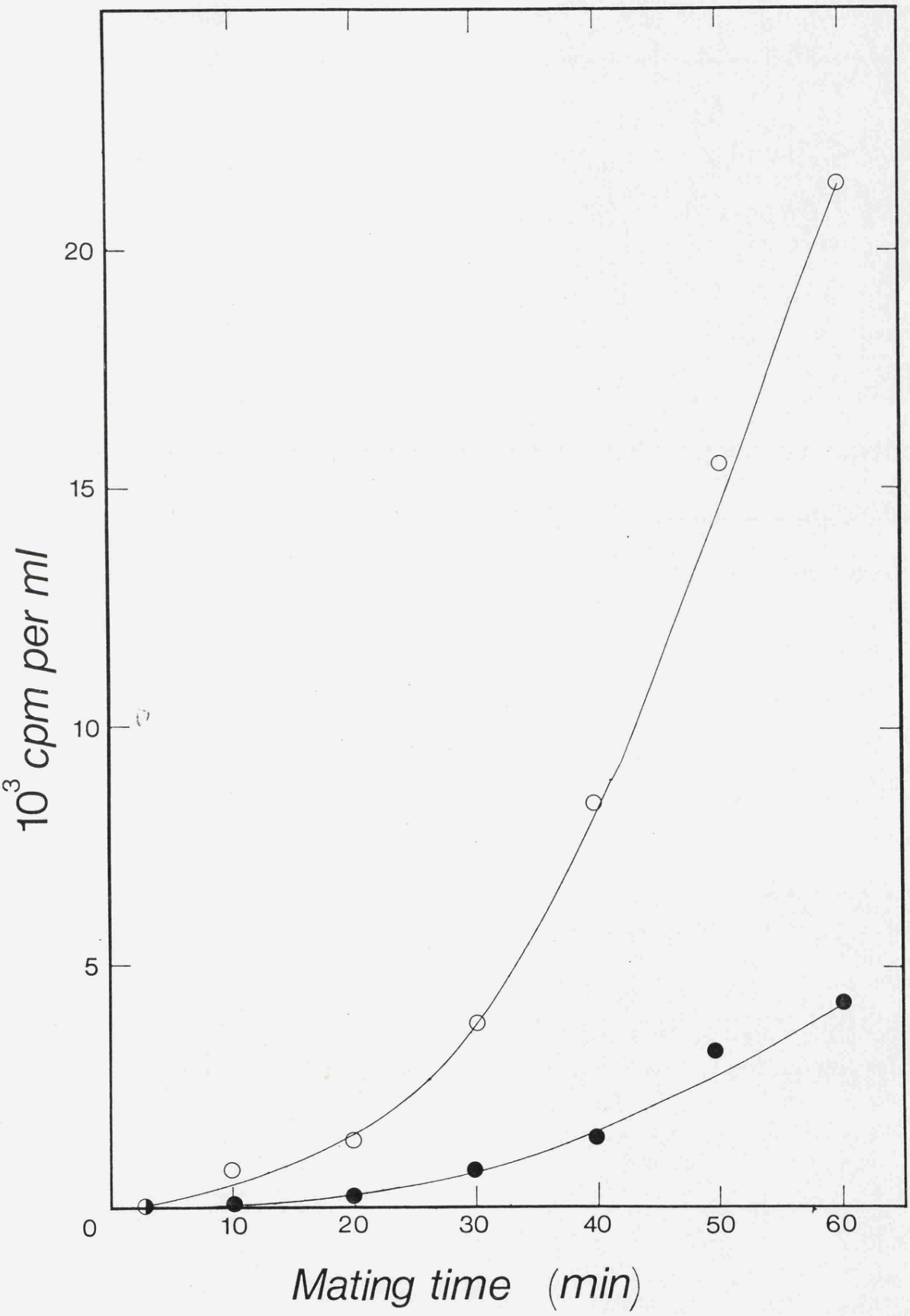
The data obtained using this method indicate that recovery of chromosomal replication is dependent on a functional conjugation system, since inhibition of DNA transfer to recipients carrying a cloned entry exclusion (*eex*) gene of ColIb-P9 caused a corresponding reduction of DNA synthesis in both UV irradiated and unirradiated recipients. Although experiments have utilized rifampicin-resistant *dna*$^+$ donors of pLG221 to maximise levels of conjugative DNA synthesis, recovery does not appear to involve the transfer of either bacterial primase or RNA polymerase from donor cells. The observation that donors harbouring the Sog$^-$ plasmid pLG250 were unable to restore DNA synthesis in *dnaG* recipient cells implicates plasmid primase as the enzyme initiating the recovery process.

6.2 dnaG protein of donor cells is not responsible for the recovery of chromosomal DNA synthesis in mated dnaG3 recipient cells

Conjugative DNA synthesis in UV irradiated and unirradiated *dnaG* recipients during rifampicin-treated matings with *dnaG* donor cells of pLG221 is described in Figure 6-1. It has been shown previously (Boulnois and Wilkins, 1979) that a 10 min incubation period at 41°C is sufficient to inactivate bacterial primase activity in temperature-sensitive BW86 (*dnaG3*) mutants and this is confirmed by the small amount of DNA synthesis detected in the unmated cells in Figure 6-1. BW101 donor bacteria carry

Figure 6-1 : Effect of UV irradiation on conjugative DNA synthesis in rifampicin-treated dnaG3 recipients during matings with dnaG3 donors of pLG221

BW101 (pLG221) was mated with rifampicin-sensitive BW86 recipients at 41°C in the presence of [^{14}C]thymine (0.5 μCi μg^{-1}) and rifampicin (100μgml^{-1}). Recipient bacteria were either irradiated with 400Jm^{-2} of UV light immediately prior to mating (•) or left untreated (o). Samples (0.5 ml) from mating mixtures were taken into TCA, and insoluble radioactivity determined. Control mixtures, consisting of parental strains, were incubated separately, and treated as for mating cultures. All control values, with the exception of unirradiated recipients, were less than 500 cpm ml^{-1} after 1h of incubation. Untreated BW86 progressively incorporated [^{14}C]thymine to a value of 1,400 cpm ml^{-1}. Results are presented after subtraction of the control values.


20
15
10
5
0
10
20
30
40
50
60
10^3 cpm per ml
Mating time (min)

the same dnaG3 mutation as BW86, since all dnaG3 strains used in this thesis were derived from the ancestral strain NY73, and so both parental cultures were held at 41°C for 10 min immediately prior to mating to achieve inactivation of bacterial primase.

Figure 6-1 shows that the relative amounts of conjugative DNA synthesis in UV irradiated and unirradiated dnaG recipients mated with BW101 donor cells are similar to those obtained with dna$^+$ donors (Figure 5-1), with approximately five-fold more synthesis detected in the unirradiated recipients. However, it should be noted that the absolute amounts of [^{14}C]thymine incorporated by BW86 recipients in these two sets of experiments differ. Irradiated and unirradiated recipient cells in matings with dnaG donors incorporated approximately 69% and 55% respectively of the radioactivity measured in equivalent matings with dna$^+$ donors. The dnaG3, rpoB donor strain BW101 used in Figure 6-1 was derived from BW82 (dnaG3, tdk) by selection on nutrient agar containing rifampicin (100μgml^{-1}), and pLG221 introduced into 4 purified clones by conjugation with BW98. All four BW101 (pLG221) strains grew more slowly than BW82 (pLG221) or BW96 (pLG221) at the permissive temperature of 31°C in both Luria broth and the salts-glucose-casamino acids (SGC) medium used in these conjugative DNA synthesis experiments, each exhibiting a doubling-time of about 1h 35 min in mid-exponential growth phase. This contrasts with mass-doubling times of 50 min and 1h for BW96 (pLG221) and BW82 (pLG221) at 31°C, and suggests that the ability of BW101 (pLG221) to support a normal rate of DNA replication at the mating temperature of 41°C is impaired. Since it is reasonable to assume that slow-growing donor bacteria will show reduced fertility compared to fast-growing cells, the lower levels of DNA synthesis detected in Figure 6-1 are attributed to the physiological

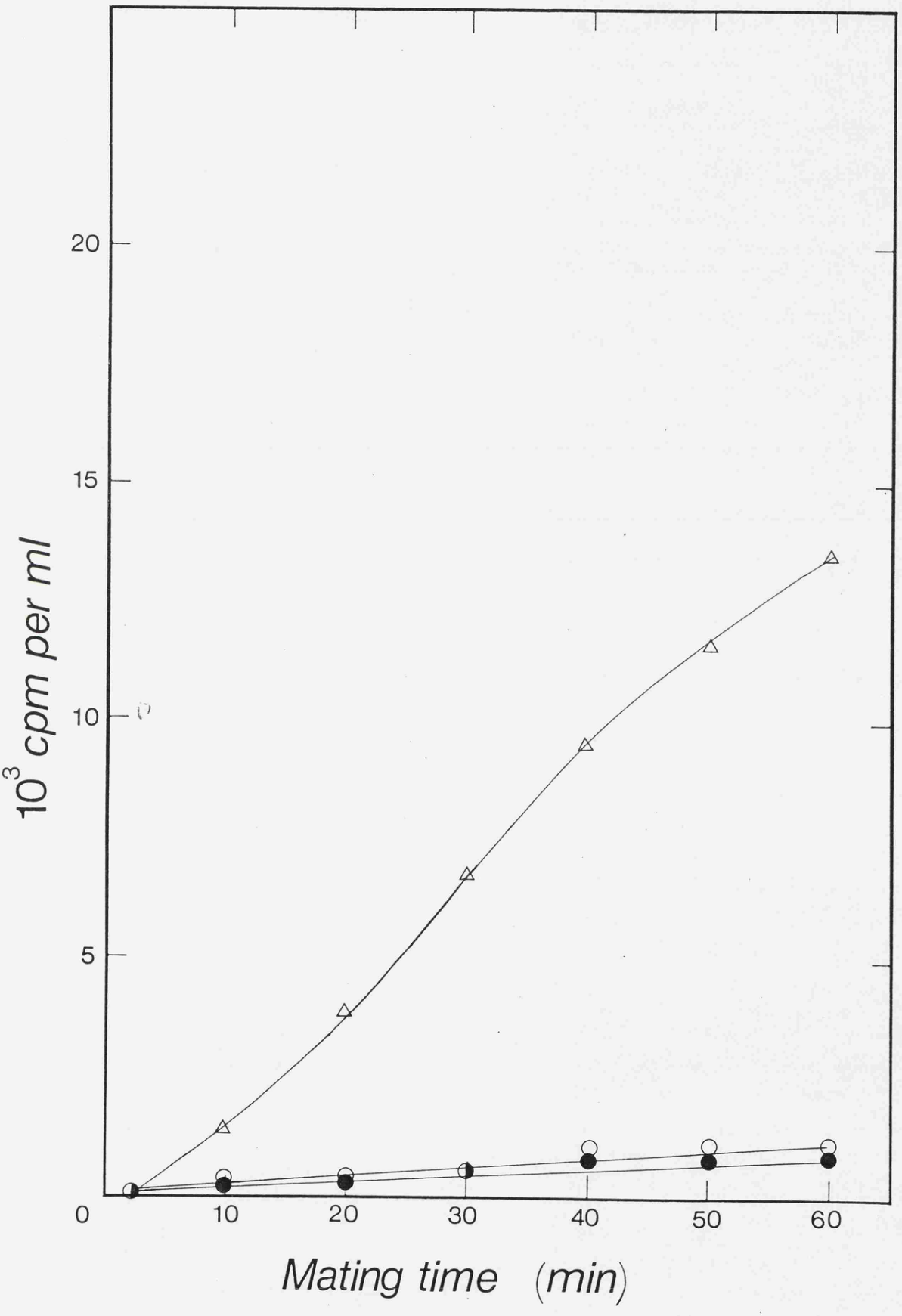
state of BW101 (pLG221) donor cells. Thus, it is inferred from the results in Figure 6-1 that recovery of chromosomal DNA synthesis by recipients does not require the activity of dnaG protein, supplied either by the recipient or the donor bacterium.

6.3 Recovery of chromosomal DNA synthesis in rifampicin-treated dnaG recipient cells requires the activity of plasmid DNA primase produced in the donor bacterium

Use of the Sog^- plasmid pLG250 in conjugative DNA synthesis experiments with UV treated and untreated dnaG recipient cells (Figure 6-2) reduced [^{14}C]thymine incorporation in both types of recipients to residual levels, comparable to the values attained by separately incubated cultures of BW86. These results show that the presence of a functional sog gene is required for the recovery process. However, it remains possible that the recovery is mediated by plasmid primase made in recipient cells following plasmid transfer; this might be effected by residual activity of the RNA polymerase in the rifampicin-treated cells, or by the transfer of drug-resistant enzyme from the rpoB donors. These possibilities were pursued by the introduction of the non-mobilisable pBR325-based Sog^+ plasmid pLG215 into BW96 (pLG250) donor cells. Previous experiments (Chapter 3) have shown that this plasmid is transferred during conjugation at the low frequency of about 10^{-4}, which may be regarded as insignificant for the purposes of conjugative DNA synthesis experiments. This co-transfer of pLG215 with pLG221 presumably reflects recA-mediated formation of cointegrates of the two plasmids, perhaps utilising the regions of homology shared by their respective sog genes, since co-transfer was not detected during equivalent matings with recA donors (Chapter 7).

Figure 6-2 : Measurements of conjugative DNA synthesis in UV irradiated and unirradiated dnaG recipients of pLG250

BW96 donors of the Sog^- Iα plasmid pLG250 were mated with either UV treated (•) or unirradiated (o) BW86 recipients in the presence of 100 $\mu g ml^{-1}$ of rifampicin. The mating conditions and procedure used for these experiments were as described for Figure 6-1. Parental control mixtures for each mating were incubated separately, and incorporated about 400 cpm ml^{-1} after 1h, with the exception of unirradiated BW86 (1,400 cpm ml^{-1}). These values have been subtracted from the presented data. As a positive control, BW96 (pLG221) was mated with irradiated BW86 (Δ).

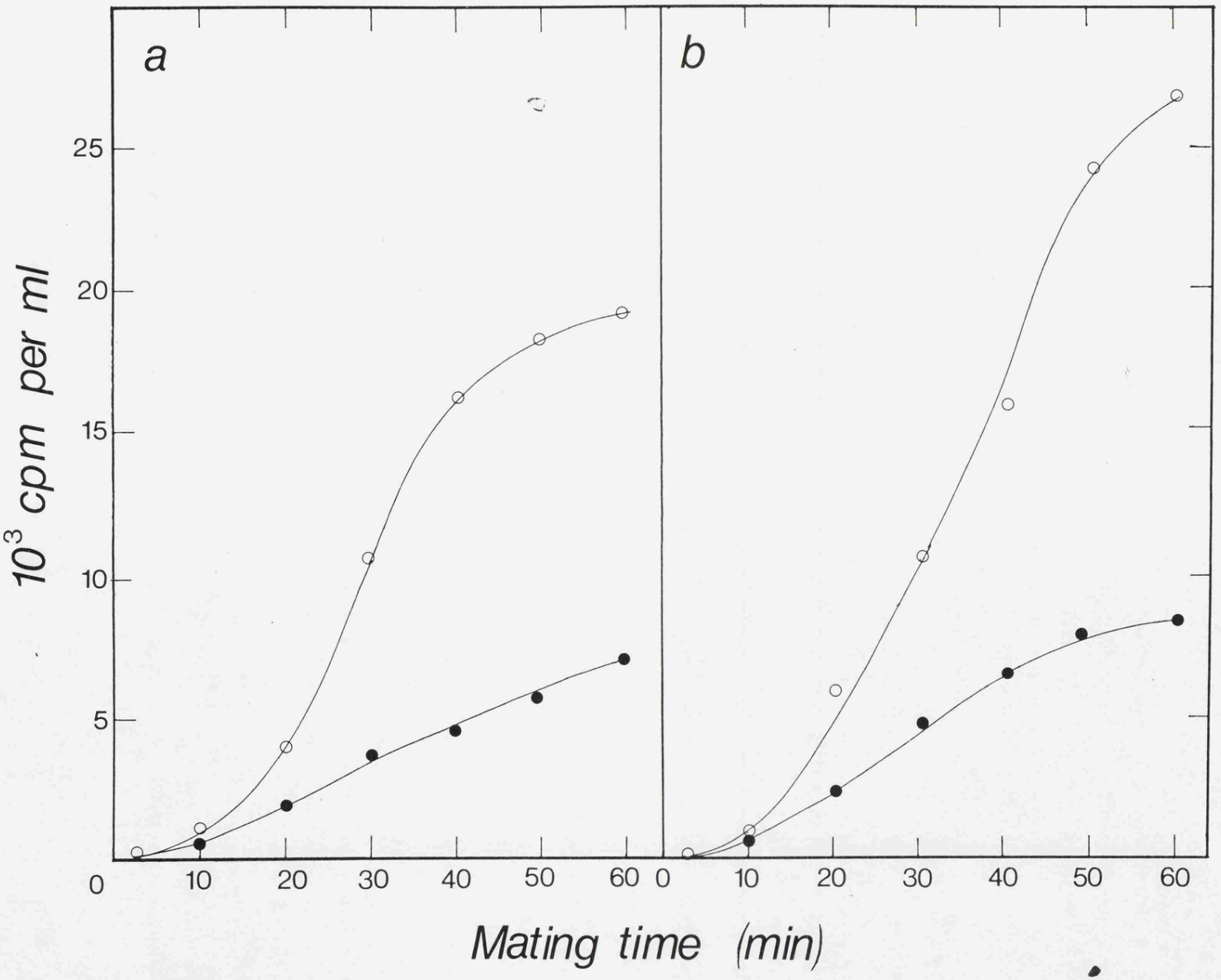

20
15
10
5
0
10
20
30
40
50
60
10^3 cpm per ml
Mating time (min)

The results described in Figure 6-3a for experiments using BW96 (pLG250, pLG215) donor cells in rifampicin-treated matings indicate that the presence of the Sog^+ recombinant plasmid is able to complement the inability of pLG250 to rectify dnaG-deficient chromosomal DNA synthesis in BW86 recipients. This finding supports the conclusion derived from Figure 6-2 that an intact sog gene is required for the recovery process. Furthermore, since the presence of this gene is required only in the donor strain in such matings, it is implied that recovery does not proceed by transcription of copies of sog transferred to recipients, either by the activity of rifampicin-resistant RNA polymerase transferred from the rpoB donors, or the hypothetical residual activity of the recipient drug-sensitive enzyme. Thus, these results indicate that transfer of plasmid primase synthesised in the donor cell, rather than the transmission of its structural gene, is required for dnaG-independent chromosomal DNA replication by recipient bacteria.

Experiments substituting the Sog^+ plasmid pLG214 for pLG215 in donor cells (Figure 6-3b) reinforce the above conclusions, as they also demonstrate the synthesis of chromosomal DNA in unirradiated recipients. pLG214 has been described in Chapter 3; it carries a deletion mutation in the cloned sog gene, which results in the production of a truncated primase that is about one third of the normal size and is more active than the wild-type enzyme in discontinuous bacterial DNA replication. The additional DNA synthesis depicted in Figure 6-3b compared with that in Figure 6-3a therefore confirms that plasmid primase is the enzyme engaged in recipient chromosomal DNA synthesis. It also indicates that transfer of only a low molecular weight form of plasmid primase is required for the priming reaction on the recipient chromosome.

Figure 6-3 : Conjugative DNA synthesis during pLG250-mediated matings involving the presence of Sog^+ recombinant plasmids in donor cells

BW96 donors of pLG250 containing either (a) the Sog^+ recombinant plasmid pLG215 or (b) the Sog^+ recombinant pLG214 were mated with unirradiated (o) and UV treated (•) BW86 recipients at 41°C in the presence of rifampicin ($100\mu g ml^{-1}$) and [^{14}C]thymine ($0.5\ \mu Ci\ \mu g^{-1}$). Conjugatively-occurring DNA synthesis in recipients was measured as before (Figure 6-1). Results are presented after subtraction of separately-incubated parental control values, which, after 1h at 41°C were approximately as follows: donors; 50 cpm ml^{-1}; UV irradiated recipients 120 cpm ml^{-1}; unirradiated recipients 1050 cpm ml^{-1}. Donor strains were grown prior to mating in the presence of tetracycline ($7.5\ \mu g ml^{-1}$) to select for the presence of pBR325-based Sog^+ recombinant plasmids. The antibiotic was removed immediately before the onset of mating by sedimentation and gentle resuspension of cells in SGC medium.


a
b
25
20
15
10
5
0
10
20
30
40
50
60
10^3 cpm per ml
Mating time (min)

6.4 DNA transfer is required for the transfer of plasmid primase to recipient cells

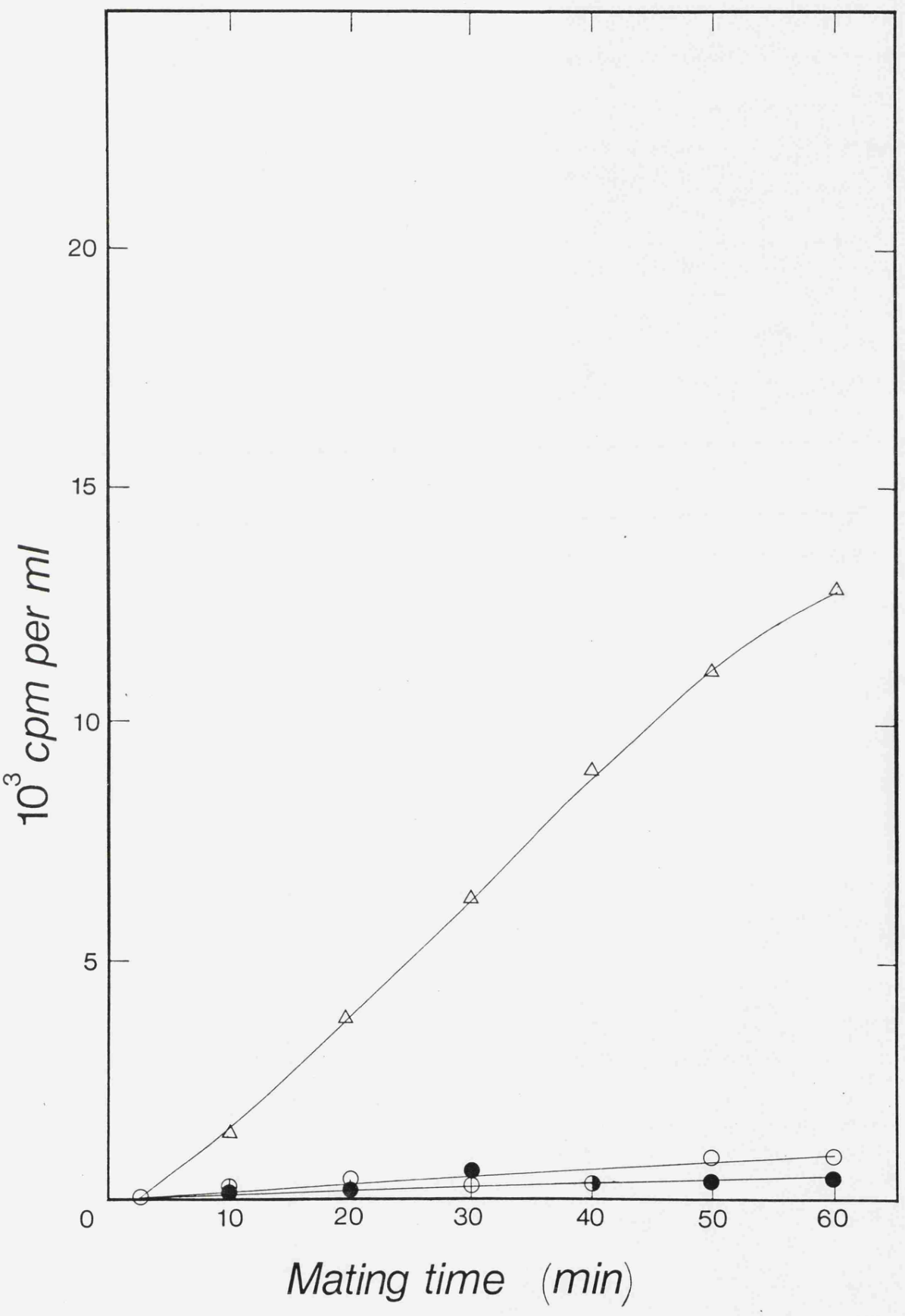
The presence of the pBR325-based recombinant plasmid pLG252, which carries a cloned entry exclusion gene from ColIb-P9*drd-1*, in rifampicin treated recipient bacteria has been shown significantly to reduce DNA transfer (Table 5-1). Measurement of conjugative DNA synthesis in both UV treated and untreated BW86 (pLG252) recipient cells under the same mating conditions is described in Figure 6-4, and the data show that, if DNA transfer is abolished by an entry exclusion mechanism, then both plasmid and chromosomal DNA synthesis in *dnaG* recipients is similarly affected. Thus an effective DNA transfer system is another requirement for the recovery process.

6.5 Discussion

In order to provide a convenient comparison of results described in this chapter, the relative amounts of [^{14}C]thymine incorporated by recipient strains in these experiments after 50 min of mating is tabulated in Table 6-1. The proportion of chromosomal DNA labelled in unirradiated recipients by this time was calculated by correcting the difference between the number of counts incorporated by unirradiated and UV-treated recipients in isogenic matings for an 11% decrease in DNA transfer to UV damaged cells (Table 5-1). Since the closely related *E.coli* K12 strains used were mated under identical mating conditions of temperature, rifampicin concentration and specific activity of [^{14}C]thymine, this form of presentation should allow valid comparison of the results. The data is clearly supportive of the hypothesis that recovery of recipient chromosomal DNA synthesis by BW86 recipients (Table 6-1; line 1) is due to the transfer of plasmid primase in an active form from donor cells.

Figure 6-4 : Conjugative DNA synthesis in UV irradiated and unirradiated dnaG recipient bacteria containing a cloned entry exclusion gene (eex) of ColIb-P9

BW96 donors of pLG221 were mated with UV treated (•) and untreated (o) BW86 (pLG252; Eex^+) recipients for 1h at 41°C in the presence of rifampicin (100 $\mu g ml^{-1}$) and [^{14}C]thymine (0.5 $\mu Ci\ \mu g^{-1}$), and DNA synthesis in these recipients examined as described for Figure 6-1. Recipients were grown in the presence of tetracycline (7.5 $\mu g ml^{-1}$) to select for the maintenance of pLG252, and the antibiotic removed with the exogeneous thymine in these cultures prior to mating. As a positive control, BW96 (pLG221) was mated with UV irradiated BW86 recipients (Δ). Results are presented after subtraction of parental control values, which each amounted to less than 450 cpm ml^{-1} after 60 min, with the exception of unirradiated BW86 (pLG252) which had assimilated about 1050 cpm ml^{-1} by this time.


20
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0
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20
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40
50
60
10^3 cpm per ml
Mating time (min)

That the presence of functional sog gene product is required for this recovery process is shown by the data in line 3 of Table 6-1. It is apparent that recovery of recipient chromosomal DNA synthesis does not involve the activity of bacterial primase transferred from the donor (line 2), since insignificant counts were incorporated by unirradiated recipients mated with dna$^+$ donors of pLG250 (line 3). Furthermore, since this deficiency can be rectified by the inclusion of a non-mobilisable Sog$^+$ recombinant plasmid in the donor strain (lines 4 and 5), we can also conclude that rifampicin-resistant RNA polymerase is not transferred from the donor to promote the recovery process either by acting directly on the recipient chromosome or by transcribing copies of sog transferred during the mating. Taken together, these results confirm that transfer of molecules of plasmid primase, in an active form, rather than of sog, is required for the recovery of dnaG-deficient recipient DNA synthesis.

The results also allow the identification of certain proteins involved in DNA replication that do not appear to be transferred in an active form during conjugation. As well as dnaG protein and RNA polymerase, the comparable amounts of DNA synthesis detected in unirradiated and UV irradiated dnaB recipients of pLG221 (line 7) imply that dnaB protein is not transferred during conjugation, since the presence of active enzyme in the donor cannot promote rescue of dnaB-deficient chromosomal DNA synthesis in the recipient. Similarly, lack of conjugative DNA synthesis in dnaE recipients of ColIb-P9drd-1 (Wilkins and Hollom, 1974) indicate that DNA polymerase III holoenzyme also cannot be transmitted between mating cells. These conclusions reinforce the opinion, first suggested by the upper limit for donor cell protein transfer of 0.3% (Silver and Ozeki, 1962), that conjugative transfer of proteins must be a selective process, involving only specific molecules.

Table 6-1 DNA synthesis in UV irradiated and unirradiated recipient bacteria

The derivation of the data is given in the discussion (section 6-5) to this chapter and conditions used for experiments are described in the Figure legends referenced in column 3.

[a] Data represents DNA synthesis after 50 min of mating in each culture.

[b] Values indicate the proportion of DNA synthesised in the unirradiated recipient cells that is estimated to be chromosomal. Estimates are corrected to allow for an 11% decrease in DNA transfer to UV treated recipients compared to equivalent untreated matings.

[c] NEG = negligible chromosomal DNA synthesis in unirradiated recipients.

Table 6-1

Strain		Reference for data (Figure)	DNA synthesis (cpm ml^{-1})[a]			
Donor	Recipient		+UV	-UV	Difference	% chromosomal DNA synthesis -UV[b]
BW96 *dna*$^+$ (pLG221 Sog$^+$)	BW86 *dnaG*	5-1	11,300	38,500	27,200	67
BW101 *dnaG* (pLG221 Sog$^+$)	BW86 *dnaG*	6-1	3,300	15,800	12,500	77
BW96 *dna*$^+$ (pLG250 Sog$^-$)	BW86 *dnaG*	6-2	900	1,400	500	NEG[c]
BW96 *dna*$^+$ (pLG250 Sog$^-$, pLG215 Sog$^+$)	BW86 *dnaG*	6-3a	5,800	18,400	12,400	65
BW96 *dna*$^+$ (pLG250 Sog$^-$, pLG214 Sog$^+$)	BW86 *dnaG*	6-3b	8,100	24,200	16,100	63
BW96 *dna*$^+$ (pLG221 Sog$^+$)	BW86 *dnaG* (pLG252 Eex$^+$)	6-4	700	1,300	600	NEG
BW96 *dna*$^+$ (pLG221 Sog$^+$)	BW69 *dnaB*	5-2	5,000	6,800	1,800	NEG

Since a functional conjugation system permitting the transfer of plasmid DNA is apparently necessary for the transmission of molecules of plasmid primase, as demonstrated by the absence of DNA synthesis in Eex^+ recipients (line 6), it is interesting to speculate upon the relationship between DNA and protein transfer. One strategy that the plasmid could utilise to attain the selective transfer of specific proteins could involve attachment of these protein molecules to the plasmid DNA strand destined for transfer to recipients. Thus only DNA-binding proteins specific for ColIb-P9 DNA would be transmitted to recipient cells, and since plasmid primase has been shown to bind to single-stranded DNA (Lanka et al., 1979), sog gene product would be included in this general class of enzymes. This mechanism could also be exploited to control the amount of protein conducted relative to the amount of DNA transferred, by the limitation or specifity of protein binding sites specified by the plasmid DNA, and would presumably rely upon DNA transfer as the driving force for transportation of protein molecules between the mating cells.

Further consideration of this and alternative models, and the candidacy of other proteins that could be included in this class, is delayed until the final chapter of this thesis, but it is important to note at this stage that the truncated 87,000 M.W. primase specified by pLG214 is able to promote chromosomal DNA recovery in recipients with a greater efficiency than the pLG215-encoded protein (lines 4 and 5). However, neither BW96 (pLG250, pLG215) nor BW96 (pLG250, pLG214) donors are able to promote levels of recovery in unirradiated recipients equal to that achieved by BW96 (pLG221), and this lack of total complementation by the recombinant plasmids may reflect competition of some kind between the mutant and wild-type forms of sog protein in the rescue process. Since preliminary

measurements of conjugative DNA synthesis in UV-treated BW86 recipients containing the Sog$^-$ recombinant plasmid pLG217 and plasmid-free BW86 cells (Chapter 3) mated with BW96 (pLG221 Sog$^+$) donors detected no competition between the two forms of the enzyme in recipients (data not shown), the failure of pLG215 and pLG214 to complement fully the sog mutation of pLG250 may reflect a competition for the hypothetical recognition sites on the transferred plasmid DNA strand. The smaller size of truncated sog product may account for the greater efficiency of rescue of chromosomal DNA synthesis in mated recipients than caused by the wild-type pLG215 product, since this might be an advantage in competing with the larger pLG250-specified protein for the same DNA-binding sites. Thus the transfer properties of plasmid primase must be specified by the portion of sog lying outside the 1.5 Kb deletion of pLG214.

Although the results of this, and the preceding chapter, indicate plasmid primase transfer, they do not by themselves rule out the possibility of concomitant transfer of RNA primers synthesised in the donor. Since the primers synthesised by plasmid primase in vitro comprise no more than about 10 ribonucleotides (Lanka and Furste, 1984), it is thought that these would be too small to be transmitted as naked RNA-DNA duplexes. Gillespie (1966) showed that RNA molecules of around 12 nucleotides were required for efficient formation of stable duplexes with single-stranded DNA under physiological conditions of temperature, ionic strength and pH. Thus, if primers are synthesised on the transferred strand of pLG221 in the donor, it is reasonable to propose that they would require some form of stabilisation. The possibility of plasmid primase fulfilling this function has already been raised earlier in Chapter 5.

There are a number of reasons why rescue of recipient chromosomal DNA synthesis is unlikely to be mediated by mobile, trans-acting RNA primer molecules generated by plasmid primase. Re-utilisation of primers after they have initiated complementary strand synthesis would require some form of processing at RNA-DNA junctions, since primers are covalently attached to nascent DNA strands after their extension by DNA polymerase from both in vivo (Sugino et al., 1972) and in vitro (Sugino and Okazaki, 1973). Thus, in order to prime a second time, the primer oligoribonucleotide would have to be cleaved from the nascent DNA and displaced from the template. No precedent exists for such a mechanism; instead, primers are thought to be degraded following extension by the exonucleolytic activities of enzymes such as DNA polymerase I or the gene 6 protein of phage T7 (Itoh and Tomizawa, 1978; 1980; Richardson et al., 1979; Shinozaki and Okazaki, 1977). Thus, a primer molecule is unlikely to prime more than a single initiation event. The notion of transferred primers acting in trans on chromosomal DNA, before they are consumed in complementary plasmid strand synthesis, is open to criticism on the grounds that the small molecules generated by plasmid primase would be prone to attack by nucleases before they could anneal to chromosomal template. It is also worth noting that this scheme would require homology between priming sites on both pLG221 and the E.coli chromosome, and that at least a portion of the chromosome be available in a single-strand form to allow association.

Only one species of trans-acting RNA molecule has so far been demonstrated to participate in the initiation of plasmid DNA synthesis, namely the 110bp RNAI molecule involved in events at the ColE1 origin of replication (Tomizawa and Itoh, 1982; Selzer et al., 1983). However, this RNA acts only in a regulative role by pairing with the RNAII molecule, and

it is stressed that RNAII, and not RNAI, functions as a primer. Furthermore, RNAII is subsequently degraded by DNA polymerase I (Itoh and Tomizawa, 1978; 1980).

It is therefore concluded that plasmid primase, transmitted from the donor cell, is responsible for the priming of chromosomal DNA synthesis in dnaG recipients of pLG221. Further consideration of this conclusion is given in Chapter 8.

CHAPTER 7

Isolation of the origin of transfer and an entry exclusion gene(s) of ColIb-P9drd-1

"To explain all nature is too difficult a task for any one man or even for any one age. 'Tis much better to do a little with certainty, and leave the rest for others that come after you, than to explain all things".

Isaac Newton

7.1 Introduction

In contrast to the previous chapters, which concentrated on the involvement of plasmid primase in conjugation, this final section of results is concerned with the transfer origin (*oriT*) and entry exclusion (Eex) system of ColIb-P9. These aspects of bacterial conjugation were considered in Chapter 1. This chapter therefore describes a side project initiated in my first year of experimental work at Leicester with the intention of isolating recombinant plasmids specifying the transfer origin and entry exclusion system of ColIb-P9*drd-1*. Of particular interact was the possible involvement of exclusion in transfer limitation (Boulnois and Wilkins, 1978). Although Eex^+ recombinants were successfully identified, it was decided that effort would be more profitably directed towards the main topic of plasmid primase, and so this hypothesis was not tested. However, one Eex^+ recombinant plasmid, pLG252, proved invaluable as a convenient control to prevent DNA transfer between mating bacteria in experiments concerning plasmid primase, and has already been referred to in the previous results chapters of this thesis.

An *oriT*-specific event, in which a single nick is generated in the plasmid strand destined for transfer, is required for the initiation of DNA transfer, while entry exclusion acts to reduce the ability of plasmid-containing cells to act as recipients in matings with donor cells harbouring an identical or closely-related plasmid with the same exclusion system. Entry exclusion is distinct from plasmid incompatibility; it acts in the initial stages of mating and prevents transfer of DNA to recipients, while incompatibility functions after DNA transfer to cause the segregation of closely-related plasmids (Novick, 1969). It was demonstrated in this laboratory (Boulnois and Wilkins, 1978) that a ColIb-P9-specified protein(s) synthesised in newly-infected recipients was responsible for limiting the amount of plasmid DNA transferred during mating. This protein(s) was proposed to also function in the entry exclusion process since it apparently destroys the competence of transconjugants to act as recipients. The inhibition of this process by the use of rifampicin to prevent transcription of plasmid DNA in drug-sensitive recipients forms the basis for the convenient amplification of DNA transfer used in the previous experimental chapters.

This chapter describes the identification of recombinant plasmids specifying the *oriT* region and *eex* gene(s) of ColIb-P9*drd-1* from a library of pBR325-based recombinant plasmids carrying *Eco*R1 endonuclease-generated fragments of ColIb-P9*drd-1* DNA, constructed by B.M. Wilkins. Individual clones harbouring recombinant plasmids were used in matings with donors of the ColIb-P9*drd-1* derivative pLG221, and measurement of transconjugant formation revealed that three out of 217 such clones possesses an Eex^+ phenotype. Another recombinant plasmid specifying *oriT* was identified by the high frequency with which it was mobilised by

pLG221 in a subsequent screening procedure. The initial characterisation and behaviour of these plasmids during pLG221-mediated conjugation is reported.

7.2 Isolation of an entry exclusion-determining DNA fragment and the origin of transfer of ColIb-P9drd-1

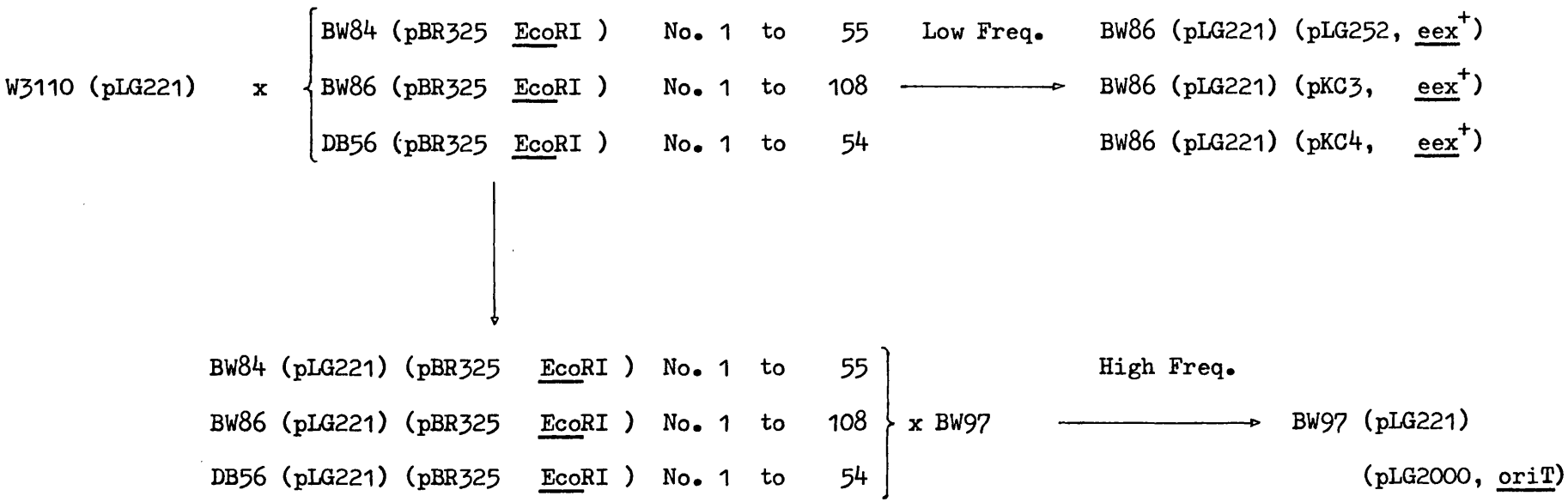
A library of transformant strains harbouring recombinant plasmids was screened for the presence of the entry exclusion gene(s) and origin of transfer of ColIb-P9drd-1. This bank of recombinant plasmids was previously constructed by B.M. Wilkins by the ligation of EcoR1-generated restriction fragments of ColIb-P9drd-1 DNA into the unique EcoR1 site in the chloramphenicol acetyltransferase resistance (Cm^R) gene of the vector plasmid pBR325 (Bolivar, 1978). The resultant recombinant plasmids, specifying resistance to tetracycline and ampicillin, were introduced into three strains of E.coli K-12 (BW84, BW86 and DB56) by transformation, and individual $Cm^S Tc^R Ap^R$ transformants isolated to generate a clone bank. The following strategy (depicted in Figure 7-1) was used to screen this bank for recombinants specifying the eex gene(s) and oriT of ColI.

pLG221 was mated from W3110 into each transformant strain in broth matings carried out in 10 ml test-tubes as described in Figure 7-1 in order to determine the exclusion phenotype of individual recipients. The frequency at which transconjugants were formed in these matings was determined by spotting serial dilutions from each mating mixture on appropriate selective media. 217 clones were screened in this way, and the majority yielded transconjugants at a frequency of 2.14×10^{8} ($\pm$ 8.1×10^{7}) cells ml^{-1}. However, in 3 of these matings, transconjugants arose at lower frequencies of between 1.8×10^{4} to 3.6×10^{4} per ml and the recombinant

Figure 7-1 : Screening procedure for the identification of recombinant plasmids specifying Eex and oriT

Donor and recipient strains were grown for 3-4 mass doublings at 37 and 30°C respectively and 0.2 ml volumes of each mixed and shaken gently for 30 min at 30°C. 20 µl samples of serial dilutions of mating mixtures were spotted onto selective agar to quantify transconjugant formation. Transconjugants generated in matings using W3110 (pLG221) as donor were assayed on nutrient agar containing thymine ($20\mu gml^{-1}$), Sm ($100\mu gml^{-1}$), Tc ($7.5\mu gml^{-1}$) and Kan ($50\mu gml^{-1}$) for BW84 and BW86 strains, and Tc and Kan for DB56 strains. Transconjugants isolated from these matings were subsequently used as donors in matings with BW97 as recipient, and BW97 transconjugants assayed on nutrient agar containing Nal ($50\mu gml^{-1}$) and Tc. The rationale for this strategy is discussed in section 7.2.

Figure 7-1


W3110 (pLG221) x
BW84 (pBR325 EcoRI) No. 1 to 55
BW86 (pBR325 EcoRI) No. 1 to 108
DB56 (pBR325 EcoRI) No. 1 to 54
Low Freq.
BW86 (pLG221) (pLG252, eex+)
BW86 (pLG221) (pKC3, eex+)
BW86 (pLG221) (pKC4, eex+)
BW84 (pLG221) (pBR325 EcoRI) No. 1 to 55
BW86 (pLG221) (pBR325 EcoRI) No. 1 to 108
DB56 (pLG221) (pBR325 EcoRI) No. 1 to 54
x BW97
High Freq.
BW97 (pLG221)
(pLG2000, oriT)

plasmids harboured by the corresponding recipient strains were tentatively assigned as exclusion-proficient and designated pKC2, pKC3 and pKC4; pKC2 was later re-designated as pLG252.

The 217 $Kan^R Tc^R$ transconjugants generated in the above procedure were next used as donors in subsequent matings with BW97 as the recipient strain to identify recombinant plasmids specifying oriT by virtue their efficient mobilisation by the conjugative plasmid pLG221. The majority of donors in these matings transferred the respective recombinants to recipients at frequencies no higher than around 3.3×10^6 per ml, but one, out of 192 individual matings, transferred Tc^R to recipients at about 1.1×10^9. The recombinant plasmid mobilised at this high frequency was designated pLG2000 (originally assigned as pKC1).

7.3 Characterisation of pLG2000

The strategy of screening for recombinant plasmids containing a cloned oriT region by their efficient co-transfer from bacteria harbouring a compatible conjugative plasmid to provide trans-acting functions has been used to isolate the transfer origins of a number of plasmids (F; Achtman et al., 1978; Thompson and Achtman, 1978; Everett and Willetts, 1982; R1, R100 and R46; see Willetts and Wilkins, 1984; RK2; Guiney and Yakobson, 1983). This approach was successful in the identification of pLG2000, a pBR325-based recombinant plasmid specifying the transfer origin of ColIb-P9drd-1, which was mobilised at a frequency 300-fold greater than the other recombinant plasmids contained in the clone bank (see previous section). Although the vector pBR325 is non-mobilisable in the sense that it lacks an oriT region (Bolivar, 1978), pBR325-specified Tc^R was inherited by recipients of pLG221 at a frequency of about one $Tc^R Kan^R$

transconjugant per 1000 Kan^R transconjugants. Since the donor strains used in these matings were *rec*$^+$, this background level of transfer was attributed to *rec*-mediated fusion involving the regions of homology between the recombinant plasmid and pLG221 to form a co-integrate molecule.

In order to confirm both the presence of *oriT* on pKC1 and the nature of the low general level of recombinant transfer, plasmid DNA was extracted from BW86(pLG2000) and from another transformant from the original clone bank, BW86(pKC5). The recombinant plasmid possessed by the latter strain is characteristic of the majority of plasmids contained in this bank, and is tranferred from pLG221 donors only at low frequency. These two plasmids were used to transform the *recA* strain J08, and pLG221 was mated into Tc^R Ap^R transformants to generate J08 (pLG221) (pLG2000) and J08 (pLG221) (pKC5). These strains were used to determine the effect of the host *recA* lesion on the co-transfer of the recombinant plasmids with pLG221.

Table 7-1 shows the yields of transconjugants obtained from 45 min broth matings using *rec*$^+$ and *recA* donor cells harbouring pLG221 and either pLG2000 or the corresponding control plasmid. It can be seen that transfer of the pKC5 control was only 0.13% of the frequency of pLG221, and that this mobilisation is dependent on the *rec* function of the donor, since no Tc^R transconjugants were obtained using J08 *recA* donor cells. In contrast, pLG2000 was mobilised by pLG221 at a frequency of about 40% in *recA* matings, thus confirming that this recombinant contains a functional *oriT* region from ColIb-P9*drd-1*. Endonuclease restriction of pLG2000 DNA (Figure 7-2) showed that this recombinant plasmid has three EcoR1 fragments of ColIb-P9 *drd-1* DNA of different sizes. It therefore appears that pLG-2000 carries two other cloned fragments in addition to that specifying *oriT*.

Table 7-1 : Mobilisation of pLG2000 by pLG221 from Rec^+ and RecA donor cells

Mating	Transconjugants per ml		Mobilisation frequency
	Kan^R	$Kan^R Tc^R$	
a) rec^+			
BW86 (pLG221) (pLG2000) x BW97	ND	5.65×10^7	
BW86 (pLG221) (pKC5) x BW97	9.74×10^7	1.26×10^5	0.0013
b) recA			
J08 (pLG221) (pLG2000) x BW97	5.27×10^7	2.08×10^7	0.395
J08 (pLG221) (pKC5) x BW97	5.48×10^7	0.0	0.0

Matings were carried out for 45 min in broth at 30°C with gentle shaking. Mating mixtures (6 ml), containing donors and recipients in a 1:5 ratio, were serially diluted and transconjugants harbouring pLG221 (Kan^R), pLG2000 (Tc^R) or pKC5 (Tc^R) were assayed by selection on kanamycin ($50 \mu g ml^{-1}$) and tetracycline ($7.5 \mu g ml^{-1}$). Plating media also contained nal. dixic acid ($50 \mu g ml^{-1}$) to provide selection against the donor strain. The frequency of mobilisation is defined as the yield of pLG2000-containing transconjugants harbouring the relevant recombinant plasmid, expressed as a proportion of transconjugants containing the conjugative plasmid pLG221. ND = not done.

Figure 7-2 Restriction endonuclease analysis of plasmids

Procedures and discussion are given in sections 2.8, 7.3 and 7.4. Tracks contain about 1μg of DNA as follows:

a) λEcoR1

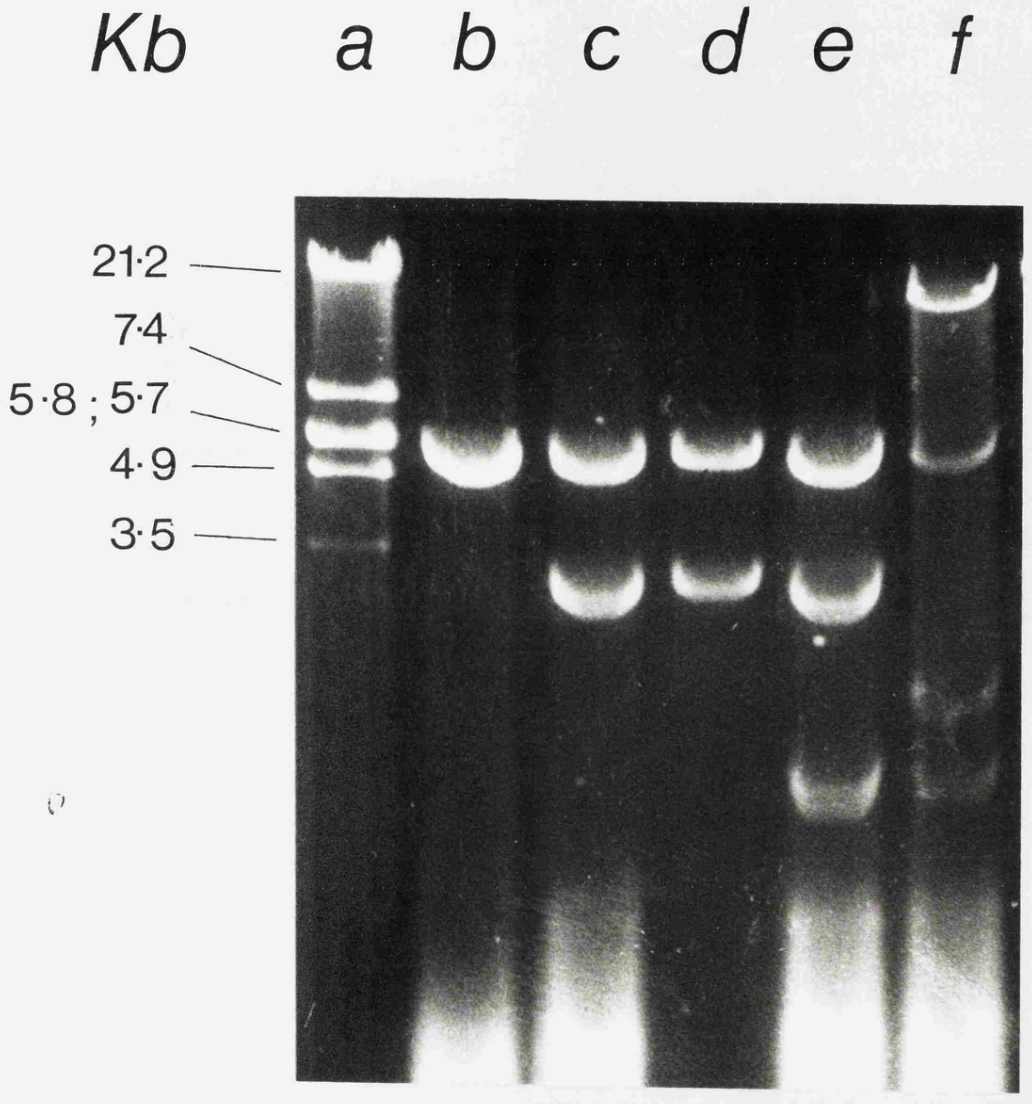
b) pBR325 EcoR1

c) pLG252 EcoR1

d) pKC3 EcoR1

e) pKC4 EcoR1

f) pLG2000 EcoR1


Kb
a
b
c
d
e
f
21·2
7·4
5·8 ; 5·7
4·9
3·5

Since pLG2000 was not investigated beyond its identification and ability to be mobilised in a recA-independent process, this plasmid will not be discussed further here. Subsequent studies involving the cloned oriT region of this plasmid were performed in this laboratory by C.C. Wymbs, and the reader is referred to his Ph.D thesis on this subject.

7.4 Characterisation of pLG252, pKC3 and pKC4

As described in section 7.2, three pBR325-based recombinant plasmids were tentatively identified as exclusion-proficient by their ability to reduce transconjugant formation. This ability is confirmed by the effect of these plasmids, designated pLG252, pKC3 and pKC4, on transconjugant formation in subsequent matings (Table 7.2). BW86 strains containing these plasmids were used as recipients in broth matings with W3110(pLG221) donor cells and the transconjugant yields compared with that obtained in parallel control matings using BW86(pBR325) as recipient, in order to determine the exclusion indices of these strains. The exclusion index of the strain under test was calculated as the ratio of the numbers of transconjugants formed per input donor in parallel Eex^- (control) and Eex^+ matings. As indicated in Table 7-2, pLG252, pKC3 and pKC4 exhibited strong exclusion of the Iα plasmid pLG221, with exclusion indices of between 316 and 208.

Agarose gel electrophoresis of EcoR1-digested pLG252, pKC3 and pKC4 DNA (Figure 7-2) revealed that all three plasmids possess a common EcoR1 fragment of ColIb-P9drd-1 DNA, and that pKC4 also carries an additional, smaller, restriction fragment. The size of the common fragment was measured at 3.5 Kb in a separate experiment, using a HindIII EcoR1 digest of phage λ DNA as a molecular weight standard (data not shown). Since pLG252 possesses only a single cloned EcoR1 fragment, it was selected as representative of the three Eex^+ recombinants.

Table 7-2

Duplicate broth matings were carried out at 30°C with gentle shaking. Mating mixtures (6ml) contained donor and recipient bacteria in a 1:5 ratio, as described in the text.

a - Values refer to the number of cells per ml of mating mixture. Donors and transconjugants were assayed respectively at t = 0 and t = 30 min by serial dilution and plating on nutrient agar containing kanamycin and tetracycline as appropriate. Samples of mating mixture were mechanically interrupted before being assayed for $Kan^R Tc^R$ transconjugants.

b - Exclusion indices are expressed as the number of transconjugants per input donor obtained in the control mating with pBR325-containing recipients divided by that obtained in the parallel mating involving the Eex^+ plasmid.

Table 7-2 : Determination of exclusion indices of pBR325-based eex^+ recombinant plasmids

Mating			Transconjugants [a]	Input donors [a]	Transconjugants per input donor	Exclusion Index [b]
Donor		Recipient				
W3110 (pLG221)	x	BW86 (pLG252)	1.3×10^5	3.4×10^7	3.8×10^{-3}	316
W3110 (pLG221)	x	BW86 (pBR325)	4.1×10^7	3.4×10^7	1.2	
W3110 (pLG221)	x	BW86 (pKC3)	1.8×10^5	3.8×10^7	4.7×10^{-3}	255
W3110 (pLG221)	x	BW86 (pBR325)	4.6×10^7	3.8×10^7	1.2	
W3110 (pLG221)	x	BW86 (pKC4)	1.8×10^5	4.7×10^7	3.8×10^{-3}	208
W3110 (pLG221)	x	BW86 (pBR325)	3.7×10^7	4.7×10^7	0.79	

7.5 Discussion

The recombinant plasmid pLG252 was judged to specify entry exclusion by its ability to reduce transconjugant formation by a factor of about 300 in matings using its host strain as recipient (Table 7-2). The possibility that pLG252 achieves this reduction in transconjugant frequency by incompatibility is ruled out by measurements of DNA transfer (Table 5-1) and conjugative DNA synthesis (Figure 6-4) in matings using pLG252-containing recipients. Both DNA transfer and conjugative DNA synthesis were virtually abolished in these matings, indicating that the exclusion system specified by both pLG252, and its parental plasmid ColIb-P9*drd-1*, acts by preventing DNA transfer. Boulnois and Wilkins (1978) demonstrated that a ColIb-P9-encoded product(s) limited the amount of plasmid DNA transferred to recipients during the course of mating. This transfer limitation involves the transcription of a plasmid gene(s) on the incoming DNA to synthesise a protein(s) which terminates DNA transfer after about 10 to 15 minutes of mating (Boulnois and Wilkins, 1978), presumably to prevent wasteful transmission of multiple copies of the plasmid. Since the cloned *eex* gene of ColIb-P9 *drd-1* carried by pLG252 prevents DNA transfer, the exclusion system of this plasmid may also function in transfer limitation. Following termination of transfer, the *eex* gene product(s) presumably prevents the newly-formed transconjugant from acting as a recipient in subsequent encounters with other ColIb-P9*drd-1*-containing cells.

One approach which might resolve the involvement of *eex* in transfer limitation is suggested by the strategy used in Chapter 3 to isolate Sog^- derivatives of pLG221. pLG252 DNA could be mutagenised, and resultant Eex^- lesions recombined into the homologous *eex* gene of pLG221 to create Eex^- Iα plasmids. Measurements of conjugative DNA synthesis in recipients during

matings mediated by these plasmids should reveal any involvement of *eex* product in transfer limitation, since one could expect to see an amplication of Eex^- Iα plasmid DNA transferred to recipients similar to that obtained by the use of rifampicin in matings using pLG221, if involvement exists. However the process may well prove to be more complicated, if other genes, which are not carried by pLG252, are involved in exclusion. Certainly, since ColIb-P9*drd-1* displays an exclusion index of 1,015 (McKenzie, 1982), and the corresponding indices of Eex^+ recombinant plasmids are some 5-fold lower in comparison (Table 7-2), this remains an important consideration in future work. Resolution might be achieved by complementation studies using pLG252 and Eex^- derivatives of Iα plasmids generated by *in vivo* mutagenesis. One such plasmid, an Eex^- derivative of R144*drd-3*, has been isolated by Boulnois (1980) using EMS mutagenesis, but presumably a number of such plasmids would have to be examined in this way to detect mutations in other Iα genes resulting in reduced exclusion.

The exclusion gene encoded by pLG252 is similar to that specified by the recombinant plasmids pRAH303 and pRAH308 constructed by Hartskeerl *et al.*, (1983). These recombinants carry a 12.3 Kb *Bgl*II fragment of R144 DNA which encodes an exclusion system that reduces the transfer of R64 and ColIb-P9 as well as R144. The cloned exclusion-determining fragments of ColIb-P9 and R144 in pLG252 and Hartskeerl's plasmids both contain adjacent 1.15 Kb *Hind*III-*Eco*R1 and 0.15 Kb *Hind*III-*Sal*I restriction fragments, and Hartskeerl *et al.* (1983) have demonstrated using Tn*5* mutagenesis that this region is required for exclusion. Thus, it appears that this exclusion system is common amongst Iα plasmids, and that the *eex* gene(s) of pLG252 specifies the same gene products as those reported for pRAH308. Hartskeerl *et al.* (1983) showed that two proteins, with MWs of 13,000 and 19,000, were

associated with the exclusion process, but it is not clear whether these are the products of one or two *eex* genes. These proteins could be encoded by overlapping genes, or result from the processing of a primary product from a single gene, since deletion and Tn*5* mutagenesis of the R144-derived exclusion fragment affects the sizes of both proteins (Hartskeerl *et al.*, 1983). These authors also reported that Eex^+ recombinant plasmids had no effect on mating-pair formation; this finding supports the hypothesis that Iα plasmid exclusion operates by preventing DNA transfer. Since R144 DNA transfer can be amplified by rifampicin-treatment of recipients in a manner analogous to that of ColIb-P9 (Boulnois and Wilkins, 1979), it is possible that the R144 exclusion proteins may also function in transfer limitation.

CHAPTER 8

Discussion

FOOL: The reason why the seven stars are no more than seven is a pretty reason.

LEAR: Because they are not eight?

FOOL: Yes indeed. Thou wouldst make a good fool.

William Shakespeare

8.1 The role of Iα plasmid primase in conjugation

IncIα plasmid primase has been identified as a single polypeptide with a MW of about 240 kdal (Wilkins et al., 1981), and since DNA primase activity requires only the N terminal third or so of the molecule (Boulnois et al., 1982), the protein is presumably multifunctional. So far three properties of this enzyme have been described; it possesses DNA primase activity and the ability to bind to single-stranded DNA (Lanka et al., 1979) and it is transferred between mating cells to promote conjugative synthesis of plasmid DNA. The observation that about 65% of the protein can be deleted without impairing any of these properties, as demonstrated by the levels of recipient DNA synthesis in matings with BW96 (pLG250, pLG214) donors (Chapter 6), provides strong evidence that other functions are likely to exist. The established roles of plasmid primase will be defined before consideration of other possible functions in subsequent sections of this chapter.

Although plasmid primase is able to generate primers on a wide variety of prokaryotic DNA templates, experiments using Sog$^-$ Iα plasmids (Chapter 4) clearly show that the normal physiological role for this enzyme lies

in the conjugative synthesis of IncIα plasmid DNA. This finding indicates the classification of sog as a transfer gene and may explain the dependence of plasmid primase on host enzymes for primer-generating activity on duplex DNA (see section 8.4). Plasmid primase acts in the donor cell to initiate conjugative synthesis of replacement DNA on the non-transferred plasmid DNA strand. Primase activity is presumably required for each round of such DNA synthesis, since DNA transfer proceeds by the successive transmission of discrete monomer strands (Fenwick and Curtiss, 1973a,b; Boulnois and Wilkins, 1979), but it is not known whether synthesis of each replacement strand is a continuous or discontinuous process. A requirement for a fresh initiation event for each round of conjugative DNA synthesis in the donor cell may reflect the unavailability of the 3'-OH terminus of the previous strand due to protein interactions at the nicked origin of transfer or the displacement of this strand prior to replacement strand synthesis. It is also not clear whether plasmid primase is involved in the transfer of pre-existing DNA. Maturin and Curtiss (1981), using cytidine starvation of donor cells to inhibit RNA synthesis, have reported that synthesis of RNA is needed to initiate DNA transfer, and since plasmid primase incorporates cytidine at the 5' terminus of its primers (E. Lanka, unpublished data in Willetts and Wilkins, 1984), it may be that primer synthesis by this enzyme initiates the displacement or transfer of the pre-existing plasmid strand. However, the transfer of pre-existing strands of pLG250 was not prevented by the Sog^- phenotype of this plasmid (see Chapter 4) and it must therefore be concluded that there is no direct evidence that plasmid primase is involved in the initiation of DNA transfer.

The role of the enzyme in recipient cells is more clearly understood. The enzyme is apparently transferred to recipients where it initiates

synthesis of DNA complementary to the transferred plasmid strand. Assuming transmission of DNA with the 5' terminus leading, complementary strand synthesis may well be discontinuous in order to minimise exposure of the template strand in the recipient. Although it is simplest to assume that primers for this conjugative DNA synthesis are made in the recipient cell by transferred enzyme, they could also be generated in the donor and transmitted to recipients attached to the transferred DNA at priming sites. The role of plasmid primase in the latter process would presumably be to stabilise such RNA-DNA complexes during transfer and it might also stimulate the extension of primers by the recipient DNA polymerase III holoenzyme. The DNA primase of phage T7 (Scherzinger et al., 1977) provides a precedent for such interactions, and it may be significant that both Iα plasmid and phage T7 primases synthesise short primer molecules which, due to their small size, are likely to require some form of stabilization on the template strand.

Identification of other functions associated with sog would appear to require analysis of the 180 kdal protein. Although this polypeptide does not possess primase activity (Wilkins et al., 1981), it is thought to share its entire amino-acid sequence with the C-terminal region of the larger 240 kdal protein, since the proteins are translated from two in-phase initiation sites on the same sog transcript (Boulnois et al., 1982). This amino-acid homology can only indicate functional similarity between the two sog products, and so elucidation of the nature and function of the 180 kdal protein should also identify the remaining properties of plasmid primase. Investigation might proceed by the isolation of Iα plasmids carrying mutations in the overlapping region of sog which mutate the 180 kdal protein but do not affect DNA primase activity. One such lesion is specified by pLG214, which carries a cloned sog gene from which about 1.4

Kb of DNA corresponding to the overlapping portion of sog has been deleted. pLG214 specifies two proteins of 87 and 42 kdal which are truncated forms of the 240 and 180 kdal sog products. Although transfer of the 87 kdal primase apparently occurred in matings using donors containing both pLG214 and pLG250, no deduction of the role of the 180 kdal protein can be made from these experiments since the appropriate wild-type protein was supplied by the sog gene of pLG250. However, complementation could be avoided if the deletion specified by pLG214 were introduced into the corresponding sog locus of a conjugative Iα plasmid such as pLG221.

The method used to isolate primase-defective Iα plasmids by the in vivo recombination between a cloned mutant sog gene and the homologous region of an Iα plasmid (Chapter 3) appeared suitable for this task, and so the following strategy was pursued in a set of pilot experiments. Both pLG221 and pLG214 were introduced into the recA$^+$ host BW96, and this strain mated with BW98 (polA1) to isolate transconjugants harbouring only the Iα plasmid. As described in Chapter 3, this procedure prevents inheritance of the recombinant plasmid by recipients since the polA gene product is required for maintenance of the vector, and it is expected that a small proportion ofthe transconjugants will have acquired the deletion formerly specified by pLG214 by a recombination event in the donor cell, and will thus synthesise truncated sog proteins. Since the phenotype of such an Iα plasmid is not known, mutants cannot be detected by functional means, and so a screening procedure was devised to identify changes in genotype. The intact sog gene of pLG215 has been mapped by restriction endonuclease digestion, and contains a 560 bp HincII restriction fragment which is located within the region deleted from pLG214 (Boulnois et al., 1982; see Figure 1-5). Transconjugants of BW98 were toothpicked onto both stock plates and nitrocellulose filters and grown to colonies for use in colony

filter hybridisation experiments by the method of Grunstein and Hogness (1975). After cell lysis and immobilisation of DNA, filters were hybridised with nick-translated [^{32}P] labelled probe DNA and washed by the method of Jeffereys and Flavell (1977). Probe DNA consisted of the 560 bp HincII fragment of pLG214; thus the probe should hybridise only to plasmids carrying an intact sog gene, and autoradiography of filters should permit identification of transconjugants harbouring derivatives of pLG221 deletant in sog. Experiments also made use of colonies of BW96 (pLG221) and BW96 (pLG214) as hybridisation controls.

About 250 transconjugants were screened by this method, but since primase-defective Iα plasmids arose with a frequency of only 2×10^{-4} in equivalent in vivo recombination experiments (Chapter 3), one would expect that at least around 5,000 colonies would have to be examined in this way. Due to this consideration, and initial difficulty with non-specific hybridisation of probe DNA to BW96 (pLG214) controls, it was felt that effort should be directed to other projects instead, and studies were not continued. However, if non-specific hybridisation could be reduced by the use of more stringent reassociation and washing conditions, this approach should yield Iα plasmids which specify the truncated forms of both sog products. Investigation of the properties of these mutants might define the role of the 180 kdal protein, and of the complementary domain of plasmid primase.

This role might be structural (see section 8.3) or concern the conjugative metabolism of plasmid DNA. An attractive possibility is provided by analogy with the primases encoded by phages T7 and T4. Phage T7 primase is a multifunctional enzyme (Hillenbrand et al., 1979) which, in addition to priming activity, also acts as a helicase on duplex DNA templates (Romano and Richardson, 1979), whereas the primase encoded by gene 61 of phage T4 acts in a complex containing one gene 61 protein and

two molecules of gene 41 product (Silver and Nossal, 1982) which also possesses helicase activity (Liu and Alberts, 1981). In this respect it may be significant that the smaller sog product is produced in higher amounts than plasmid primase (Wilkins et al., 1981). Since helicase activity would not be required on single-stranded template during synthesis of the complementary strand in recipients, it may serve in the donor in displacement of the pre-existing plasmid strand destined for transfer, while the primase activity of the larger protein initiates replacement strand synthesis. The possibility that the region of sog encoding the 180 kdal protein is responsible for helicase activity might explain the reported requirement for a primer-generating enzyme in the initiation of DNA transfer (Maturin and Curtiss, 1981) and would support the canditature of plasmid primase as the primer-generating enzyme responsible. It might also explain the transfer of pre-existing DNA from donors of pLG250, since the lesion in sog carried by this plasmid affects only primase activity.

8.2 Protein transfer during conjugation

The major conclusion drawn from Chapters 5 and 6 is that molecules of plasmid primase are conjugatively transmitted to recipient cells, where they participate in the initiation of recipient DNA synthesis. In this section I shall firstly discuss the implications of protein transfer on models of conjugation, and then consider the transfer of plasmid primase as a specific example of this process. In comparison with the plethora of information regarding the transfer of DNA, there are few reports in the literature concerning the conjugative transmission of proteins. Indeed, with the sole exception of plasmid primase, no protein species has yet been demonstrated to undergo conjugative transfer despite previous claims to the contrary. For instance, indirect induction, a process whereby

λ prophage can be induced by mating lysogenic recipient cells with UV-irradiated donors of either F (Borek and Ryan, 1958) or ColIb-P9 (Monk and Devoret, 1964), was initially attributed to the cytoplasmic transfer of an inducer molecule, until it was realised that induction occurred in response to the UV-irradiated plasmid DNA transferred to recipients (Monk, 1967). Similarly, the apparent transmission of a λ immunity factor from lysogenic donor cells during F-mediated matings (Fisher, 1962) is also attributable to the transfer of sex factor DNA, rather than to the prophage or its products (Fisher; pers. comm. in Rosner et al., 1967). Rosner et al. (1967) selected the enzyme β-galactosidase for study in this respect, since this protein was considered unlikely to be transferred between cells in the absence of conjugation. Using heat induction of thermo-inducible λ lysogens to achieve selective lysis of mating cells, these authors were able to demonstrate that only about 0.05% of the β-galactosidase present in donor cells was detected in recipients during F-mediated matings. This value is within the upper limit for protein transfer estimated by Silver (Silver, 1963; Silver et al., 1965), and emphasises that protein exchange between mating cells is not a general process. We can also deduce, as argued in Chapter 6, that the host-specified DNA replication proteins DNA polymerase III, RNA polymerase, and dnaB and dnaG proteins are not transferred to recipients, since strains deficient in one or more of these activities did not evidence recovery of DNA synthesis when mated with appropriate donor cells.

The conjugative transfer of proteins thus appears to be a highly selective process which excludes the general conveyance of host-specified enzymes and involves only those products required in newly-infected recipient cells for processing of the transferred strand of plasmid DNA. Indeed, since some plasmid proteins (such as the eex gene product of ColIb-

P9; see Chapter 7) are apparently synthesised in newly infected recipients from the immigrant plasmid, plasmid primase may typify only a very small class of protein capable of conjugative exchange between mating cells. The identities of other proteins which may be included in this class will be considered later in this section.

Any model which attempts to explain the mechanism of protein transfer during conjugation must account not only for the selectivity of the process, but also for the physical requirements imposed by the large size (240 kdal) of plasmid primase, and the driving force responsible for the conveyance of large protein molecules. An attractive possibility is that plasmid primase achieves these requirements by binding to the plasmid DNA strand destined for transfer. The enzyme has been demonstrated to bind to single-stranded DNA (Lanka et al., 1979) and this property would also be predicted from its priming function. This possibility is particularly attractive when one considers that the primary function of the enzyme in newly-infected recipients is the initiation of complementary strand synthesis, using the transferred strand as template. In this scheme, plasmid primase could achieve rapid and efficient initiation of DNA synthesis, thus minimising the time that the template strand is exposed in the recipient. Transmission of plasmid primase would presumably be driven by the force responsible for DNA transfer, and discrimination would take the form of selection for proteins capable of binding to the transferred plasmid strand.

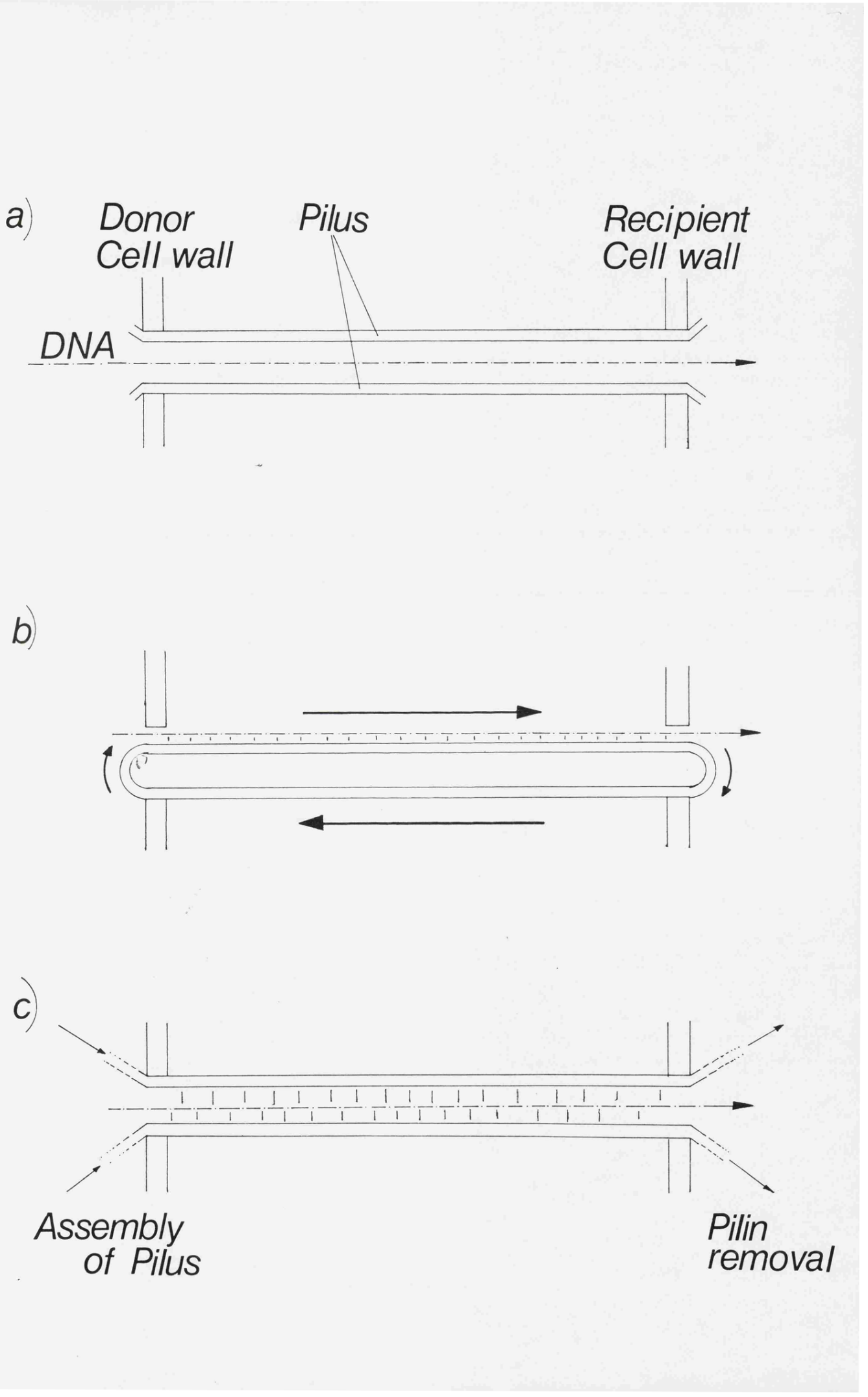
An important corollary of this model is that protein molecules must be transmitted by the same route as DNA, and as will be shown, this proposition is incompatable with certain models regarding the conduction of DNA during mating. The pilus model of DNA transfer was proposed by Brinton (Brinton et al., 1964; Brinton, 1965) following the discovery of F-specified

sex pili and their involvement in fertility (Brinton, 1959). This model, whereby DNA was conducted through the pilus was later presented in a revised form (Brinton, 1971); it is this reference which forms the basis for the three versions of this model presented in Figure 8-1. Although experimental evidence supporting this model was derived from experiments involving F, an essentially similar process was envisioned for Iα plasmids since chromosomal DNA transfer from a pilus-deficient Hfr strain could be rescued by the additional presence of an I pilus-determining R plasmid (Meynell and Cook in Curtiss, 1969) or ColIb-P9 (Clowes, 1963) in the donor strain.

Brinton envisaged conjugative plasmids as 'viruses whose infectious form is obligatorily cell attached', and proposed the term 'epivirus' to underline the analogy with single-stranded filamentous phages such as M13. In this elegant scheme, the pilus represents a modification of the protein coat of the viral ancestor of the plasmid, and the evolutionary advantage to be gained by such a strategy is the release of constraint on genomic size imposed by packaging DNA into the virus particle. Furthermore, since the conjugative act is taken to represent the infectious form of the 'epivirus', this analogy would fit comfortably with the observation that only one specific (+) strand of DNA would be transferred. It is stressed that the model does not preclude the pilus from acting as a form of initial contact between donor and recipient bacteria, and because of the difficulty of differentiating between the dual proposed roles for the pilus, much of the evidence presented in support of the pilus conduction model, such as the effects of both F and I pilus removal and the inhibitory effect of antibodies on conjugative ability (Mulezyk and Duguid, 1966; Meynell et al., 1968; Brinton, 1965; 1971; Harden and Meynell, 1972) together with the observed connection between mating pairs and conjugation (Brinton,

Figure 8-1 The pilus conduction model of DNA transfer

The figure, based on suggestions of Brinton (1971), depicts models of DNA transfer via an F pilus, which is presented as two parallel chains of F pilin (heavy horizontal lines). In the conduction model (1) DNA is transferred through the pilus via its axial hole, and the pilus functions purely as a non-mobile and structural organelle. In contrast, the conveyer belt (2) and carrier (3) models require both the binding of plasmid DNA (broken horizontal line) to the pilus, and the subsequent movement of this organelle in order to transport the DNA. In the case of (2), the two pilin strands are proposed to move relative to one another, such that the DNA, bound to only one strand, is conducted to the recipient cell. In (3) the pilus is extended by polymerisation at the donor cell membrane, and degraded at the recipient junction in order to carry the DNA between the mating cells.


a)
Donor
Cell wall
Pilus
Recipient
Cell wall
DNA
b)
c)
Assembly
of Pilus
Pilin
removal

1965), is circumstantial. The model also rests heavily on the ability of single-stranded RNA and DNA phages to achieve cellular entry of their nucleic acids by attachment to sex pili. However, the direction of DNA transfer in this case would be opposite to that taken by plasmid DNA during conjugation, and a totally different mechanism may operate in each case. Finally, and most importantly, the model requires an axial hole running the length of the pilus with a diameter large enough to provide passage for single-stranded DNA. In the case of F pili, the existence of such an internal hole has been demonstrated, and its diameter of between 2.0 and 2.5 nm (Brinton, 1965; Folkhard et al., 1979) is just large enough to admit single-stranded DNA. To appreciate the significance of this value, the diameter of double-helical DNA is 2.0 nm (Watson and Crick, 1953) and the smallest dimension of a molecule of β-galactosidase (MW = 125,000) is 7.0 nm (Karlsson et al., 1964). Thus, as Brinton (1971) stresses, protein transfer is incompatible with the F pilus conduction model due to size restraints.

In comparison, Iα plasmids specify two distinct types of pilus; a thin flexible pilus with an external diameter of 6.0 nm to which bacteriophages If1, Iα and PR64FS adsorb, and a thick rigid pilus of around 10 nm diameter (Bradley et al., 1983; Bradley, 1984). The isolation of mutant plasmids determining either thick pili only or thick pili at a repressed level and thin pili constitutively has allowed some elucidation of their function (Bradley, 1984). Thin pili are required for the formation of contacts between bacteria in liquid matings; in their absence thick pili alone can mediate contact and transfer only in surface-supported matings. Thus, thick pili also appear to play a role in contact formation, presumably in co-operation with thin pili in a two-stage process (Bradley, 1980; 1984). Since thin pili are not required for DNA transfer, these

organelles cannot be responsible for conduction of DNA, and so only thick pili can be considered as prospective agents for DNA transfer. In this regard, it is not yet known whether thick I pili have an axial hole, but if we accept that these pili are about 10.0 nm in external diameter and assume that the pilin subunits are arranged as for F pili, then this hole would not be expected to be larger than 3.0 nm in diameter. Thus, the same physical constraints with regard to conduction of protein appear to apply to both F and thick I pili.

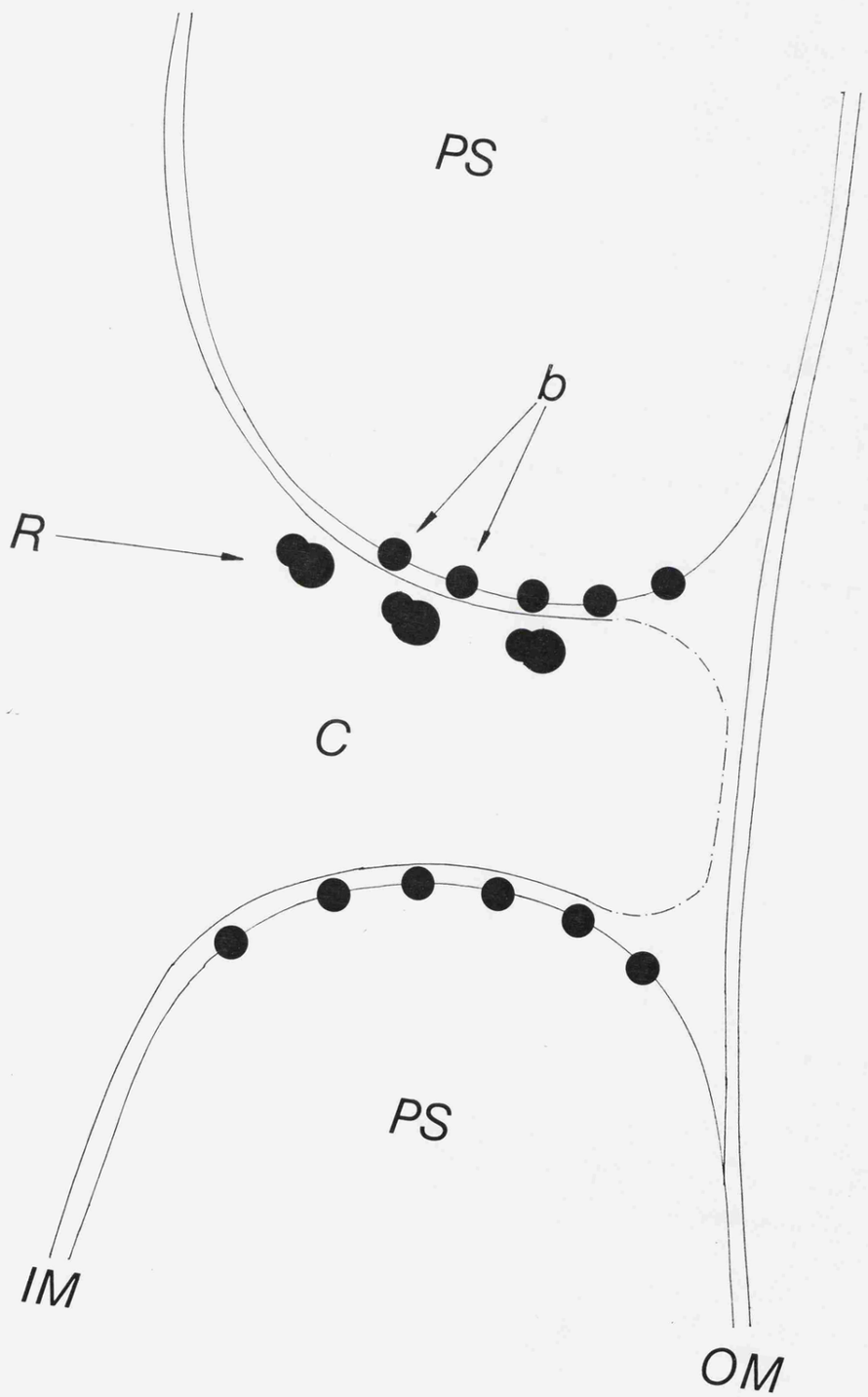
In the light of current evidence, it therefore appears that transfer of plasmid primase does not proceed via an extended pilus, and unless one invokes separate routes of transport for proteins and DNA between mating bacteria, this conclusion must constitute a serious criticism of the pilus conduction model. This contention is supported by the inability to demonstrate the presence of DNA inside the pilus (Wendt et al., 1966) and the observation that depolymerisation of F pili with SDS after mating has started does not interrupt the conjugative process (Achtman et al., 1978). As an alternative to the pilus model, Curtiss (1969) and Marvin and Hohn (1969) proposed that wall to wall contact was necessary between mating bacteria in order to promote a localised fusion of cellular membranes leading to a cytoplasmic bridge. These authors also suggested that the role of sex pili might be to retract in order to bring about the initial wall to wall contact. This hypothesis has since received support from the retraction of F pili on treatment with phage f1 (Jacobson, 1972), NaCN (Novotny and Fives-Taylor, 1974) and heat (Fives-Taylor and Novotny, 1976), and there is indirect evidence that I pili retract (Lawn and Meynell, 1972). Furthermore, close wall contact has been reported many times for mating cells (Lederberg, 1956; Ou and Anderson, 1970; Achtman et al., 1978) and this is maintained for longer periods in Hfr rather than F^+ matings (Achtman et al., 1978).

The most suitable candidates for cytoplasmic or membrane bridges between conjugating cells are the adhesion sites demonstrated in E.coli by Bayer (1967; 1968; Bayer and Starkey, 1972). These structures (see Figure 8-2) represent a localised fusion between inner and outer cell membranes and are present over about 5% of the cellular surface (Bayer, 1979). They appear to serve as growing zones for the insertion of macromolecules into the cell wall and outer membrane, and act as sites for the entry of certain bacteriophages (Bayer, 1979). Most significantly, such fusion sites have been observed at the base of F pili (Bayer, 1975), and it is thought likely that this represents the region at which the pilin subunits are assembled and the growing pilus is extended through the outer membrane. Once the tip of the extended pilus has attached to the recipient cell, perhaps at a similar adhesion site, it is easy to envisage a retraction of the pilus, mediated by depolymerisation of the organelle at one or both of its ends, bringing the outer membranes of donor and recipient into close contact. The nature of the bridge formed at such a junction must remain speculative but since it must allow the transport of DNA and certain protein molecules without permitting a general mixing of cellular contents, it would presumably be a highly developed structure. In this regard, thick I pili have been observed to possess membraneous structures attached to one end which vary in size from small knobs to vesicle-like sacks (Bradley, 1984). These structures reacted with antibodies to bacterial membranes and hence are not plasmid-specified; thus they might represent a plasmid-mediated conversion of a host adhesion site. Since conjugatively-replicating R64drd-11 (Fenwick and Curtiss, 1973c; Davis and Henry, 1982) and RIdrd-4 (Falkow et al., 1971) DNA has been shown to associate with the inner membrane, these structures may also serve as anchorage for the strand of

Figure 8-2 Morphology of an adhesion site of Escherichia coli K-12

Key:		
	IM	Inner membrane
	OM	Outer membrane
	PS	Periplasmic space
	C	Cytoplasm
	R	Ribosomes
	b	Beaded appearance of IM

Discussion is provided in the text. The diagram is taken from Bayer (1979).


PS
b
R
C
PS
IM
OM

plasmid DNA retained in the donor cell. Electron micrographs of adhesion sites estimate the junction between inner and outer membranes at about 20 to 30 nm (Bayer, 1979), a dimension more conducive to the transport of protein molecules than that of a sex pilus.

Figure 8-3 depicts a schematic model of Iα plasmid transfer, based on the available information concerning I pili and E.coli adhesion sites. The precise nature of the 'pore' connecting the mating cells is unknown, and so the presence of proteins and other molecules which may be associated with this site have been omitted from the diagram for clarity. However, the traD, traY and traM gene products of F have been shown to be membrane-associated and location of these proteins at a pore would offer advantages in the processing of F DNA during its conjugative transfer (Willetts, 1981). Apart from proteins involved in the nicking and recircularising of plasmid DNA, some machinery must also be present to guide the DNA through the pore without permitting ravelling of the strand. Thus, the pore must be considered as a complex involving host membranes and plasmid DNA and proteins.

As well as plasmid primase, other plasmid-encoded proteins may be transmitted between mating cells. Since single-stranded DNA binding proteins (SSBs) function in both bacteriophage and chromosomal DNA replication, it is reasonable to assume an analogous role for SSBs in the conjugative replication of plasmid DNA. A number of conjugative plasmids, notably F, R1, R100, R64drd-11, and RP4 (Kolodkin et al., 1983; personal communication from E. Golub and B. Low cited in Willetts and Wilkins, 1984; Barth, 1984), encode proteins capable of suppressing temperature-sensitive host ssb mutations in E.coli. In the case of R64, suppression requires de-repression of transfer genes, supporting a role for the putative IncIα plasmid-specified SSB in conjugation. An attractive possibility is that the protein coats the plasmid DNA strand undergoing transfer, and is

Figure 8-3 Schematic representation of the route of Iα DNA transfer

The model, discussed fully in section 8.3, presents the opposing cell walls and inner and outer membranes with their respective structures prior to mating (1). Thin flexible pili then attach to lipopolysaccharide receptors on the recipient OM, and form an initially weak contact (2) which is subsequently stabilised. Retraction of these pili, perhaps by depolymerisation at their bases, then brings cells closer together, and the thick rigid pilus forms a connection with a recipient adhesion site (3). Further retraction (4) brings the cells into a very stable wall to wall contact. Figure 5 represents the fusion of outer membranes between the two opposing adhesion sites to form a cytoplasmic bridge. Although the inner membrane is known to fuse with the outer membrane at adhesion sites, it may or may not occlude the pore.

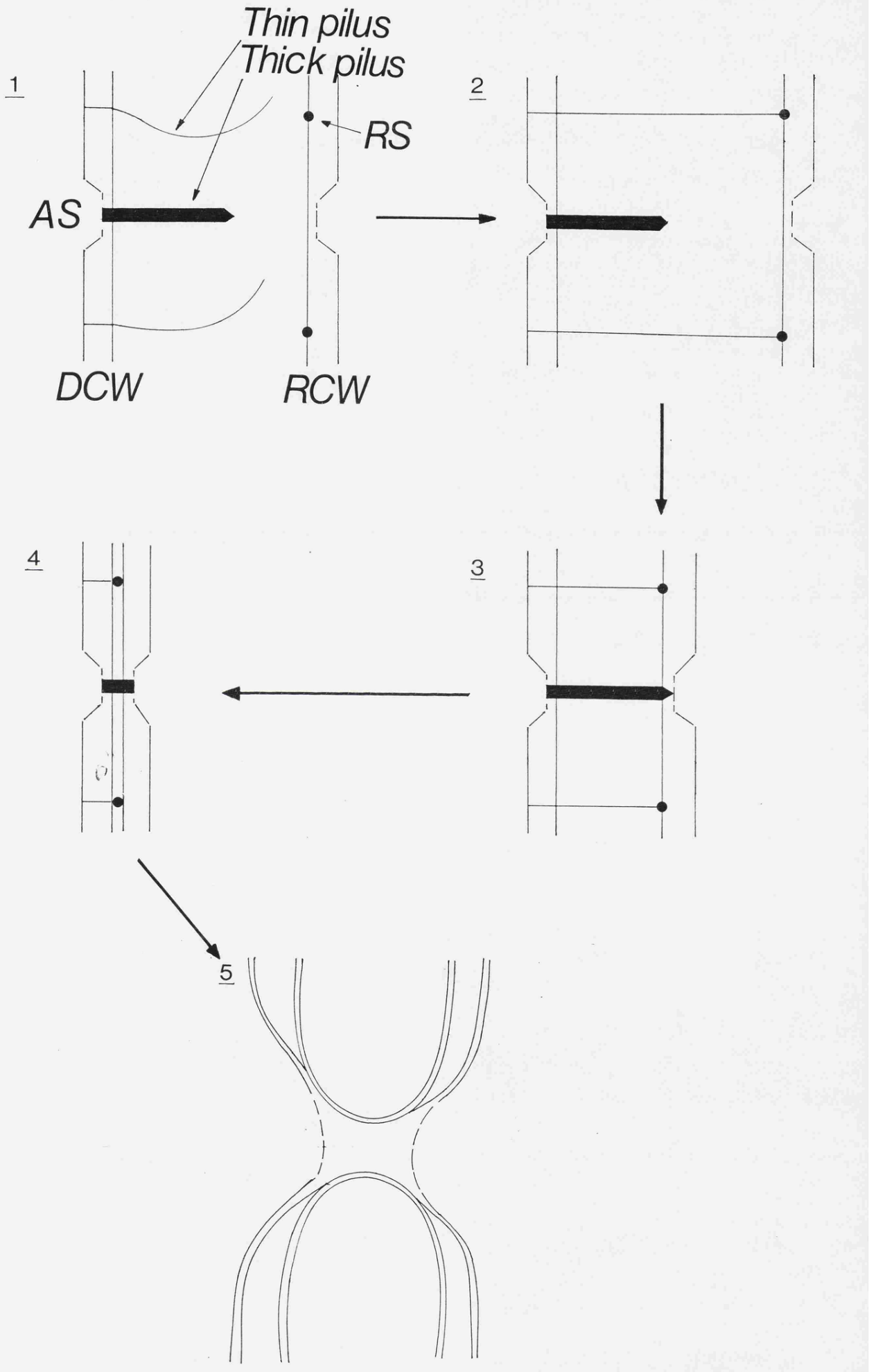
Key:

AS = Adhesion Site

RS = Receptor Site

DCW = Donor Cell Wall

RCW = Recipient Cell Wall


Thin pilus
Thick pilus
1
RS
AS
DCW
RCW
2
3
4
5

transmitted by attachment to the DNA to recipients where it participates in conjugative DNA metabolism.

Since recircularisation of ColIb-P9 DNA in recipients is independent of transcription of the transferred DNA (Boulnois and Wilkins, 1978), this process may also be reliant upon donor-supplied products. Although this observation can be explained by a transfer of the necessary protein machinery, perhaps by covalent attachment to the terminus of the transferred plasmid strand, recircularisation could also occur if the protein(s) involved formed a structural component of the pore linking the mating cells. Thus, recircularisation may take place at the membrane junction between the mating cells, rather than in the recipient cell itself.

8.3 Processing of plasmid primase

Although plasmid primase has been identified as a single polypeptide of about 240,000 MW, primase activity requires only the N-terminal third or so of this molecule (Boulnois *et al.*, 1982). The part of the 240 kdal protein that is inessential for primase activity includes that sequence shared with the 180 kdal *sog* product; the function of this sequence is presumably equivalent to that of the smaller protein and is at present unknown. Since the evidence in favour of plasmid primase transfer rests on detection of activity in recipient cells, only the unique N-terminal region of the 240 kdal protein can be deduced to be transferred and so it is a possibility that the remainder of the protein is removed prior to or during the transfer process.

Apart from advantages that might arise from decreasing the size and amount of proteins that are transferred via an energy-dependent and possibly size-restrictive conjugation system, this prospect is consistent with the observation that the truncated primase specified by BW96 (pLG250,

pLG214) donor cells is efficiently transmitted to recipients (Chapter 6), despite the loss of about 65% of the normal amino-acid sequence. Thus there is no reason to assume that the 240,000 dal form of plasmid primase is transferred in its entirety. It has been suggested that the genetic organisation of the sog locus arose as a result of fusion between two separate genesto allow regions common to both polypeptides to interact and so form multifunctional complexes (Boulnois et al., 1982). If the role of the 180 kdal protein were structural, perhaps contributing to the base of the pilus or to the transmembrane pore, then interaction may guide the primase moiety to the site of transfer. Processing of the enzyme might occur after this stage at the membrane, followed by transfer of a truncated primase protein. The traA gene product of F, which is converted from a 14,000 dal form into 7,000 dal molecules of F pilin by the membrane-located traQ product (Ippen-Ihler et al., 1984) is an example of the processing of plasmid products in this way, and proteolytic cleavage has been described for host proteins in their transport across the inner membrane to the outer membrane (see Silhavy et al., 1983 for a recent review).

Until the role of the 180 kdal region has been established, such models must remain speculative. The only experimental evidence indicative of processing of sog products is the isolation of a 140,000 MW form of sog primase by Lanka et al. (1979) instead of the 240,000 MW form detected in crude cell extracts (Wilkins et al., 1981; Boulnois et al., 1982). This novel size may indicate that processing can occur at sites within the region corresponding to the 180 kdal polypeptide, and suggests that cleavage could occur at more than one site, and perhaps involve the 180 kdal protein as well.

Progress requires resolution of whether the 240 kdal form of plasmid primase, or a truncated polypeptide with priming activity, is transmitted to recipients, and elucidation of the function of the 180 kdal sog product. Analysis of proteins transmitted to rifampicin-treated recipient cells might be feasible using the selective phage T6 lysis of donors used in DNA transfer experiments (Chapter 5) to remove donor proteins from the samples. Recipients containing the Eex$^+$ recombinant pLG252 could be used in control matings to test the suitability of this approach. Proteins in recipient cells could be analysed by SDS-polyacrylamide gel electrophoresis and sog proteins transferred from donors identified by a number of different means. Selective labelling of donor proteins with [^{35}S]methionine might be successful in this respect if used to allow autoradiography of SDS-polyacrylamide gels in order to avoid the significant background radioactivity assimilated by recipients (Silver, 1963; Silver et al., 1965). Alternatively, polypeptides could be transferred to nitrocellulose and treated with antisera raised against plasmid primase or the 180 kdal protein. Use might also be made of recombinant plasmids with various deletions affecting the C-terminal portion of plasmid primase (Boulnois et al., 1982) as a source of antiserum specific only for the N-terminal primase domain of the molecule.

8.4 Plasmid primase and suppression of dnaG mutations

The ability of certain conjugative plasmids to suppress the temperature-sensitive phenotype of dnaG3 mutants has been reported for members of the Iα, Iγ, Iγ, I_2, B, C, J, K, P and U incompatibility groups (Wilkins, 1975; Sasakawa and Yoshikawa, 1978; Lanka et al., 1979; Ludwig and Johansen, 1980; Lanka and Barth, 1981; Dalrymple et al., 1982; Dalrymple and Williams, 1982), although in at least some cases it appears that a mutational change affecting the plasmid-encoded primase is

necessary for the detection of this ability by colony formation at restrictive temperature, (Ludwig and Johansen, 1980; Lanka et al., 1984). This mutation is presumably separate from that reported by Sasakawa and Yoshikawa (1978). These authors showed that in addition to an authentic derepressing mutation another plasmid mutation was required for complete dnaG suppression, but this is apparently separate from the mutational change in the primase gene since it results in a greater derepression of transfer functions.

IncIα plasmid primase appears to achieve suppression by directly substituting for E.coli primase in the priming of chromosomal DNA synthesis. The enzyme functions on single-stranded DNA templates independently of dnaB and dnaC proteins (Lanka et al., 1979) but is unable to bypass the requirement for these proteins on duplex DNA (Fenwick and Curtiss, 1973a; Boulnois and Wilkins, 1979). Thus plasmid primase might co-operate with certain bacterial enzymes in order to generate primers for chromosomal DNA replication and it might be incorporated into the host primosome complex to achieve this action. The large size of plasmid primase (240 kdal) as compared with dnaG protein (60 kdal) might make this interaction inefficient, unless the protein were processed to generate a smaller primase molecule (Section 8.3). In this respect, it may be relevant that the truncated primase (87 kdal) specified by pLG214 is capable of suppressing dnaG3 mutations with almost 100% efficiency (Chapter 3). Since dnaB protein is thought to act as a mobile promoter, acting to recognise and modify sites for utilisation by primase (McMacken et al., 1977; Arai and Kornberg, 1981a), the plasmid-specified enzyme may require dnaB protein to recognise sites for primer synthesis on bacterial DNA. Alternatively, as plasmid primase generates short oligoribonucleotide primers, it might be expected that regions of DNA complementary to the primer sequences on the Iα

plasmid may also exist on the host chromosome, allowing the enzyme to initiate chromosomal replication from these sites rather than at sequences utilised by dnaG protein. The role of dnaB product in this scheme would lie in some other aspect of DNA replication, such as destabilisation of the helix (Staudenbauer et al., 1979).

Wang and Iyer (1977, 1978) have interpreted genetic experiments to indicate that many conjugative plasmids encode an analogue of the host dnaB protein, similar in nature to the phage P1 ban protein (Schuster et al., 1975). These authors demonstrated that a number of plasmids, including R64 and R144, could partially suppress the temperature-sensitive phenotype of four different dnaB alleles, and that still others caused an enhancement of the temperature-sensitivity. In keeping with this latter class of plasmid, it was observed during the course of this work that both pLG221 and pLG250 slightly enhanced the temperature-sensitivity of DNA synthesis in the dnaB70 strain BW68 during control measurements at 43°C. Residual DNA synthesis in this strain was reduced from 7% to 2% by the inclusion of either pLG221 or pLG250 (data not shown). The nature or significance of this effect is not known, but it is unlikely to be caused by a plasmid-encoded dnaB-like protein, since attempts to purify the putative dnaB analogue from R64drd-11-containing cells and to show that the plasmid has sequences homologous with the host dnaB gene have proved fruitless (Lanka et al., 1979; V.N. Iyer, personal communication to B.M. Wilkins). Instead of detecting a plasmid-encoded dnaB analogue, the studies demonstrated that the protein responsible for complementation in some temperature-sensitive dnaB strains harbouring R64drd-11 was indistinguishable from the host dnaB product except for a 6% reduction in apparent molecular weight. Furthermore, since the presence of the plasmid was not required for the continued

synthesis of this protein, it appears that the formation of thermo-resistant colonies by R64*drd-11*-containing cells may be due to the plasmid acting as a mutator to promote change of the original *dnaB* lesion and hence allow growth at high temperature.

While ColIb-P9 does not appear to specify a *dnaB*-like protein capable of action on bacterial DNA templates, the possibility that it may encode some other product involved in the suppression process is raised by the observation that pLG215 suppressed the host *dnaG3* mutation when paired with pLG250 in the same cell (Table 3-1). As explained in section 3.4, the gene for this cofactor might be identified by a cloning strategy using plasmid-free segregants of BW86 (pLG215) grown at 40°C, or by genetic mapping of the chromosomal mutation carried by such segregants. Investigation of both the existence and nature of cofactors may shed light on the mechanism of *dnaG* suppression.

8.5 Evolution of plasmid-encoded DNA primases

Plasmid-specified priming enzymes are fairly ubiquitous amongst diverse groups of conjugative plasmids (Lanka and Barth, 1981) and at least three different primase genes have been identified so far. Despite the apparent unrelatedness of these loci, as shown by lack of DNA-DNA hybridisation and absence of antigenic cross-reactivity between polypeptides (Lanka and Barth, 1981; Dalrymple, 1982; Dalrymple *et al.*, 1982), the nature and organisation of these loci and their products are remarkably similar. All are thought to consist of overlapping genes that specify two sequence-related proteins of similar relative sizes. Plasmid-located primase genes are not only widespread but appear to have arisen by at least three different evolutionary pathways. Divergent evolution from a common ancestral gene is unlikely, considering the lack of DNA homology and anti-

genic relatedness. Furthermore, while the RP4 *pri* locus encodes two primer-generating enzymes with the primase domain occupying the C-terminal region of each, only the N-terminus of the larger *sog* product has DNA primase activity (Lanka and Barth, 1981; Boulnois *et al.*, 1982; Lanka *et al.*, 1984). The functional similarities between the different classes of plasmid-encoded primases therefore presumably reflect convergent evolutionary histories.

Selection pressure favouring the development of a plasmid-encoded primase may arise as a result of different vegetative and conjugative DNA replication strategies of the plasmid. If plasmid replication is a unidirectional process, synthesis of multiple primers will only be necessary on one strand, namely the template for the lagging strand (Figure 1-1). It seems reasonable to suppose that the other strand, which serves in replication as template for the leading strand, will be deficient in sites recognisable by host primer-generating enzymes, with the possible exception of the *oriV* region. If this second strand is transferred during conjugation, efficient complementary DNA synthesis in the recipient cell will require some conjugation-specific priming mechanism to compensate for the lack of sites recognisable by host enzymes and a plasmid-encoded DNA primase would fulfil this function.

The properties of RP4 are consistent with this hypothesis. DNA transfer proceeds in the opposite direction to unidirectional DNA replication (see Lanka and Barth, 1981; Al-Doori *et al.*, 1982; Lanka *et al.*, 1983) and, if we assume by analogy with F that a specific strand of RP4 is transferred during conjugation with its 5' terminus leading, the transferred strand will be the one that serves as template for leading strand synthesis during vegetative replication (Wilkins *et al.*, 1984). Thus, the DNA primase encoded by this plasmid may have arisen from the need for discontinuous synthesis of the complementary strand in the recipient cell and the inefficiency of host

Table 8-1 Properties of some DNA primase-specifying conjugative plasmids

Discussion is provided in section 8.5. For the sake of consistency, I have used the term Iα instead of I_1 with reference to incompatibility, and plasmids are referred to by their wild-type designation to avoid listing the numerous drd mutants of the sample plasmid that were often used in separate experiments.

a Data from Coetzee et al. (1982); Bradley (1984).

b Data from Dalrymple et al. (1982).

c Unpublished dataof C.C. Wymbs and B.M. Wilkins.

d Parentheses represent an intermediate amount of reactivity with antiserum (Bradley, 1984).

Table 8-1

Plasmid	Incompatibility group [a]	Primase gene type [b]	Characteristics of thin pili				Characteristics of thick pili		Mobilisation of pLG2000 [c]
			Sensitivity to phage [a]		Reaction with antisera to thin pili [a]		Reaction with antisera to thick pili [a]		
			Iα	PR64FS	R64	R16	R144	R16	
ColIb-P9	Iα	sog	+	+					+
R64	Iα	sog	+	+	+	-	+	-	+
R144	Iα + B	sog	+	+	+	+	+	-	+
R864a	Iα + B	sog	+	+	+	-	(+) [d]	-	+
R621a	B	sog	+	+	+	+	(+)	-	+
R1P72	B	sog	-	-					-
R16	B	pri	-	-	(+) [d]	+	-	+	
TP125	B	pri							-
R805a	Iα + B	pri	-	+	(+)	+	-	+	-

enzymes to generate primers for this process. Although neither the mode of replication nor the orientation of the transferred strand of an IncIα plasmid has been determined, sog may also have arisen in response to such selective pressures.

The presence of sog-like sequences in different conjugative plasmid is also of interest. Sequences homologous with the ColIb-P9 sog gene have been detected in R64 (IncIα), R144 and R864a (IncIα + B), together with R621a and RIP72 (IncB), as shown in Table 8-1. Although all these plasmids are closely related, sog is clearly not associated with plasmids of one particular incompatibility group. Furthermore, the small recombinant plasmid pLG2009, which was derived from pLG2000 (unpublished work of C.C. Wymbs) and carries the oriT region of ColIb-P9drd-1, is mobilised efficiently by the above conjugative plasmids that contain sequences homologous with sog, with the exception of RIP72 (Table 8-1). Thus, sog-like sequences are not limited to plasmids specifying trans-acting functions that recognise the transfer of ColIb-P9.

Plasmids R64, R144, R864a and R621a (which possess sog-like sequences) also determine closely related thick pili as demonstrated by electron microscopic examination and serological analysis (Bradley, 1984; Table 8-1). In contrast, R805a specifies thick pili which are serologically related to those produced by the IncB plasmid R16. Since R805a has an R16-like pri gene, it appears that each class of primase gene may be associated with genes for a particular type of thick pilus. This relationship may reflect a functional interdependence between the products of the primase locus and the thick pilus, thus supporting the notion of an interaction between sog proteins and the base of the thick I pilus (see Section 8.3). It should be noted, however, that sog shows no such association with the genes for thin pili, since

those pili encoded by RIP72 differ in bacteriophage adsorption from those produced by ColIb-P9, R64, R144 and R864a (Table 8-1). Thus sog-like sequences are not associated with the determinants of a specific type of thin pilus, and this would agree with the idea that only thick pili are required for the formation of the bridge by which plasmid primase gains egress to the recipient cell (Section 8.3).

The association of sog with a range of genes for conjugation functions on different, but closely related plasmids could be most easily explained by recombination or transposon-mediated events between replicons resulting in the exchange or acquisition of new plasmid functions. Clustering of genes with related functions and their co-ordinate expression would facilitate this process, and many mechanisms by which discrete segments of DNA can be rearranged in this manner have already been described (see Campbell, 1981 and Levin and Lenski, 1983 for recent reviews of plasmid evolution). In this scheme the plasmid is viewed as a transient entity, exchanging discrete, internally conserved DNA sequences with other plasmids. Since this requires that any two plasmids or replicons undergoing such an exchange of genetic information must share the same host cell, the host range of the plasmids involved must be a contributary factor towards this process. This may explain why the plasmids listed in Table 8-1 as harbouring sog-like genes share a relatively narrow host range (Jacob et al., 1977) and also account for the possession of sog by RIP72.

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ABSTRACT

Title ROLE OF PLASMID COLIb-P9 DNA PRIMASE

by Lee K. Chatfield

The sog locus of ColIb-P9 is known to encode a DNA primase that generates RNA primers for DNA synthesis on a variety of DNA templates and it promotes bacterial DNA replication in primase-defective (dnaG3) mutants of Escherichia coli K-12. The thesis reports that the physiological role of the enzyme is in conjugative metabolism of plasmid DNA.

Derivatives of ColIb-P9drd-1 carrying defined sog mutations were constructed by in vivo recombination. The mutant plasmids were maintained stably, showing that the primase is inessential for vegetative DNA replication, but they were deficient in transconjugant formation during bacterial mating. Amounts of conjugative DNA synthesis in matings involving mutants and complementing plasmids imply that the primase is required for efficient DNA synthesis on the plasmid strand transferred to the recipient cell, and that the enzyme may act in the donor cell to promote synthesis of a replacement strand.

Recipient dnaG3 bacteria, treated with rifampicin to inhibit transcription, recovered some ability to synthesise chromosomal DNA during mating with donors of a Sog^+ conjugative plasmid. Recovery was dependent on plasmid primase and an active DNA transfer system but it did not require transmission of a functional sog gene to the recipient cell. It is argued that the recovery reflects conjugative transfer of plasmid primase, that the transferred enzyme normally acts to initiate DNA synthesis on the plasmid strand transmitted from the donor cell, and that sog primase is selectively transferred during conjugation, possibly in association with plasmid DNA.

Isolation of recombinant plasmids carrying the origin of transfer or an entry exclusion gene(s) of ColIb-P9drd-1 is described.