STUDIES ON THE TRANSFER OF IncIα PLASMIDS IN *ESCHERICHIA COLI* K-12

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by

GRAHAM J. BOULNOIS B.Sc.

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

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CHAPTER 1

INTRODUCTION

The phenomenon of bacterial conjugation was first described over 30 years ago (Lederberg and Tatum, 1946a, b). Despite a plethora of subsequent publications on the subject we remain largely ignorant of the details of the process. Although much of the work has concentrated on the archetypal conjugative plasmid F, first defined by Hayes (1953), it is now clear that conjugative plasmids are ubiquitous among the enterobacteria (Datta, 1975a).

An attempt to classify these plasmids, based on their inability to coexist stably within the same cell, has been undertaken (Datta, 1975b). Such incompatibility is thought to reflect close phylogenetic origins and incompatible plasmids are placed in the same compatibility group. Plasmids belonging to the IncF1 and F11 groups have extensive DNA sequence homology primarily within the region of the plasmid that encodes functions involved in conjugation (Sharp et al., 1973). In contrast there is little sequence homology between plasmids of different compatibility groups (Grindley et al., 1973; Falkow et al., 1974; Anderson et al., 1975). These observations have led Achtman and Skurray (1977) to speculate that as many different conjugation systems may exist as there are compatibility groups. At the present 32 compatibility groups have been identified among plasmids originating from the Enterobacteriaceae alone (Jacob et al., 1977).

For historical reasons F plasmid transfer has been studied in the most detail and has been extensively reviewed (Curtiss, 1969; Willetts,

1977a; Curtiss et al., 1977; Achtman and Skurray, 1977; Rowbury, 1977; Manning and Achtman, 1979). Representative plasmids of the other major compatibility groups have been studied but in much less detail. The primary concern of this thesis is the mechanism of transfer of the IncI$_{\alpha}$ group of plasmids and in particular the metabolism of plasmid DNA during the transfer process. However any consideration of bacterial conjugation must involve a detailed discussion of the F plasmid. I will first describe the genetical organisation and possible functions of the F transfer genes and before considering the metabolism of F DNA during conjugation I will digress to outline current ideas on certain aspects of the mechanism of DNA synthesis in bacteria. Following a short discussion of the mechanism of mobilisation of ColE1 by conjugative plasmids, I will describe in detail what is known of IncI$_{\alpha}$ plasmid transfer.

F PLASMID TRANSFER

Conjugation mediated by the F plasmid is a complex series of events which culminates in the establishment of the plasmid in the recipient. Studies on the molecular biology of F plasmid-mediated conjugation have been facilitated by two separate but complementary approaches. The isolation and characterisation of numerous mutants carrying point mutations causing transfer deficiency (Achtman et al., 1971) and the in vitro construction of recombinant plasmids between EcoRI restriction fragments of F and a small plasmid (Skurray et al., 1976b; Clark et al., 1976) have provided a detailed genetic map of the F transfer genes (Fig. 1-1) (Achtman, 1973a; Willetts, 1977a; Achtman and Skurray, 1977; Rowbury, 1977).

The mutant F plasmids and the cloned fragments of F have been used to dissect the process of conjugation and a detailed (but still vastly

incomplete) understanding of this process is now available (Achtman and Skurray, 1977; Manning and Achtman, 1979). An outline of the mating cycle is given in Fig. 1-2. Donors and recipients are brought into wall to wall contact and unstable mating aggregates are formed. The majority of cells in a mating population are in the form of aggregates rather than pairs (Achtman, 1975). These aggregates are stabilised and DNA transfer ensues. Expression of the plasmid in the newly infected recipients results in the disaggregation of mating bacteria (Achtman, 1977) and the conversion of the recipients into competent donors. These newly established donors are unsuitable as recipients in a further mating with the same or a closely related plasmid because surface exclusion is established (Lederberg et al., 1952).

In this discussion I will concern myself primarily with a consideration of DNA transfer and the genes that are involved in this process. I intend discussing in some detail the disaggregation of mating bacteria and the establishment of surface exclusion as these two phenomena are intimately connected to DNA transfer. I will not consider aggregate formation and the interested reader is referred to a recent review by Manning and Achtman (1979) which exhaustively documents the cell to cell interactions that occur during conjugation.

Organisation and function of F transfer genes

Approximately one third of the F plasmid is taken up by transfer genes (Fig. 1-3). The remainder of the plasmid encodes a variety of functions but large regions remain unmapped. Between kilobase co-ordinates 93 and 17.6 is located a series of insertion sequences which are involved in the formation of F' plasmids and Hfrs (Davidson et al.,

1975). Genes for a variety of functions have been mapped between co-ordinates 33 and 49. These include inhibition of phage T7 multiplication (pif) (Skurray et al., 1976b; Palchoudhuri and Maas, 1977), the origin of vegetative replication (oriV) (Eichenlaub et al., 1977), functions involved in replication (frp) (Skurray et al., 1976a; Eichenlaub, 1979) and incompatibility (Santos et al., 1975; Skurray et al., 1976b).

Seventeen genes (Fig. 1-1) have been defined by mutation as being required for conjugation. Presumably more genes are involved because protein products from this region that do not correspond with known tra genes have been identified (6c, 6d, 6e, and 2b) and moreover this region has not been mapped to saturation (Kennedy et al., 1977; Thompson and Achtman, 1979). Most of these genes are arranged in a single, large operon (Helmuth and Achtman, 1975) which is under the positive control of the traJ gene product (Willetts and Achtman, 1972; Achtman, 1973b). This has recently been shown to be required for transcription of the transfer operon (Willetts, 1977b). However it is interesting to note that traJ gene product, a polypeptide of about 23,500 daltons, is an outer membrane protein, a location which would appear to contradict its role as a transcription control factor (Kennedy et al., 1977).

The majority of plasmids isolated from nature do not express their full potential for conjugal transfer (Meynell et al., 1968). In the case of many F-like plasmids this is a consequence of the activity of a plasmid-encoded fertility inhibition system. Fertility inhibition exerts negative control on the expression of traJ and hence of the tra operon (Finnegan and Willetts, 1973; Willetts, 1977b. This control is thought to be mediated by three genes finO, finP and traO (Finnegan and Willetts, 1971). The finO and finP gene products are both required for the inhibition of traJ. Mutations in traO are cis-dominant, prevent repression

and map close to traJ (Willetts et al., 1976; Willetts, 1977a). These mutants may define the traJ promoter which may be the site of action of finO and finP products (Willetts et al., 1976). The F plasmid is a naturally occurring finO mutant and therefore expresses transfer genes constitutively.

At least twelve transfer gene products of F (traA, E, K, B, V, W, C, U, F, H and part of traG) are involved in pilus biosynthesis (Achtman et al., 1971, 1972; Willetts and Achtman, 1972; Miki et al., 1978). F pili are specialised filaments on the surface of F^+ bacteria that can be identified immunologically (Ørskov and Ørskov, 1960), by electron microscopy (Crawford and Gesteland, 1964) and are the adsorption site for a variety of RNA and DNA bacteriophages (Crawford and Gesteland, 1964; Caro and Schnös, 1966). The properties and possible biological role of sex pili have been the subject of several comprehensive reviews (Brinton, 1971; Paranchych, 1975; Tomoeda et al., 1975). The pilus consists of one subunit (termed pilin) of about 11,200 daltons (Brinton, 1971; Date et al., 1977; Helmuth and Achtman, 1978) and contains a single D-glucose and two phosphate residues (Brinton, 1971). The pilin subunits are arranged in helices; each pilus is composed of 4 interwound helices with a central hole of 20Å diameter (Folkhardt et al., 1979).

The majority of the evidence suggests that pili serve to establish initial contacts between donors and recipients (Achtman and Skurray, 1977). It has also been suggested that F pili retract during conjugation (Curtiss, 1969) since F pili may be induced to retract by treating donors in a variety of ways including infection by DNA-containing bacteriophages (Jacobson, 1972), cyanide or low temperature (Novotny and Fives-Taylor, 1974). Retraction may serve to bring donor and recipient cell surfaces into close contact. In contrast to the idea that F pili are the

recognition site for recipients, Brinton (1965, 1971) has suggested that DNA transfer occurs through the pilus. This was suggested because DNA transfer was claimed to have occurred between cells connected by pili but not in wall to wall contact (Ou and Anderson, 1970). This suggestion seems unlikely because at concentrations of sodium dodecyl sulphate (SDS) sufficient to dissolve pili, DNA transfer occurs normally within mating aggregates (Achtman et al., 1978b).

There is some controversy regarding which transfer gene encodes pilin. Evidence exists which suggests that either traA or traJ is the pilin structural gene. Suppression of amber traA mutations resulted in the production of F pilin with an altered amino acid sequence (Min kley et al., 1976) suggesting traA to be the pilin structural gene. In contrast Manning and Achtman (1979) have argued that traJ is the pilin structural gene. The protein product of the traA gene is not precipitated with anti F pilin antiserum (Kennedy et al., 1977) and the tryptic finger print of purified traA product does not resemble that of F pilin whilst that of traJ does (Helmuth, cited in Manning and Achtman, 1979). The assignment of traJ as the F pilin structural gene is consistent with its location in the outer membrane of the cell envelope (Kennedy et al., 1977). If traJ is ultimately proven to be the pilin structural gene one has then to account for the findings that traJ product is required for transcription of the tra operon (Willetts, 1977b). Manning and Achtman (1979) have proposed that an unidentified protein monitors the amount of traJ gene product in the cell envelope and triggers transcription of the tra operon when traJ product is present in an unprocessed form.

The role of the other transfer genes that are involved in pilus biosynthesis is unknown. They may be involved in phosphorylation or glucosylation of pilin or in pilus assembly. Since both traA gene product

(13,700 daltons) and traJ gene product (23,500 daltons) are larger than pilin (11,200 daltons) purified from the pilus, one or more of the transfer genes may encode enzymes that are involved in the post transcriptional modification of pilin precursor.

The traG gene product appears to be bifunctional, because bacteria harbouring some traG mutants do not synthesise pili whilst others do but are transfer defective (Achtman et al., 1971, 1972; Willetts and Achtman, 1972). Piliated bacteria containing traG mutants form few mating aggregates with recipients suggesting a role for this gene product in the stabilisation of mating aggregates (Achtman and Skurray, 1977).

Five other genes have been identified which are involved in transfer but are not required for pilus biosynthesis. The products of traD, traI, traM, traN and traY may be involved in the metabolism of plasmid DNA during transfer (Achtman et al., 1971, 1972; Willetts and Achtman, 1972; Achtman et al., 1979; Miki et al., 1978).

Plasmid transfer is associated with DNA synthesis and before considering this aspect of conjugation I will digress to consider current knowledge of some relevant aspects of DNA synthesis in bacteria.

Some aspects of the enzymology of DNA synthesis

In this section I will describe some of the basic features of DNA synthesis which are pertinent to a consideration of the mechanism of DNA synthesis associated with plasmid transfer. This conjugal synthesis of plasmid DNA is an asymmetric process occurring in both donor and recipient. It resembles, in a formal sense, the mechanism of DNA synthesis observed during the infection of E.coli by single-stranded DNA phages rather than vegetative synthesis of E.coli chromosomal DNA. For this reason I will not discuss the mechanism by which a duplex DNA molecule is replicated

but will consider DNA synthesis on single-stranded templates. This type of DNA synthesis and the mechanism of vegetative DNA replication have been the subject of numerous reviews (Kornberg, 1974, 1976, 1977, 1978; Gefter, 1975; Geider, 1976; Alberts and Sternglanz, 1977; McMacken et al., 1977b; Wickner, 1978; Zechel, 1978).

The starting point for this discussion is the finding that no known DNA polymerase can initiate the synthesis of DNA de novo (Kornberg, 1974). E.coli DNA polymerases demand a correctly base-paired residue at the 3'OH terminus of the strand to which nucleotides are to be polymerised. It has been argued that the requirement for this primer terminus is a consequence of the need for high fidelity copying of the template (Alberts and Sternglanz, 1977).

A search for the primer-generating enzymes was undertaken using in vitro or semi in vitro DNA synthesising systems which exploit the single-stranded DNA of certain small bacteriophages as templates (Geider, 1976). The first enzyme capable of synthesising a primer was identified when the DNA of the bacteriophage fd (M13) was used in these systems. The replication of these filamentous DNA phages has been reviewed recently by Ray (1977). The synthesis of DNA on the viral DNA of phage fd is sensitive to rifampicin (Brutlag et al., 1971; Wickner, W. et al., 1972; Geider and Kornberg, 1974) implicating DNA dependent RNA polymerase in the priming process. The synthesis of fd replicative form (RF) from viral DNA requires four ribonucleotides (Wickner, W. et al., 1972) and is initiated at a unique site on the template (Wickner, W. et al., 1972; Tabak et al., 1974). In an in vitro DNA synthesis system involving purified components, this DNA synthesis requires RNA polymerase, DNA binding protein (DBP), DNA polymerase III holoenzyme, DNA polymerase I

and DNA ligase (Geider and Kornberg, 1974).

It is thought that DBP covers most of the single-stranded phage DNA leaving an essentially double-stranded region of DNA some 125 nucleotides long (Schaller et al., 1976). This region contains two hair pin-like structures (Gray et al., 1978) and in the presence of ribonucleoside triphosphates and RNA polymerase, a short transcript is synthesised at one of these hairpin structures. The purified RNA has a length of about 30 nucleotides (Geider et al., 1978). DNA polymerase III initiates DNA synthesis on this primed template and a complementary strand is synthesised with an RNA fragment at the 5' terminus (Westergaard et al., 1973). The 5'→3' exonuclease activity of DNA polymerase I is thought to remove the primer molecule and fill the gap created. Ligase joins the ends of the newly synthesised DNA to generate an intact circular DNA molecule (Geider and Kornberg, 1974). In the absence of DNA polymerase I a small gap is created in the newly synthesised strand (Geider and Kornberg, 1974) which corresponds to the in vivo origin of replication (Horiuchi and Zinder, 1976).

In contrast to phage fd, the conversion of the single-stranded DNA of phages G4 and ØX174 to the duplex form is not inhibited by rifampicin (Scheckman et al., 1972, 1974). Whilst it is clear that RNA polymerase does not feature in the initiation of DNA synthesis on these templates, an RNA synthetic event and a possible role for this molecule in priming DNA synthesis has been identified (Scheckman et al., 1972).

Studies involving phage G4 viral DNA have identified a protein that can synthesise primers in a rifampicin-resistant process. In vitro studies showed that synthesis of a complementary strand to G4 viral DNA requires only three components : DBP, DNA polymerase III holoenzyme and one other component (Zechel et al., 1975). This unidentified protein has a

molecular weight of about 60,000 and is resistant to N-ethyl maleimide, properties which suggested it may be the product of the dnaG gene (Wickner et al., 1973). This gene product had been implicated previously in the initiation of nascent (or Okazaki) replication fragments (Lark, 1972; Olivera and Lark, 1973). Bouché et al. (1975b) reported the purification of this enzyme and identified this protein as a rifampicin-resistant RNA polymerase. This enzyme catalyses the incorporation of ribonucleoside triphosphates into an oligoribonucleotide synthesised from a unique region of G4 DNA coated with DBP. The oligoribonucleotide is covalently linked to the 5' terminus of the newly synthesised complementary strand (Bouché et al., 1975a, 1978). The dnaG gene product, now termed primase (Rowen and Kornberg, 1978a), uses either ribo- or deoxyribonucleoside triphosphates as substrates (McMacken et al., 1976a; Kornberg, 1977; Wickner, 1977; Rowen and Kornberg, 1978b). In the presence of ribonucleoside triphosphates the primer molecule, made at the origin of G4 DNA synthesis, is about 29 residues in length, contains a hairpin region (Bouché et al., 1978) and is reduced to about 6 residues in length when mixed ribo- and deoxyribonucleoside triphosphates are used as substrate (Rowen and Kornberg, 1978b). Priming occurs at a specific site (Hourcade and Dressler, 1978; Fiddes et al., 1978) which contains a DNA sequence complementary to the sequence of the RNA primer (Sims and Dressler, 1978).

A third and complex priming system has been identified using the single-stranded DNA of phage ØX174 as a template for DNA synthesis. The duplication of ØX174 DNA has recently been reviewed by Denhardt (1977). Like phage G4, conversion of the viral DNA of phage ØX174 to the duplex RF is resistant to rifampicin, but in contrast this DNA synthesis requires numerous host proteins defined by both mutation and biochemical analysis.

DNA synthesis on a template of ØX174 DNA requires DNA polymerase III holoenzyme, dnaB, dnaC and dnaG products and is independent of RNA polymerase (Scheckman et al., 1972; Wickner, R.B. et al., 1972). In vitro in addition to these proteins it also requires DBP and two or three E.coli proteins which have been termed x, y and z (Wickner and Hurwitz, 1974) or n and i (Schekman et al., 1975). Factor y is a single-stranded DNA dependent ATPase and dATPase (Wickner and Hurwitz, 1975c). The product of the dnaB gene has been purified in a number of laboratories as protein of 48-55,000 daltons (Lanka et al., 1978; Rehakrantz and Hurwitz, 1978a; Ueda et al., 1978) which exists as a multimer (Lark and Weschler, 1975; Kogoma, 1976) of 6 subunits within the cell (Rehakrantz and Hurwitz, 1978a). The dnaB product is a single-stranded DNA-dependent ribonucleoside triphosphatase (Wickner et al., 1974; Rehakrantz and Hurwitz, 1978b) which in the presence of ATP forms a complex with dnaC gene product (Wickner and Hurwitz, 1975a).

The conversion of ØX174 viral DNA to the duplex form may be described as a series of enzymic steps involving each of the components detailed above (McMacken and Kornberg, 1978). Viral DNA coated with DBP is converted into a replication intermediate containing dnaB, dnaC proteins and proteins i and n (or x and y) (Wickner and Hurwitz, 1974; Weiner et al., 1976; Wickner and Hurwitz, 1975b). The dnaB product, once fixed in the intermediate, may migrate along the DBP-covered template strand using ATP hydrolysis as an energy source and participate in initiating several primers along the template strand, perhaps acting as a motile promoter for primase action (McMacken et al., 1977b).

Any of the three DNA polymerases in E.coli can initiate DNA synthesis on a primed template strand : DNA polymerase I (Kornberg, 1974), DNA polymerase II (Sherman and Gefter, 1976) or DNA polymerase III. DNA

polymerase III, the product of the polC (dnaE) gene is the primary replication enzyme since DNA synthesis in vitro and the replication of the E.coli chromosome in vivo are thermosensitive in temperature-sensitive polC strains (Gefter et al., 1971). Moreover nonconditional mutations have been isolated in polA and polB, the genes for DNA polymerase I and II respectively (De Lucia and Cairns, 1969; Campbell et al., 1972). The polC gene product is part of the DNA polymerase III holoenzyme because when the holoenzyme is purified from a polC (dnaE) thermosensitive mutant it is thermolabile (Gefter et al., 1971). The mechanism of DNA synthesis mediated by DNA polymerase III will not be discussed. This aspect of DNA synthesis has been reviewed recently by Wickner (1978).

Possible involvement of these various enzymes in the synthesis of plasmid DNA associated with transfer will now be considered.

Metabolism of F plasmid DNA during transfer

Processing of F plasmid DNA during transfer has been studied in some detail. A specific strand of the plasmid is transferred from donor to recipient (Cohen et al., 1968a,b; Ohki and Tomizawa, 1968; Rupp and Ihler, 1968; Vapnek and Rupp, 1970), and by examining λ prophage transfer from F' and Hfr donors it was demonstrated that the DNA enters the recipient with its 5' terminus leading (Ohki and Tomizawa, 1968; Rupp and Ihler, 1968). The complement to the transferred strand of DNA is conserved in the donor where it acts as a template for the synthesis of a replacement strand (Vapnek and Rupp, 1970). DNA complementary to that transferred from the donor is synthesised in the recipient (Ohki and Tomizawa, 1968).

Transfer is initiated therefore by a strand specific nicking event. A cis-dominant site, termed oriT (origin of transfer), which is required for transfer, has been mapped on the F plasmid (Fig. 1-1, 1-3) (Willetts, 1972; Guyer and Clark, 1976). This site, which has been assigned to a 250 base pair region of F (Achtman et al., 1979), may be the site of nicking.

From an examination of F plasmid transfer during Hfr-mediated matings Wollman and Jacob (1958) concluded that Hfr donors transfer part of the F plasmid early and part late. This is consistent with the position of oriT and the insertion sequences on the map of F (Fig. 1-3). These conclusions have been confirmed by Guyer and Clark (1977) who demonstrated that F replication genes (frp) were transferred early in an Hfr mating.

Little is known about the biochemistry of the nicking reaction. Nicking apparently does not occur in bacteria carrying Flac traI and traM mutants (Kingsman and Willetts, 1978) suggesting a role for these gene products in this reaction. It has been suggested that the traI gene product may be a specific endonuclease which acts at the origin of transfer (Reeves and Willetts, 1974). However studies by Achtman et al. (1979) have suggested a novel role for traI and traM gene products and have identified a site tirO which maps very close and to the left of oriT. Two plasmid-specified proteins, termed InhIp and InhMp, were postulated to exert inhibition of plasmid transfer. During conjugation traI and traM gene products were proposed to antagonize the inhibitory effects of InhIp and InhMp. In the presence of all other conjugation proteins, the result of this antagonism is the initiation of DNA transfer and conjugal DNA synthesis.

These results for the processing of F plasmid DNA during transfer have been incorporated into several more detailed models for plasmid transfer

(a) The rolling circle model. The rolling circle model for conjugal transfer and replication (Gilbert and Dressler, 1968) is a well established model and is based on two separate lines of evidence. Fulton (1965) demonstrated linkage, late in an Hfr cross, between markers that border oriT, suggesting that chromosome transfer was continuous with no defined terminus. In agreement with this idea, F and F' DNA isolated from recipients was claimed to be in the form of a linear duplex DNA molecule that was longer than the unit length of the parental plasmid (Matsubara, 1968; Ohki and Tomizawa, 1968). In its original form the rolling circle model (Fig. 1-4) (Gilbert and Dressler, 1968; Ohki and Tomizawa, 1968) involves strand specific cutting of the circular plasmid molecule (a) to create a 3'OH terminus (b) which is extended by DNA synthesis (c). The displaced 5' terminus is transferred to the recipient (c) and continuous DNA synthesis in the donor generates multimeric lengths of single-stranded DNA (d). This material is converted to the duplex form (d, e) and a unit length circular molecule is generated by recombination between the repeated regions of plasmid DNA (f) (Matsubara, 1968).

Subsequent studies have shown this classical rolling circle model to be an over-simplification and have led to a second model.

(b) A modified rolling circle model. Conjugal synthesis of Flac in the donor is mediated by DNA polymerase III (Kingsman and Willetts, 1978), the primary replication enzyme of E.coli, in a process that is independent of dnaB gene product (Bresler et al., 1968, 1973; Vapnek and Rupp, 1971; Wilkins and Hollom, 1974). Kingsman and Willetts (1978) found that conjugal DNA synthesis in a dnaB donor is abolished by rifampicin, a specific inhibitor of RNA polymerase (Wehrli et al., 1968), but is unaffected by the protein synthesis inhibitor, spectinomycin. These

results suggest the involvement of an untranslated species of RNA in some aspect of the transfer process. RNA synthesis is not involved in the displacement of the strand destined to be transferred because transfer occurs in the presence of rifampicin. RNA synthesis may therefore be required to prime the synthesis in the donor of DNA to replace the transferred material (Kingsman and Willetts, 1978). Why this process should require such priming by an RNA molecule is unclear. The 3'OH group created by nicking at oriT should be capable of acting as a primer terminus, as proposed in the original rolling circle model (Fig. 1-4), but presumably this terminus is inaccessible to DNA polymerase III. A similar mechanism of DNA synthesis is associated with the synthesis of single-stranded viral DNA of phage M13 from its replicative form (Olsen et al., 1972; Staudenbauer and Hofschneider, 1972; Staudenbauer, 1974), a process formally analogous to donor conjugal synthesis. In order that this system may allow the generation of multimeric lengths of plasmid DNA in the recipient, the postulated RNA primer has to be removed and the gap created filled by DNA synthesis.

None of the transfer gene products identified to date appear to be involved directly in donor conjugal DNA synthesis. Synthesis does not occur in bacteria harbouring Flac plasmids mutant at traI or traM (Kingsman and Willetts, 1978). However these gene products have been implicated in the nicking reaction at oriT (Willetts and Maule, 1979; Achtman et al., 1979) an event which is a prerequisite for donor conjugal synthesis. Conjugal synthesis occurs at a reduced rate in Flac traD mutants and this gene product may facilitate the removal of the strand to be transferred from the template for conjugal DNA synthesis (Kingsman and Willetts, 1978). Adsorption of male specific phage to Flac traD

bacteria is normal but intracellular penetration is reduced (Achtman et al., 1971) suggesting that this gene product may be involved in the penetration of phage RNA into the cell. Willetts (1977) has argued that by analogy it may also be required for the penetration of the cell surface by plasmid DNA during transfer. Membrane penetration and removal of the strand to be transferred from the template for donor conjugal DNA synthesis may be closely related phenomena. Flac traN mutants conjugally synthesise plasmid DNA in donors but are transfer defective, suggesting that the traN gene product may function in DNA transfer (Kingsman and Willetts, 1978).

In the recipient a complement to the transferred strand is synthesised by DNA polymerase III (Wilkins and Hollom, 1974). These workers found that conjugal synthesis of Flac in recipients in which dnaB product was thermally inactivated was sensitive to rifampicin. However in the presence of active dnaB product the process was resistant to the drug. These findings suggest that conjugal synthesis of Flac in recipients may be primed by two interchangeable priming systems involving RNA polymerase or primase. The rifampicin sensitivity of this DNA synthesis in dnaB bacteria indicates that primers can be made by RNA polymerase. This system is presumably analogous to that operating during conversion of M13 viral DNA to the duplex form. However the rifampicin resistance of conjugal DNA synthesis in $dnaB^+$ bacteria suggests that the priming reaction can also be mediated by dnaB product in a rifampicin-resistant process. Such a primer synthesising system has been identified in the conversion of single-stranded ØX174 DNA into replicative form and involves primer synthesis by primase acting with dnaB product in a multicomponent system. Thus in a dnaB recipient, conjugal synthesis is primed by

RNA polymerase and in a rifampicin-treated *dnaB*$^+$ recipient it is initiated by primase activity in conjunction with *dnaB* product. Marinus and Adelberg (1970) have reported the detection of conjugal synthesis of F*lac* in bacteria carrying temperature-sensitive *dnaG* mutations. This is not surprising if this synthesis can be initiated by RNA polymerase.

Interchangeability of primer synthesising systems has been observed in phage ØX174 replication. Dumas *et al*. (1975) found that in a *dnaC* mutant ØX174 RF formation involves a rifampicin-sensitive initiation process which allows phage replication at the non-permissive temperature. This reaction also occurs *in vitro* (Wickner and Kornberg, 1974). This is not a simple substitution between RNA polymerase and primase because RNA polymerase cannot replace primase activity as shown by the finding that phage replication is blocked in both *dnaG* and *dnaB* mutants at the non-permissive temperature (McFadden and Denhardt, 1974; Dumas and Miller, 1974).

A central feature of the rolling circle model for conjugal transfer is the generation in recipients of a linear duplex molecule greater than the unit length of the parental plasmid. In the original model (Fig. 1-4) circularisation of this material was envisaged to occur by a recombinational event between the repeated regions of plasmid DNA. However since the formation of circular molecules is apparently normal in a *recA* recipient (Clark and Margulies, 1965) an alternative process must also be available. It has been postulated that circle formation may be mediated by a site-specific recombinational system specified by F (Kingsman and Willetts, 1978; McIntire and Willetts, 1978). However transcription of F DNA in the newly infected recipients is not required for the formation of covalently closed circular molecules (Hiraga and Saitoh, 1975). It is

possible that circularisation in the recipient may be mediated by functions supplied by the donor. This possibility is discussed in the following section.

(c) F plasmid transfer involving transmission of molecules of unit length

The experimental evidence upon which the rolling circle model was based has recently been criticised (Achtman and Skurray, 1977). These authors suggest that the observations of Fulton (1965) may be explained by multiple transfer events from more than one donor to a single recipient (Achtman et al., 1978b) accounting for the linkage of proximal and distal markers late in an Hfr mating. The experiments of Matsubara (1968) and Ohki and Tomizawa (1968) are subject to the criticism that techniques designed to differentiate unit length, supercoiled or open circular molecules from linear multimers were not employed. Furthermore, in apparent contradiction to a rolling circle model Falkow et al. (1971) were unable to demonstrate multimeric linear molecules as intermediates in the transfer of the F-like R plasmid R1drd-19. Moreover transfer of multiple lengths of single-stranded Flac DNA is not a prerequisite for inheritance of the plasmid by recipients because when conjugal DNA synthesis in the donor is inhibited by rifampicin, transfer of preexisting plasmid DNA to recipients occurs normally (Kingsman and Willetts, 1978). Under such conditions a precise monomeric length of Flac DNA is presumably transferred, replicated in the recipient and circularised. Such a finding also argues against the notion that conjugal DNA synthesis in the donor provides the driving force for DNA transfer (Jacob et al., 1963).

These findings have been encompassed in a model for transfer involving transmission of precise monomeric lengths of plasmid DNA (Kingsman and Willetts, 1978). Nicking at oriT was proposed to result in complexing of

the 3'OH terminus created by the nicking event. The complexed terminus would then be unable to function as a primer terminus for conjugal DNA synthesis in the donor and provides an explanation for the requirement for an RNA primer (Kingsman and Willetts, 1978). It was also proposed that the 5' terminus is complexed in a manner analogous to the cisA protein-DNA complex in ØX174 DNA replication (Eisenberg et al., 1977). The cisA protein is a multifunctional protein that is thought to mediate nicking of duplex and circular phage DNA and the joining of the 5' and 3' ends of the displaced single-stranded phage DNA to generate viral DNA (Eisenberg et al., 1977). In F transfer the complexed termini may be able to promote the direct ligation of the 5' and 3' ends to achieve circularisation in the recipient (Kingsman and Willetts, 1978). A similar model has been proposed by Warren et al. (1978) and Staudenbauer (1978) for the mobilisation of ColE1 (see Fig. 1-5) by a sex factor. This model for F plasmid transfer predicts that an intact oriT sequence in the donor is required for subsequent circularisation of the transferred DNA in the recipient. However McIntire and Willetts (1978) were able to demonstrate that deletions of Flac which removed the entire transfer region and oriT were capable of circularisation in recA recipients, following mobilisation by a tra^+ F plasmid from transient heterozygotes.

Active dis aggregation and surface exclusion

Shortly after DNA transfer is complete, expression of the plasmid in recipients has at least two effects : mating bacteria actively dis - aggregate (Achtman, 1977; Achtman et al., 1978b) and surface exclusion is established (Lederberg et al., 1952). The majority of cells in a mating population of bacteria are found in large aggregates (Achtman, 1975). In matings between an Hfr and an F^- recipient mating aggregates are

not broken down (Achtman, 1977), whilst in matings involving an autonomous F plasmid mating bacteria rapidly dis aggregate after DNA transfer (Achtman et al., 1978b). Because part of the F plasmid is inherited by the recipient late in an Hfr mating (Guyer and Clark, 1977), it was proposed that the genes responsible for promoting dissaggregation are located in this region of F (Achtman, 1977). Despite an extensive analysis of active dissaggregation the plasmid gene(s) responsible have not been identified (Achtman, 1977). However it is clear that traS product, which promotes surface exclusion, is not involved because dissaggregation occurs in matings involving donors of an Flac traS mutant. The biochemical basis of dissaggregation is completely obscure but Achtman (1977) has suggested that the immigrant plasmid directs the synthesis of enzymes that modify the cell envelope and render mating aggregates unstable.

As a consequence of recipients inheriting the F plasmid, they are no longer able to act as recipients in further matings with the same or a related plasmid (Lederberg et al., 1952; Willetts and Maule, 1974). This property of F^+ bacteria is termed surface or entry exclusion (Novick, 1969). Surface exclusion is not a consequence of bacteria bearing pili because mutants of Flac have been isolated which do not direct the synthesis of pili but are still proficient in surface exclusion (Achtman et al., 1972).

Eckerson and Reynard (1977) have claimed that surface exclusion acts at a stage subsequent to the formation of mating aggregates. They found that when both parents exhibited surface exclusion the number of mating aggregates was unaffected while transconjugant formation was markedly reduced. However a number of observations suggest that surface exclusion

acts at both the level of aggregate formation and DNA transfer. Achtman et al. (1972) have demonstrated a correlation between the expression of surface exclusion and a diminution in the formation of mating aggregates as measured by a Coulter counter. Two genes, traS and traT have been identified as jointly mediating surface exclusion (Achtman et al., 1977; Kennedy et al., 1977). Mutations in traS or traT reduce but do not abolish surface exclusion (Achtman et al., 1977) whilst traJ mutants are wholly defective (Achtman et al., 1971). It was concluded (Achtman et al., 1977) that surface exclusion is a phenomenon mediated independently but cumulatively by traS and traT gene products. The traT gene product primarily inhibits stable aggregate formation and the traS gene product limits DNA transfer between aggregated bacteria.

It has been suggested that traG gene product in the donor may be involved in the stabilisation of mating aggregates (Achtman and Skurray, 1977). Few aggregates are formed in an Flac traG x F^- mating as in an F^+ x F^+ mating, and those that do form are unstable. Achtman and Skurray (1977) have proposed that traT gene product, an outer membrane protein (Minkley and Ippen-Ihler, 1977; Kennedy et al., 1977), acts at the same stage in the stabilisation of mating aggregates as traG gene product, perhaps by inhibiting the interaction between a cell-surface component and the traG product. The site of action of traS gene product, an inner membrane protein (Manning and Achtman, 1979) is unknown.

SEX FACTOR - PROMOTED MOBILISATION OF ColE1

Since the genes required for conjugation occupy a 15-20 megadalton region of the F plasmid (Helmuth and Achtman, 1975), most conjugative plasmids are of necessity large, normally ranging from 50-100 megadaltons in size. Conversely small plasmids of less than 10 megadaltons cannot be

sex factors. Transfer (or mobilisation) of small non-self transmissible plasmids may be promoted by the presence of a conjugative plasmid. ColE1 is one such plasmid and its mobilisation is promoted efficiently by IncF and IncIα plasmids (Reeves and Willetts, 1974). Studies of the mobilisation process have provided an insight into the events occurring at the origin of transfer.

A number of observations suggest that mobilisation is not by the formation of a transient cointegrate. Firstly when ColE1 is mobilised to a recipient by ColI, many recipients inherit ColE1 without ColI (Smith et al., 1963). Secondly traI mutants of the F plasmid efficiently mobilise ColE1 (Willetts, 1972).

The metabolism of ColE1 DNA during mobilisation has not been studied in detail. It is unclear whether a specific strand of the plasmid is transferred and if conjugal synthesis occurs in the donor and recipient. Kingsbury and Helinski (1973) examined ColV-promoted transfer of ColE1 between polA (ts) mutants. These workers found that at the restrictive temperature ColE1 transfer was dramatically reduced if the donor was polA (ts), whilst it was abolished if the recipient was polA (ts). These results have been interpreted as suggesting that ColE1 transfer involves conjugal synthesis of plasmid DNA in both the donor and recipient (Staudenbauer, 1978).

If such a pattern of asymmetric transfer and DNA synthesis occurs (and this needs confirming) then a nicking event is implicated in the donor. A likely candidate for the nicking enzyme is the relaxation complex. Covalently closed circular ColE1 DNA may be purified in a form that, when subjected to a variety of treatments that affect protein structure, is nicked at a specific site in a specific strand (Clewell and

Helinski, 1969, 1970a; Lovett et al., 1974). This protein DNA complex has been termed the relaxation complex. The ColE1 relaxation complex has been purified and three protein components of molecular weights 60,000, 16,000 and 11,000 are found associated with plasmid DNA (Lovett and Helinski, 1975). Relaxation results in the dissociation of the smaller proteins from the DNA, whereas the larger protein remains covalently associated with the 5' terminus of the nicked DNA strand (Blair and Helinski, 1975; Guiney and Helinski, 1975). These proteins may or may not be specified by the ColE1 plasmid (Dougan and Sherratt, 1977; Meagher et al., 1977).

It was originally proposed that the relaxation complex functioned in vegetative replication but more recently a correlation between relaxation and mobility has been established. An insight into the biological role of the complex was obtained using deletion and Tn1 insertion mutants of ColE1. Some of the mutants are both non-transmissible and non-relaxable (Dougan and Sherratt, 1977; Inselburg, 1977). The transfer defect is complemented by wild-type ColE1 (Warren and Sherratt, 1977). When the site of the relaxation nick is inserted into a non-mobilisable plasmid, the resulting hybrid plasmid is mobilised by a sex factor in the presence of ColE1 (Warren et al., 1978).

These findings have been summarised in two independent but similar models for the mobilisation of ColE1 (Staudenbauer, 1978; Warren et al., 1978). The model presented by Warren et al. (1978) is described in Fig. 1-5. The supercoiled plasmid DNA-protein complex in the donor is relaxed and one of the relaxation proteins (60,000 daltons) becomes covalently attached to the 5' end of the nicked strand (a). The 5' terminus begins to be transferred to the recipient (b) and a replacement strand is synthesised by a rolling circle type mechanism in the

donor (b). Transfer of a unit length of ColE1 is completed and complementary strand synthesis in the recipient is initiated (c). This synthesis recreates the relaxation site in the recipient and the relaxation protein attached to the 5' terminus mediates a second nicking reaction (d). Circularisation is achieved by ligation of the two ends in the recipient by the 5' attached relaxation complex protein (e). Complementary strand synthesis in the recipient is completed and the transferred plasmid is supercoiled. In this model the 60,000 dalton relaxation complex protein functions in a manner analogous to that proposed for the cisA protein of ØX174 (Eisenberg et al., 1977).

This model predicts that conjugal DNA synthesis in the donor is a prerequisite for inheritance of the plasmid by recipients. Such a requirement is not observed for Flac transfer (Kingsman and Willetts, 1978).

IncIα PLASMID TRANSFER

The work in this thesis has involved IncIα plasmids. The prototype plasmid is ColIb-P9 and was first identified in Shigella sonnei P9 (Fredericq, 1965) by mating this strain with Salmonella typhimurium LT2 and E.coli K12. The transconjugants were subsequently shown to be able to transmit the property of colicin Ib production to another recipient (Ozeki and Howarth, 1961; Clowes, 1961). However the efficiency with which ColI is transmitted from an established strain is much lower than the prototype sex factor F. In contrast when the ability of newly formed transconjugants to transmit ColI was examined, the frequency of transfer was increased by almost three orders of magnitude (Stocker et al., 1963). Bacteria in these two states have been termed LFT and HFT (high/low

frequency of transfer) (Monk and Clowes, 1964a). This transition from the LFT state to HFT state was proposed to be a consequence of ColI transfer being subject to negative control by repression (Clark and Adelburg, 1962). Thus upon transfer to a recipient, the state of repression is temporarily relieved and HFT cultures result. After a period of growth repressor is synthesised and the LFT state established. This hypothesis was strengthened when ColI mutants that expressed transfer functions constitutively (drd) were isolated by Dowman and Meynell (1970) using a method developed by Meynell and Datta (1967). It seems likely therefore that IncIα plasmid transfer is controlled by a system analogous to that controlling F plasmid transfer. The extent of the similarity remains to be ascertained.

A number of observations suggest that the F and I transfer systems may be different. The I compatibility group comprises several sub groups (Hedges and Datta, 1973) many of which encode a pilus which is distinct from the F pilus. Pili specified by I group plasmids do not adsorb F pilus-specific phages and conversely F pili will not absorb I specific phage (Meynell and Lawn, 1968). F and I type pili do not cross react serologically (Lawn and Meynell, 1970) and are structurally distinct (Lawn et al., 1967). Studies on E.coli mutants that manifest a reduced ability to act as recipients in matings with F and I plasmid donors have identified different components in the cell envelope that are involved in conjugation with the different donors (reviewed by Manning and Achtman, 1979).

Transfer-deficient mutants of the IncIα plasmid R64drd-11 are not complemented by Flac and several Flac Tra$^-$ mutants are not complemented by R64drd-11 (Cooke et al., 1970; Willetts, 1970). Furthermore the

transfer inhibition system of the I group plasmids does not repress the transfer of F type plasmids that are expressing transfer functions constitutively (Lawn et al., 1967; Rowero and Meynell, 1969). The surface exclusion systems specified by F and the I group conjugative plasmids also show different specificities (Monk and Clowes, 1964b).

Although these findings presumably reflect an underlying difference in the two transfer systems, the I group plasmids show the same asymmetric pattern of plasmid transfer and conjugal DNA synthesis described for F plasmids (Vapnek et al., 1971). Transfer of the IncI$_\alpha$ plasmid R64drd-11 has been examined in detail by Fenwick and Curtiss (1973a,b,c) in a mating system that involves donors mutant at dnaB and minicells as recipients. Exploitation of DNA-deficient minicells as recipients allowed the separation of donor and recipient bacteria from the mating mixture by sucrose density gradient centrifugation, thus facilitating an analysis of the transferred material. Replacement strand synthesis was monitored in the dnaB donors in the absence of chromosomal DNA synthesis (Vapnek and Rupp, 1971; Bresler et al., 1973). R64drd-11 DNA isolated from the recipient minicells was found to be single-stranded and judged by sedimentation analysis to be the same length as the parental R64 plasmid. DNA greater than unit length was not observed (Fenwick and Curtiss, 1973a,b). In addition a delay of several minutes occurred between transfer of one copy of the plasmid and the transfer of its replacement strand (Fenwick and Curtiss, 1973a). These two observations are incompatible with a classical rolling circle type of mechanism for the conjugal transfer and synthesis of R64drd-11.

Involvement of new protein and RNA synthesis in the transfer of R64drd-11 was also examined by treating donors with either the protein synthesis inhibitor chloramphenicol, or rifampicin, a specific inhibitor

of RNA polymerase. Addition of chloramphenicol reduced plasmid transfer by 20-40% and the DNA made in the donors to replace the transferred material was not in turn transmitted. Fenwick and Curtiss (1973b) concluded that in the majority of donors all proteins required for transfer are available before recipient cells are encountered. During transfer of the preexisting strand of the plasmid one or more proteins are consumed and de novo protein synthesis is required before DNA made to replace the transferred strand may itself be transmitted.

Inhibition of RNA synthesis in the dnaB donors by treatment with rifampicin reduced transfer by 70-90%. When the drug was added five minutes after mating had begun, 80% of the donors transferred plasmid DNA (Fenwick and Curtiss, 1973b). The effects of rifampicin were not a result of an impaired ability of the drug-treated bacteria to form stable mating aggregates (Curtiss, 1975). Involvement of a species of RNA that is active in chloramphenicol and synthesised in response to contact with recipients was suggested to be a feature of R64drd-11 transfer. This untranslated RNA molecule, whose synthesis is required for transfer, was postulated to function as a primer for conjugal synthesis of R64 DNA in the donor. An RNA primer has also been implicated in the conjugal replication of Flac in donors, but in this case primer synthesis is not essential for plasmid transfer (Kingsman and Willetts, 1978).

Two models for the transfer of R64 have been proposed (Curtiss and Fenwick, 1975). One model invokes endonucleolytic cleavage of the covalently closed circular plasmid molecule to yield a linear duplex molecule with complementary and overlapping termini. DNA synthesis generates a linear molecule with flush ends. Synthesis of RNA displaces the strands destined to be transferred and also initiates replacement strand synthesis. Therefore recipients receive a single strand of plasmid DNA that is

slightly larger than unit length. Following its conversion to the duplex form, a process which was also postulated to involve an RNA primer, exonucleolytic digestion of the 3' termini (digestion of 5' termini would also suffice) generates a molecule with cohesive termini which facilitate circularisation. Reformation of circular molecules in the donor was envisaged to occur by an essentially similar process.

In an alternative model, transfer was postulated to occur from an open circular plasmid molecule (Curtiss and Fenwick, 1975). The 3'OH group created by nicking at oriT is extended by RNA synthesis resulting in displacement of the strand to be transferred. DNA synthesis is initiated on the 3' terminus of the RNA molecule and sometime before transfer is complete a hypothetical nuclease nicks between the 3' end of the RNA primer and the 5' end of the conjugally synthesised DNA. Consequently recipients receive a unit length of plasmid DNA with an RNA molecule attached to the 3' end. The RNA moiety was postulated to form a hairpin loop which acts as a primer for complementary strand synthesis. Ribonucleolytic digestion before completion of complementary strand synthesis removes the RNA molecule and generates a linear molecule with cohesive termini which promote circularisation.

In apparent contradiction to the findings of Fenwick and Curtiss (1973b) it has been reported that rifampicin has little effect on the transfer of a drd mutant of the prototype IncIα plasmid, ColI, from dna$^+$ bacteria (Wilkins and Hollom, 1974). The reasons for these conflicting results is discussed in Chapter 4.

The conjugal synthesis of ColIdrd-1 in recipients has also been studied but the detailed process is unclear. DNA polymerase III is thought to mediate this DNA synthesis in a process that is independent of both dnaB product and RNA polymerase (Wilkins and Hollom, 1974).

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Therefore this DNA synthesis is not initiated either by RNA polymerase, as in the synthesis of M13 RF DNA or by primase in a multienzyme system involving dnaB product, as in the conversion of ØX174 DNA to RF. The mechanism of primer synthesis is unclear but it is discussed in greater detail in Chapter 5.

AIMS AND METHODOLOGY

The primary aim of this study was to examine the role of primase and RNA polymerase in the conjugal synthesis of IncIα plasmids in recipients. This has been approached using two different but complementary techniques designed to measure transfer from donors and conjugal DNA synthesis in recipients. Because these methods are central to the experiments in this thesis, they will be discussed at this stage.

Conjugal DNA synthesis in recipients can be measured using a technique based on that developed by Freifelder and Freifelder (1968). It involves mating thymine-requiring recipients (mutant at thyA and deoB) with donors in the presence of radioactive thymine. Uptake of labelled thymine by the donors is prevented by exploiting bacteria deficient in thymidine kinase (tdk). Replication of the resident DNA in recipients, which will mask conjugal DNA synthesis, can be eliminated in two ways. The chromosome of the recipient may be rendered an unsuitable template for DNA synthesis by irradiation. Using a strain defective in DNA repair by the excision process (uvr), the bacteria are extensively irradiated with ultraviolet (UV) light before mating (Freifelder and Freifelder, 1968). Radioactive thymine is primarily incorporated in this system into conjugally synthesised DNA in the recipients (Freifelder and Freifelder, 1968; Falkow et al., 1971; Wilkins and Hollom, 1974). Vegetative replication of the incoming plasmid in these irradiated recipients is

thought not to occur because the amount of DNA transferred from the donor has been shown to equal the amount of conjugal DNA synthesis in the recipient (Wilkins and Hollom, 1974; Chapter 3). Furthermore in matings involving F, label is predominantly detected in one strand of the plasmid which is the complement to that transferred from the donor (W.D. Rupp, personal communication to B.M. Wilkins).

Alternatively replication of the chromosome of the recipient may be prevented by exploiting bacteria harbouring a temperature-sensitive dnaB mutation and mating at the non-permissive temperature. At high temperature replication of the chromosome ceases abruptly in such mutants (Weschler and Gross, 1971). Recipients mutant at dnaB and mated at the non-permissive temperature retain the capacity both to receive plasmid DNA during mating and to synthesise a complementary strand (Bresler et al., 1968, 1973; Marinus and Adelberg, 1970; Vapnek and Rupp, 1971; Wilkins and Hollom, 1974; Kingsman and Willetts, 1978).

The amount of plasmid DNA transferred from the donor can be measured by growing and mating thyA deoB donors in the presence of radioactive thymine. Such donors, which must be sensitive to infection by bacteriophage T6, are mated with tdk recipients resistant to phage T6. After mating the donors are destroyed using a system developed by Ohki and Tomizawa (1968) and Matsubara (1968) and involving the addition of a large excess of phage T6 to lyse donors from without. The exposed DNA is removed by addition of nucleases and extensive washing. The amount of radioactivity resisting T6 lysis reflects the extent of DNA transfer during mating (Ohki and Tomizawa, 1968; Matsubara, 1968; Vapnek and Rupp, 1970; Wilkins and Hollom, 1974; Kingsman and Willetts, 1978).

Successful measurements of DNA transfer and conjugal DNA synthesis in recipients relies on the use of very fertile plasmids. The transfer

genes of naturally occurring plasmids are generally repressed and transfer occurs at too low a frequency to be detected in these systems. Therefore plasmids that transfer at a high frequency by virtue of a mutation to derepression need to be used in these experiments.

To determine the role of RNA polymerase in conjugal DNA metabolism, rifampicin-treated bacteria have been used as both donors and recipients. Rifampicin is an antibiotic that specifically inhibits RNA polymerase activity (Wehrli et al., 1968) by blocking the initiation of RNA synthesis in E.coli (Sippel and Hartman, 1968). The drug binds stoichiometrically (Wehrli et al., 1968) to the β subunit of the polymerase (Heil and Zillig, 1970) and blocks, at least in vitro, the translocation event that occurs after the formation of the first phosphodiester bond (McClure and Cech, 1978).

The role of primase in conjugal DNA synthesis was examined in matings involving bacteria carrying the thermosensitive dnaG3 mutation. This maps at about 60 min (Chen and Carl, 1975) on the recalibrated linkage map of E.coli (Bachman et al., 1976). Mutants carrying dnaG3 were used in the purification of dnaG gene product and its identification as primase. This mutation is the prototype dnaG mutation; to my knowledge the other putative dnaG mutations (dna-308 and dna-399) (Wechsler and Gross, 1971) have not been shown to be in dnaG by complementation tests.

Before matings involving rifampicin treatment of donors and recipients could be performed, the effects of rifampicin on plasmid transfer had to be elucidated. Wilkins and Hollom (1974) reported that when rifampicin-treated recipients were mated with drug-resistant donors of ColIdrd-1, conjugal DNA synthesis was enhanced five fold. Furthermore there are conflicting reports of the effect of rifampicin-treatment of donors on the transfer of IncIα plasmids. Fenwick and Curtiss (1973b) claimed

that transfer of R64drd-11 between dnaB bacteria was sensitive to the drug, whilst Wilkins and Hollom (1974) reported that rifampicin had little effect on ColIdrd-1 transfer between dna$^+$ bacteria. Therefore before rifampicin could be used with confidence these effects of the drug had to be resolved.

In Chapter 3 I will consider the effects of rifampicin-treatment of recipients on both DNA transfer and conjugal DNA synthesis in the recipients. Chapter 4 is addressed to the effects of rifampicin-treatment of donors on DNA transfer. In Chapter 5 the possible roles of RNA polymerase and primase in the conjugal synthesis of IncIα plasmids are examined. The final chapter of results (Chapter 6) reports the beginnings of a genetical and functional analysis of an IncIα plasmid. In a general discussion (Chapter 7) all the results obtained in this study are brought together to allow reevaluation of the mechanism of IncIα plasmid transfer.

Figure 1-1: TRANSFER GENES OF THE F PLASMID

The cistrons encoding the various tra proteins are assigned to the DNA according to Kennedy et al. (1977); Achtman et al. (1978a,c); Thompson and Achtman (1978, 1979); Achtman et al. (1979) using the kilobase coordinates of the F plasmid (see Fig. 1-3). The boxes represent the amount of DNA required to encode the appropriate protein. The figure is adapted from Manning and Achtman (1979).

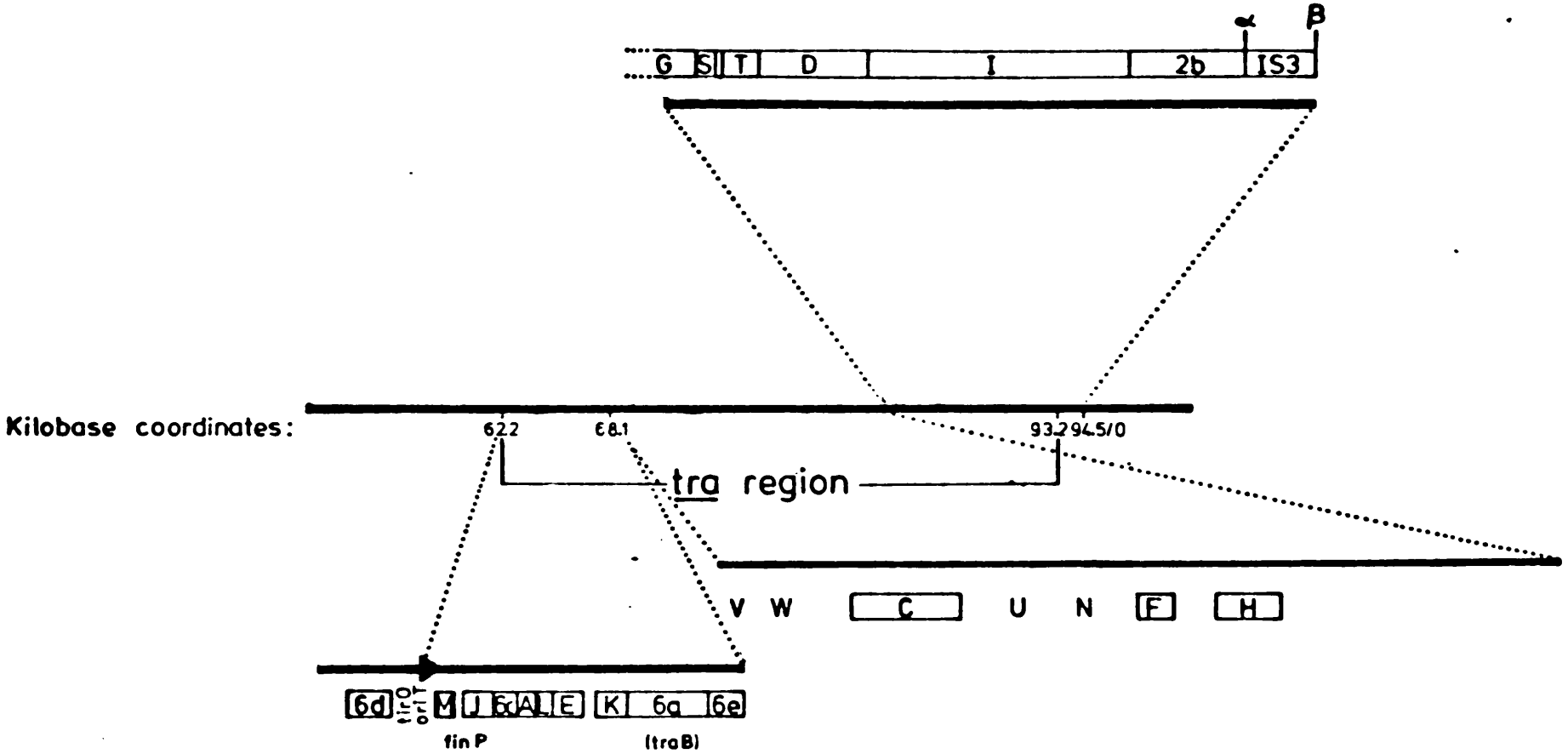

α
β
G
S
T
D
I
2b
IS3
Kilobase coordinates:
62.2
68.1
93.2
94.5/0
tra region
V
W
C
U
N
F
H
6d
oriT
M
J
K
A
L
E
K
6a
6e
fin P
(traB)

Figure 1-2: THE MATING CYCLE

The stages in the cycle are described in the text. Donors are represented as rod-shaped whilst recipients are shown as round: x represents receptors for F pili, which are depicted as curved projections from the donor cells. The figure is adapted from Achtman and Skurray (1977).

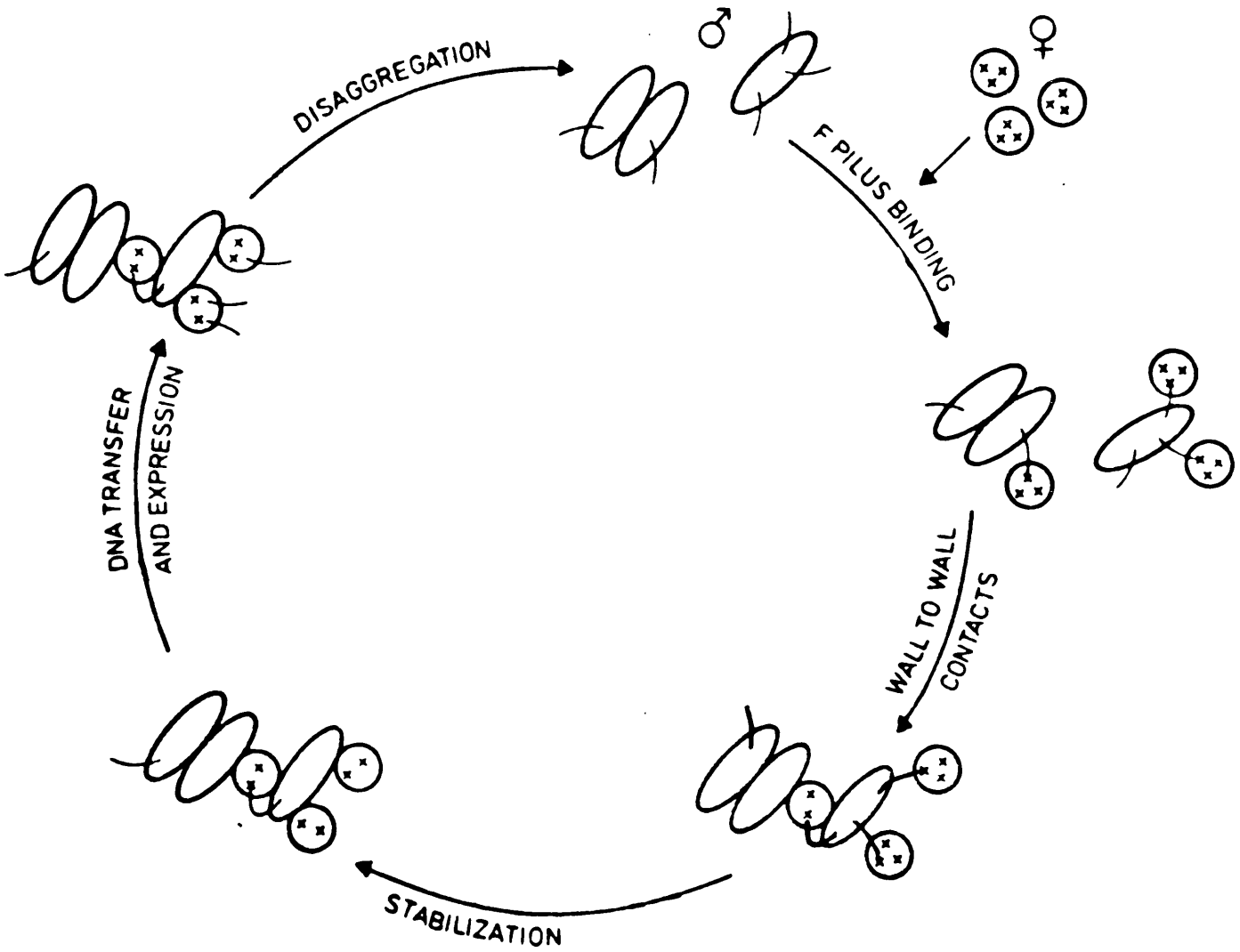

DISAGGREGATION
♂
♀
F PILUS BINDING
WALL TO WALL
CONTACTS
STABILIZATION
DNA TRANSFER
AND EXPRESSION

Figure 1-3: A CURRENT MAP OF THE F PLASMID

The majority of mnemonics are explained in the text. IS2, IS3 and γ - δ are insertion sequences. The position of the various genes is according to Skurray et al. (1976), Achtman et al. (1978c), Thompson and Achtman (1978), and located according to the kilobase coordinates of the F plasmid (0-94.5). The arrow indicates the origin and direction of transfer. The figure is adapted from Manning and Achtman (1979).

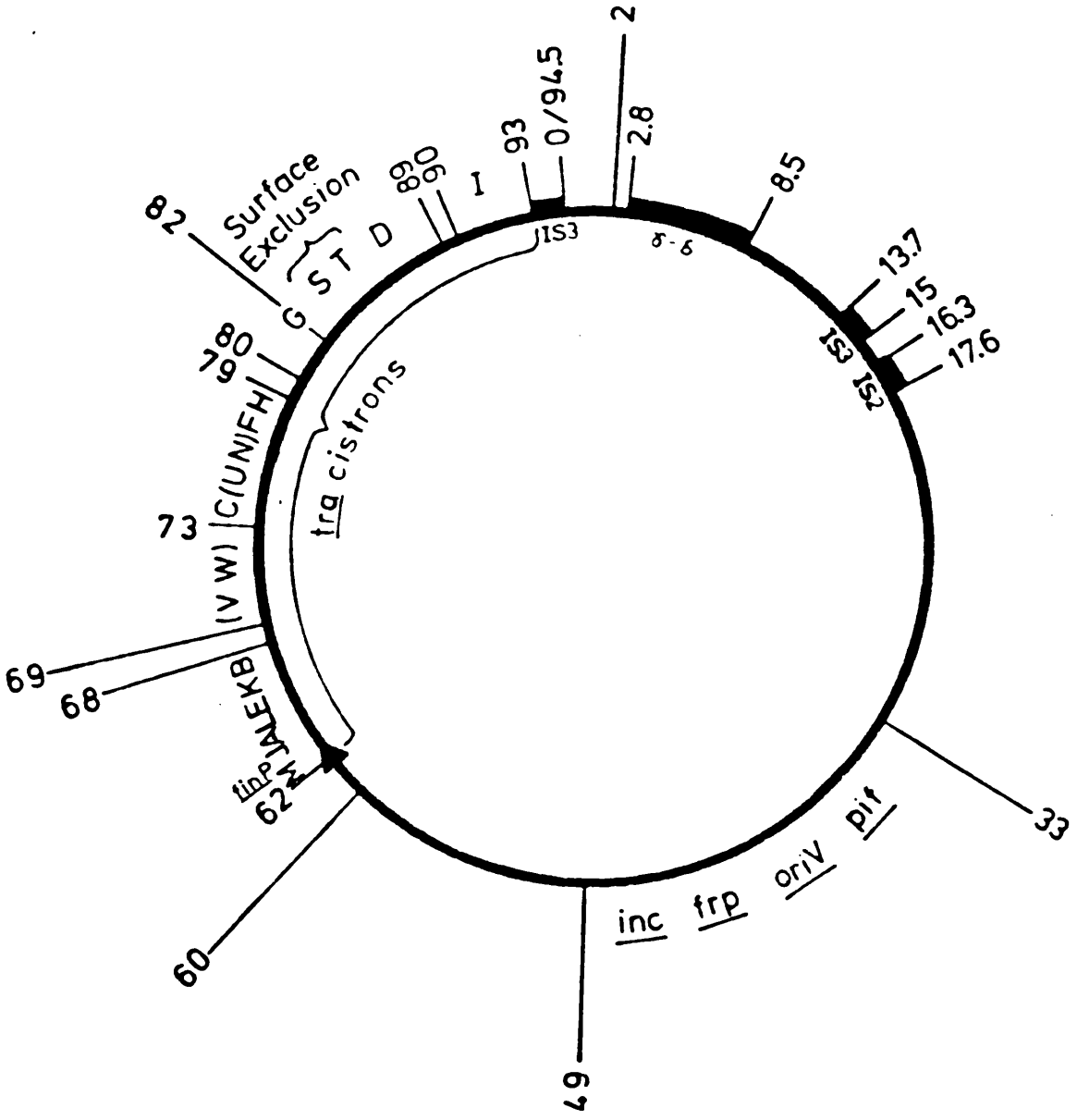

2
2.8
0/94.5
93
I
80
80
Surface Exclusion
82
G S T D
IS3
δ-δ
8.5
13.7
15
16.3
17.6
IS3 IS2
trq cistrons
98
100
C(UN)FH
73
(V W)
69
68
tinP JALEKB
62
60
49
inc frp oriV pif
33

Figure 1-4: THE ROLLING CIRCLE MODEL FOR TRANSFER

The details are discussed in the text. Heavy lines represent DNA synthesised during mating.

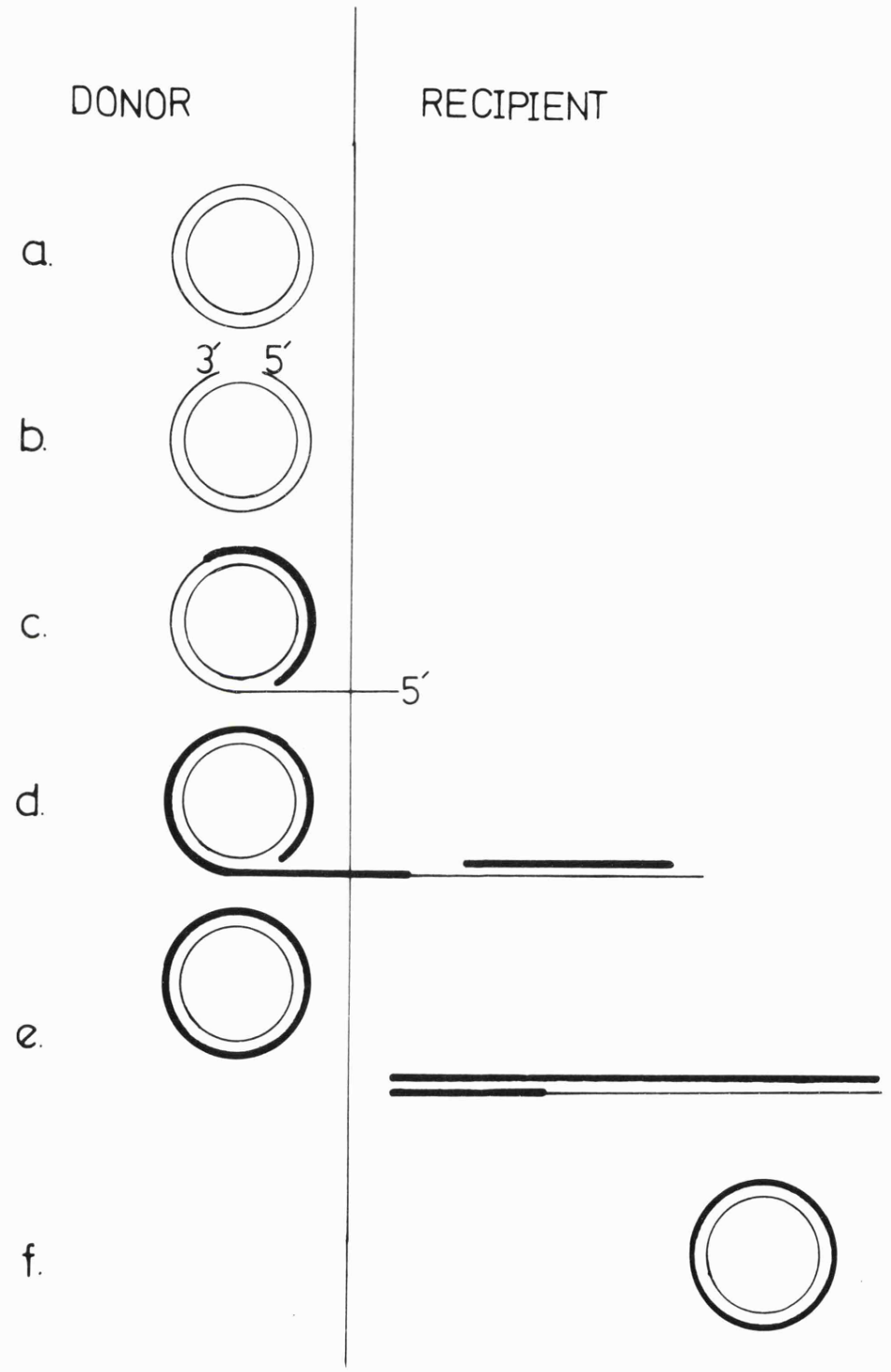
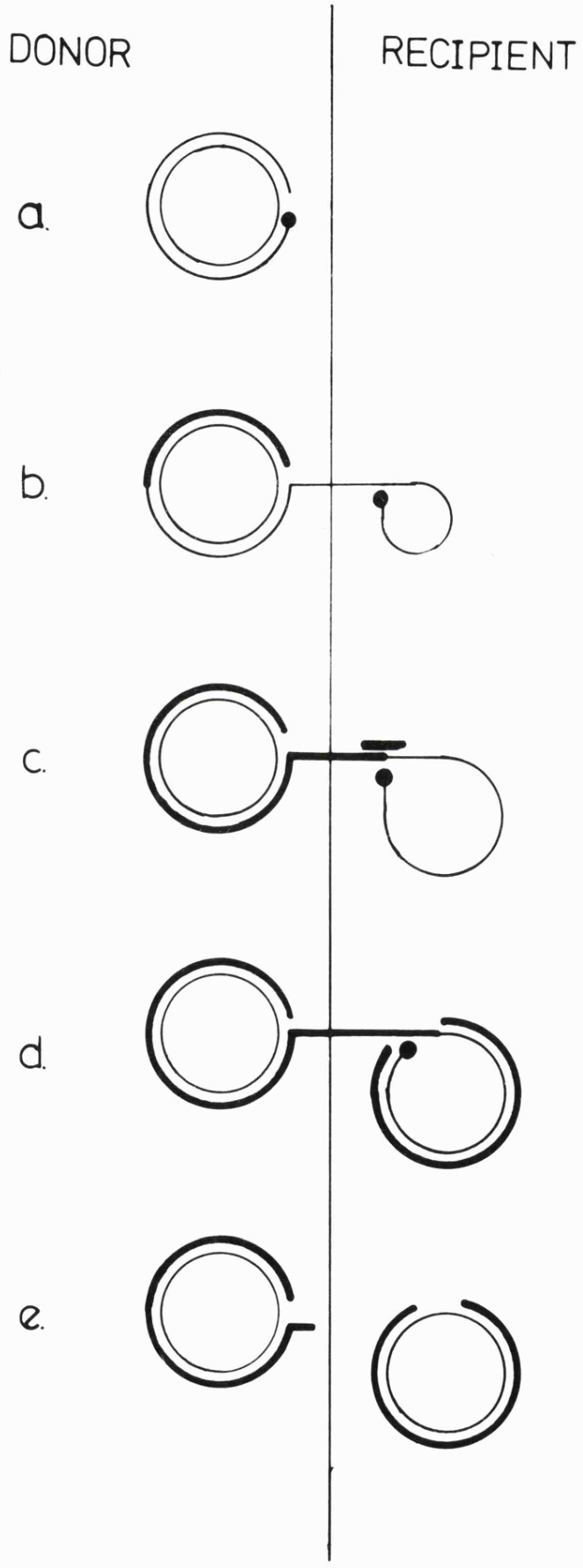

DONOR
RECIPIENT
a.
3′
5′
b.
c.
5′
d.
e.
f.

Figure 1-5: MODEL FOR THE MOBILISATION OF COLE1

Redrawn from Warren *et al.* (1978). The details are discussed in the text. Heavy lines represent newly synthesised DNA.


DONOR
RECIPIENT
a.
b.
c.
d.
e.

CHAPTER 2

MATERIALS AND METHODS

Bacterial strains and plasmids

The plasmids used in this study are summarised in Table 2-1. The properties of ColIdrd-1 and R144drd-3 have been described before (Wilkins and Hollom, 1974; Wilkins, 1975) and their sources are given in Table 2-1. The Flac plasmid was JCFLO (Achtman et al., 1971). pLG205 is a ColIdrd-1 prime plasmid carrying the lac region of the E.coli K-12 chromosome and was obtained from M.I. Sedgwick.

Bacteriophages T6 and MS2 were obtained from B.M. Wilkins and BF23 from I.B. Holland.

The bacterial strains used in this study were all derivatives of Escherichia coli K-12 and are listed in Table 2-2. BW82 and BW84 were obtained by mating BW63 and BW61 (both metE rpoB polA rpsL and derivatives of NY73) with CGSC 4315 (met^+ $rpsL^+$, a derivative of HfrJ4) for 35 min at 33°C and selecting met^+ rpsL transconjugants. These were then screened for the presence of the unselected $rpoB^+$ and $polA^+$ genes, determined respectively by sensitivity to 20 µg rifampicin (Sigma) per ml and resistance to both ultraviolet (UV) light and methylmethane sulphonate (Sigma) according to Wilkins and Hollom (1974). CGSC 4315 is an unstable Hfr that spontaneously breaks down to give F^+ bacteria (B. Bachmann, personal communication). As a consequence all the recombinants tested had also become F^+ transconjugants. F^- derivatives were isolated by a two stage procedure. F^- phenocopies of these transconjugants were mated with KLF1/PA211a, an F' leu^+ strain, selecting rpsL

leu$^+$ transconjugants. F$^-$ phenocopies were generated by growing the appropriate strain at 25°C for 28 h. The F*leu*$^+$ transconjugants were then cured of the F' plasmid by treatment with acridine orange (Hopkin and Williams) in Luria broth at pH 7.8 (N. Willetts, personal communication). Attempts to cure using acridine orange (Sigma) according to Miller (1972) proved to be unsuccessful. F$^-$ derivatives (BW82 and BW84) were tested for resistance to MS2 at 34°C on medium supplemented with Giemsa, according to McIntire and Willetts (1978). Loss of the F'*leu* plasmid was confirmed by analysis of cleared lysates (Davis and Vapnek, 1976) of the appropriate strains on 0.7% agarose gels.

Deletions in the *chlA-bio* region were isolated as spontaneous chlorate-resistant mutants by plating bacteria on nutrient agar supplemented with 2 mg potassium chlorate per ml and 0.2% glucose and incubation under anaerobic conditions (Adhya *et al.*, 1968). Chlorate-resistant mutants were then screened for pronounced sensitivity to UV light.

Bacteria resistant to rifampicin and nalidixic acid (Winthrop Laboratories) were obtained as spontaneous mutants after plating the appropriate strains on nutrient agar supplemented with 100 µg per ml of the relevant drug. Resistance to high levels of rifampicin and nalidixic acid are assumed to be due to a mutation in *rpoB* and *nalA* respectively.

Temperature-resistant revertants were obtained by plating bacteria at 40°C.

Strains resistant to phage T6 were obtained as spontaneous mutants by plating bacteria on agar plates previously spread with 0.1 ml of a suspension of T6 at a titre of 10^{10} plaque-forming units per ml.

Spontaneous mutations to colicin Ib resistance were selected by plating bacteria on agar on which bacteria that produce colicin Ib had

been grown overnight. The colicin-producing strain was scraped off the agar and the surface of the plate was exposed to chloroform vapour to kill residual bacteria and the plate overlayed with soft nutrient agar. Each resistant strain was subsequently checked to ensure that it had not inherited the ability to produce colicin. It is not known whether this resistance is due to mutation in cir or tonB.

The appropriate plasmids were introduced into the relevant strain by mating with a suitable donor strain in Luria broth. In general donors and recipients were mixed in the ratio 3:1 and mated for 30 min at a suitable temperature before plating on medium selective for trans-conjugants.

Chemicals and enzymes

The majority of chemicals were purchased from Fisons (Scientific) Ltd and were of analytical grade. Restriction enzymes EcoRI and HindIII were obtained from the Microbiological Research Establishment, Porton Down and T4 DNA ligase and PstI were supplied by Boehringer. All radio-chemicals were supplied by the Radiochemical Centre, Amersham.

Media

Nutrient agar contained per litre of distilled water 25g Oxoid No.2 nutrient broth, 14.5g Davis agar. When soft nutrient agar was used the amount of Davis agar added was reduced to 6g.

Luria broth contained 10g tryptone, 5g yeast extract, 5g NaCl per litre of distilled water and was adjusted to pH7.0.

SGC medium contained per litre of distilled water : 6g Na_2HPO_4, 3g KH_2PO_4, 1g NH_4Cl, 0.5g NaCl, 0.12g $MgSO_4$, 11mg $CaCl_2$, 4g glucose,

2g Casamino acids (Difco), 1mg thiamine hydrochloride. The latter 5 components were added separately from stock solutions after autoclaving.

Minimal agar was made up as for SGC medium except that Casamino acids were omitted and it contained 2g glucose. All chemicals were added to distilled water containing 15g/l Davis agar after autoclaving. Minimal agar was supplemented with 2 μg/ml of appropriate amino acids (Sigma).

Giemsa agar (McIntire and Willetts, 1978) contained 10g yeast extract (Difco), 10g tryptone (Difco), 8g NaCl, 15g agar (Difco), 10g glucose. After sterilisation of this mixture, $CaCl_2$ was added to 2mM and Giemsa stain (improved R66 solution, Gurr) was added to a final concentration of 0.5%.

Antibiotics were added to molten agar (at about 50°C) as follows: streptomycin (200 μg/ml), spectinomycin (100 μg/ml), tetracycline (15 μg/ml) and kanamycin (25 μg/ml). Rifampicin was added to molten agar as a 10 mg/ml solution in 50% ethanol and 0.05M K_2CO_3 to the appropriate concentration. Nalidixic acid was added as a 10 mg/ml solution in 1M NaOH and the NaOH neutralised by addition of an equal volume of 1M HCl.

Buffers

Phosphate buffer contained per litre of distilled water : 3g KH_2PO_4, 7g Na_2HPO_4, 4g NaCl, 0.1g $MgSO_4.7H_2O$.

T2 adsorbtion buffer contained per litre of distilled water : 8g NaCl, 10g K_2SO_4, 3g KH_2PO_4, 3.2g Na_2HPO_4, 20mg gelatin. After adjusting to pH 7.1 and sterilisation, $MgSO_4$ and $CaCl_2$ were added to 20 mM and 2mM respectively.

Preparation of phage T6

Two 200 ml cultures of E.coli B were grown at 37°C to a cell concentration of 1.5×10^8 per ml in SGC medium and 3×10^8 phage T6 particles were added to each culture. Incubation was continued with vigorous aeration and when the absorbance had fallen to about 20% of its maximum value, growth was stopped by the addition of chloroform (4 ml per culture) with vigorous shaking. The chloroform was allowed to settle, and the aqueous phase was decanted off and centrifuged at 8000 rpm for 10 min in a Sorvall GSA rotor to remove bacterial debris. The supernatant was collected, stored overnight at 4°C and centrifuged at 17,000 rpm for 2.5 h at 0°C in a Sorvall SS-34 rotor. The supernatant was discarded, 6 ml of T2 adsorption buffer added and the phage pellet was allowed to resuspend overnight. The resuspended phages were shaken gently for 20 min to promote disaggregation. Chloroform (0.5 ml per 10 ml of phage suspension) was added to sterilise the suspension and subsequently removed by decanting the aqueous phase into a fresh tube. Any residual chloroform was removed by gentle aeration at 37°C. The number of plaque-forming units (pfu) was determined by plating serial dilutions of the phage suspension on E.coli B. Normal yields were about 6 ml of phage suspension at 4×10^{12} pfu per ml.

Conjugal DNA synthesis

Overnight cultures in SGC medium were diluted and grown for at least 3 mass doublings before use in mating. Thymine-requiring strains were grown in SGC medium supplemented with 2 µg thymine (Sigma) and 200 µg 2'-deoxyguanosine (Sigma) per ml. *dna*$^+$ bacteria and *dnaB70* mutants were grown at 37°C and 33°C respectively. Bacteria mutant at *dnaG* and carrying either ColI*drd-1* or R144*drd-3* were grown at 37°C

(Wilkins, 1975) and dnaG3 bacteria without a plasmid were grown at 33°C.

When UV-irradiated recipients were used, bacteria were harvested by centrifugation when the culture contained about $3x10^8$ bacteria per ml. The sedimented bacteria were resuspended in phosphate buffer at $2x10^8$ bacteria per ml and irradiated in glass petri dishes with 400 J/m^2 under a Hanovia bacteriocidal discharge tube emitting radiation principally at 254 nm. Dose rates were monitored with a dosimeter constructed by R. Latarjet (Latarjet et al., 1953). The irradiated bacteria were sedimented and resuspended in SGC medium containing deoxyguanosine (200 µg/ml) at about $5x10^8$ bacteria per ml. For matings described in Chapter 3 these bacteria were mixed immediately with donors. For all other matings irradiated bacteria were incubated at the appropriate temperature for 10 min before mating. When rifampicin or chloramphenicol (Sigma) was used, the irradiated bacteria were incubated for 5 min at the mating temperature and antibiotic was added in prewarmed SGC medium to give 100 and 200 µg per ml respectively. After a further 5 min incubation, donors were added to initiate conjugation. When dnaB70 bacteria were used as recipients they were prewarmed for 10 min at 43°C (Chapter 3) or 15 min (Chapter 4) at 42°C. Antibiotics were added usually 5 min before mating was initiated.

Donors were grown for at least three mass doublings to a density of $3x10^8$ cells per ml. They were incubated at the temperature to be used in mating in general for 10 min before mixing with recipients. When experiments involved dnaB donors (Chapter 4) they were incubated at 42°C for 15 min before mating. When rifampicin or chloramphenicol were used the drugs were added in SGC medium supplemented with deoxyguanosine (200 µg/ml) 5 min before mating to give concentrations of 100 and 200 µg per ml respectively.

Mating mixtures varied between 3.5 ml and 5.0 ml in volume depending on the number of samples to be taken, and contained about 10^8 bacteria of each parental strain per ml and either 2 μg (Chapter 3) or 1 μg (Chapters 4 and 5) thymine per ml and 200 μg deoxyguanosine per ml. [2-^{14}C] thymine or [methyl-^{3}H] thymine were added to the specific activities indicated in the legends to the figures. Before use in matings the radiochemicals were always preconditioned using the following procedure. Radiochemicals were added to a small volume of exponentially growing BW40 at about 10^8 bacteria per ml in SGC medium. After incubation at 37°C for about 30 min, bacteria were removed by filtration and the filtrate stored on ice. This procedure removed trace amounts of undefined labelled compounds that were rapidly incorporated into trichloracetic acid (TCA) - precipitable material by both tdk and thyA bacteria (Wilkins and Hollom, unpublished data).

Matings were gently swirled in 150 ml Erlenmeyer flasks. Samples (0.5 ml) were removed into ice-cold TCA (final concentration 5%) containing 100 μg of both thymine and thymidine per ml.

To determine the background incorporation of label into unmated bacteria the parental cultures were either incubated separately (for experiments in Chapter 3) or mixed in the presence of 100 μg SDS per ml. Samples were removed into TCA accordingly.

Frequency of transconjugants

The indicated donor strain was mated with the appropriate unirradiated recipient using exactly the same procedure described for measurements of conjugal DNA synthesis. Samples were taken at intervals, blended for 5 sec (Low and Wood, 1965) and treated as follows. (1) For Fig. 3-1: An equal volume of phage T6 suspension (10^{12} pfu/ml) was added and the

mixture was incubated at 43°C to allow lysis of phage-sensitive donors. Recipients were diluted, plated and following overnight incubation were tested for colicin production (see below). (II) For Fig. 5-2, 5-3 : The blended bacteria were plated on nutrient agar containing nalidixic acid (100 μg/ml) to select against the donors. The resulting colonies were tested for the ability to produce colicin (see below).

Measurements of DNA transfer

Donor bacteria were grown in SGC medium containing 2 μg thymine and 200 μg deoxyguanosine per ml and generally supplemented with radioactive thymine at the specific activity to be used in the mating, (see Figure legends). Matings which were 3.5 ml in volume were set up as described for measurements of conjugal DNA synthesis. After 60 min of mating donors were selectively destroyed using methods developed by Ohki and Tomizawa (1968) and Matsubara (1968). A 2.5 ml volume of mating mixture was added to 0.6 ml of 10mM Tris pH 8 containing 10mM KCN and 40 μg tryptophan and 100 μg of thymine per ml. The mixture was blended for 5 sec (Low and Wood, 1965) and added to 0.5 ml of T2 adsorption buffer containing 10^{12} phage T6 particles per ml. The phage had been irradiated with 80 J/m^2 of UV light. After 5 min at 37°C, deoxyribonuclease (Calbiochem, B) in 50mM Tris-HCl pH 8 and ribonuclease (Calbiochem, A) in 50mM Tris-HCl pH 8 were added to a final concentration of 150 and 100 μg per ml respectively. The mixture was incubated at 37°C for 15 min after which Pronase (Calbiochem, B) in water, predigested for 60 min at 37°C, was added to a final concentration of 375 μg per ml. After a further 15 min of incubation Brij 58 (Honeywell Atlas) in distilled water was added to 5 mg/ml and the mixture was vortexed and cooled to 0°C. About 2×10^8 BW40 cells, UV-irradiated with 500 J/m^2, were added as carrier to promote

pellet formation. The recipients were washed three times at 4°C by sedimentation in a Sorvall SS-34 rotor for 3 min at 7,000 rpm and resuspension in buffer containing 100 µg of thymine per ml. For experiments described in Chapter 3 this buffer was supplemented with 5mM KCN. The pellets were finally resuspended in 2.5 ml of buffer. Samples (0.5 ml) were removed into TCA (final concentration of 5%) and assayed for TCA-precipitable radioactivity.

Pulse labelling

Bacteria were grown as for mating. To measure the rate of DNA synthesis in BW86 following a temperature shift, a series of 0.5 ml samples was added to equal volumes of SGC medium containing 0.05 µg [methyl-^{3}H]thymidine (9.7 Ci/mmol), starting immediately before a shift from 33°C to 41°C. After incubation at the relevant temperature for 2 min, incorporation was stopped by the addition of 1 ml of ice-cold 10% TCA containing unlabelled thymidine (100 µg/ml) and cooling on ice. To measure the effect of rifampicin on the rate of RNA synthesis in BW86, a series of samples were taken before and after the addition of the drug. [5-^{3}H]uridine (325 Ci/mmol) or [5-^{3}H]uracil (27 Ci/mmol) was added to each to give 3 µg uridine or 1 µg uracil per ml. After 2 min, incorporation was stopped by adding ice-cold TCA (to 5%) containing unlabelled uridine or uracil (100 µg/ml) followed by cooling. To measure the effect of chloramphenicol on protein synthesis a series of samples was taken before and after addition of the antibiotic (200 µg/ml). L-[^{35}S] methionine (290 Ci/mmol) was added to 5 µCi/ml and after 2 min, incorporation was stopped by adding ice-cold TCA to 5% and unlabelled methionine to 100 µg per ml.

Determination of TCA-precipitable radioactivity

After at least 1 h in TCA the resulting precipitate was collected on Sartorius membrane filters (0.45 μm pore size, 25mm diameter) that had been soaked in a 2 mg/ml solution of either thymine, uridine, uracil or methionine depending on the radiochemical used. Filters were washed with either ten 5 ml volumes of boiling water for DNA and RNA determinations or ten 5 ml volumes of cold water for protein measurements. The filters were dried and placed in toluene-based scintillation fluid [PPO (2,5-diphenyl oxazole), 5g per litre; dimethyl-POPOP (1,4-bis-2-[4-methyl-5-phenyl oxazolyl]-benzene), 0.3g per litre] and counted in a Packard 3255 liquid scintillation spectrophotometer.

Sedimentation analysis of transferred DNA

Matings were performed exactly as described for measurements of DNA transfer. Selective lysis of the donors was accomplished as described previously except that the washed recipients were resuspended in 0.7 ml of lysis buffer (50mM NaCl, 20mM EDTA, 20mM Tris-HCl pH 9.1) according to Freifelder et al. (1971). The yield of covalently closed circular (CCC) DNA was measured after addition of 0.1 ml of the resuspended cells to 0.05 ml of 1% SDS in 0.8M NaOH (Fenwick and Curtiss, 1973a) which had been layered on top of a preformed alkaline sucrose gradient. Each gradient consisted of 4.1 ml of 5 to 20% (wt/vol) sucrose in 0.7M NaCl, 0.3M NaOH and 10mM EDTA on a 2.5 ml shelf of 70% sucrose. The gradients were centrifuged (35,000 rpm) in a Beckman SW56 rotor at 20°C. Alternatively 0.1 ml of cells in lysis buffer were added to 0.1 ml of alkaline SDS layered on top of a 12.5 ml 5 to 20% alkaline sucrose gradient above a 0.7 ml shelf of 70% sucrose. These gradients were centrifuged in a Beckman SW40 rotor for 3 h at 30,000 rpm.

Gradients were fractionated by puncturing the base of the nitrocellulose centrifuge tube and collecting drops on filter paper. When 4.1 ml gradients were fractionated, 5 drop fractions were collected on 2.5 cm square pieces of Whatman No.1 filter paper and 10 drop fractions from 12.5 ml gradients were collected on 2.5 cm diameter circles of Whatman No.3 filter papers.

Filters were dried and about 100 were added to 200 ml ice-cold 5% TCA containing 100 µg of thymine per ml. After 15 min the TCA was discarded and a second 200 ml volume of TCA was added and kept at $4^{\circ}C$ for 10 min. After a third TCA wash, 200 ml of ice-cold methanol was added. After 10 min the methanol was replaced by 200 ml of ice-cold acetone. Filters were removed after 10 min, dried and added to toluene scintillation fluid and counted as described above.

Preparation of plasmid marker DNA

Thymine-requiring strains carrying the appropriate plasmid were grown in SGC medium supplemented with either $[^{3}H]$ thymine (25 µCi/µg) or $[^{14}C]$ thymine (0.25 µCi/µg) and 200 µg deoxyguanosine per ml. The final concentration of thymine was always 2 µg/ml. The culture (7.5 ml) was grown until the cell density reached $2x10^{8}$ per ml. 6 ml of this culture was harvested by centrifugation and washed three times with ice-cold TES (50mM Tris-HCl, 5mM EDTA, 50mM NaCl, pH 8) and finally resuspended in 1 ml of 10% sucrose in 0.1M Tris-HCl pH 8.0. Freshly prepared lysozyme (Sigma) in 0.1M Tris-HCl pH 8 was added to a concentration of 600 µg/ml. The mixture was kept on ice for 10 min. EDTA (pH 8) was added to 50mM and the mixture was held on ice for 5 min. Sarkosyl (Geigy) in water was added to a final concentration of 4 mg/ml and the mixture was held at room temperature to achieve lysis of the spheroplasts. The resulting

clear and viscous lysate was vortexed for 15 sec. The lysate (0.75 ml) was added to a saturated solution of caesium chloride in TES containing ethidium bromide (Sigma) to give a final density of CsCl of 1.605g per cm^3 and 300 µg ethidium bromide per ml. This mixture was centrifuged at 20°C in a Beckman type 65 rotor at 40,000 rpm for 40 h. The gradients were fractionated by puncturing the bottom of the nitrocellulose tubes and 11 drop fractions collected into the wells of plastic microtitre plates. Radioactivity in each fraction was determined by applying 10 µl samples of each fraction on to Whatman No.1 filters which were dried, and washed with TCA as described above. Fractions containing the major plasmid peak were pooled and ethidium bromide extracted by two successive treatments with CsCl-saturated propanol. The aqueous layer was extensively dialysed against ice-cold TES pH 7. Typical plasmid preparations contained 3.9×10^5 cpm per ml of ^3H and 2×10^4 cpm per ml of ^{14}C.

Large scale plasmid preparation

(i) After Guerry et al. (1973). 100 ml cultures were grown overnight in SGC medium. Cells were harvested by centrifugation and resuspended in 1 ml of cold 25% sucrose in 0.25M Tris-HCl, pH 8. Lysozyme (freshly made in 0.25M Tris-HCl, pH 8) was added to a concentration of 0.8 mg/ml and the mixture kept on ice for 5 min. EDTA (0.25M, pH 8) was added to a final concentration of 62.5 mM and the incubation on ice was continued for a further 5 min. SDS (5% in water) was added to 1% and lysis achieved at 37°C for 20 min. NaCl (5M) was added to a final concentration of 1M and the mixture was left overnight at 4°C, after which it was centrifuged in a Sorvall SS-34 rotor at 14,000 rpm for 30 min at 4°C. The supernatant was removed and stored at -20°C.

(ii) After Sharp et al. (1972). Bacteria were grown in 250 ml of SGC medium to 10^9 cells per ml, harvested by centrifugation and resuspended in 12.5 ml 10% sucrose in TES. 6.25 ml of a 2% solution of SDS in TES was added and the cells allowed to lyse (about 15 min). The exposed DNA was sheared by passing through a 20 ml plastic syringe (without needle). Sheared DNA was denatured by raising the pH to 12.2 by addition of 4M NaOH with vigorous stirring. The pH was monitored with a micro pH electrode. The lysate was held at pH 12.2 for 3 min after which it was rapidly reduced to pH 8 by addition of 2M Tris-HCl pH 7. The lysate was adjusted to 0.3M NaCl and bulk nitrocellulose (6.35g, grade FHX8.13 or FHX30.50, I.C.I.) was added and the mixture was gently agitated at 4°C for 1 h. The nitrocellulose was removed by centrifugation at 5,000 rpm for 10 min at 4°C in a Sorvall SS-34 rotor.

(iii) After Davis and Vapnek (1976). A litre culture of the appropriate bacterial strain was grown to $8x10^8$ cells per ml. Bacteria were harvested by centrifugation at 4°C and resuspended in a total volume of 120 ml 10% sucrose in 50mM Tris-HCl pH 8. Lysozyme (24 ml, 5 mg/ml in 0.25M Tris-HCl pH 8) and 8 ml of 0.2M EDTA pH 8 were added. After 10 min at 4°C the mixture was incubated at 37°C for about 10 min to allow spheroplast formation. 4 ml of Sarkosyl (2% in water) was added to each flask which was gently shaken to ensure dispersal of the detergent prior to lysis. After lysis was complete the lysate was incubated at 37°C for about 15 min. The lysate was centrifuged for 2 h at 4°C in a Beckman type 19 rotor at 17,000 rpm. The supernatant was removed and twice extracted with an equal volume of phenol mixture (100g Analar phenol, 0.1g 8-hydroxyquinoline, 4 ml iso-amyl alcohol and 100 ml chloroform). The phenolic and aquous phases were separated by centrifugation at 6,000 rpm for 30

min in a Sorvall GS3 rotor. The aqueous phase was removed and 2 volumes of absolute ethanol (-20°C) were added. After standing overnight at -20°C the precipitated DNA was sedimented by centrifugation at 8,000 rpm for 20 min at 0°C in a Sorvall GS3 rotor. Pellets were suspended in 8 ml of a solution containing 50mM Tris-HCl, 5mM EDTA pH 8 (TE). The sample was mixed with a saturated CsCl solution and a 5 mg/ml solution of ethidium bromide to a final density of 1.605g per cm^3 and 100 µg of ethidium bromide per ml. After centrifugation at 35,000 rpm for 40 h at 20°C in a Beckman type 40 rotor the lower band in the gradient, viewed by exposure to long wave UV light, was removed by puncturing the side of the nitrocellulose tube with a syringe. Ethidium bromide was extracted twice with propanol saturated with CsCl. The CsCl was removed by extensive dialysis against TE. Between 20-40 ug of ColI DNA was routinely prepared in this way.

Restriction and ligation

Digestion of DNA (2 µg) with PstI was performed in a reaction mixture containing 10mM Tris-HCl, pH 7.4, 10mM $MgCl_2$, 10mM NaCl and 1mM dithiothreitol (DTT). Cleavage with EcoR1 was carried out in 100mM Tris-HCl pH 7.4, 10mM $MgCl_2$, 0.5mM DTT and 50mM NaCl. HindIII cleavage of DNA was performed in 6mM Tris-HCl pH 7.4, 6mM $MgCl_2$, 50mM NaCl and 0.5mM DTT. All digestions were performed at 37°C for 3 h and followed by a 10 min heat pulse at 65°C.

Ligation mixtures, containing 1 unit of T4 DNA ligase, were 100 µl in volume and contained 1 µg of PstI cleaved vector DNA (pBR322) and 0.2 µg of PstI cleaved ColI DNA in 7.5mM Tris-HCl pH 7.4, 7.5mM $MgCl_2$, 10mM DTT and 1mM ATP. Ligation was carried out at 14°C overnight.

Transformation

A 50 ml culture of recipient bacteria was grown in Luria broth to $6x10^8$ cells per ml and then well chilled in ice-water. Bacteria were sedimented and washed in 50 ml ice-cold, 30mM $CaCl_2$. After harvesting by centrifugation at 0°C, cells were again resuspended in 50 ml $CaCl_2$ and kept on ice for 20 min. These bacteria were then sedimented and resuspended in 5 ml 30mM $CaCl_2$. DNA (1 µg), 0.1 ml 30mM $CaCl_2$ and 0.2 ml cells in $CaCl_2$ were mixed in cold tubes and kept on ice for 30 min. Bacteria were transferred to 42°C for 3 min. Luria broth (3 ml) supplemented with 0.2% glucose and 10 µg thiamine per ml was added and the tubes incubated at 37°C for 90 min before plating on the appropriate selective medium.

Agarose gel electrophoresis

DNA samples were mixed with agarose beads in the ratio of 1.5:1. These samples were loaded into slots, of various sizes depending on the volume of the sample, in horizontal agarose slab gels made in Tris-acetate buffer (40mM Tris-acetate pH 7.7, 1mM EDTA). The concentration of agarose used is detailed in the Figure legends. Samples were subjected to electrophoresis at room temperature at 50V for 30 min and then 200V for about 1 h.

Agarose beads were prepared according to Schaffner et al. (1976). Agarose (100 mg) and glycerol (5g) were dissolved by heating in 50 ml of 10mM Tris-HCl pH 7.5 and 2 ml of 0.5M EDTA with a small amount of bromophenol blue. The mixture was allowed to set at room temperature and then extruded through a syringe until liquid.

After electrophoresis was complete the gel was stained in ethidium bromide (0.5 µg/ml) for about 30 min, viewed over a UV light (260 nm)

and photographed using 35mm HS23 (Ilfodata) film.

Screening transformants for recombinant plasmids

Transformants were screened by using two different techniques. (1) After Eckhardt (1978). Single colonies were lysed in the wells of a vertical agarose (0.7%) slab gel (110 x 140 x 6mm) with slots of 6.5x 15 x 6 mm in dimension. Agarose was prepared in Tris-borate electrophoresis buffer (89mM Tris, 2.5mM EDTA, 89mM boric acid, pH 8.2). 25 µl of a mixture containing lysozyme 7500 u/ml, ribonuclease 0.3 u/ml (pretreated at 80°C for 10 min in 0.4M sodium acetate, pH 4.0) and 0.05% bromophenol blue in 20% Ficoll 400,000 (Pharmacia) in electrophoresis buffer was added to each well. One or two single colonies were resuspended in the lysozyme mixture. After about 5 min, 35 µl of 0.2% SDS in 10% Ficoll 400,000 in buffer was carefully layered onto the bacterial suspension in each well. The contents of each well was gently stirred using a toothpick and 50 µl of 0.2% SDS in 5% Ficoll made up in electrophoresis buffer was added to each well. Wells were sealed with 0.7% agarose and the samples were subjected to electrophoresis at 50mA for about 3 h. After electrophoresis the gel was stained with ethidium bromide, viewed over a UV light and photographed.

(2) Alternatively transformants were screened for recombinant plasmids using a cleared lysate technique. Bacteria were picked from a fresh agar plate and resuspended in 0.1 ml of 25% sucrose in 50mM Tris-HCl pH 8, 10mM EDTA contained in an Eppendorf reaction tube (1.5 ml). 5 µl of 10% SDS was added and cellular lysis performed at room temperature for 5 min. The DNA released by this treatment was sheared by vortexing and a clearing spin performed in an Eppendorf micro centrifuge for 15 min. A sample of the supernatant was removed, added to agarose beads and subjected

to electrophoresis in a 0.7% horizontal agarose slab gel as described above.

Mutagenesis and replica plate matings

Cultures of GB10 (R144drd-3) were treated with ethyl methane sulphonate (EMS) (Sigma) in three different ways. (i) Bacteria were grown in 5 ml Luria broth at 37°C to $5x10^8$ cells per ml, harvested by centrifugation and resuspended in 1 ml of phosphate buffer. 0.2 ml EMS was added and the bacteria incubated at 37°C for 20 min. The treated cells were collected by centrifugation and resuspended in 1 ml Luria broth. The culture was diluted .10 fold in broth and 1 ml volumes dispensed in a series of tubes. The bacteria were grown overnight and plated on appropriate media (Achtman et al, 1971). (ii) 5 ml cultures were grown in Luria broth to $5x10^8$ cells per ml, harvested by centrifugation and resuspended in 2.5 ml SGC medium lacking glucose. 0.1 ml of these cells was added to 5 ml SGC without glucose and containing 0.1 ml EMS. Bacteria were incubated at 37°C for 30 min, diluted 10 fold in Luria broth and 1 ml aliquots dispensed, grown overnight, and then plated on an appropriate medium (M. Achtman, personal communication). (iii) A 4 ml culture was grown in SGC medium to $3x10^8$ bacteria per ml. Cells were collected by centrifugation and resuspended in 2 ml SGC medium without glucose but containing 0.2M Tris-HCl, pH 7.5. EMS (0.03 ml) was added and vigorously aerated at 37°C for 2 h. Treated bacteria were diluted 10 fold in Luria broth, dispensed in 1 ml aliquots, incubated overnight and plated appropriately (Miller, 1972).

When survivors of the mutagenesis were to be screened for transfer and surface exclusion proficiency, the overnight cultures were plated on minimal medium supplemented with threonine and leucine and containing

25 μg kanamycin per ml. After 48 h of growth any resulting colonies were patch plated on prewarmed (37°) nutrient agar plates containing kanamycin (25 μg/ml) and grown for 5 h at 37°C. The resulting patches were mated in two different replica plate mating systems. (i) The patches on nutrient agar-kanamycin plates were replica plated onto nutrient agar plates containing kanamycin (25 μg/ml) and spectinomycin (100 μg/ml) which had been spread with $3x10^7$ cells of GB11 and allowed to dry for 5 min. Following replica plating, these plates were incubated overnight at 37°C and failure to detect transconjugants identified transfer-defective (Tra^-) mutants of R144. Transconjugants resulting from this mating were replica plated on minimal medium plates supplemented with histidine, tryptophan and lysine and containing streptomycin (200 μg/ml) and kanamycin (25 μg/ml) which had been spread with $3x10^7$ cells of BW66 (replica plate mating 2, Fig. 6-1). The plates were incubated for 48 h and amber-suppressible Tra^- mutants of R144drd-3 were identified by the absence of transconjugants.

Survivors of the mutagenesis that had become good recipients (i.e. surface exclusion (Sex^-) mutants) were identified by replica plating bacterial patches on nutrient agar-kanamycin plates onto minimal medium plates containing threonine, streptomycin and kanamycin and spread with $3x10^7$ cells of the donor strain GB11 (pLG205) in exponential phase of growth. Formation of leu^+ recombinants identifies putative Sex^- mutants of R144drd-3.

Survivors of the mutagenesis were also screened for kanamycin resistant clones that did not produce colicin Ib.

Testing for colicin production and resistance

(i) Colicin production. Patches or single colonies resulting from overnight growth of the relevant strain were killed by exposing the surface of the plate to chloroform vapour for about 15 min. Plates were exposed to the air (about 10 min) to allow dispersal of residual chloroform vapour, and overlayed with a suitable colicin-sensitive indicator strain in soft nutrient agar. If the agar plates used for overnight growth of bacteria contained no antibiotic, BW40 was used as an indicator. If agar plates contained nalidixic acid then the indicator was strain 711.

(ii) Colicin resistance. A strain producing colicin Ib was streaked across a nutrient agar plate and grown overnight. The resulting bacterial growth was scraped from the plate and residual bacteria killed by chloroform treatment as described above. Bacteria to be tested for colicin resistance/immunity were streaked at right angles to the area that had supported growth of the colicin-producing strain. Sensitive bacteria do not grow across this region.

Table 2-1: PLASMIDS

Plasmid	Phenotype [a]	Reference
ColIb-P9*drd-1*	Tra$^+$ Dps[b](Ifl) Cib Uv Phi[c] (T5, BF23) (derepressed mutant of ColIb-P9)	Dowman and Meynell (1970)
R144*drd-3*	Tra$^+$ Dps(Ifl) Cib Uv Km (derepressed mutant of R144)	Meynell and Datta (1967)
JCFLO	Tra$^+$ Dps(f1, f2, MS2) Lac$^+$	Achtman *et al.* (1971)
pL205	Tra$^+$ Cib Lac$^+$	M.I. Sedgwick
pBR322	Ap Tc	Bolivar *et al.* (1977)
pBRR325	Ap Tc Cm	Bolivar (1978)
ColE1	Cel Ice	R. Diaz
pSF2124	Ap Cel Ice	So *et al.* (1975)

[a] Phenotypic symbols are defined in Novick *et al.* (1976) and Jacob *et al.* (1977)

[b] Dps = Donor phage sensitivity : the relevant phages are indicated in parentheses.

[c] Phi = Phage inhibition : the relevant phages are indicated in parentheses.

Table 2-2: BACTERIAL STRAINS

Strain number	Genotype [a]	Source/Reference
BW26	proA2 thr-1 leu-6 argE3 his-4 thi-1 lacY1 galK2 ara-14 mtl-1 xyl-5 uvrB5 rpsL31 tsx-33 sup-37 thyA deoB	Wilkins and Hollom (1974)
BW40	proA2 thr-1 leu-6 argE3 his-4 thi-1 lacY1 galK2 ara14 mtl-1 xyl-5 uvrB5 rpsL31 tsx-33 tdk-1	"
BW50	proA2 thr-1 leu-6 argE3 his-4 thi-1 galK2 ara-14 mtl-1 xyl-5 uvrB5 rpsL31 sup-37 thyA deoB	From BW26 (Wilkins and Hollom, 1974)
BW51	proA2 thr-1 leu-6 argE3 his-4 thi-1 galK2 ara-14 mtl-1 xyl-5 uvrB5 rpsL31 tdk-1	From BW40 (Wilkins and Hollom, 1974)
BW56	dnaB70 polA1 lacY14 rha rpsL thyA deoB deoC tsx	Wilkins and Hollom (1974)
BW58	dnaB70 polA1 lacY14 rha rpsL deoA deoC tdk tsx	Wilkins and Hollom (1974)

Strain number	Genotype [a]	Source/Reference
BW61	dnaG3 polA1 leu metE thyA deoB rpoB rpsL Col I^{R} [b]	Wilkins (1975)
BW63	dnaG3 polA1 leu metE deoA deoC tdk rpoB rpsL Col I^{R}	Wilkins (1975)
BW66	his trp lys gal lacΔx74 rpsL tsx Col I^{R}	B.M. Wilkins
BW67	dnaB70 lacY14 rha rpsL deoA deoC tdk tsx Col I^{R}	From BW58
BW68	dnaB70 lacY14 rha rpsL thyA deoB deoC Col I^{R}	From BW56
BW69	dnaB70 lacY14 rha rpsL thyA deoB deoC. Δ (chlA- uvrB -bio)	From BW68
BW72	proA2 thr-1 leu-6 argE3 his-4 thi-1 galK2 ara-14 mtl-1 xyl-5 uvrB5 rpsL31 tdk-1 rpoB	From BW51
BW73	proA2 thr-1 leu-6 argE3 his-4 thi-1 galK2 ara-14 mtl-1 xyl-5 uvrB5 rpsL31 sup-37 thyA deoB rpoB Col I^{R}	From BW74

Strain number	Genotype[a]	Source/Reference
BW74	proA2 thr-1 leu-6 argE3 his-4 thi-1 galK2 ara-14 mtl-1 xyl-5 uvrB5 rpsL31 sup-37 thyA deoB rpoB	From BW50
BW82	dnaG3 leu deoA deoC tdk rpsL ColI^R	From BW63
BW84	dnaG3 leu thyA deoB rpsL ColI^R	From BW61
BW85	dna$^+$ leu thyA deoB rpsL ColI^R	From BW84
BW86	dnaG3 leu thyA deoB rpsL ColI^R Δ (chlA-uvrB)	From BW84
BW89	dna$^+$ leu thyA deoB rpsL ColI^R Δ (chlA-uvrB)	From BW86
BW91	dna$^+$ leu deoA deoC tdk rpsL ColI^R	From BW82
BW94	dna$^+$ leu deoA deoC tdk rpsL ColI^R tsx Δ (chlA-uvrB)	From BW91
BW95	dnaG3 leu deoA deoC tdk rpsL rpoB ColI^R	From BW82
BW96	dna$^+$ leu deoA deoC tdk rpsL rpoB ColI^R	From BW95

Strain number	Genotype[a]	Source/Reference
BW97	*dna*$^+$ *leu deoA deoC tdk rpsL rpoB nal* Col I^R	From BW96
C600	*thr-1 leu-6 thi-1 supE44 lacY1*	B.M. Wilkins
BW162	*thr-1 leu-6 thi-1 supE44 lacY1* Col I^R	Boulnois *et al.* (1979)
GB10	*thr-1 leu-6 thi-1 supE44 lacY1 rpsL* Col I^R	From BW162
GB11	*his trp spc lac*Δ *x74 tsx ton imm*λ Col I^R	B.M. Wilkins
RR1	*pro leu thi rpsL lacY hsdM hsdR*	Bolivar *et al.* (1977)
711	*nal* prototrophic	P.H. Williams

[a] Genotypic symbols are defined in Bachman *et al.* (1976).

[b] Phenotypic symbol for colicin resistance. It is not known whether the mutation is in *cir* or *tonB*.

Plasmid containing strains are described by the notation : host strain number (plasmid).

CHAPTER 3

RIFAMPICIN TREATMENT OF RECIPIENTS ENHANCES TRANSFER OF ColI*drd-1*

1. INTRODUCTION

An underlying theme of this thesis is the effect on IncI$^{\gamma}$ plasmid transfer and conjugal synthesis of treating either donors or recipients with rifampicin. This chapter describes the effects on plasmid transfer of treating recipients with the drug. Donors that were resistant to rifampicin were used to limit the effects of the drug on the expression of functions required for conjugal transfer.

Wilkins and Hollom (1974) found that addition of rifampicin to a mating involving sensitive recipients and drug-resistant donors of ColI *drd-1* enhanced conjugal synthesis of the plasmid in recipients some five fold. More recently, Beddoes and Wilkins (in Boulnois and Wilkins, 1978) have shown that this effect could be reproduced using the protein synthesis inhibitor, streptomycin. Therefore the enhanced conjugal DNA synthesis in rifampicin-treated recipients reflects an effect of the drug on the synthesis of translated RNA. Because these experiments always involved drug-resistant donors and drug-sensitive recipients, both antibiotics presumably act by inhibiting events in the recipients. In confirmation, rifampicin had little effect on conjugal synthesis when matings involved drug-resistant recipients (Beddoes and Wilkins, in Boulnois and Wilkins, 1978).

Two possible explanations for these effects of rifampicin on conjugal synthesis in recipients have been advanced (Wilkins and Hollom, 1974). The drug may enhance the amount of transfer from the resistant donors and consequently increase the number of template strands in the recipient that

are available for conjugal DNA synthesis. Alternatively rifampicin may allow replication of the immigrant plasmid in the newly infected recipients. This might result if replication of ColI is under negative control as has been suggested for other replicons (Pritchard et al., 1969; Rosenberg et al., 1969), the drug inhibiting synthesis of the replication repressor.

In this chapter I describe studies on the phenomenon of enhanced conjugal DNA synthesis induced by the inhibition of synthesis of a protein or proteins in the recipients. Protein synthesis has been inhibited indirectly by the use of rifampicin to block transcription, rather than inhibiting protein synthesis directly with streptomycin, because the effects of rifampicin are much more rapid than streptomycin (Beddoes and Wilkins, in Boulnois and Wilkins, 1978). The results indicate that enhanced conjugal DNA synthesis in recipients is a result of enhanced ColIdrd-1 transfer, a consequence of inhibiting the synthesis of a plasmid specified protein or proteins in the recipient. The F plasmid does not appear to encode an analogous protein.

Ability to enhance transfer by inhibiting protein synthesis in recipients has been used to examine possible intermediates of transfer. The results of these experiments, involving a sedimentation analysis of the transferred DNA, suggest that transfer of ColIdrd-1 involves the transmission of unit lengths of the plasmid.

2. RESULTS

The effects of rifampicin on conjugal synthesis of ColIdrd-1 in recipients

The effect of rifampicin on conjugal synthesis of ColIdrd-1 was determined by mating dnaB70 recipients with drug-resistant (rpoB), tdk donors at 43°C. The results of two such experiments are summarised in Table 3-1 (lines 1 and 2). After 60 min of mating five times more [^{14}C] thymine had been incorporated in the treated mating than in the untreated mating, in agreement with previous data (Wilkins and Hollom, 1974). The control values show the incorporation of [^{14}C] thymine into the parental cultures when they were incubated separately in this type of experiment. About 130 cpm per ml were incorporated and thus about 86% of the counts in the untreated mating and at least 97% of those incorporated in the treated mating can be attributed to the synthesis of plasmid DNA in the recipients.

One interpretation of the enhanced [^{14}C] thymine incorporation in the presence of rifampicin is that the drug increased the amount of DNA transferred (Wilkins and Hollom, 1974). This hypothesis was tested indirectly by adding bacteriophage T6 at a high multiplicity of infection to half of the matings at 30 min to lyse the T6^{s} donors. The recipients were resistant to infection by T6. If enhanced conjugal synthesis in rifampicin reflects enhanced transfer, then destruction of donors by phage T6 should curtail the drug-induced stimulation of [^{14}C] thymine incorporation. When T6 was added to the untreated mating in preliminary experiments, [^{14}C] thymine incorporation was surprisingly enhanced about five times, amounting at 60 min to 5584 cpm per ml of mating mixture. The reason for this enhanced [^{14}C] thymine incorporation following addition of phage T6 is unclear. It may reflect host-range mutants of phage T6 that were capable of infecting the phage-resistant recipients or incomplete

lysis from without of donor bacteria. Productive infection of the tdk donors by T6 may result in the incorporation of [^{14}C] thymine into phage DNA because early in infection phage T6 directs the synthesis of a phage-specified thymidine-kinase (Hiraga et al., 1967). Replication and expression of phage DNA may be abolished by irradiation of the phage with ultra-violet (UV) light. Therefore in all subsequent experiments involving T6 lysis of donors, UV-irradiated T6 was used.

Fig. 3-1 shows the effects of UV-irradiated phage T6 on the kinetics of [^{14}C] thymine incorporation in the presence and absence of rifampicin in an experiment essentially identical to those described in Table 3-1. Rifampicin treatment of the mating again stimulated conjugal synthesis in the recipient about four times. The line of data at the top of Fig. 3-1 indicates the percentage of recipients that had acquired at least one copy of the plasmid at different times in the untreated mating. UV-irradiated T6 was added to half of the treated and untreated matings after 30 min. An equivalent volume of T2 adsorption buffer was added to the remaining half of each mating. Addition of UV-irradiated T6 abruptly stopped [^{14}C] thymine incorporation showing that intact donors are essential for a continued stimulation of DNA synthesis in the rifampicin-treated recipients. Requirement for intact donors implies that enhanced conjugal DNA synthesis results from a rifampicin-induced stimulation of plasmid transfer. In contrast to the rifampicin-treated mating, incorporation of [^{14}C] thymine continued to increase slowly after addition of UV-irradiated T6 to the untreated mating. This may reflect the transconjugants becoming active donors as the mating proceeds. If this explanation is valid, the slight incorporation following addition of phage T6 will overestimate the relative amount of secondary transfer because the transconjugants, being mutant at thyA, will incorporate thymine

into the DNA synthesised to replace any plasmid DNA transferred from them. This DNA synthesis will not be detected in the primary matings because donors were tdk.

The effects of rifampicin on ColIdrd-1 transfer

Experiments described in the preceding section suggest that inhibition of protein synthesis via blocked transcription in the recipients results in amplified transfer. Since conjugal DNA synthesis is only an indirect assay of plasmid transfer, transfer was measured directly by exploiting the methods developed by Ohki and Tomizawa (1968) and Matsubara (1968).

The effect of rifampicin on the transfer of ColIdrd-1 to dnaB recipients is shown in Table 3-2. The presence of the dnaB70 allele in recipients was inessential in these experiments but was included to make the strain as isogenic as possible with recipients described in Table 3-1 and Fig. 3-1. The total amount of DNA transferred was measured with donors that were grown before and during mating in the presence of $[^{14}C]$ thymine and is shown in Table 3-2 (lines 1-3) in the form of radioactivity resisting T6 lysis. Addition of rifampicin to the mating mixture was found to quadruple the total amount of DNA transferred. The number of counts agrees closely with the amounts of conjugal DNA synthesis detected in experiments summarised in Table 3-1 and Fig. 3-1. It is therefore concluded that rifampicin enhances conjugal DNA synthesis by promoting transfer from the donor.

This experimental procedure can be adapted in two ways in order to measure either the transfer of plasmid DNA that was made in the donors before mating or to measure transfer of DNA made in the donors during

the period of mating. Transfer of preexisting DNA was measured by labelling the DNA of donors before mating only. Unincorporated [^{14}C] thymine was removed from the donor culture by washing before donor and recipient bacteria were mixed. Mating was conducted in the presence of an excess of unlabelled thymine. To determine the amount of transfer of DNA made in the donor during the mating period, donor bacteria were grown before mating in the presence of non-radioactive thymine and [^{14}C] thymine was added immediately before donor and recipient bacteria were mixed. The results of these experiments (Table 3-2, lines 4-7) show that rifampicin doubled the amount of preexisting plasmid DNA transferred but enhanced transfer of DNA made during mating some eight fold. Thus the drug primarily enhances transfer by promoting the transmission of DNA that was made during mating.

The effects of rifampicin on the transfer of ColI*drd-1* to UV-irradiated recipients

Rifampicin (and presumably streptomycin) may enhance plasmid transfer by preventing either expression of plasmid or bacterial genes in the recipient. Discrimination between these two possibilities was attempted by exploiting UV-irradiated recipients. The bacteria, defective in the repair of UV-damaged DNA by the excision process, (*uvr*), were irradiated with a high dose of UV light before mating. Irradiation of bacteria renders the resident DNA an unsuitable template for RNA synthesis. However irradiated cells retain both the capacity to synthesise RNA and protein from templates introduced after irradiation, (Hendrix, 1971) and to participate as recipients in conjugation (Freifelder and Freifelder, 1968; Falkow *et al.*, 1971; Wilkins and

Hollom, 1974). Thus if rifampicin increases transfer to drug-treated recipients by inhibiting transcription of the immigrant plasmid, transfer should still be increased if the recipients are also irradiated before mating.

Table 3-3 shows that rifampicin enhanced DNA transfer by more than four times when donors of ColIdrd-1 were mated with recipients defective in excision repair and irradiated with UV light. Matings were incubated at 37°C and 43°C to ensure the temperature independence of the effect. Since irradiation reduces RNA synthesis to 1% as measured by the incorporation of [^{14}C]uracil into TCA-insoluble material, from a 2 min pulse (Beddoes and Wilkins, unpublished data), the results suggest that inhibition of transcription of plasmid genes in recipients allows plasmid transfer to continue.

Studies with a second IncIα plasmid

The phenomenon of enhanced transfer induced by rifampicin is not limited to ColIdrd-1 mediated matings. The drug also enhanced conjugal synthesis in recipients of a second IncIα plasmid, R144drd-3, in matings involving rifampicin resistant donors. When BW51 (R144drd-3) was mated with BW68 at 43°C in three separate experiments, conjugal synthesis was enhanced 3.9 times (Table 3-1, lines 3 and 4). No direct measurements of R144drd-3 transfer were performed and it is assumed that enhanced conjugal synthesis of R144drd-3 reflects enhanced transfer.

The effect of rifampicin on Flac transfer

Inhibition of the expression of plasmid genes in newly formed transconjugants may allow increased transfer of other types of conjugative

plasmids. In this section I describe experiments with the wild-type Flac plasmid JCFL0 (Achtman et al., 1971). It is essential to exploit dna$^+$ recipient bacteria in these experiments because Flac conjugal synthesis is sensitive to rifampicin when dnaB product is thermally inactivated in recipients (Wilkins and Hollom, 1974).

Conjugal synthesis of Flac was measured at 37°C in UV-irradiated BW26 mated with BW51 (JCFL0). In three separate experiments [^{14}C] thymine incorporation was enhanced by an average of 26% when the recipients were treated with rifampicin (Table 3-4). The effect of rifampicin on the transfer of JCFL0 to irradiated, drug-sensitive, dna$^+$ recipients (BW40) at 37°C was measured directly by using BW73 (JCFL0) as a donor. Irradiation of recipients in these experiments was inessential but was included to make the experiments as similar as possible to those allowing measurement of conjugal DNA synthesis in recipients (Table 3-4). The results (Table 3-3) show that the drug increased transfer, as measured by the amount of radioactivity resistant to T6 lysis of the donors, by an average of about 20%. Values obtained in five separate experiments ranged from a reduction in transfer of 12% to an increase of 41%. Therefore rifampicin treatment of recipients has little or no effect on Flac transfer, in marked contrast to matings involving ColIdrd-1.

UV-irradiation has been reported to curtail the capacity of BW40 to receive Flac. A dose of 400 Jm^{-2} reduced by about 50% the amount of DNA transferred in a 60 min mating (Wilkins and Hollom, unpublished data). Therefore a possible explanation of the failure to detect an enhanced transfer of Flac to irradiated, drug-treated recipients is that irradiation inhibits extensive plasmid transfer. To examine this

possibility the Flac mediated matings described in Table 3-3 were repeated using unirradiated recipients. BW50 (JCFL0) was mated with BW40 and rifampicin treatment of these recipients failed to cause a marked increase of Flac transfer (Table 3-5). In two separate experiments the average stimulation of [^{14}C] thymine-incorporation was 52%.

Sedimentation analysis of transferred ColIdrd-1 DNA

To determine whether the increased transfer to rifampicin-treated recipients was associated with the appearance of novel species of plasmid DNA the sedimentation properties of the transferred ColIdrd-1 DNA was analysed in 5 to 20% alkaline sucrose gradients. Thymine-requiring ColIdrd-1 donors were labelled with [^{14}C] thymine prior to and throughout mating with tdk dnaB recipients. Mating was interrupted at 60 min and the donors were selectively lysed with phage T6 as described before. Washed recipients were lysed in alkaline SDS on top of 4.1 ml alkaline sucrose gradients which were centrifuged for 37 min at 30,000 rpm. To indicate the expected sedimentation position of dimeric molecules in these gradients pLG205 DNA was added as a marker. pLG205 is a ColIb-prime plasmid carrying E.coli K12 DNA and has a molecular weight of 1.2×10^8 (M.I. Sedgwick, personal communication) twice the reported molecular weight of ColI (6.2×10^7) (Clewell and Helinski, 1970b).

DNA transferred during the untreated mating was found in two approximately equal peaks after centrifugation (Fig. 3-2). From the sedimentation patterns of marker ColIdrd-1 and pLG205 DNA, the rapidly sedimenting material was identified as covalently closed circular (CCC) ColI. In alkaline sucrose gradients CCC DNA forms a compact random coil that sediments 3-4 times faster than circular or linear species of DNA (Vinograd et al., 1965; Rhoades and Thomas, 1969). Most of the more

slowly sedimenting material cosedimented with single strands of ColIdrd-1 marker DNA. Marker DNA was isolated as CCC molecules, the single strands presumably result from nicking of the CCC molecule during preparation and storage of the marker DNA.

The transferred DNA isolated from rifampicin-treated recipients gave the same two peaks, but the fraction of label in CCC DNA was less than in the control. The difference in the relative amounts of CCC DNA is attributed to experimental variation. It does not reflect inability of recipients to circularise the extra DNA that is transferred in the rifampicin-treated matings because when the experiment was repeated using donors labelled solely during mating, the size of the two peaks was equal (Fig. 3-3). These results therefore show that rifampicin does not inhibit the conversion of the transferred DNA into CCC molecules. Furthermore the drug does not promote the formation of detectable amounts of covalently closed dimeric circles.

Amplification of transfer by rifampicin provides a very sensitive system for the examination of transfer intermediates. In preliminary experiments the sedimentation properties of the transferred DNA that sedimented more slowly than CCC moleculeswere examined by centrifuging the gradients described in Fig. 3-3 for a longer period. This approach was unsuccessful for two reasons. Firstly the number of counts in this slowly sedimenting material was too low to afford reasonable resolution of circular and linear material and secondly these gradients were prone to overloading. Overloading the gradients by adding too large a sample caused the marker DNA to sediment in the gradients in a very erratic manner.

To overcome these problems the procedure described for Fig. 3-2 was slightly modified. $[^3H]$thymine was used as a DNA label because it

was available at a much higher specific activity than [^{14}C] thymine, and secondly, to accommodate more DNA, larger gradients were used (12.5 ml). Recipients isolated from a 45 min mating were lysed on top of a 12.5 ml 5 to 20% alkaline sucrose gradient and centrifuged for 3 h at 35,000 rpm (Fig. 3-4). Radioactivity at the bottom of the gradient is assumed to be in CCC DNA. The majority of the remaining labelled material from either the untreated or treated mating was found in two unequal peaks. The smaller of the two peaks had sedimented 1.1 times further than the larger peak. From an analysis of the circular forms of polyoma DNA (Vinograd et al., 1965) and P22 DNA (Rhoades and Thomas, 1968) the sedimentation coefficients of circular DNA species relative to linear species was found to be 1.12 in alkaline sucrose gradients. Therefore the material in the smaller and faster sedimenting peak may be identified as single-stranded circular DNA and that in the larger, more slowly sedimenting peak as linear ColIdrd-1 DNA. There was no evidence for the accumulation of higher or lower molecular weight DNA in rifampicin-treated recipients. In a separate experiment, the chromosomal DNA of the recipients was found to sediment at the bottom of such gradients.

3. DISCUSSION

Conjugal synthesis of ColIdrd-1 in recipients was stimulated four -fold when rifampicin-treated recipients were mated with drug-resistant donors (Wilkins and Hollom, 1974; Table 3-1; Fig. 3-1). This enhanced conjugal DNA synthesis was caused by increased plasmid transfer because it was associated with a corresponding four fold stimulation in the amount of DNA transferred from the donors (Table 3-1). In such a drug-treated mating the average recipient receives at least seven single-stranded equivalents of ColIdrd-1 within 60 min. This calculation is based on the data for conjugal DNA synthesis at 10 min and the percentage of recipients that had inherited the plasmid at that time (Fig. 3-1). At 10 min, 52% of the recipients had inherited at least one copy of the plasmid and 340 cpm/ml had been incorporated into DNA synthesised as a result of mating.

The effect of rifampicin on conjugal transfer and DNA synthesis could be reproduced by treating recipients with the protein synthesis inhibitor streptomycin. The drug at a concentration of 30 μg per ml enhanced conjugal DNA synthesis of ColIdrd-1 in recipients four-fold (Beddoes and Wilkins, in Boulnois and Wilkins, 1978). Enhanced transfer therefore results from inhibition of synthesis of at least one species of protein in the recipient. In an untreated mating this protein(s), synthesised in newly infected recipients must normally limit the amount of DNA transferred. One cannot exclude the formal possibility that transfer normally occurs at the enhanced level and the first plasmid to be transferred directs the synthesis of a nuclease that degrades further transferred material. Rifampicin and streptomycin would inhibit the breakdown of the extra transferred material according to this model. This idea lacks appeal because it is energetically extravagant. Results of experiments with UV-irradiated recipients

(Table 3-2) suggest that this species of protein, which is postulated to limit the amount of DNA transferred during mating, is specified by the incoming plasmid rather than the chromosome of the recipient. Specification of a transfer-limiting function may be a general property of IncIα plasmids because enhanced conjugal synthesis in rifampicin-treated recipients was observed in matings mediated by R144*drd-3*. Conjugal synthesis of the IncIα plasmid R64*drd-11* was not measured because in my hands this plasmid, obtained from three different sources, was not sufficiently fertile to perform the experiment.

The mode of action of such a protein is unclear. Elucidation of the mechanism of transfer limitation will require the isolation of suitable mutants of IncIα plasmids. In order to make use of the numerous *Flac* mutants that are available, the possibility that a similar protein acts to limit the amount of DNA transferred in an F mediated mating was examined. Rifampicin treatment of recipients only increased *Flac* DNA transfer by 19% (Table 3-3) and conjugal DNA synthesis in recipients was enhanced by 21% (Table 3-4). Wilkins and Hollom (1974) detected a rifampicin-induced enhancement of conjugal DNA synthesis of about 80%. This small increment of transfer and conjugal DNA synthesis is too small to allow exploitation of existing F mutants to analyse its nature.

A clue to the role of the IncIα plasmid-specified protein is provided by the observation that rifampicin treatment doubled the amount of transfer of ColI*drd-1* DNA that existed in the donors before mating (Table 3-2). This observation implies that only half of the pre-existing plasmids had the opportunity to transmit DNA in a normal, untreated mating. The protein may therefore limit transfer by destroying the competence of newly formed transconjugants to act as recipients. Its activity is detectable in a population of recipients within 10 min

of mating (Fig. 3-1). In a formal sense the process of transfer limitation is analogous to the phenomenon of surface exclusion. This property of plasmid-containing cells reduces their ability to act as recipients in matings with bacteria harbouring the same or a related plasmid (Novick, 1969). Surface exclusion specified by the F plasmid is mediated by two plasmid genes traS and traT. The traS gene product has little effect on the formation of mating aggregates but effectively prevents DNA transfer to traS$^+$ cells (Achtman et al., 1977) perhaps acting directly at the level of DNA transfer. Transfer limitation and surface exclusion may be the same phenomenon. This suggestion is subject to the criticism that whilst both F and ColIdrd-1 cause efficient surface exclusion (Meynell, 1969; Novick, 1969) the effects of rifampicin on the transfer of these plasmids differ. Such an objection assumes that surface exclusion specified by F and IncIα plasmids are by similar mechanisms. Further analysis of this phenomenon may require the isolation of transfer-proficient, exclusion-defective mutants of IncIα plasmids (see Chapter 6).

Transfer limitation may be related to the process of active disaggregation of mating aggregates, a process that is triggered after successful DNA transfer (Achtman, 1977). After transfer, expression of a plasmid gene(s) in recipient cells is postulated to produce enzymes that modify the cell surface rendering mating aggregates unstable. However both F and IncIα plasmid transfer is followed by active disaggregation of mating aggregates and moreover, the process was not fully inhibited by treating the mating with the protein synthesis inhibitor, chloramphenicol (Achtman et al., 1978b). It therefore seems unlikely that the process of transfer limitation is related to active disaggregation. The molecular mechanism of transfer limitation awaits further analysis.

Fenwick and Curtiss (1973b) have reported that the transferred DNA of an IncI$_{\alpha}$ plasmid, R64drd-11, was of unit size when isolated from recipient minicells. Results in Fig. 3-2, 3-3 and 3-4 also suggest that ColI transfer involves the transmission of unit length DNA. Transferred ColIdrd-1 DNA in recipients cosedimented in alkaline sucrose gradients with monomeric units of ColIdrd-1 DNA. Even when transfer was amplified by rifampicin treatment, no evidence for the accumulation of high molecular weight plasmid DNA was obtained. Transmission of unit length single strands of DNA may therefore be a general feature of the transfer of IncI$_{\alpha}$ plasmids.

The reactions involved in the formation of covalently closed circular molecules from unit length single-stranded DNA are not understood. Formation of CCC molecules occurred normally when recipients were treated with rifampicin indicating that the process does not require transcription of the immigrant plasmid. Curtiss and Fenwick (1975) have postulated two models based on the transfer of DNA that either is slightly greater than monomeric length or has an RNA molecule attached at the 3' terminus. Two alternative models were proposed by Boulnois and Wilkins (1978) and are summarised in Fig. 3-5a,b. The essential feature of both of these models is the existence of two pairs of inverted complementary DNA sequences in the plasmid. Circularisation may be achieved by a scheme outlined in Fig. 3-5a. One pair of the hypothetical complementary sequences in the transferred strand (1) may promote synthesis of the complementary strand (2), whilst the other, following nicking (3), promotes the formation of terminal redundancy (3,4) by a process essentially similar to that proposed by Cavalier-Smith (1974) to describe replication of chromosome ends. The molecule generated (5) is circularised by some form of recombinational event. The nicking event

would presumably be mediated by a plasmid-encoded enzyme, and since transcription of the transferred DNA in the recipient is not required, this hypothetical nuclease would be derived from the donor.

Alternatively, circularisation (see Fig. 3-5b) may be achieved by a scheme proposed originally for the closure of the viral strand of single-stranded DNA phages (Kornberg, 1974). By utilising the hypothetical inverted complementary DNA sequences the transferred strand (1) can take the configuration shown in (2). Sealing of the gap by DNA ligase followed by complementary strand synthesis results in the formation of a circular duplex in the recipient.

Since these models were proposed, subsequent studies have shown that closure of the viral strands of phage ØX174 involves the phage cisA gene product (Eisenberg et al., 1977). A more detailed discussion of this process is presented in the introduction, and a model for transfer invoking a similar activity is described in the general discussion (Chapter 7).

Table 3-1: EFFECT OF RIFAMPICIN ON CONJUGAL SYNTHESIS OF ColI*drd-1* AND R144*drd-3* IN *dnaB70* RECIPIENTS

Donors were mated with BW68 at 43°C for 1 h. The volume of the mating mixture was 3.5 ml and contained [^{14}C] thymine (0.25 μCi/μg). After 1 h of mating four replicate samples (0.75 ml) were removed into TCA and insoluble radioactivity determined.

a Donors were BW51 (ColI*drd-1*)

b Donors were BW51 (R144*drd-3*)

Plasmid	Rifampicin (μg/ml)	No. of Expts.	^{14}C incorporation (cpm per ml)			+Rif/-Rif
			Mating (M)	Control (C)	M-C	
ColI*drd-1* [a]	0	2	1050	131	919	
	200	2	4837	135	4702	5.1
R144*drd-3* [b]	0	3	1783	274	1509	
	200	3	6026	170	5856	3.9

Table 3-2: EFFECT OF RIFAMPICIN ON THE TRANSFER OF ColIdrd-1 TO dnaB RECIPIENTS

BW73 (ColIdrd-1) was mated with BW67 for 1 h at 43°C. The amount of TCA-precipitable radioactivity was determined after selective lysis of the donors. The controls consisted of the parental cultures incubated separately for 1 h and then mixed immediately before lysis of the donors.

a Donors were grown and mated in the presence of [^{14}C] thymine (0.25 µCi/µg).

b The donors were incubated in the presence of [^{14}C] thymine (0.25 µCi/µg) for three to four mass doublings. Immediately before mating, they were sedimented and then resuspended and mated in SGC medium containing 50 µg unlabelled thymine per ml.

c The donors were grown before mating for three to four mass doublings in the presence of non radioactive thymine. [^{14}C] thymine (0.25 µCi/µg) was added to the mating mixture.

	Labelling procedure	Rifampicin	cpm per ml		Transfer
		µg/ml	Mating (M)	Control (C)	M-C
1.	Before and during mating [a]	0	1169	8	1611
		120	4484	100	4384
		200	4584	2	4582
2.	Before mating only [b]	0	738	2	736
		200	1461	0	1461
3.	During mating only [c]	0	347	30	317
		120	2542	30	2512

Table 3-3: EFFECT OF RIFAMPICIN ON PLASMID TRANSFER TO UV-IRRADIATED, DRUG-SENSITIVE RECIPIENTS

Plasmid	Temp (°C)	cpm transferred/ml		+Rif/-Rif
		-Rif	+Rif	
ColIdrd-1[a]	43	924	4134	4.47
	37	518	2270	4.38
JCFLO[b]	37	456	542	1.19

In all matings the recipients were irradiated with 400 J/m^2 of UV light and the DNA of the donors was labelled before and during mating with [^{14}C] thymine (0.25 μCi/μg). Counts transferred were measured after selective lysis of the donors. The data have been corrected to account for the radioactivity resisting T6 lysis in the control experiments (about 60 cpm per ml). In these controls, the parental cultures were incubated separately for 1 h and were then mixed immediately before selective lysis of the donors.

[a] BW73 (ColIdrd-1) was mated with a colicin Ib-resistant mutant of BW40 for 1 h at either 43 or 37°C. Each line contains the values derived in a single experiment. Rifampicin was added to give 200 μg/ml.

[b] BW74 (JCFLO) was mated with BW40 for 1 h at 37°C. The data are the average of five separate experiments. Rifampicin was added to give 100 μg/ml.

Table 3-4: EFFECT OF RIFAMPICIN ON CONJUGAL SYNTHESIS OF JCFLO IN UV-IRRADIATED RECIPIENTS AT 37°C

Rifampicin	^{14}C incorporation per ml			+Rif/-Rif
(μg/ml)	Mating (M)	Control (C)	M-C	
0	736	47	689	
200	915	78	837	1.21

BW72 (JCFLO) was mated with UV-irradiated BW26 at 37°C. The mating mixtures (3.5 ml) contained $\left[^{14}C\right]$thymine at a specific activity of 0.25 μCi/μg. The controls consisted of the parental bacteria incubated separately. After 1 h, samples (0.75 ml) were taken in quadruplicate and TCA-insoluble radioactivity determined. The data are the average of three separate experiments.

Table 3-5: EFFECT OF RIFAMPICIN ON JCFLO TRANSFER TO UNIRRADIATED RECIPIENTS

Rifampicin	^{14}C incorporation (cpm per ml)			+Rif/-Rif
(μg/ml)	Mating (M)	Control (C)	M-C	
0	1096	50	1046	
200	1623	44	1589	1.52

BW73 (JCFLO) was grown and mated with BW40 in the presence of [^{14}C] thymine (0.25 μCi/μg) at 37°C. After selective lysis of the donors, TCA-precipitable radioactivity was determined. The controls consisted of the parental cultures incubated separately for 1 h and then mixed before lysis of the donors. The data are the average of two separate experiments.

Figure 3-1: EFFECT OF RIFAMPICIN ON CONJUGAL DNA SYNTHESIS IN DRUG-SENSITIVE *dnaB* BACTERIA

BW72 (ColI*drd-1*) was mated with BW68 at 43°C in the presence of [^{14}C] thymine (0.25 μCi/ug) in the presence (o) or absence (•) of rifampicin (200 μg/ml). The volume of the mating mixture was 8 ml. At 30 min, 2 ml of each mating was added to 2 ml of SGC medium containing [^{14}C] thymine and either 4.3×10^{11} phage T6 particles, irradiated with 120 J/m^2 of UV light (Δ , + Rif; ▲ , - Rif), or T2 adsorption medium (o, + Rif; •, - Rif). Samples (0.5 ml) were taken into TCA and insoluble radioactivity determined. The counts incorporated after 30 min have been corrected to account for the dilution. The % Cib$^+$ values indicate the percentage of recipients that had acquired the plasmid at 0, 10, 20, 30, 40 and 60 min in such a mating. These values were obtained in an interrupted mating experiment. After interruption the donors were eliminated by the addition of T6, and the recipients were plated on nutrient agar. The resulting colonies (about 350) were scored for the Cib$^+$ (colicin Ib-producing) phenotype.

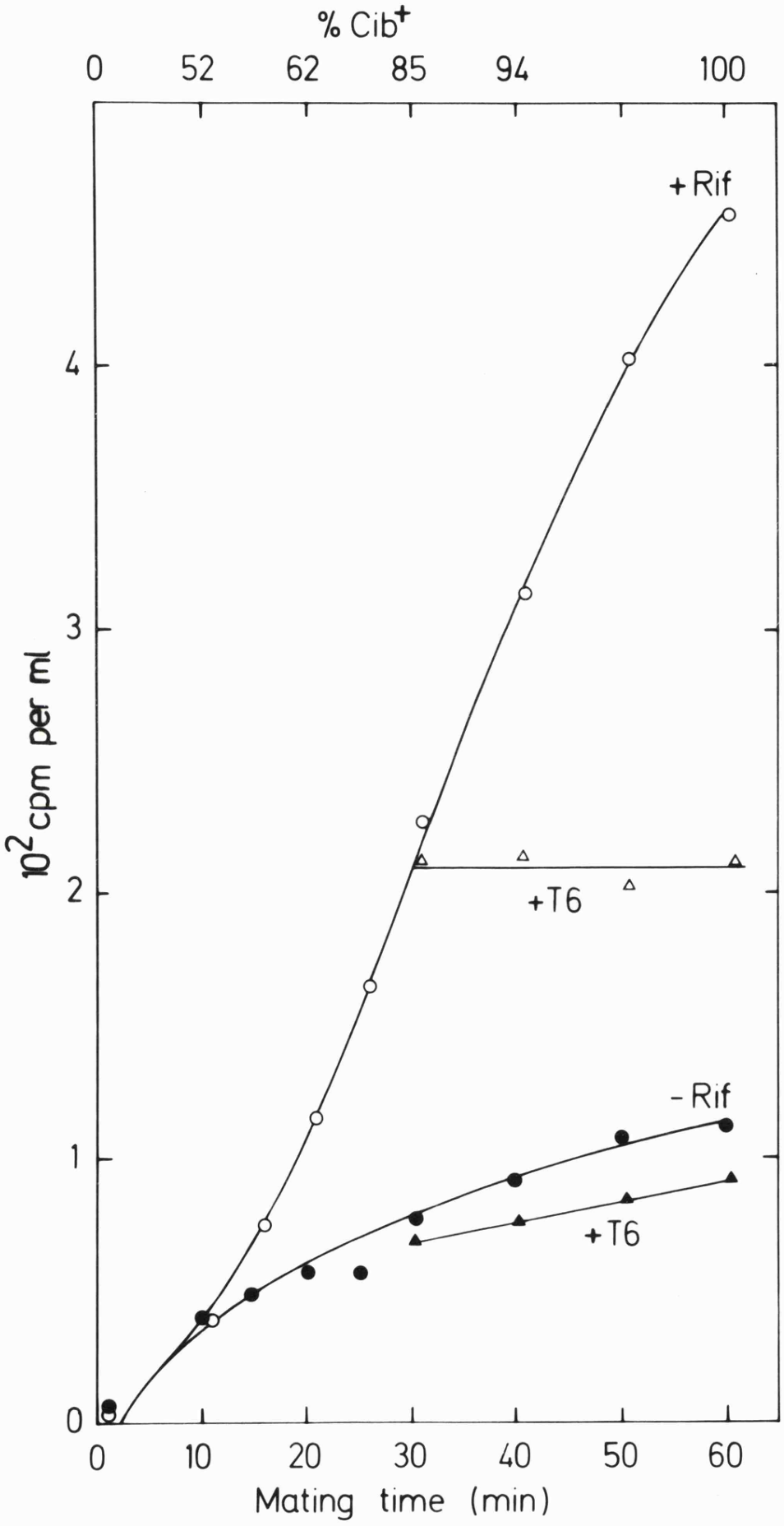

% Cib+
0
52
62
85
94
100
+ Rif
+T6
- Rif
+T6
10^2 cpm per ml
0
1
2
3
4
0
10
20
30
40
50
60
Mating time (min)

Figure 3-2: SEDIMENTATION ANALYSIS OF TRANSFERRED ColIdrd-1

BW73 (ColIdrd-1) was mated with BW67 for 1 h at 43°C in [^{14}C] thymine (0.25 μCi/μg) in the presence (•) or absence (o) of 200 μg rifampicin per ml. The matings were 3.5 ml in volume. Donors were grown before mating in [^{14}C] thymine. After selective lysis of the donors, the washed recipients were lysed in alkaline SDS on top of 4.1 ml, 5 to 20% alkaline sucrose gradients. The gradients were centrifuged for 37 min at 30,000 rpm. The direction of sedimentation was from right to left. The arrows refer to the fractions containing peaks of rapidly sedimenting, labelled DNA when ColIdrd-1 and pLG205 isolated as CCC material, were sedimented accordingly. Sedimentation rates cannot be calculated from these gradients because the SDS on the gradients reduced the volume of the drops corresponding to the top 10 fractions.

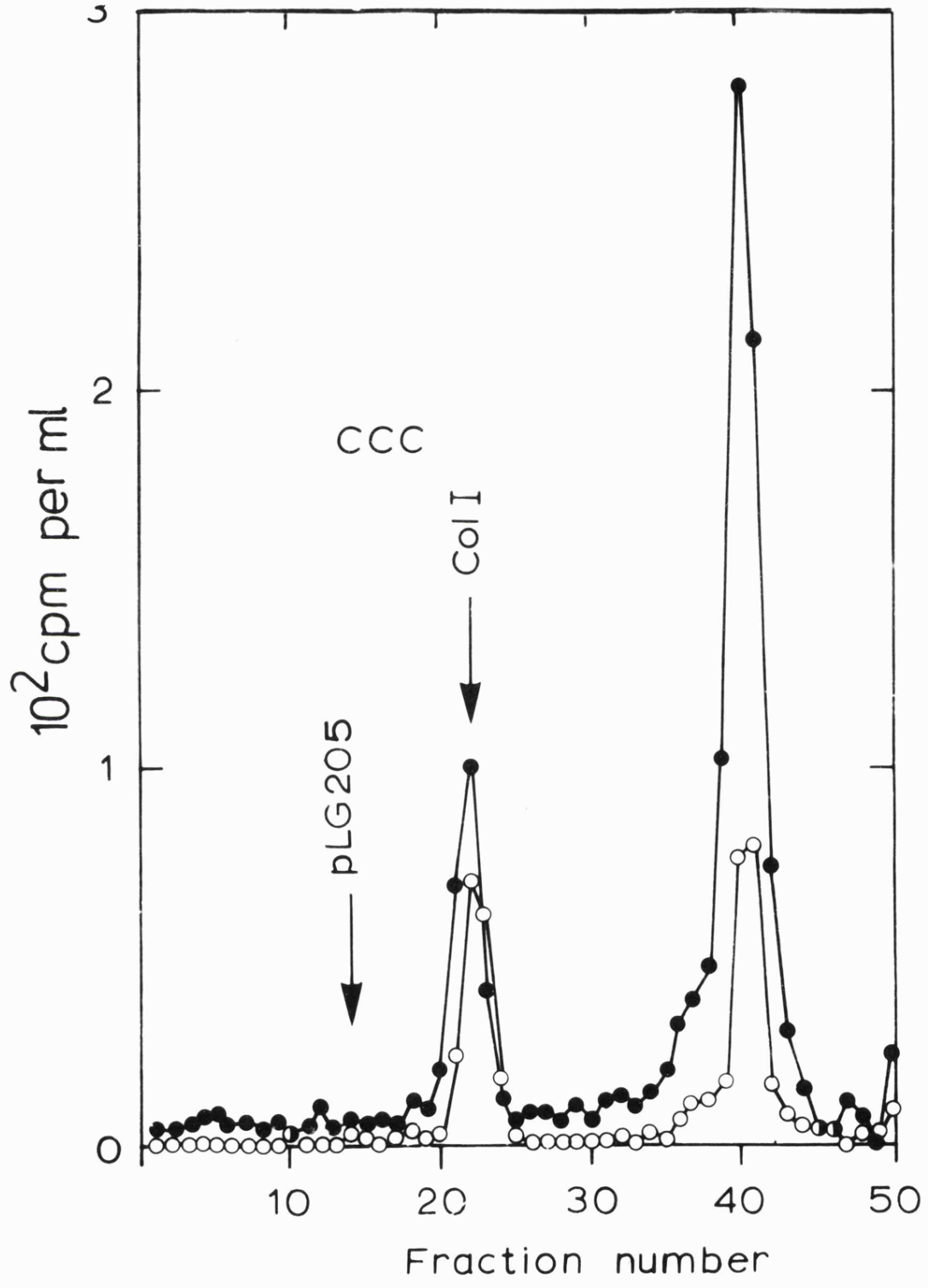

10^2cpm per ml
0
1
2
3
CCC
Col I
pLG205
10
20
30
40
50
Fraction number

Figure 3-3: SEDIMENTATION ANALYSIS OF TRANSFERRED ColIdrd-1 DNA MADE IN THE DONOR DURING MATING

BW73 (ColIdrd-1) was mated with BW67 in the presence of 200 μg rifampicin per ml. The procedure was the same as for Fig. 3-2 except donors were labelled with [^{14}C] thymine (0.25 μCi/μg) only during the 1 h mating.

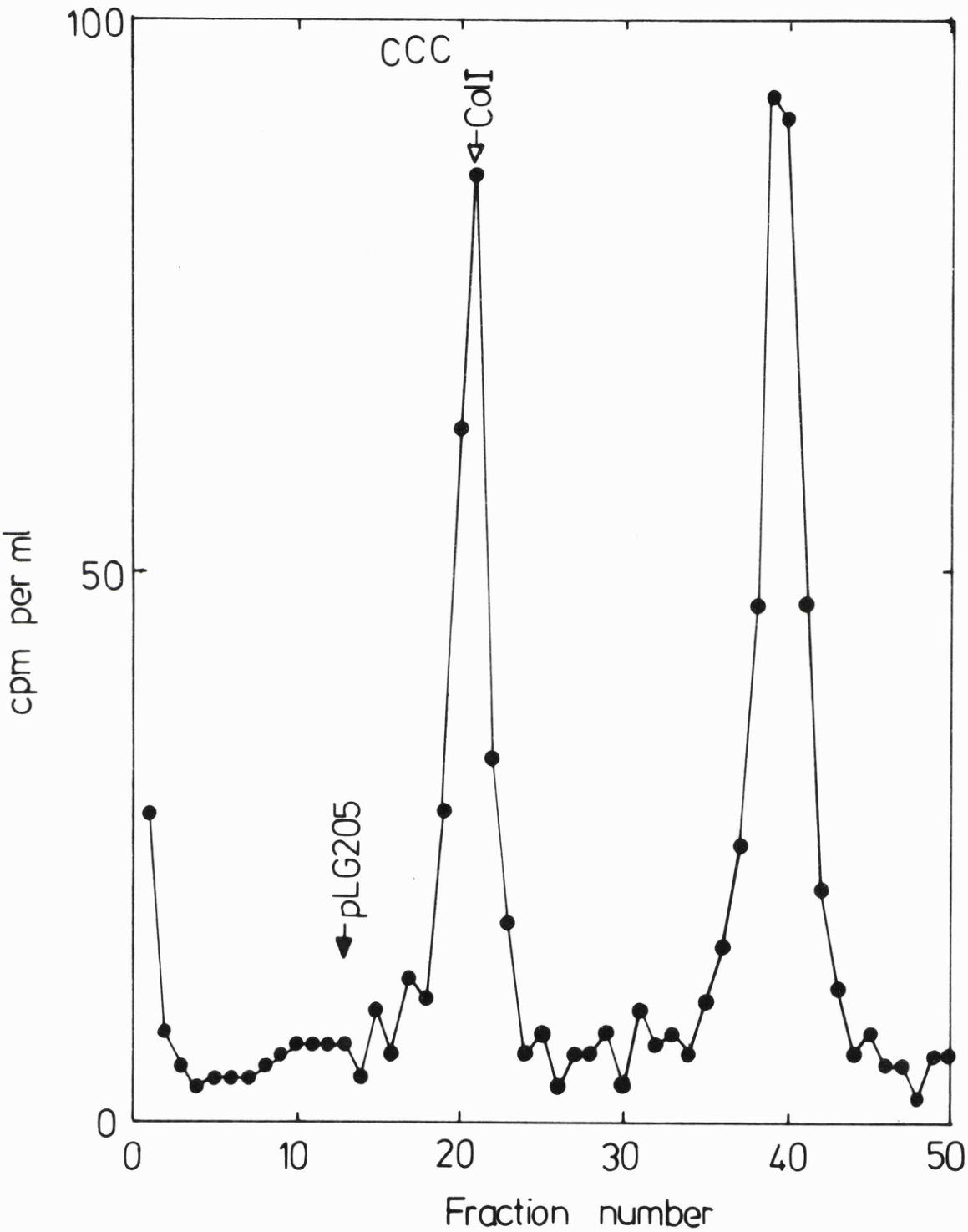

100
50
0
cpm per ml
CCC
ColI
pLG205
0
10
20
30
40
50
Fraction number

Figure 3-4: SEDIMENTATION ANALYSIS OF TRANSFERRED ColIdrd-1 DNA

BW73 (ColIdrd-1) was mated with BW67. The procedure was the same as for Fig. 3-2, except that matings were for 45 min in the presence of [^{3}H] thymine (10 μCi/μg). The donors were also grown prior to mating in ^{3}H thymine. 12.5 ml gradients were used and centrifuged for 3 h at 35,000 rpm. The arrows refer to the fractions containing peaks of radioactivity when ColIdrd-1 and pLG205 DNA were sedimented accordingly. Each reference plasmid gave two peaks which are assumed to correspond to circular (C) and linear (L) single-stranded DNA (Vinograd et al., 1965; Rhoades and Thomas, 1968).

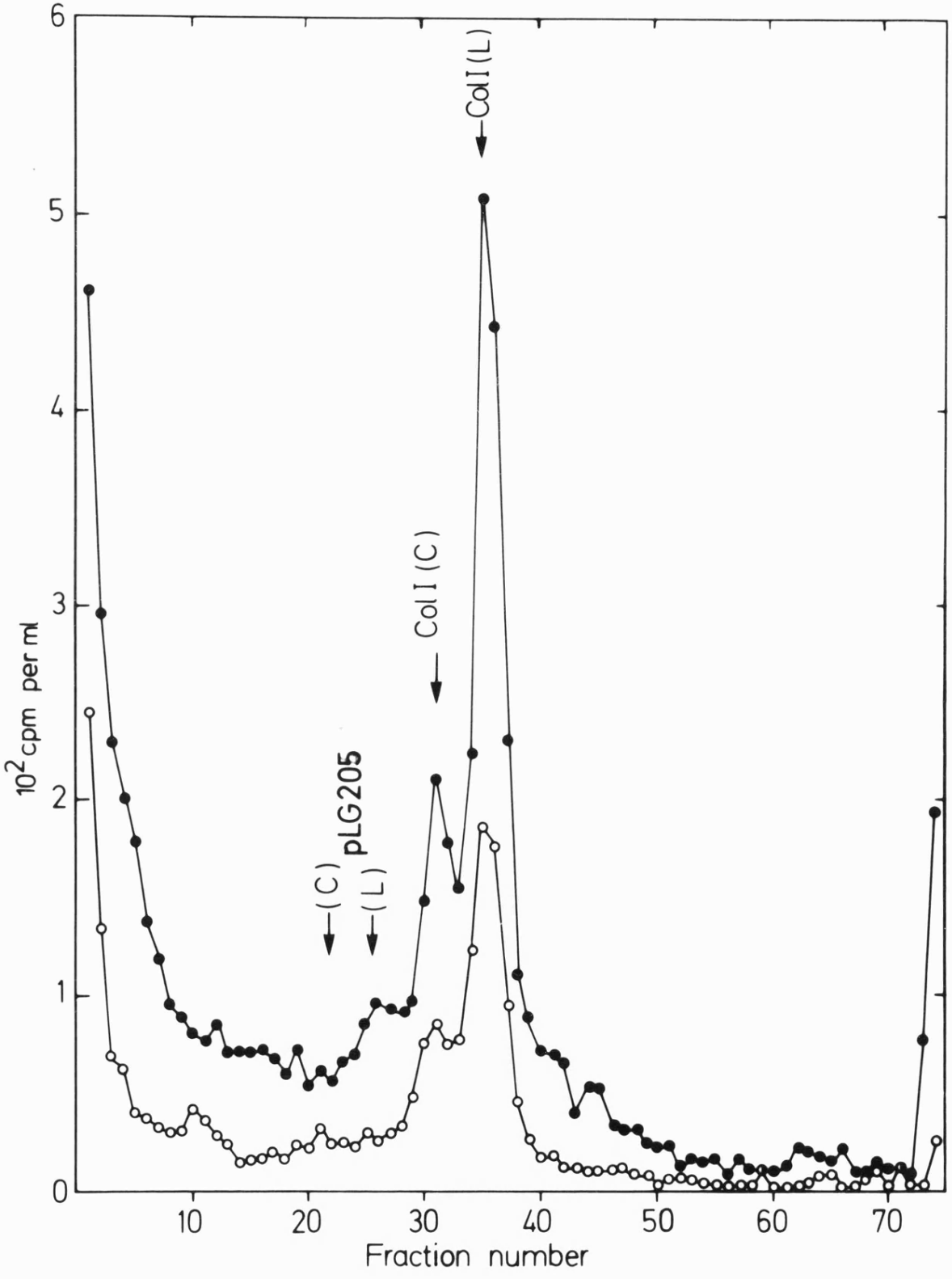
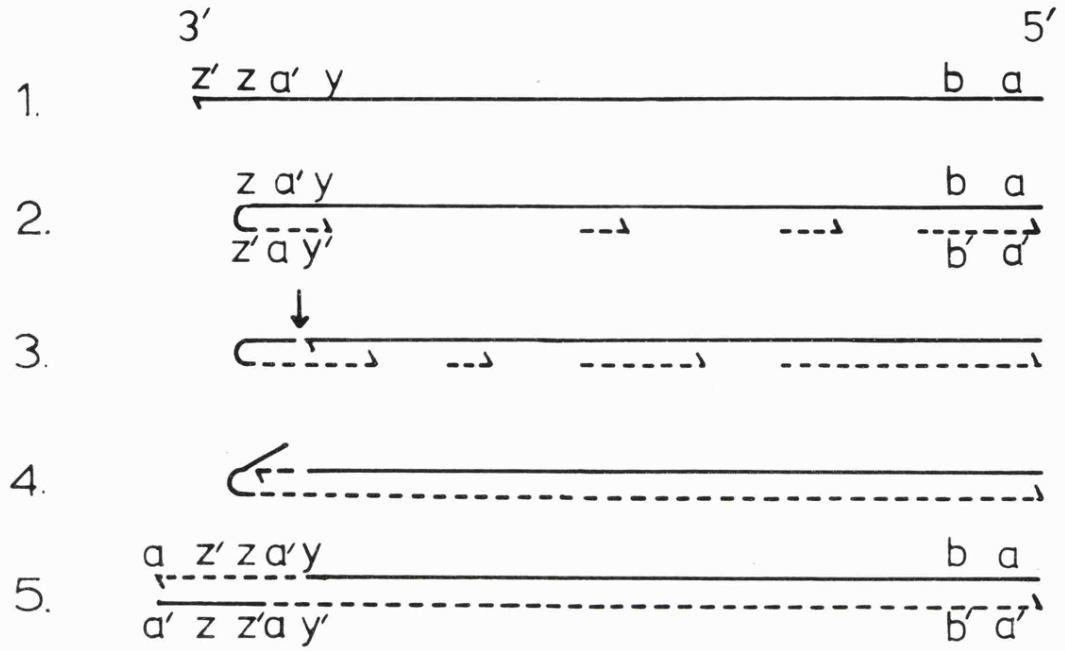

6
5
4
3
2
1
0
10^2cpm per ml
ColI (L)
ColI (C)
(C)
(L)
pLG205
10
20
30
40
50
60
70
Fraction number

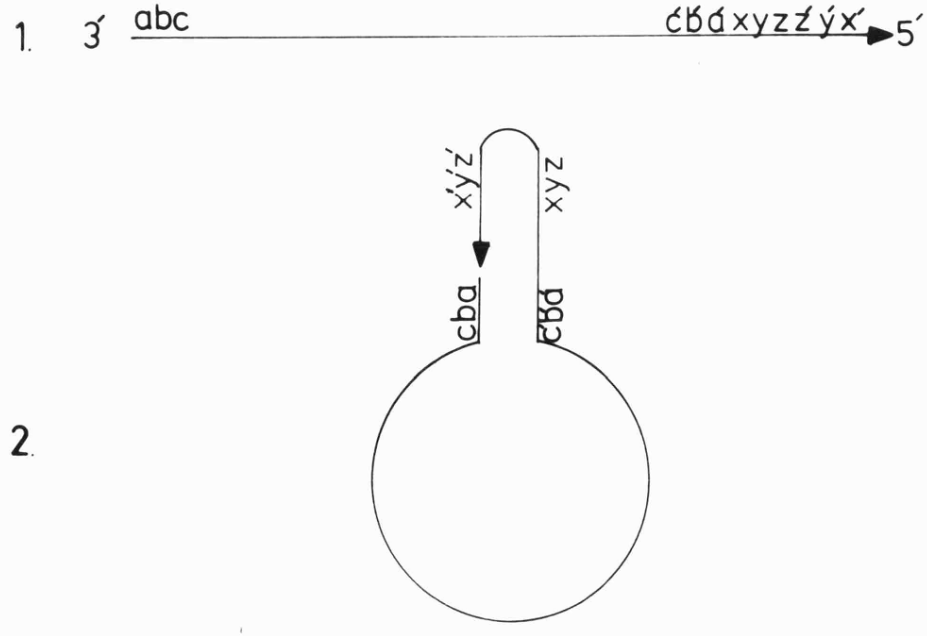
Figure 3-5: TWO MODELS FOR IncIα PLASMID TRANSFER

A detailed discussion of both models is presented in the text. The dashed lines in Fig. 3-5a represent DNA synthesised during conjugation.

(a)


3′
5′
1.
z′ z a′ y
b a
2.
z a′ y
b a
z′ a y′
b′ a′
3.
4.
a z′ z a′ y
b a
5.
a′ z z′a y′
b′ a′

(b)


1.
3′
abc
c̀b́á xyz ź ý x́
5′
2.
x′y′z′
xyz
cba
c̀b́á

CHAPTER 4

RIFAMPICIN TREATMENT OF DONORS OF ColIdrd-1 DISRUPTS PLASMID TRANSFER

1. INTRODUCTION

In the previous chapter I have described some of the effects on IncIα plasmid transfer of treating recipients with rifampicin in matings that involved drug-resistant donors. This chapter describes the effect on plasmid transfer of treating both donors and recipients with rifampicin. This has been examined before. Fenwick and Curtiss (1973b) have claimed that transfer of an IncIα plasmid, R64drd-11 from dnaB donors to recipient minicells at 42°C was more sensitive to rifampicin than chloramphenicol, an inhibitor of protein synthesis. These findings have been interpreted as defining a role in transfer for an RNA primer, made in the donor by RNA polymerase, in response to contact with recipients. This interpretation has led to two important and contraversial models for R64drd-11 transfer (Curtiss and Fenwick, 1975). In contrast Wilkins and Hollom (1974) have reported that transfer of a derepressed mutant of the prototype IncIα plasmid, ColI, between dna^+ bacteria at 37°C was unaffected by rifampicin, at least in the early period of mating.

In view of these conflicting reports and because in the following chapter matings involving rifampicin-treatment of drug-sensitive donors and recipients are discussed, it is essential to establish the effect of the drug on plasmid transfer. Rifampicin may exert an effect on transfer by inhibiting the synthesis of either translated or untranslated RNA. To distinguish between these two possible effects of rifampicin, the

effects of chloramphenicol on transfer have also been studied. If rifampicin affects transfer by inhibiting the synthesis of RNA destined for translation, the same result should be obtained in the presence of chloramphenicol.

Whilst these studies were in progress Beddoes and Wilkins (in Boulnois et al., 1979) found that rifampicin, but not chloramphenicol, had unexpected side effects on chromosomal DNA synthesis in bacteria that harbour IncI$_\alpha$ plasmids. Rifampicin treatment of such bacteria led to a general disruption of DNA metabolism. In this chapter I will present evidence that suggests that conjugal DNA metabolism is also subject to the same disruptive effects of the drug. It is suggested that the apparent rifampicin-sensitivity of R64drd-11 transfer (Fenwick and Curtiss, 1973b) can be attributed to these unexpected side effects of the drug on DNA metabolism, rather than a specific role for RNA polymerase in some aspect of the transfer process.

2. RESULTS

Plasmid transfer in the presence of rifampicin and chloramphenicol

ColI transfer was measured directly essentially as described in the previous chapter, except that the tdk recipients were defective in repair of UV-induced damage (uvr), and had been irradiated with UV light before mating. Irradiation, which is inessential in these experiments, was included to enable a comparison of results obtained in experiments designed to measure conjugal DNA synthesis in recipients and discussed in the following section.

In the previous chapter incorporation of [^{14}C] thymine into unmated bacteria was measured by incubating donors and recipients separately. However in this and the following chapter the unmated controls involved mixing donors and recipients in the presence of 100 µg SDS per ml. This modification facilitates a kinetic analysis of conjugal DNA synthesis in recipients in a series of matings performed concurrently. In F-mediated matings, SDS inhibits transfer by preventing aggregation of bacteria (Achtman and Helmuth, 1975). The detergent also prevents transfer of IncI$_\alpha$ plasmids, as determined by measurement of plasmid transconjugants. In a 45 min mating between GB10 (R144drd-3) and GB11, 7.3×10^8 transconjugants per ml were formed, but addition of SDS (100 µg/ml) reduced the number of transconjugants to less than 1×10^4 per ml. This concentration of SDS had no detectable effect on either the growth or viability of the parental cultures.

To determine the effects of rifampicin and chloramphenicol on ColI transfer, drug-sensitive donors and recipients were incubated in the appropriate antibiotic for 5 min before and throughout matings. Rifampicin and chloramphenicol were used at concentrations of 100 and 200 µg per ml respectively.

The effect of 100 μg rifampicin per ml on the rate of RNA synthesis in BW91 (ColIdrd-1) was measured by following the incorporation at 41°C of [^{3}H] uracil from a 2 min pulse into TCA-insoluble material. The incorporation was 14,750 cpm per ml immediately before addition of rifampicin and was reduced to 65 cpm per ml after 5 min in the drug. Therefore the preincubation in rifampicin is sufficient to abolish RNA synthesis in parental bacteria before mating was initiated. The rate of protein synthesis in BW91 (ColIdrd-1) treated with chloramphenicol was measured by following the incorporation at 41°C of [^{35}S] methionine from a 2 min pulse into TCA precipitable material. 48,650 cpm per ml were incorporated immediately prior to addition of chloramphenicol and after 5 min in the drug the incorporation was 3504 cpm per ml, a reduction of about 93%. Incorporation was not significantly reduced by continued incubation in the presence of the drug. For example the incorporation after 10 min was 3484 cpm per ml. This residual incorporation presumably reflects incorporation of [^{35}S] methionine into methionyl tRNA since the experimental procedure did not include a step in which RNA was hydrolysed.

Mating bacteria were incubated at 37°C and 41°C to determine whether any effects of the drugs were temperature dependent. 41°C was chosen because this is the highest permissible temperature for an ancestor of the strains used (J.A. Wechsler, personal communication).

The results in Table 4-1 show the effect of rifampicin and chloramphenicol treatment of donors on the transfer of ColIdrd-1 between dna^{+} bacteria. Transfer was only slightly more sensitive to rifampicin than to chloramphenicol. Rifampicin and chloramphenicol-treated donors retained the capacity to donate about 50% of the normal amount of DNA in the one hour matings, irrespective of whether the mating bacteria

were incubated at 37°C or 41°C.

Conjugal DNA synthesis in recipients

Adaptation of the experimental approach discussed in the previous section to study the kinetics of transfer in three simultaneous matings is not practical because the lysis procedure is too cumbersome for processing a large number of samples. A kinetic study may be attempted by measuring conjugal DNA synthesis in UV-irradiated recipient bacteria. This synthesis, which reflects formation of DNA complementary to the transferred strands, has been shown to be a reasonable estimate of DNA transfer in the case of F-mediated matings (see Chapter 3).

Conjugal DNA synthesis in recipients was measured essentially as described in the previous chapter. Donors mutant at tdk were mated with thymine-requiring recipients in the presence of labelled thymine. Incorporation of label into the chromosome of the recipient was limited by irradiating the repair-defective recipient bacteria with UV light prior to mating.

Fig. 4-1 shows the kinetics of conjugal DNA synthesis in dna$^+$ recipients mated with dna$^+$ donors of ColIdrd-1. The data are representative of three separate experiments. The amounts of synthesis when the bacteria were treated with rifampicin or chloramphenicol is also shown. The values at 60 min are in reasonable accord with the amount of DNA transferred in 1 hr (Table 4-1). Therefore conjugal DNA synthesis in this system is a reasonably accurate estimate of DNA transfer. In the chloramphenicol-treated matings, the rate of synthesis progressively declined over about 40 min. However in the rifampicin-treated matings normal amounts of synthesis were detected for about 10 min after which

[^{14}C] thymine incorporation ceased abruptly. The 10 min mating time corresponds to 15 min incubation in the presence of the drug because the parental bacteria were treated with antibiotics for 5 min before mixing.

This capacity of rifampicin-treated donors to transfer ColI*drd-1* early in mating was not eliminated if they were incubated in rifampicin for 15 min prior to mating (Fig. 4-2). The experimental protocol was identical to Fig. 4-1b with the exception that donors were incubated in the presence of the drug for an extra 10 min. Conjugal synthesis at 41°C was detected for about 8 min in the presence of rifampicin and at 60 min the number of counts incorporated in the rifampicin-treated mating was 38%. Incubation of the donors in chloramphenicol for 15 min before mating did not alter the pattern of conjugal synthesis when compared to that observed in Fig. 4-1.

The same pattern of results was obtained when the experiment was repeated with bacteria of widely different ancestry. The data in Fig. 4-3 show the effect of rifampicin and chloramphenicol on [^{14}C] thymine incorporation in unirradiated *dnaB* recipients mated with BW51 (ColI*drd-1*) at 43°C. After one hour of mating rifampicin and chloramphenicol had reduced conjugal DNA synthesis in recipients by 53% and 26% respectively. In the presence of rifampicin [^{14}C] thymine incorporation ceased abruptly between 10 and 20 min. Therefore the effect of rifampicin on the transfer of ColI*drd-1* does not appear to be strain dependent.

Conjugal DNA synthesis in *dnaB* bacteria

In contrast to the results described above, Fenwick and Curtiss (1973b) found that transfer of R64*drd-11* from *dnaB43* donors was abolished by rifampicin. Therefore the sensitivity of ColI*drd-1* transfer to rifampicin may be increased if the donors are mutant at *dnaB* rather than *dna*$^+$. To test this possibility, transfer from *dnaB* donors was followed by measuring conjugal DNA synthesis in rifampicin-sensitive, *dnaB70* recipients. This approach was justified by previous studies (Boulnois and Wilkins, 1978; see Chapter 3) which show that conjugal synthesis of ColI*drd-1* in *dnaB* recipients is both an accurate reflection of transfer and is unaffected by rifampicin.

In preliminary experiments designed to measure conjugal DNA synthesis in matings between *dnaB* bacteria, incorporation of [^{14}C] thymine into conjugally synthesised DNA was followed. For reasons which are not clear the amount of radioactivity incorporated was not great enough to afford the desired resolution. Therefore [^{3}H] thymine was used in these experiments because it is available at a higher specific activity than the ^{14}C labelled compound.

Results in Fig. 4-4b show the effects of rifampicin and chloramphenicol on conjugal DNA synthesis in thymine-requiring *dnaB* recipients at 42°C mated with *tdk dnaB* donors of ColI*drd-1*. Donors were treated with the drugs for 5 min before and throughout mating. Incorporation of [^{3}H] thymine in the rifampicin-treated mating was normal for about 10 min when it stopped. Thus transfer of ColI*drd-1* between *dnaB* mutants at 42°C appears to be no more sensitive to rifampicin than between *dna*$^+$ bacteria at 37°C or 41°C (Fig. 4-1b).

Transfer of plasmid DNA is normally associated with the synthesis of replacement strands in the donor. Conjugal synthesis in the donors

can be followed by mating thymine-requiring dnaB70 donors of ColIdrd-1 with dnaB70 tdk recipients at 42°C in the presence of [^{3}H] thymine. The dnaB70 allele in the recipient is inessential in this experiment but serves to make the system as similar as possible to that described in Fig. 4-4b. Incorporation of label into TCA-insoluble material in this mating primarily reflects the synthesis of DNA in donors to replace that transferred (Wilkins and Hollom, 1974).

Rifampicin uncouples transfer of Flac from conjugal DNA synthesis in dnaB70 donors (Kingsman and Willetts, 1978). This raises the possibility that, although substantial transfer of ColIdrd-1 occurs from rifampicin-treated dnaB70 donors (Fig. 4-4b), the bacteria may be unable to synthesise replacement strands. The results in Fig. 4-4a, which show the effects of rifampicin and chloramphenicol on donor conjugal synthesis, do not support this hypothesis. Synthesis in the rifampicin-treated donors occurred for about 10 min and the amount of synthesis at this time matched that detected in the reciprocal experiment (Fig. 4-4b) which provides an estimate of DNA transfer.

The results in Fig. 4-4a which are representative of data obtained in three separate experiments, show that DNA made during mating in the rifampicin-treated donors is unstable, because the number of counts diminished after peak incorporation at about 10 min. In contrast, the DNA made in the rifampicin-treated recipients (Fig. 4-4b) appears to be stable.

3. DISCUSSION

Two models have been proposed for the conjugal transfer of R64drd-11 (Curtiss and Fenwick, 1975) a plasmid closely related to ColI (Lawn et al., 1967; Hedges and Datta, 1973; Falkow et al., 1974). Both models involve a requirement for an RNA primer, made in the donor in response to contact with recipients, but transfer of DNA that was made before mating is independent of de novo protein synthesis. These two features were suggested by the observation that transfer of R64drd-11 DNA that existed before mating was more sensitive to rifampicin than chloramphenicol (Fenwick and Curtiss, 1973b).

In the previous chapter it was demonstrated that when protein synthesis was inhibited at the level of either transcription or translation in the recipient, transfer of ColIdrd-1 from the donor was enhanced. In contrast, results described in this chapter show that when protein synthesis in both the donor and recipient was inhibited by chloramphenicol, not only did amplification of transfer not develop but the amount of transfer was reduced by about 50%. This finding is consistent with the notion that proteins required for transfer are consumed in the donor during the transfer of ColI DNA that was made before mating was initiated. However the significant transfer from rifampicin-treated dna$^+$ donors conflicts with an essential requirement for untranslated RNA in ColIdrd-1 transfer, because RNA synthesis was effectively inhibited at the time of mating by the preincubation in the drug.

The amount of transfer at 60 min from rifampicin-treated donors of ColIdrd-1 almost equalled that detected in the chloramphenicol-treated mating (Table 4-1). However the kinetics of transfer, followed by measuring conjugal synthesis in recipients, were different in rifampicin and chloramphenicol (Fig. 4-1, 4-2, 4-3). Transfer was apparently normal

in the presence of rifampicin for about 10 min when it stopped abruptly. Increasing the preincubation time to 15 min in rifampicin only slightly affected this pattern of results (Fig. 4-2). The capacity of chloramphenicol-treated donors to transmit DNA gradually diminished over a longer period, implying that the effect of rifampicin on transfer does not reflect inhibition of protein synthesis at the level of transcription. Evidence is presented in the following chapter which shows that R144drd-3 also shows this same pattern of transfer in rifampicin. Similar studies were not undertaken with strains carrying R64drd-11 because I have found them to be insufficiently fertile to afford the required resolution in these types of experiments.

A clue to the reason for the different effects of the drugs on transfer may be provided by the observation that rifampicin but not chloramphenicol disrupts the metabolism of bacterial DNA when the cells harbour an IncIα plasmid (Beddoes and Wilkins, in Boulnois et al., 1979). This rather surprising result was obtained in a series of experiments which were designed to examine the rifampicin-sensitivity of DNA synthesis in bacteria mutant at dnaG and carrying ColIdrd-1 (Wilkins, 1975). Beddoes and Wilkins were examining the hypothesis that DNA synthesised in plasmid-suppressed dnaG mutants was mediated by a plasmid-specified enzyme that whilst being analogous to primase in function was sensitive to rifampicin.

It is well documented that addition of rifampicin or chloramphenicol to E.coli prevents initiation of new rounds of DNA replication whilst allowing rounds already in progress to continue towards completion (Lark, 1972; Messer, 1972; Helmstetter, 1974; Pato, 1975; Bremer and Churchward, 1977). This pattern of results was not observed by Beddoes and Wilkins when the bacteria carried a variety of IncIα plasmids

(see Boulnois et al., 1979). At all temperatures used from 33°C to 41°C, DNA replication ceased prematurely in rifampicin-treated dna$^+$ bacteria if they also carried ColIdrd-1. DNA synthesis stopped some 15 min after the addition of rifampicin. In the plasmid-free strain at 37°C, DNA replication ceased after about 70 min, in the presence of rifampicin.

At 41°C, but not 37°C or below, this premature cessation of DNA replication was followed by extensive DNA breakdown. These effects were detected with bacteria harbouring other IncIα plasmids, namely R144drd-3 and R64drd-11, and in strains of E.coli of different ancestry (Beddoes and Wilkins, in Boulnois et al., 1979). These disruptive effects of rifampicin were not detected when IncIα plasmid-containing strains were incubated in the presence of chloramphenicol. In this case the rate of accumulation of DNA gradually diminished over about 70 min (Beddoes and Wilkins, in Boulnois et al., 1979).

Plasmid DNA metabolism during conjugation may also be subject to the same disruptive effects of rifampicin treatment. The coincidence between the abrupt cessation of transfer after about 15 min in rifampicin (Figs. 4-1, 4-2, 4-3) and the sudden inhibition of chromosomal DNA replication, also after about 15 min in the drug points to this conclusion. Furthermore, DNA synthesised in the rifampicin-treated donors at 42°C to replace the transferred strands tends to be degraded (Fig. 4-4a) as is chromosomal DNA in unmated bacteria incubated in the presence of the drug at high temperature.

DNA made during mating in the rifampicin-treated recipients is stable, a conclusion supported by other studies involving rifampicin-resistant donors and described in Chapters 3 and 5. This is not surprising since plasmid-encoded products are presumably required for

promoting DNA breakdown and they will not be synthesised in the newly infected recipients because rifampicin will inhibit transcription of the relevant plasmid genes.

It is proposed therefore that the effect of rifampicin in limiting transfer of IncIα plasmids between drug-sensitive bacteria can be attributed to the disruptive effects of the drug on DNA metabolism in the donor rather than a specific involvement of RNA polymerase in the transfer process. Rejection of a requirement for untranslated RNA in the transfer process allows simpler models for conjugal transfer to be considered and will be discussed in the final chapter.

The data in Fig. 4-4a also bear on the requirements for priming of conjugal DNA synthesis in donors. Synthesis of replacement strands in the dnaB donors occurred with normal efficiency in the early period of mating in the presence of rifampicin (Fig. 4-4a). Therefore donor conjugal DNA synthesis is independent of RNA polymerase and dnaB product as is conjugal synthesis in the recipient (Wilkins and Hollom, 1974; see also Chapter 3). This point will be considered in the general discussion (Chapter 7).

The interactions whereby rifampicin disrupts DNA metabolism in IncIα plasmid-containing bacteria are obscure. At least one plasmid-specified product is required. In addition, the binding of rifampicin to RNA polymerase is also involved. This enzyme is implicated because the disruptive effects of the drug on DNA metabolism were not detected in rifampicin-resistant mutants (Boulnois et al., 1979). Such mutants presumably have an RNA polymerase altered in the β subunit (Rabussay and Zillig, 1969; Heil and Zillig, 1970). Involvement of transfer gene products, which are expressed constitutively by plasmid drd mutants, is unlikely but cannot be excluded (Boulnois et al., 1979). Wild-type

plasmids and their drd mutants promoted premature cessation of DNA replication and DNA breakdown in rifampicin-treated cells but the effects were consistently less pronounced in strains containing wild-type plasmids.

Although a number of observations may be relevant in a consideration of the mechanism of rifampicin-induced disruption of DNA synthesis in bacteria containing IncIα plasmids, no obvious explanation is available. It has been reported (Romero et al., 1971) that when certain R factors, including representatives of the IncIα group, are introduced into some rifampicin-resistant mutants of E.coli, the minimal inhibitory concentration (m.i.c.) of rifampicin is markedly reduced. Maximal reduction in the m.i.c. of the drug was correlated with derepression of the transfer genes, although this was not rigorously tested. The reduction in the m.i.c. of rifampicin was attributed to an increased permeability of the resistant bacteria to the drug rather than an elevated sensitivity of RNA polymerase itself in the R^+ bacteria. Increased permeability was attributed to cell surface alterations induced by the R factor. In agreement with this idea Dowman and Meynell (1970) have reported that drd mutants of IncIα plasmids have pleiotropic effects on the host bacteria, including a change in the bacterial cell wall which renders the bacteria susceptible to lytic agents. Increased permeability to rifampicin may be a contributory factor in promoting the drug-induced disruption of DNA synthesis.

Inhibition of RNA synthesis by rifampicin is known to have adverse effects on the organisation of the bacterial chromosome in plasmid-free strains. The bacterial chromosome is observed as a compact nucleo-protein structure, the nucleoid (Hecht et al., 1975; Stonington and

Pettijohn, 1971). This can be disrupted by the addition of rifampicin in vivo (Pettijohn and Hecht, 1973; Dworsky and Schaecter, 1973). One might envisage that this disruption may be more severe in the presence of an IncIα plasmid, perhaps as a consequence of an increased permeability of the bacteria to the drug.

How disruption of the nucleoid structure of the chromosome in the presence of an IncIα plasmid may impede the progress of replication forks is open to speculation. One possibility is that disruption of the nucleoid structure coupled with a plasmid-induced modification of the bacterial cell envelope may be sufficient to block the progression of replication forks. There is evidence for the association of replicating DNA with the membrane (Smith and Hanawalt, 1967). Alternatively rifampicin-inactivated RNA polymerase may bind efficiently but unproductively to the unfolded chromosome and block the movement of the replication machinery. A similar idea has been suggested by Pato (1975) to explain the slightly slower progress of replication forks in bacteria treated with rifampicin. This model is in principle the same as that proposed to explain the dominance of rifampicin sensitivity over resistance in merodiploids of E.coli. It was envisaged that rifampicin-inactivated RNA polymerase, which can bind to DNA (Hinkle et al., 1972) does so unproductively and retards the movement of drug-resistant RNA polymerase along the template (Austin et al., 1971; Hayward, 1976). The notion that the unfolded nucleoid is more susceptible to this blockade is lent credence by the proposal that regions of DNA within the nucleoid undergoing active transcription are looped out into the cytoplasm (Ryter and Chang, 1973).

An alternative and intriguing model for the rifampicin-induced

disruption of DNA synthesis in bacteria carrying an IncI$_{\alpha}$ plasmid involves the postulate that the plasmid encodes a product that modifies RNA polymerase. This modified RNA polymerase may respond abnormally to rifampicin, perhaps bringing about DNA blockade more efficiently. It has been suggested previously that ColI encodes a product that modifies RNA polymerase (Herman and Moyer, 1975). This proposal arose from studies on the mechanism of ColI-mediated abortive infection of E.coli by bacteriophages T5 and BF23 (Strobel and Nomura, 1966; Nisioka and Ozeki, 1968; Moyer et al., 1972). During infection of E.coli by phage T5, a phage-specified protein is thought to interact with host RNA polymerase (Szabo et al., 1975; Szabo and Moyer, 1975) and perhaps act as a sigma-like subunit to direct phage transcription (Chinnadurin and McCorquodale, 1974). One may envisage that the T5-specified protein that modifies RNA polymerase may not be able to interact with an already modified RNA polymerase in ColI-containing bacteria and thereby result in abortive infection. Such a model predicts rifampicin-induced disruption of DNA metabolism would not be detected in bacteria harbouring a mutant ColI plasmid that allows normal infection by T5. Secondly one would expect that if the ColI genes involved in promoting abortive infection of T5 were cloned into a suitable vector, then this recombinant plasmid would also promote rifampicin-induced disruption of DNA synthesis.

Recently the notion that abortive infection by T5 involves a transcriptional control system has been reexamined. It has been claimed that membrane damage is associated with abortive infection by T5 of bacteria containing ColI (Cheung and Duckworth, 1977). This membrane damage, which causes leakage of metabolites and proteins, was proposed to result in abortive infection. However it was not clear whether

abortive infection was a consequence of membrane damage, or membrane damage was a result of abortive infection. Quite clearly the basis for rifampicin-induced disruption of DNA synthesis in bacteria containing IncIα plasmids requires a more detailed study.

It is equally unclear why DNA in the presence of rifampicin and an IncIα plasmid is unstable at 41°C but not at 37°C or less. Breakdown of DNA in plasmid-containing strains occurs some 40 min after addition of rifampicin. Therefore DNA degradation can be distinguished from the premature cessation of DNA synthesis by time of onset and incubation temperature. Breakdown does not require prior replication of DNA in the presence of rifampicin because it was detected in *dnaB* mutants in which replication was thermally inactivated at the time of addition of the drug (Beddoes and Wilkins, in Boulnois *et al.*, 1979).

Disruption of the nucleoid, induced by rifampicin treatment, may be enhanced when the bacteria are incubated at temperatures above 37°C. The chromosome in this extended state may be a substrate for a plasmid-specified nuclease or a host nuclease activated by the plasmid.

Table 4-1: EFFECT OF RIFAMPICIN AND CHLORAMPHENICOL ON ColIdrd-1 TRANSFER BETWEEN *dna*$^+$ BACTERIA

Temp (°C)	Drug	Transfer [a] cpm/ml	+drug/-drug
37	None	2815	
	CAM	1295	0.46
	RIF	1190	0.42
41	None	2472	
	CAM	1319	0.56
	RIF	1215	0.49

BW85 (ColIdrd-1) was labelled with [^{14}C] thymine (0.5 μCi/μg) before and during mating with BW94. Mating was for 1 h at the appropriate temperature. The antibiotics, chloramphenicol (CAM) and rifampicin (RIF) were added to a final concentration of 200 μg and 100 μg per ml respectively. The amount of TCA-insoluble radioactivity was determined after selective lysis of the donors.

[a] The unmated controls consisting of the parental cultures mixed in the presence of SDS, retained on average 76 cpm/ml following selective lysis of donors. The values for the controls have been subtracted to give the data in column 3.

Figure 4-1: EFFECT OF RIFAMPICIN AND CHLORAMPHENICOL ON CONJUGAL DNA SYNTHESIS IN IRRADIATED RECIPIENTS

Donor strain BW91 (ColIdrd-1) was mated with BW89 in the presence of [^{14}C] thymine (0.42 μCi/μg) at either 37°C (a) or 41°C (b). The volume of the mating mixture was 5.0 ml. TCA-insoluble radioactivity in 0.5 ml samples was determined. The symbols refer to : •, mating with 100 μg rifampicin per ml; △ , mating with 200 μg chloramphenicol per ml; o, mating without drugs; ▲ , control incubation of donors and recipients in the presence of SDS. Similar values for the controls were obtained when rifampicin or chloramphenicol was also present.

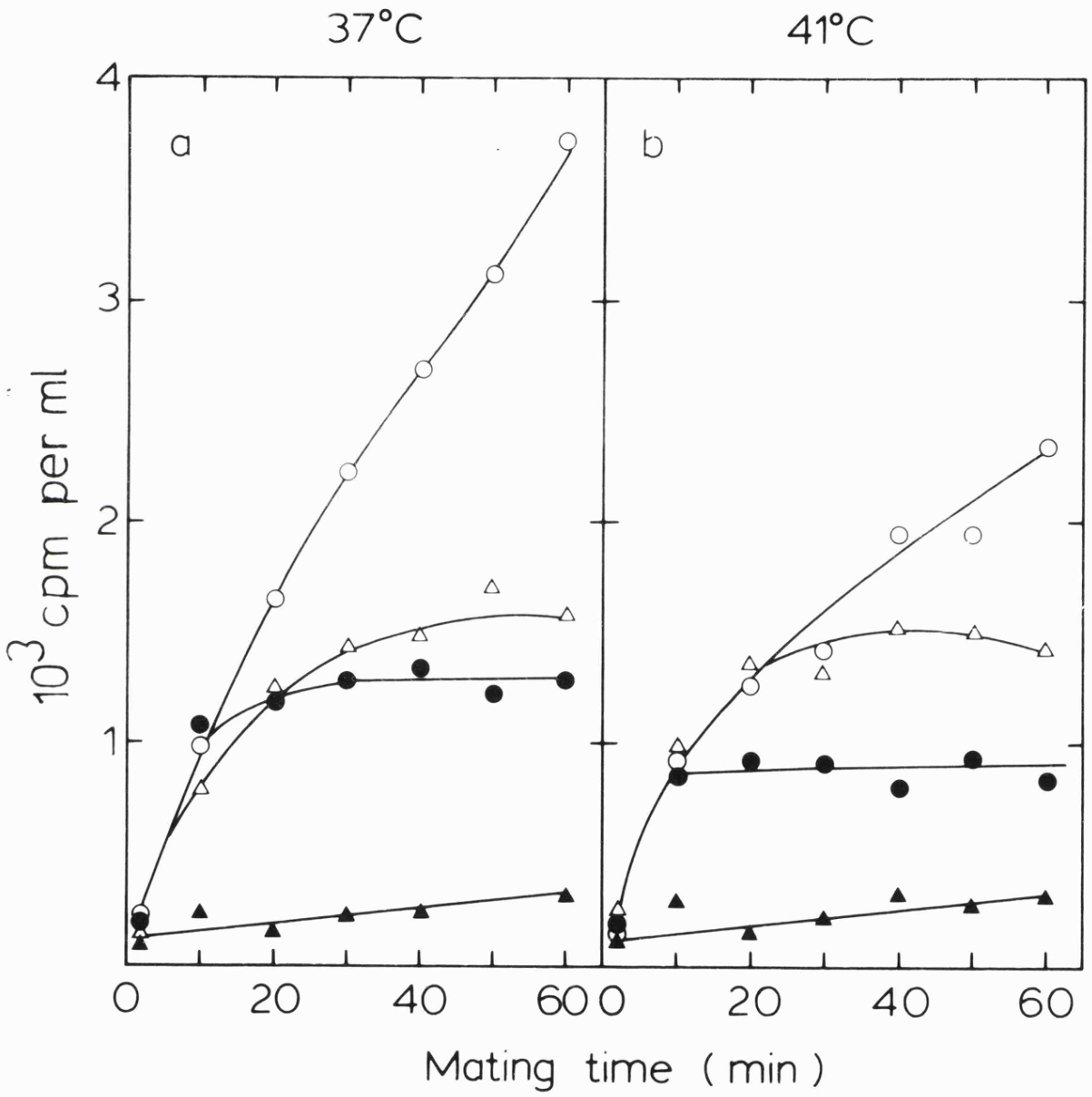

37°C
41°C
a
b
4
3
2
1
0
20
40
60
10^3 cpm per ml
Mating time (min)

Figure 4-2: EFFECT OF A 15 min PRETREATMENT OF PARENTAL BACTERIA WITH RIFAMPICIN OR CHLORAMPHENICOL ON CONJUGAL DNA SYNTHESIS IN IRRADIATED RECIPIENTS

Donor strain BW91 (ColIdrd-1) was mated with UV-irradiated BW89. The procedure was the same as Fig. 4-1 except the parental cultures were incubated in the presence of rifampicin (100 µg/ml) or chloramphenicol (200 µg/ml) for 15 min before mixing. The symbols refer to : ●, mating with rifampicin; △ , mating with chloramphenicol; o, mating without drugs; ▲ , control incubation of donors and recipients in the presence of SDS.

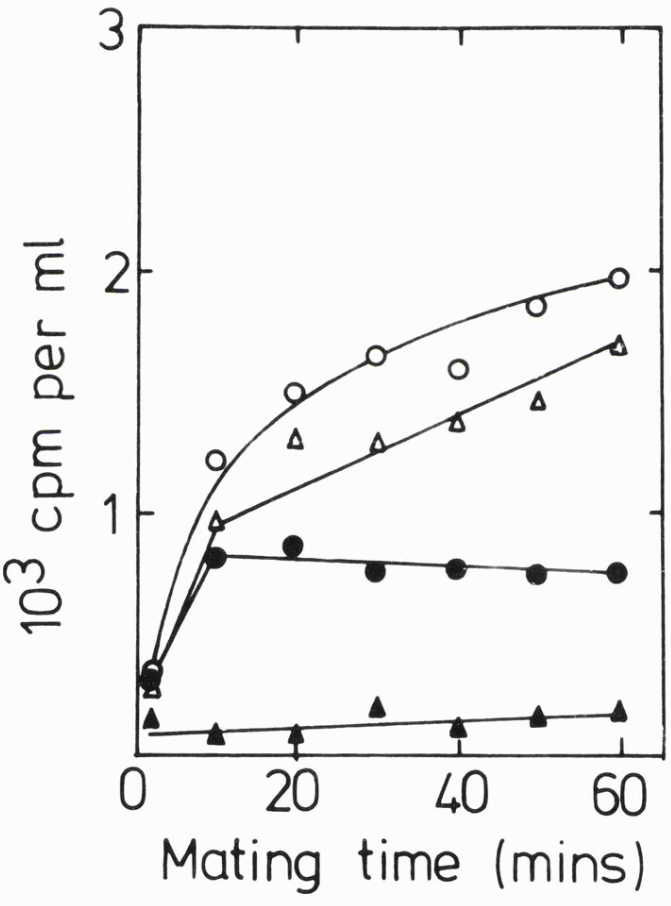

3
2
1
10^3 cpm per ml
0
20
40
60
Mating time (mins)

Figure 4-3: EFFECT OF RIFAMPICIN AND CHLORAMPHENICOL ON CONJUGAL DNA SYNTHESIS IN *dnaB* RECIPIENTS

BW51 (ColI*drd-1*) was mated with BW68 in the presence of [^{14}C] thymine (0.42 μCi/μg) at 43°C. The volume of the mating mixture was 5 ml. Samples (0.5 ml) were taken into TCA and insoluble radioactivity determined. Matings were in the presence of 100 μg rifampicin per ml (●); 200 μg chloramphenicol per ml (△) or untreated (○). The controls (▲) were obtained by incubating the parental bacteria in the presence of SDS.

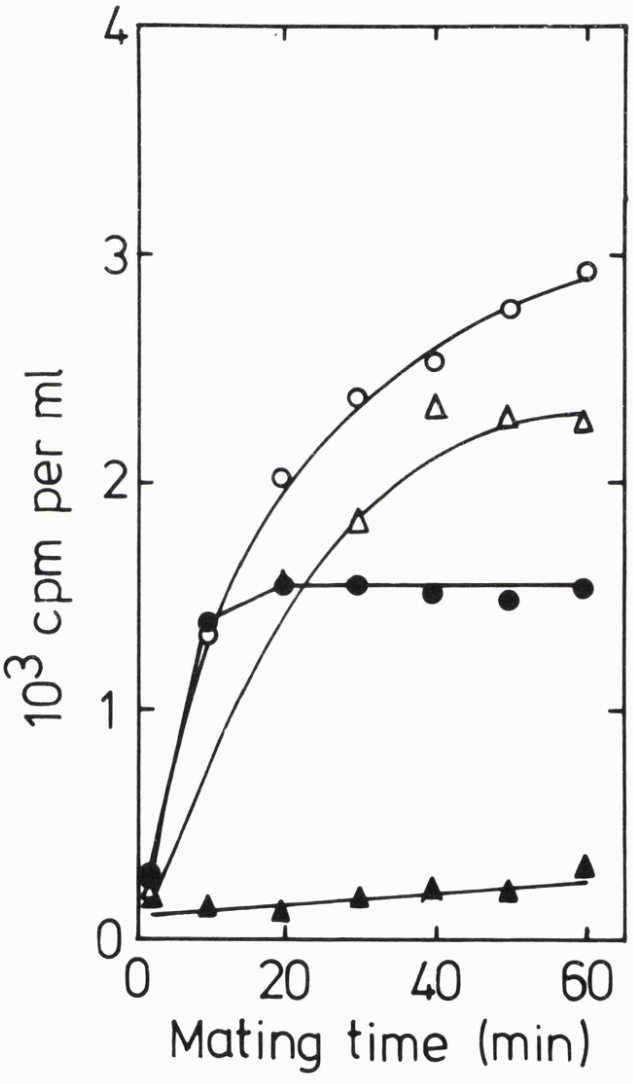
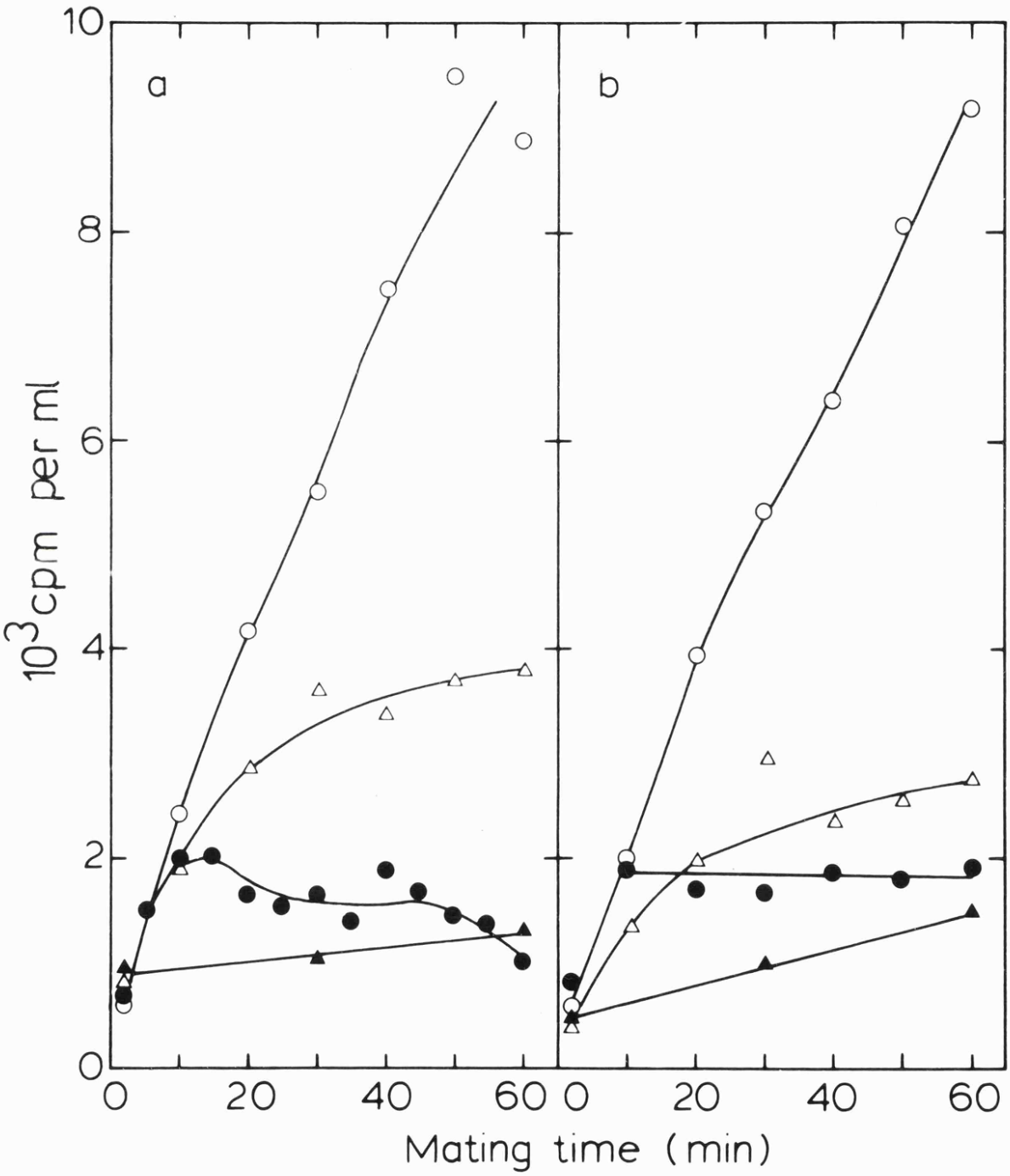

4
3
2
1
0
10^3 cpm per ml
0
20
40
60
Mating time (min)

Figure 4-4: EFFECT OF RIFAMPICIN AND CHLORAMPHENICOL ON CONJUGAL DNA SYNTHESIS IN *dnaB* BACTERIA

(a) Synthesis in donors: BW68 (ColI*drd-1*) was mated with BW67 in the presence of [^{3}H]thymine (20 μCi/μg) at 42°C. The volume of the mating mixture was 5 ml. Samples (0.5 ml) were taken into TCA and insoluble radioactivity determined. Samples from the rifampicin-treated mating were 0.3 ml.

(b) Synthesis in recipients: as in (a) but BW67 (ColI*drd-1*) was mated with BW68. Matings were in the presence of 100 μg rifampicin per ml (•); 200 μg chloramphenicol per ml (Δ); or untreated (o). The controls (▲) were obtained by incubating donors and recipients in the presence of SDS and rifampicin.


a
b
10
8
6
4
2
0
0
20
40
60
10^3cpm per ml
Mating time (min)

CHAPTER 5

A NOVEL PRIMING SYSTEM FOR CONJUGAL SYNTHESIS OF IncIα PLASMIDS IN RECIPIENTS

1. INTRODUCTION

In recipients conjugal synthesis of ColIdrd-1 (and presumably other IncIα plasmids) is mediated by DNA polymerase III (Wilkins and Hollom, 1974), an enzyme that requires a primer terminus to initiate DNA synthesis on a template strand (Kornberg, 1974). The mechanism of primer synthesis during conjugal synthesis of IncIα plasmids is unclear. Primers are not made by primase acting in a multienzyme system involving dnaB product as in phage ØX174 DNA replication, or by RNA polymerase. These two priming systems are ruled out because conjugal synthesis of IncIα plasmids is resistant to rifampicin in recipients defective in dnaB product (Wilkins and Hollom, 1974; see also Chapters 3 and 4). An attractive feature of a model for R64drd-11 transfer proposed by Curtiss and Fenwick (1975) was the priming of conjugal DNA synthesis in recipients by an RNA molecule attached to the 3' terminus of the transferred strand of plasmid DNA. However in the previous chapter I have argued that the results obtained by Fenwick and Curtiss (1973b) upon which the existence of such an RNA molecule was based had been incorrectly interpreted. Therefore there is no reason to suppose that priming of conjugal DNA synthesis occurs in the way postulated by Curtiss and Fenwick (1975).

The possibility remains that conjugal DNA synthesis in recipients involves primase in a reaction similar to that operating during conversion of G4 viral DNA to the duplex form. In this chapter the role of primase

in the conjugal synthesis of IncIα plasmids in recipients is considered. Conjugal DNA synthesis was measured in recipients in which both primase and RNA polymerase were inactive. Recipients simultaneously defective in the activity of these enzymes were exploited because evidence has been presented (Wilkins and Hollom, 1974) that conjugal synthesis of Flac may involve priming by either RNA polymerase or primase. Such recipients were mated with donors deficient in the activities of RNA polymerase and/or primase. RNA polymerase activity was inhibited by treating bacteria with rifampicin and primase was inactivated by exploiting bacteria carrying the thermosensitive dnaG3 mutation and mating at a nonpermissive temperature.

From studies described in previous chapters interpretation of matings involving rifampicin treatment is complicated by two contrasting effects of the drug on plasmid transfer. When the activity of RNA polymerase was inactivated only in the recipients, transfer from donors was enhanced. Amplified transfer results from inhibiting the synthesis of IncIα plasmid-specified proteins in newly infected recipients. This protein(s) normally acts to limit the amount of plasmid DNA transferred (Boulnois and Wilkins, 1978; and Chapter 3). In contrast, treatment of both donors and recipients with rifampicin results in a reduction of plasmid transfer. Plasmid transfer from drug-treated donors occurs only in the initial 10-15 minutes of mating. Rifampicin-treated donors are thought to lose their ability to transmit DNA because of a general disruption of DNA metabolism in these cells (Boulnois et al., 1979; and Chapter 3).

Evidence is given in this chapter that synthesis of DNA complementary to the transferred strands of R144drd-3 occurs when both RNA polymerase and primase are simultaneously inactivated in both donors and recipients.

It is proposed that this conjugal DNA synthesis in the recipient is primed by a plasmid-specified protein supplied by the donor parent.

In this chapter I will also consider the possible role of RNA polymerase and primase in the conjugal synthesis of Flac in recipients. Wilkins and Hollom (1974) have presented data that implicates either RNA polymerase or primase in the priming of conjugal synthesis of Flac (see Chapter 1). Therefore to confirm this interpretation I have measured Flac conjugal synthesis in rifampicin-treated dnaG3 recipients mated with drug-resistant dna^+ donors. The results of these matings, whilst being inconclusive, demonstrate an involvement of recipient primase and RNA polymerase in the conjugal synthesis of Flac.

2. RESULTS

Before use in matings donor and recipient bacteria were routinely incubated at 41°C for 10 min to inactivate primase in dnaG3 bacteria. The kinetics with which the rate of DNA synthesis decreases in dnaG3 bacteria as a result of such treatment is shown in Fig. 5-1a. The rate of DNA synthesis was measured by determining the incorporation of [^{3}H] thymidine into TCA-insoluble material from a 2 min pulse. The rate of DNA synthesis at 33°C, immediately before a shift to 41°C (t=0) was taken as 100%. Following the temperature shift, samples of the culture were pulse labelled starting at the times indicated. After 10 min at 41°C the rate of DNA synthesis in BW86 was reduced to about 1.5%. Continued incubation at 41°C did not significantly reduce this residual DNA synthesis. Synthesis of a similar small amount of DNA at high temperature is a property of the original dnaG3 mutant, PC3 (Carl, 1970). However the immediate ancestor of the dnaG3 strains used here is unable to support primase-dependent bacterial and phage DNA synthesis at 40°C (Lark, 1972; McFadden and Denhardt, 1974). Thus if primase is required for conjugal DNA synthesis, the latter should be markedly diminished or undetectable in the mutant dnaG strains under the conditions used here.

When matings involved rifampicin treatment, the drug was added to the bacteria at 41°C for 5 min before mating. The effect of this treatment on the rate of RNA synthesis in drug-sensitive bacteria was measured by following the incorporation of [^{3}H] Uridine into TCA-precipitable material from a 2 min pulse. The incorporation immediately before addition of the drug (t=0) was taken as 100%. A 5 min treatment of BW86 with rifampicin was sufficient to reduce the rate of RNA synthesis to 1% (Fig. 5-1b). Continued incubation in the drug rendered RNA synthesis undetectable by seven minutes.

Conjugal synthesis of IncIα plasmids in recipients

The experimental protocol for measuring conjugal DNA synthesis in recipients was the same as that described before. Donors mutant at tdk were mated with UV-irradiated recipients. Irradiation is essential for the detection of conjugal DNA synthesis in dna$^+$ and dnaG3 bacteria. UV-irradiation prevents replication of the DNA in dna$^+$ bacteria and reduces the residual incorporation of radioactive thymine observed in dnaG3 bacteria at 41°C (Fig. 5-1a). Irradiation also prevents the considerable amount of chromosomal DNA synthesis detected in dnaG3 bacteria at 41°C as a consequence of inheriting IncIα plasmids. This DNA synthesis occurs because these plasmids encode a product that suppresses the phenotype of dnaG mutations (Wilkins, 1975).

The results in Fig. 5-2a show the effect of rifampicin on the conjugal synthesis of R144drd-3 in drug-sensitive, dna$^+$ recipient bacteria at 41°C. These recipients were mated with donors that were also dna$^+$ but drug resistant. Rifampicin-resistant donors were used to prevent the disruption of transfer that results from the rifampicin treatment of sensitive donors of IncIα plasmids (Chapter 4). Rifampicin treatment of the mating induced a 2.7 fold increase in $[^{14}C]$ thymine-incorporation. This finding is in agreement with previous studies involving drug-resistant donors and sensitive recipients and presumably reflects enhanced transfer of plasmid DNA from the donor (Chapter 3).

To relate $[^{14}C]$ thymine incorporation to the number of plasmid strands synthesised in the average recipient, plasmid transfer from the untreated donors was measured genetically in an interrupted mating (values above Fig. 5-2a). After 10 min of mating, every recipient had inherited at least one copy of the plasmid and 1,200 counts per min per ml had been incorporated. Assuming that this value reflects the synthesis of one

complementary strand in each recipient, it is concluded that the average recipient in the rifampicin-treated mating made almost 10 single-stranded equivalents of the plasmid by 60 min.

To determine whether this conjugal synthesis of plasmid DNA in rifampicin-treated *dna*$^+$ recipients requires primase of the recipient, the experiment in Fig. 5-2a was repeated using a recipient mutant at *dnaG*. The kinetics of conjugal DNA synthesis in such a recipient is shown in Fig. 5-2b. The extent and pattern of [^{14}C] thymine-incorporation in the presence and absence of rifampicin matches that observed in the control mating involving *dna*$^+$ recipients (Fig. 5-2a). Similar results were obtained in matings involving ColI*drd-1*. [^{14}C] thymine-incorporation in both *dna*$^+$ and *dnaG3* recipients (Fig. 5-2a,b) was not altered by pretreatment of the bacteria at 41°C for 15 min. Moreover this incorporation was unaffected by pretreatment and mating of the bacteria at 43°C. The substantial amount of conjugal DNA synthesis in rifampicin-treated *dnaG3* cells therefore implies that a recipient can synthesise complements to several transferred strands of an IncIα plasmid even when the resident primase and RNA polymerase are simultaneously inactivated.

Since the experiments described above involve donors in which RNA polymerase and primase were functional it is possible that conjugal DNA synthesis in recipients involves one of these enzymes derived from the donor. To test the hypothesis that donor primase primes conjugal synthesis of R144*drd-3* in recipient donors that were mutant at *dnaG* and resistant to rifampicin were employed. These donors were mated at 41°C with drug-sensitive *dnaG3* recipients. The effect of rifampicin on the conjugal synthesis of R144*drd-3* in such a mating is shown in Fig. 5-2c. In the absence of rifampicin almost the same levels of [^{14}C] thymine were incorporated as in matings involving *dna*$^+$ donors. This result suggests

that donor primase is not involved in conjugal DNA synthesis in recipients. In the presence of rifampicin this conjugal DNA synthesis was enhanced at 60 min by only 40% in contrast to the 2.7 fold stimulation observed when *dna*$^+$ donors were used (Fig. 5-2a,b). There are two possible explanations for this slight deficiency. Firstly conjugal DNA synthesis in recipient bacteria may involve donor primase to a small extent or secondly, transfer from the primase-deficient donors may be reduced. Conjugal DNA synthesis in UV-irradiated recipients has proved a reliable estimate of the amount of DNA transferred from the donor (Chapter 4). Thus the deficiency of conjugal DNA synthesis detected in matings involving donors mutant at *dnaG* probably reflects a reduction in transfer. If such an interpretation is correct then restoration of primase activity in recipients by exploiting *dna*$^+$ bacteria should give the same pattern of conjugal DNA synthesis as that observed when *dnaG3* recipients were used. The effect of rifampicin on the conjugal synthesis of R144*drd-3* in a *dna*$^+$ recipient mated with a rifampicin-resistant, *dnaG3* donor of R144 *drd-3* is shown in Fig. 5-2d. The similarity of the results in Figs. 5-2c and 2d support the notion that *dnaG3* donors of R144*drd-3* are deficient in transfer. A similar result was obtained in matings involving ColI*drd-1*.

Although *dnaG3* donors were deficient in transmitting the plasmid when the recipients were treated with rifampicin, the donors transfer R144*drd-3* with almost normal efficiency under conditions which allow the phenomenon of transfer limitation to be expressed in newly formed transconjugants. This conclusion is indicated by the similarity between the pattern with which recipients inherit the plasmid from *dnaG3* donors in an untreated mating (see % Cib$^+$ data, Fig. 5-2d) and that observed when rifampicin resistant *dna*$^+$ donors were used (% Cib$^+$ data, Fig. 5-2a).

From the % Cib$^+$ data (Fig. 5-2d) it is estimated that the average rifampicin-treated *dna*$^+$ recipient received about 5 single-stranded equivalents o:

the plasmid following 60 min of mating with the dnaG3 donor strain. Because the amount of conjugal DNA synthesis was identical when dnaG3 recipients were used (Fig. 5-2c) it is concluded that this synthesis of R144 drd-3 in recipients does not involve primase of the donor or recipient.

The matings described above involved donors that, by virtue of a mutation at rpoB, retained active RNA polymerase in the presence of rifampicin. It is possible that conjugal DNA synthesis in recipient bacteria requires this enzyme activity of the donors. To exclude this possibility conjugal DNA synthesis was measured in rifampicin-sensitive dnaG3 recipients mated with drug-sensitive dnaG3 donors of R144drd-3 (Fig. 5-3a). Extensive [^{14}C] thymine incorporation occurred in the untreated mating confirming the conclusion that conjugal DNA synthesis is independent of donor and recipient primase. Interpretation of the results of the rifampicin-treated mating is complicated by two contrasting effects of the drug on plasmid transfer. The initial stimulation of thymine-incorporation presumably reflects the amplification of transfer that occurs when the expression of plasmid genes is inhibited in the newly infected recipients (Chapter 3). The abrupt cessation of thymine-incorporation after 10-20 min of mating presumably results from the rifampicin-treated donors losing their ability to transmit the plasmid. Such transfer inhibition was detected with other donors of IncIα plasmids and coincides with a general disruption of DNA metabolism in the donor cells (Chapter 4).

Despite the limitation on transfer imposed by treating donors with rifampicin, about 1,750 counts per min per ml were incorporated by 30 min when both parental strains were deficient in primase and RNA polymerase (Fig. 5-3a). This is in excess of the amount required for the synthesis of one single-stranded equivalent of the plasmid in each

recipient. This can be estimated to be about 2,100 counts per min per ml from the amount of thymine incorporated when the recipients had inherited the plasmid in the comparable mating involving the same donors but *dna*$^+$ recipients (Fig. 5-3b).

Conjugal synthesis of F*lac* in recipients

In this section the role of RNA polymerase and primase in the conjugal synthesis of F*lac* in recipients is considered. This was examined by measuring conjugal DNA synthesis in rifampicin-treated, *dnaG3* recipients irradiated with UV-light and mated with drug-resistant *dna*$^+$ donors of the plasmid. The donor strain used in these experiments was BW72 (JCFLO). Strains descended from BW82, and used in the previous section, were not exploited because, for reasons that are not understood, they proved to be poor donors of JCFLO.

The results in Fig. 5-4a, which are representative of two experiments, show the effect of rifampicin on conjugal synthesis of F*lac* in drug-sensitive *dna*$^+$ recipients at 41°C in a mating with BW72 (JCFLO). After 60 mins of mating, 25% more [^{14}C] thymine had been incorporated in the rifampicin-treated mating than in the untreated mating. This small increment of conjugal DNA synthesis is consistent with previous results (Wilkins and Hollom, 1974; and Chapter 3). The pattern of conjugal synthesis was markedly altered when the mating was repeated using *dnaG3* recipients (Fig. 5-4b). In the untreated mating at 41°C [^{14}C] thymine-incorporation was reduced by 26% compared to the mating involving *dna*$^+$ recipients (Fig. 5-4a). Addition of rifampicin to the mating reduced [^{14}C] thymine-incorporation even further. Thymine incorporation was 53% of that detected in a rifampicin-treated *dna*$^+$ recipient (Fig. 5-4a). These data are representative of three separate experiments.

3. DISCUSSION

In this chapter I have described experiments which were designed to test a possible involvement of primase and RNA polymerase in the conjugal synthesis of R144*drd-3* in recipients. The results in Fig. 5-2b clearly suggest that this DNA synthesis is independent of the two defined bacterial priming systems of the recipient. Rifampicin treatment of the *dnaG3* recipients enhanced conjugal DNA synthesis, presumably by amplifying transfer from the drug-resistant donors (see Chapter 3) and the amount of synthesis was normal when compared with the control mating involving *dna*$^+$ recipients (Fig. 5-2a).

The possibility that the defined bacterial priming proteins of the donor are responsible for initiating conjugal DNA synthesis in the recipient is also ruled out. When both parents were mutant at *dnaG* and treated with rifampicin, the average recipient synthesised at least one single-stranded equivalent of the plasmid within the limited period that the drug-sensitive donors retained the ability to transmit DNA (Fig. 5-2a). The amount of conjugal DNA synthesis in this mating was not deficient when compared with results obtained with rifampicin-treated *dna*$^+$ strains (see Fig. 4-1, Chapter 4).

The capacity of recipients to support conjugal DNA synthesis in the absence of the defined bacterial priming systems implies the existence of a novel priming process. The obvious candidate is the product of the gene in IncIα plasmids that suppresses the temperature sensitivity of vegetative DNA replication in *dnaG* mutants (Wilkins, 1975). The determinant has been designated *sog* (suppression of *dnaG*) (Boulnois and Wilkins, 1979). It has been tentatively classified as a transfer gene because its expression is controlled co-ordinately with other genes required for conjugation (Wilkins, 1975; Sasakawa and Yoshikawa, 1978).

It is suggested that sog product primes conjugal DNA synthesis in recipients.

Transcription of sog or other plasmid genes in the newly infected recipients is not a prerequisite for synthesis of DNA complementary to the transferred strands because this synthesis is not eliminated by rifampicin. Therefore if sog product promotes conjugal DNA synthesis in the recipient, it must be supplied by the donor. Primers may be made in the donor and transmitted with the transferred DNA or the protein itself may be transferred between mating cells to act in the recipient. Transfer of replication proteins has not been detected in the past. DNA polymerase III was not transferred from donors to rectify the deficiency of conjugal DNA synthesis in dnaE recipients (Wilkins and Hollom, 1974). Furthermore neither RNA polymerase nor dnaB product was transferred during Flac mediated matings to restore conjugal DNA synthesis in rifampicin-treated dnaB recipients (Wilkins and Hollom, 1974). It might be expected that sog product would be efficiently transferred if it can recognise initiation sites in the strand destined for transfer.

It has recently been shown that bacteria harbouring various IncIα plasmids contain an enzyme that can substitute in vitro for primase and RNA polymerase in the priming reaction for DNA polymerase III on the viral DNA of phages fd, G4 and ØX174 (Lanka et al., 1979). This enzyme was found to synthesise oligoribonucleotides on a template in a reaction that is rifampicin-resistant and independent of dnaB and dnaC products. It has been termed a DNA primase and is a highly anisometric protein with a molecular weight of 140,000. It may be sog product. The highly anisometric nature of this protein may suggest that it is transferred to the recipient during mating.

It is not possible to exclude other possibilities for priming conjugal DNA synthesis in recipients. The transferred strand may be able to form a hairpin-like structure at its 3' terminus to allow initiation of DNA synthesis (Fig. 3-5a) or alternatively the recipient may encode a novel rifampicin-resistant RNA polymerase. Such an activity has been detected in cytoplasmic membranes and DNA-protein complexes isolated from E.coli (Ohashi and Tsugita, 1976). However this activity, which is distinct from dnaG product, was not shown to function as a primase. Confirmation and further analysis of the proposed role of sog product in priming conjugal DNA synthesis in recipients awaits the isolation of a mutant defective in this gene product.

The mechanism of priming of conjugal synthesis IncI plasmids is clearly different from that which initiates conjugal synthesis of Flac. Synthesis of DNA complementary to the transferred strands of Flac was found to be sensitive to rifampicin in dnaB recipients but resistant in dna$^+$ bacteria (Wilkins and Hollom, 1974). I have suggested (Chapter 1) that this demonstrates that in dna$^+$ bacteria, conjugal synthesis of Flac is primed by a system analogous to that operating during ØX174 RF1 formation and involving primase and dnaB product. When this system is inoperative as a result of thermal inactivation of dnaB product, conjugal DNA synthesis is initiated by RNA polymerase. This interpretation predicts that conjugal synthesis of Flac should be sensitive to rifampicin in a recipient deficient in primase.

The results in Fig. 5-4b support this suggestion but indicate that the system is more complex. When primase was inactivated in recipients conjugal synthesis of Flac at 60 min was reduced by 26% (Fig. 5-4b), whilst addition of rifampicin reduced synthesis by 47% compared to the same mating involving recipient bacteria in which primase was active

(Fig. 5-4a). Quite clearly primase and RNA polymerase of the recipient play a role in the conjugal synthesis of F*lac*.

The nature of the residual conjugal DNA synthesis in rifampicin-treated *dnaG3* recipients is unclear. It is unlikely that it is initiated by the residual primase activity in the recipients because related *dnaG3* strains appear to be wholly defective in supporting primase-dependent DNA replication at the lower temperature of 40°C (Lark, 1972; McFadden and Denhardt, 1974). The synthesis may reflect some priming of conjugal synthesis of F*lac* in recipients by primase derived from the donor. If this explanation is correct the finding that conjugal synthesis of F*lac* is abolished by rifampicin-treatment of *dnaB* mutants (Wilkins and Hollom, 1974) must be explained. One hypothesis is that primase is transferred from the donor to the recipient where it requires the activity of recipient *dnaB* product. To accommodate the data of Wilkins and Hollom (1974) it must be proposed that donor *dnaB* product cannot be transferred to the recipient. This hypothesis was not tested because derivatives of the *dnaG3* strain NY73 donate F*lac* poorly at 33°C and 40°C to *dna*$^+$ strains as measured by transconjugant formation (data not given).

Figure 5-1: (a) RATE OF DNA SYNTHESIS IN A dnaG3 MUTANT FOLLOWING A SHIFT TO THE NON-PERMISSIVE TEMPERATURE

At zero time a 0.5 ml sample of a culture of BW86 growing at 33°C was taken and labelled with [^{3}H] thymidine (20 µCi/µg) for 2 min at 33°C. Incorporation of radioactivity into TCA insoluble material was determined. The remaining culture was shifted to 41°C immediately following removal of the zero time sample. Samples (0.5 ml) were taken at the indicated times and labelled as before. Incorporation of radioactivity into TCA-insoluble material in these samples was expressed as a percentage of the incorporation in the zero time sample. This sample contained 63,643 cpm/ml.

(b) RATE OF RNA SYNTHESIS IN A dnaG3 MUTANT AT 41°C FOLLOWING THE ADDITION OF RIFAMPICIN

A culture of BW86 growing at 33°C was placed at 41°C for 5 min at which time rifampicin was added to 100 µg/ml. A 0.5 ml sample was taken immediately prior to the addition of the drug and labelled with [^{3}H] Uridine (1.3 µCi/µg) at 41°C for 2 min in the absence of rifampicin (time zero sample). Samples were removed from the rifampicin-treated culture at the indicated times and labelled as above but in the presence of the drug. Incorporation in these samples was expressed as a percentage of the incorporation in the zero time sample. This sample contained 26,672 cpm/ml.

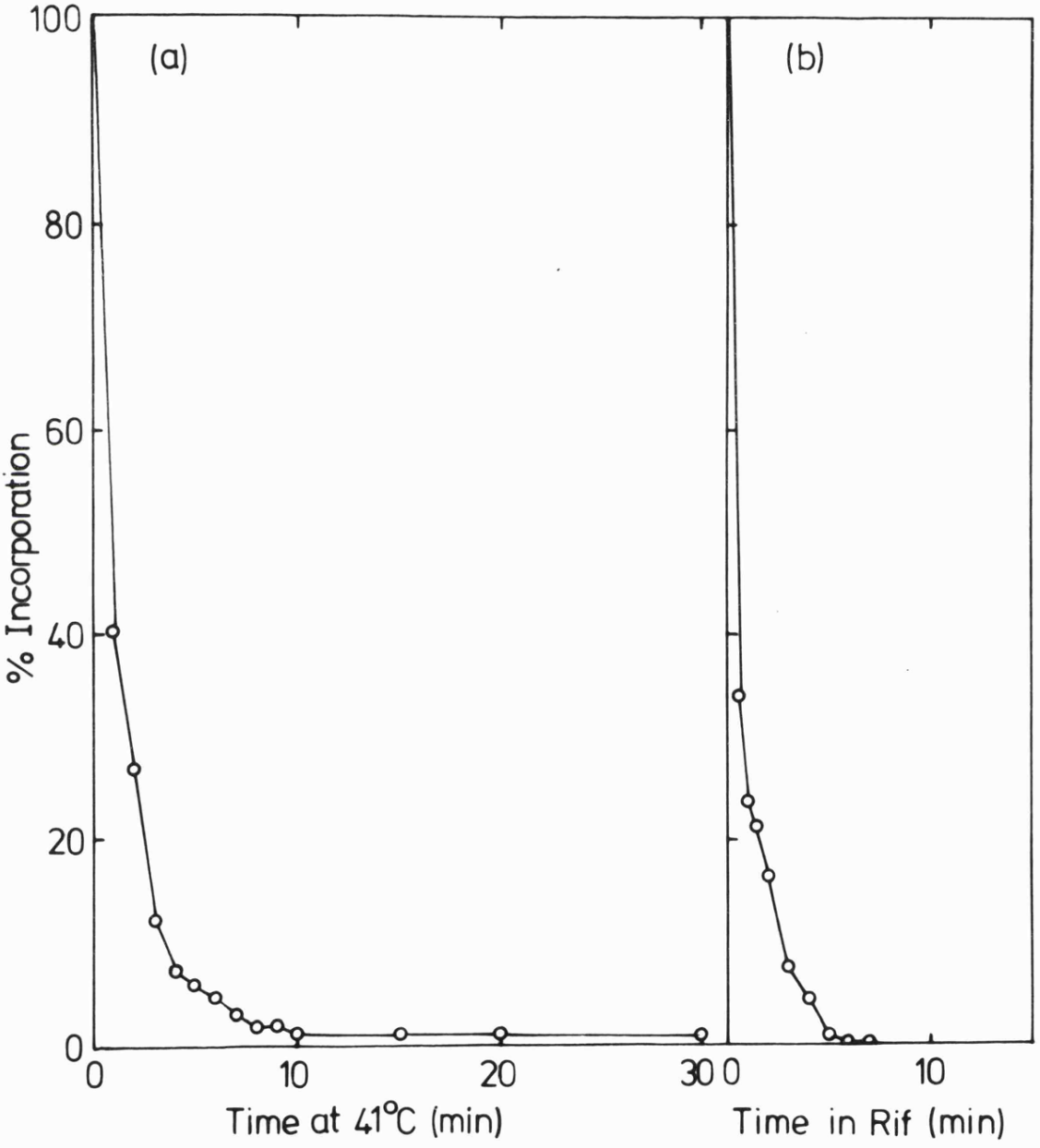

100
80
60
40
20
0
% Incorporation
(a)
(b)
0
10
20
30
0
10
Time at 41°C (min)
Time in Rif (min)

Figure 5-2: CONJUGAL DNA SYNTHESIS IN RIFAMPICIN-TREATED *dna*$^+$ AND *dnaG3* RECIPIENTS MATED WITH DRUG-RESISTANT DONORS OF R144*drd-3*

Donors and recipients were mated in the presence of [^{14}C] thymine (0.5 μCi/μg) at 41°C. The volume of the mating mixture was 4.7 ml. Samples (0.5 ml) were removed into TCA and precipitated radioactivity determined. BW96 (R144*drd-3*) was mated with BW89 (a) and BW86 (b). BW95 (R144*drd-3*) was mated with BW86 (c) and BW89 (d). Matings were performed in the presence of 100 μg rifampicin per ml (•) or untreated (o). The controls (▲) were obtained by mixing donors and recipients in the presence of SDS. % transconjugants indicates the percentage of recipient cells converted to plasmid transconjugants at the indicated times in the untreated matings.

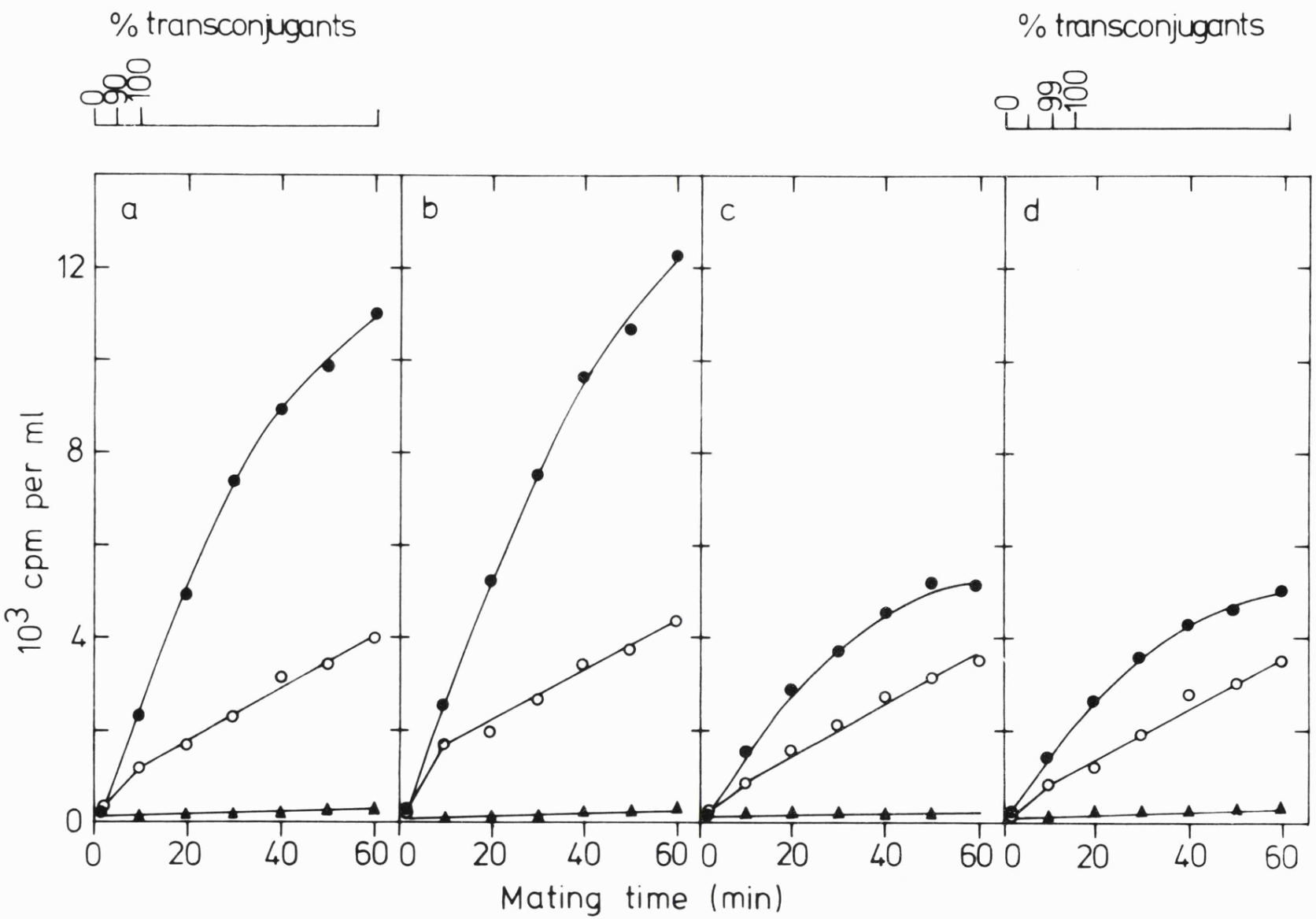

% transconjugants
0
90
100
% transconjugants
0
99
100
a
b
c
d
12
8
4
0
10^3 cpm per ml
0
20
40
60
Mating time (min)

Figure 5-3: CONJUGAL DNA SYNTHESIS IN RIFAMPICIN-TREATED *dnaG3* AND *dna*$^+$ RECIPIENTS MATED WITH DRUG-SENSITIVE *dnaG3* DONORS OF R144*drd-3*

BW82 (R144*drd-3*) was mated with BW86 (a) and BW89 (b) in the presence of [^{14}C] thymine (0.5 μCi/μg) and at 41°C. The volume of the mating mixture was 4.7 ml. Samples (0.5 ml) were taken into TCA and insoluble radioactivity determined. [^{14}C] thymine incorporation was measured in the presence (•) or absence (o) of rifampicin (100 μg/ml). Unmated controls (▲) consisted of parental bacteria mixed in the presence of SDS. % transconjugants indicates the percentage of recipient cells converted to plasmid transconjugants at the indicated times in the untreated matings.

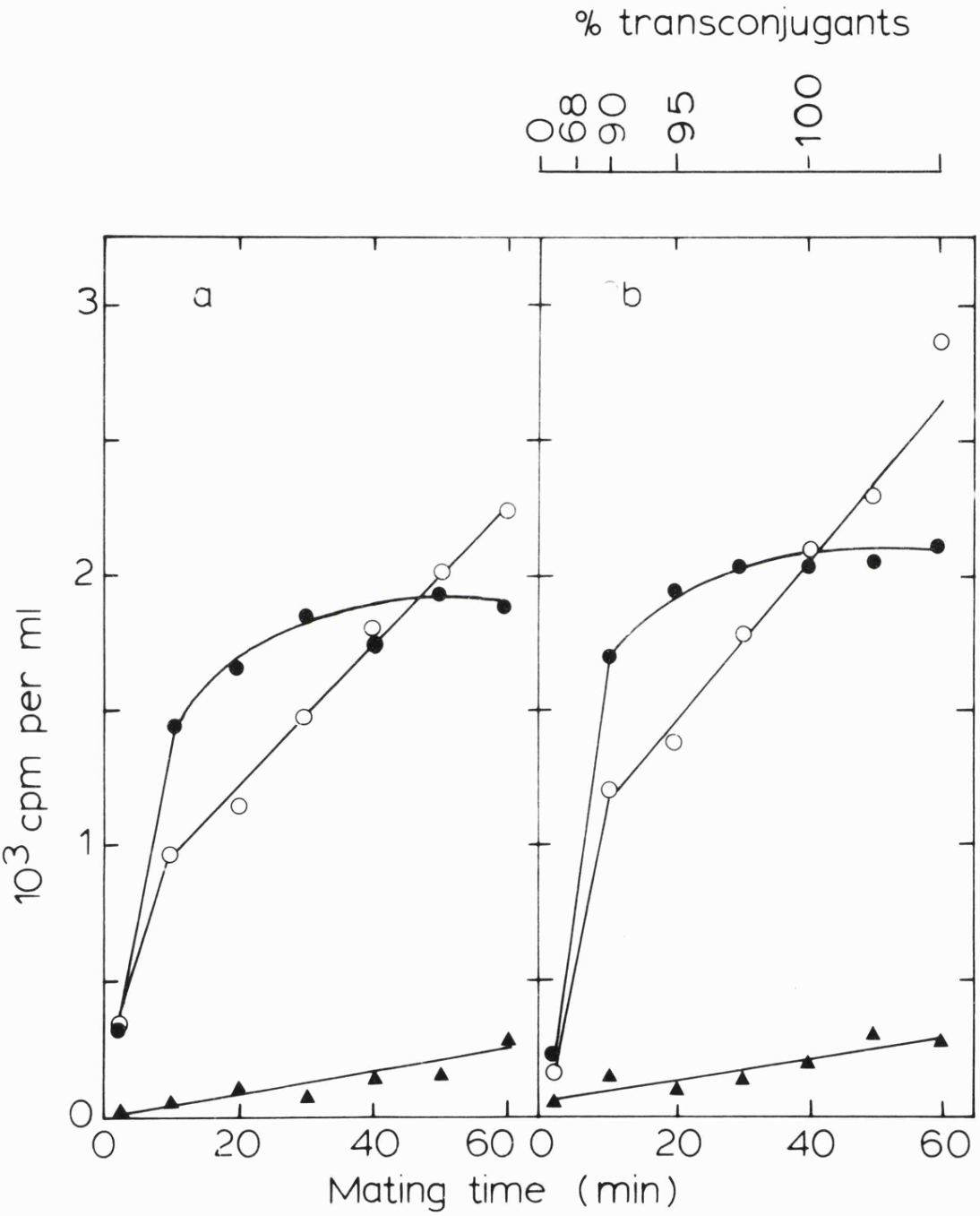
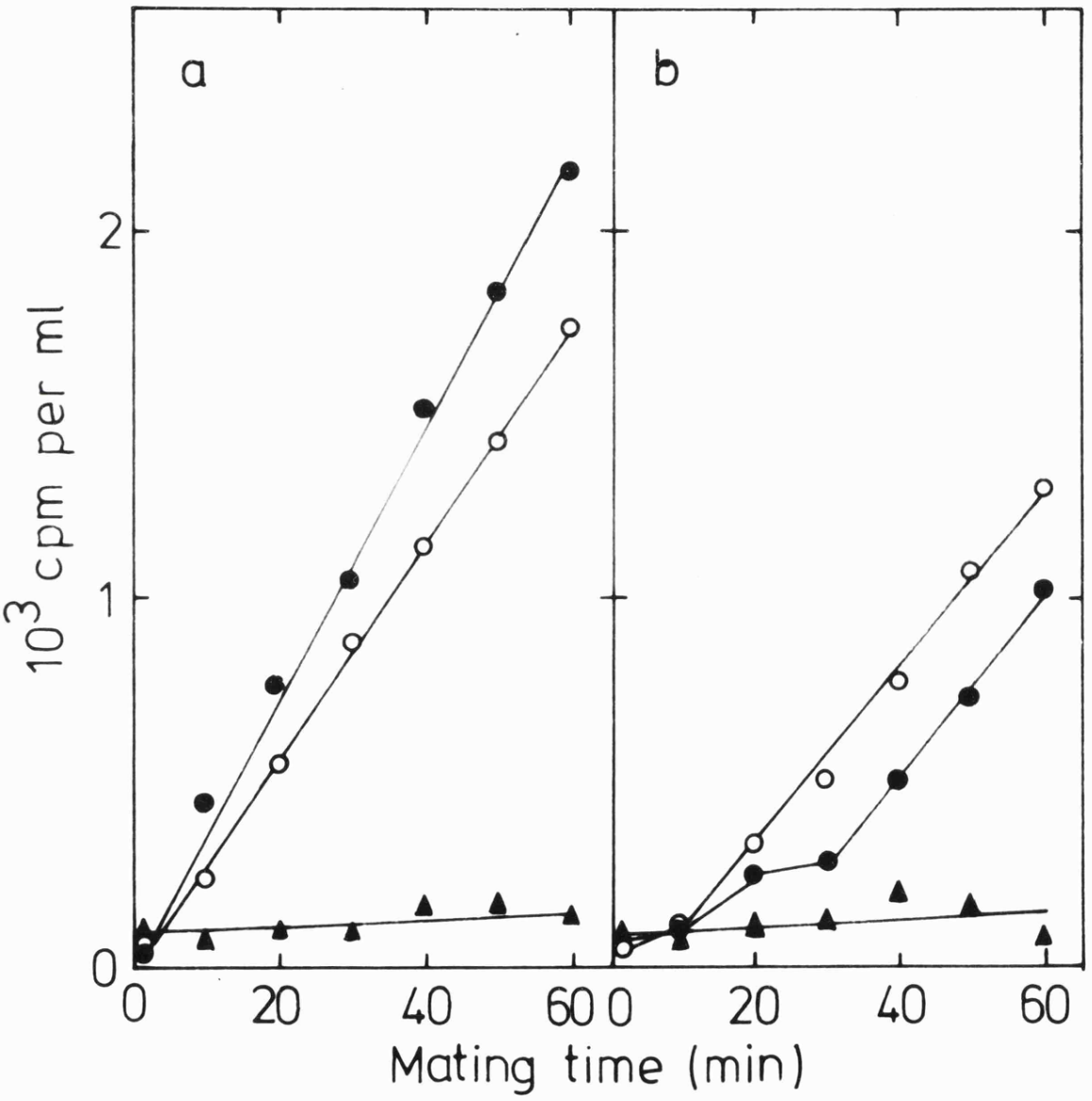

% transconjugants
0
68
90
95
100
a
b
3
2
1
0
10^3 cpm per ml
0
20
40
60
Mating time (min)

Figure 5-4: CONJUGAL DNA SYNTHESIS IN RIFAMPICIN-TREATED *dna*$^+$ (a) AND *dnaG3* (b) RECIPIENTS MATED WITH DRUG-RESISTANT DONORS OF F*lac*

BW72 (JCFLO) was mated with BW89 (a) and BW86 (b) in the presence of $[^{14}C]$ thymine (0.5 μCi/μg) at 41°C. The volume of the mating mixture was 4.7 ml. Samples (0.5 ml) were taken into TCA and precipitated radioactivity determined. Matings were in the presence of 100 μg rifampicin per ml (•) or untreated (o). The controls (▲) involved mixing of parental bacteria in the presence of SDS.


a
b
2
1
0
10^3 cpm per ml
0
20
40
60
0
20
40
60
Mating time (min)

CHAPTER 6

BEGINNING A GENETIC ANALYSIS OF IncIα PLASMIDS

1. INTRODUCTION

Studies on the molecular biology of F plasmid transfer have been greatly facilitated by a knowledge of the genetic organisation of the plasmid. IncIα plasmid transfer has been studied in considerable detail but nothing is known about the genetic organisation of these plasmids. IncIα plasmid-specified products have been implicated in the process of transfer limitation (Boulnois and Wilkins, 1978; Chapter 3), rifampicin-induced disruption of conjugal and vegetative DNA synthesis (Boulnois et al., 1979; Chapter 4) and the priming reaction for conjugal DNA synthesis in recipients (Boulnois and Wilkins, 1979; Chapter 5). Confirmatory and more detailed studies of these processes will require the availability of appropriate mutants of IncIα plasmids and some understanding of the functional organisation of this class of plasmids. Therefore, it seems an appropriate time to initiate a study of the genetics of IncIα plasmids.

A number of different approaches may be employed to analyse plasmid structure and function. Mutagenesis of plasmid DNA *in vivo* by treatment with ethyl methyl sulphonate (EMS) or nitrosoguanidine (NG) has been particularly successful in the analysis of F-mediated conjugation (Achtman et al., 1971; reviewed by Willetts, 1977a; Achtman and Skurray, 1977). However this approach is time consuming, requiring the extensive screening of clones surviving mutagenesis. In terms of total cellular DNA the target site for mutagenesis is small, an objection that may be

circumvented by performing the mutagenesis of purified plasmid DNA *in vitro* using hydroxylamine (Humphreys *et al.*, 1976).

Alternatively isolation of mutant plasmids by insertion of a transposon into the plasmid molecule has been particularly successful in the study of the IncP group plasmid, RP4 (Barth and Grinter, 1977; Barth *et al.*, 1978). Selection can be made for insertion of the transposon providing a rapid means of isolating mutants. Insertion of a transposon within a gene completely inactivates expression of that gene and if this gene is part of a larger transcriptional unit (or operon) it may also prevent expression of genes distal to the point of insertion (Kleckner, 1977). Such polar mutants preclude the use of transposons as agents for the generation of point mutants. However some transposons, notably Tn 1, have been described that only exert polar effects when they are integrated in one orientation with respect to the direction of transcription of the operon (Reubens *et al.*, 1976). A variety of genetic rearrangements which includes deletion formation are associated with the presence of a transposon which may be useful in genetic analysis (Kleckner, 1977; Nevers and Saedler, 1977). Such an approach has been used in the mapping of the transfer genes of R100*drd-1* (Foster and Willetts, 1976, 1977; Kehoe and Foster, 1977).

Another potentially invaluable approach to the study of plasmid structure and function is the use of DNA cloning (Timmis *et al.*, 1978c). If partial and complete restriction endonuclease digestion products are cloned into a suitable plasmid vector the position of each restriction endonuclease fragment produced may be mapped relative to other fragments. Phenotypic markers may then be assigned to particular fragments allowing the formation of a functional map of the plasmid. Each separately cloned fragment may then be used in a multitude of different ways to explore

almost any facet of plasmid biology. Such an approach has proved invaluable in studies of plasmid F (Clark et al., 1976; Skurray et al., 1976; Achtman and Skurray, 1977) and R6-5 (Achtman et al., 1978a; Timmis et al., 1978a,b).

It was originally proposed to develop each of these different techniques to study the genetic organisation of IncIα plasmids. This type of study is obviously a long term exercise and in this chapter I will describe the very beginning of the analysis. Consequently most of the data are incomplete and require extensive confirmatory work.

2. RESULTS AND DISCUSSION

Mutagenesis *in vivo* using EMS

The generation of point mutations in the IncIα plasmid, R144drd-3, was attempted by treating bacteria carrying the plasmid with EMS. A number of protocols are available for EMS-induced mutagenesis and the efficiency with which mutations are generated using each of the protocols was monitored by reversion studies of the leu6 mutation in GB10.

Method 1 A culture of GB10 (R144drd-3) was treated with EMS according to the protocol of Achtman *et al.* (1971) and involved EMS treatment of bacteria in phosphate buffer for 20 min at 37°C.

Method 2 Mutagenesis was performed in SGC medium lacking glucose as a carbon source for 30 min at 37°C.

Method 3 Mutagenesis was performed according to Miller (1972) in SGC minus glucose but containing 0.2M Tris pH 7.5 for 2 h.

The mutagenised cultures were diluted 10 fold in Luria broth and small volumes distributed in several tubes and incubated overnight at 37°C. Aliquots were plated on minimal medium selecting for leu^+ revertants. Methods 1 and 2 enhanced reversion to leu^+ by 10 and 5 fold respectively. The third method derived by Miller (1972) caused a 100 fold enhancement of reversion. In subsequent experiments methods 1 and 3 were used.

GB10 (R144drd-3) was treated with EMS according to Achtman *et al.* (1971) and 1200 surviving clones were screened for transfer proficiency (Tra^+), surface exclusion (Sex^+) and colicin production (Cib^+). Screening for Tra^- clones was performed in a replica plate mating system (Fig. 6-1). Tra^- mutants were detected by the inability of clones to transmit kanamycin resistance to GB11 (replica plate mating 1, Fig. 6-1). The kanamycin-resistant transconjugants were then screened for their ability to transmit kanamycin resistance to BW66 (Replica plate mating 2,

Fig. 6-1). Amber Tra$^-$ mutants would be transferred efficiently from GB10 because of the supE48 mutation, but not from the suppressor-free strains GB11. Therefore both amber and non conditional mutations may be identified in this mating system. R144drd-3 was transmitted with high efficiency through both matings when an untreated culture of GB10 (R144drd-3) was used.

To screen for Sex$^-$ mutants, survivors of the mutagenesis which were still resistant to kanamycin, were mated with GB11 (pLG205) to identify clones that had become good recipients in mating (replica plate mating 3, Fig. 6-1). GB11 (pLG205) is a leu$^+$ strain harbouring a ColIdrd-1' lac$^+$ plasmid which mobilises leu$^+$ early in mating and with high efficiency (M.I. Sedgwick personal communication). The formation of leu$^+$ rpsL recombinants that were kanamycin resistant would identify Sex$^-$ mutants of R144drd-3. In control replica plate matings involving GB10 and GB11 (pLG205), leu$^+$ rpsL recombinants were readily detected. No recombinants were formed when GB10 (R144drd-3) was used in a control plate mating.

Colicin Ib production was determined by overlaying patch plates, that had been exposed to chloroform to kill the bacterial growth, with a colicin sensitive indicator strain (for example BW40).

Of the 1200 survivors tested from the mutagenesis no Tra$^-$, Sex$^-$ or Cib$^-$ mutants were obtained. When the mutagenesis was repeated using Method 3 (Miller, 1972), 1500 survivors were screened and no Tra$^-$ or Cib$^-$ mutants were isolated. One putative Sex$^-$ mutant was obtained. In a subsequent liquid medium mating using the same strains this mutant showed only a 20 fold reduction in surface exclusion in comparison to a strain carrying R144drd-3.

It is unclear why the yield of plasmid mutants was so low. It seems likely that under these conditions the plasmid is poorly affected by EMS.

In a pilot experiment GB10 (R144drd-3) was treated with EMS according to Miller (1972) and the surviving clones tested for reversion of the thr and leu-6 markers of the host and mutation of the plasmid genes specifying kanamycin resistance and colicin production. The yield of Thr^+ and Leu^+ revertants was enhanced 100 fold but out of 3000 survivors tested none were Cib^- and 6 had become sensitive to kanamycin. Since all of the drug-sensitive bacteria were obtained from the same overnight culture they may have been siblings.

The efficiency with which plasmid mutants are isolated may be improved if the mutagenesis is performed in vitro using purified plasmid DNA and hydroxylamine (Humphreys et al., 1976). Hydroxylamine modifies cytosine so that during replication this base pairs with adenine rather than guanine (Freese et al., 1961). Following mutagenesis, the treated DNA is introduced into a suitable host by transformation. This method relies on the availability of a reasonably large supply of purified plasmid DNA.

Large scale plasmid purification

A variety of methods have been published for the isolation of large quantities of plasmid DNA. A particularly simple protocol has been devised by Guerry et al. (1973). This procedure involves cellular lysis with SDS and subsequent removal of the detergent and chromosomal DNA by addition of NaCl to 1M. Following a clearing spin, DNA in the supernatant, which is enriched for plasmid DNA, is subjected to caesium chloride-ethidium bromide density gradient centrifugation or is analysed for its plasmid content by agarose gel electrophoresis. Using this procedure Guerry et al. (1973) reported the successful purification of R144 DNA. However in my hands this procedure, which worked well for F and small plasmids like

ColE1, was unsuccessful when R144, R144*drd-3*, ColI and ColI*drd-1* containing strains were used. Purification of IncI$_{\alpha}$ plasmids was not achieved by varying the concentration of salt (to 0.5 and 0.25 M) or by treating the lysate with RNAase and Pronase.

An alternative procedure devised by Cohen and Miller (1968) and used in the purification of F DNA (Sharp *et al.*, 1972; Ohtsubo *et al.*, 1974; Skurray *et al.*, 1977) was used. This procedure involves adsorption of sheared single-stranded chromosomal DNA to bulk nitrocellulose. Single-stranded chromosomal DNA was generated by denaturation in NaOH followed by a rapid return to neutrality. Double-stranded plasmid molecules form compact random coils in alkali (Vinograd *et al.*, 1965) which upon a return to neutrality reform double-stranded plasmid molecules. These double-stranded DNA molecules are not adsorbed by nitrocellulose and hence when the nitrocellulose is removed from the lysate by centrifugation, the supernatant is enriched for plasmid DNA. When this procedure was used with strains harbouring various IncI$_{\alpha}$ plasmids, no evidence for the presence of plasmid DNA following addition of nitrocellulose (Grade FHX8-13, I.C.I) was obtained. A prominent band of DNA, which migrated in agarose gels with chromosomal DNA of a plasmid-free strain, was always observed. This suggests that the nitrocellulose step was not working. In an attempt to improve the technique, the bacterial lysate was extensively deproteinised by repeated phenol extraction before the addition of nitrocellulose. This approach was also unsuccessful as was the use of a different grade of nitrocellulose (Grade FHX30-50, I.C.I). The critical step in this procedure is the alkaline denaturation and rapid neutralisation which are particularly difficult steps to perform because of the high viscosity of the lysate. It seems likely that problems with these operations accounted for the failure of this preparative technique.

A protocol developed by Davis and Vapnek (1976) has been used in the purification of R538-1 plasmid DNA (Alton and Vapnek, 1978) and has been successfully used in the purification of a wide variety of plasmids up to a molecular weight of 10^8 (D. Vapnek, personal communication). This method involves overnight lysis of spheroplasts with sarkosyl and a 2 h clearing spin at 17,000 rpm in a Beckman Type 19 rotor. The cleared lysate is deproteinised by phenol extraction and the DNA in the supernatant is precipitated with ethanol. The precipitate is resuspended in an appropriate buffer and can be analysed by agarose gel electrophoresis or purified further by CsCl-ethidium bromide density gradient centrifugation. I have found this procedure to be successful for the purification of ColI*drd-1*, JCFL0 and a variety of small, high copy number plasmids.

ColI*drd-1* DNA was isolated using the method of Davis and Vapnek (1976) and CsCl-ethidium bromide centrifugation. Plasmid DNA was removed from the gradient and ethidium bromide extracted with CsCl-saturated propanol. CsCl was removed by dialysis and the DNA concentrated either by ethanol precipitation or lyophilisation followed by resuspension in a small volume of Tris buffer (10mM Tris-HCl pH7.4). The yields of plasmid DNA using this procedure were low, about 20-40 µg of DNA were obtained from a 1 litre culture, but they were sufficient for subsequent manipulation.

The reasons why the purification of ColI DNA is difficult is unclear. Skorupska *et al*. (1979) have also reported difficulties with the isolation of this plasmid which they attribute to the plasmid's susceptibility to nucleases. These authors claim that purification was facilitated when the host strain was mutant at *recB recC* and *sbcB*.

Restriction analysis of ColIdrd-1

ColIdrd-1 DNA, prepared using a modification of the method of Davis and Vapnek (1976), was cleaved with restriction endonucleases EcoRI or PstI. EcoRI-cleaved DNA was subjected to electrophoresis through a 0.5% agarose (Fig. 6-2a) and PstI-cleaved DNA was analysed on a 0.7% (Fig. 6-2b) or 1.5% (Fig. 6-2c) agarose gel. ColIdrd-1 DNA is cleaved into greater than 30 fragments with PstI and into 15 fragments with two minor bands by EcoRI.

The pattern of fragments generated by cleavage of ColIdrd-1 DNA with EcoRI closely resembles that observed by Roussel et al. (1979) during studies of another IncI$_{\alpha}$ plasmid (pIP173) isolated from Salmonella ordonez and a series of derivatives of this plasmid. Fifteen EcoRI fragments were found in common to each derivative and these correspond well to those shown in Fig. 6-2a. The EcoRI cleavage pattern of ColI DNA reported by Skorupska et al. (1979) appears to show a number of differences from the patterns reported by Roussel et al. (1979) for pIP173 and that in Fig. 6-2a. The extent of, and reasons for, these differences are unclear and require further study.

Cloning of PstI-generated fragments of ColIdrd-1

The vector chosen was pBR322, a 2.6×10^6 dalton plasmid (Bolivar et al., 1977). This plasmid is a relaxed replicating unit and carries resistance genes to ampicillin and tetracycline. There is a single PstI site in the ampicillin-resistant gene. Insertion of PstI-generated fragments at this site inactivates the ampicillin-resistance gene.

ColIdrd-1 DNA was cleaved with the restriction endonuclease PstI, mixed with pBR322 DNA also cleaved with the enzyme and joining was achieved by adding T4 DNA ligase. C600 was then transformed with the ligated mixture using the procedure of Cohen et al. (1972) and selection made for

tetracycline-resistant transformants. Recombinant molecules were identified by screening the tetracycline-resistant transformants for ampicillin sensitivity. Using this procedure about 20% of the total tetracycline-resistant transformants were identified as ampicillin sensitive.

Bacteria containing putative recombinant plasmids were screened for the presence of plasmids that were larger than pBR322 using the single colony lysate procedure of Eckhardt (1977) or a mini-cleared lysate system. Fig. 6-3 shows the result of one screening experiment using the technique of Eckhardt (1977). A small amount of bacterial growth was removed from a nutrient agar plate and resuspended in a Ficoll-lysozyme mixture in the slot of a vertical 0.7% agarose gel. Cellular lysis was achieved by addition of SDS to the slot. Following electrophoresis the gel was stained and viewed over a UV light.

Slot A contains a lysate of C600 recently transformed with pBR322 that had previously been cleaved with PstI and religated. Recombinant plasmids were easily identified as a species of plasmid DNA that migrates more slowly than the original cloning vector, pBR322. Therefore bacteria lysed in slots D, F, G, H and I contain a recombinant plasmid.

An interesting feature of this gel is the identification of a plasmid species that migrated faster than pBR322 (Slot B) and is therefore smaller than the original vector. One possible explanation of the origin of such a molecule is that during the transformation step, bacteria were transformed at a low frequency with a linear species of pBR322 remaining after the ligation procedure. Once inside the cell the ends of this molecule may have been subject to nucleolytic degradation before the selection for tetracycline resistant bacteria was imposed. Circularisation of these deletion mutants presumably occurred in two stages. Incorrect pairing

of the ends of the linear molecule followed by repair of the mismatched base pairs would generate a viable plasmid that encoded tetracycline resistance but not resistance to ampicillin. Transformation with linear plasmid DNA has been used as a method for generating deletion mutants extending from the termini of the linear plasmid molecule (Thompson and Achtman, 1979).

Fig. 6-4 shows the result of a screening experiment involving cleared lysates. Bacteria containing putative recombinant plasmids were scraped off the surface of an agar plate and resuspended in a small volume of buffer and lysed by the addition of SDS. The exposed DNA was sheared by vortexing and cleared by centrifugation in an Eppendorf bench centrifuge. The supernatant was subject to electrophoresis through a horizontal agarose slab gel (0.7%). Tracks A, B and C of the gel (Fig. 6-4) contain lysates of C600 (pBR322), C600 (ColE1) and C600 (pSF2124) which acted as molecular weight markers. The two plasmid species observed in Track B are assumed to be open circular and CCC plasmids. There will be a large proportion of ColE1 plasmid DNA in the open circular form because the lysis procedure will induce relaxation of ColE1 (Clewell and Helinski, 1969). Tracks D-H contain lysates of bacteria containing putative recombinant plasmids. No plasmid band is visible in Track G, presumably because the plasmid comigrated with the chromosomal DNA.

Functional analysis of recombinant plasmids

C600 bacteria harbouring recombinant derivatives of pBR322 which contained PstI-generated fragments of ColI were next examined for some of the phenotypic properties conferred on cells by ColI*drd-1*.

In a screening of bacteria containing recombinant plasmids, bacteria which produced colicin Ib and were immune to killing by the colicin were identified. Ability to produce colicin Ib was tested by overlaying chloroform-treated colonies with a colicin-sensitive indicator strain. Immunity to the colicin was tested by cross streaking a suspension of each clone over a streak of colicin Ib that had been released into the agar during overnight growth of a standard colicin Ib-producing strain (Table 6-1). Since the host strain (C600) used in the cloning experiment was susceptible to killing by colicin Ib it was expected that colicin-producing bacteria would also have to be insensitive to the colicin. Two colicin producing strains were identified out of 40 and these were found to be also insensitive. This property may have resulted from the simultaneous cloning of the colicin immunity gene or by a spontaneous mutation of the host. Since the structural genes for colicin Ib and immunity may be closely linked the former possibility seems most plausible. To confirm, a suitable colicin-sensitive strain must be transformed with plasmid DNA purified from these bacteria. If insensitivity towards the colicin is a consequence of resistance then the yield of transformants will be low and if it is due to immunity normal yields of transformants will be obtained.

E.coli is sensitive to infection by the bacteriophage BF23. However when bacteria harbour ColI the plating efficiency of the phage is reduced by about 5 orders of magnitude (Strobel and Nomura, 1966). Recombinant plasmids that reduce the plating ability of BF23 were identified by cross streaking suspensions of bacteria across a streak of phage BF23 suspension at 4×10^9 plaque forming units per ml (Table 6-1). It should be stressed that this is only a qualitative test that requires

confirmation in a more rigorous and quantitative assay.

This test allowed the detection of two classes of plasmid that appeared to confer different levels of immunity to infection by BF23 : some streaks exhibited confluent bacterial growth whereas others showed less dense growth in the region of the phage. These clones may have arisen by the insertion of the cloned fragment in different orientations with respect to the cloning vector. One orientation may be more favourable for transcription, particularly if the transcription of this fragment relies on a promoter of pBR322.

An interesting feature of this cloning experiment was the isolation of recombinant plasmids of different molecular weight but which conferred the same phenotype on the host bacteria. A possible explanation of this observation is that during cloning more than one fragment of ColI*drd-1* was inserted into the cloning vector. Since the original cleavage of ColI*drd-1* with *Pst*I was complete it is unlikely that these cloned fragments are contiguous in ColI*drd-1*. This may also explain why different levels of immunity to BF23 infection was observed.

The clones were also given to Dr. E. Lanka (Max-Planck-Institut, Berlin) who tested them for production of the plasmid-specified primase identified by Lanka *et al*. (1979). Recombinant plasmids producing this enzyme were not identified. This is not surprising : the *Eco*RI-generated fragment of ColI*drd-1* encoding this enzyme has subsequently been cloned by Beddoes and Wilkins (unpublished data), has a molecular weight of 5.5×10^6 and 9 *Pst*I cleavage sites (Boulnois and Wilkins, unpublished data). Since the primase protein has a molecular weight of 140,000 it seems likely that the structural gene for this enzyme contains at least one *Pst*I recognition sequence.

A shortage of time prevented a screening for other ColI*drd-1* encoded functions such as surface exclusion, origins of transfer and DNA replication and incompatibility.

Whilst few hard data have emerged from this exercise in gene cloning, the results obtained do bear on a recent model to account for abortive infection of ColI-containing bacteria by phage BF23. McCorquodale *et al.* (1979) have proposed that a BF23-specified protein inactivates the colicin immunity protein of ColI, resulting in the killing of the cells by internally liberated colicin. This hypothesis was suggested by the finding that the phage could productively infect bacteria containing mutants of ColI that were defective in colicin production. In addition, the cellular changes occurring during abortive infection of ColI$^+$ bacteria closely resemble those that result from cell killing by colicin Ib (McCorquodale *et al.*, 1979).

The hypothesis that colicin Ib is involved in abortive infection of ColI$^+$ bacteria is not supported by the results summarised in Table 6-1. Recombinant plasmids that caused abortive infection of *E.coli* by BF23 were isolated; these did not specify either colicin production or immunity. Moreover recombinant plasmids were constructed that whilst specifying both colicin production and immunity did not cause BF23 to abortively infect. These findings are not consistent with the suggestion that abortive infection involves colicin Ib and suggest that the mutant ColI plasmids isolated by McCorquodale *et al.* (1979) may be defective in more than one function.

Table 6-1: PROPERTIES OF SOME STRAINS CONTAINING RECOMBINANT PLASMIDS

Strain (plasmid)	Phenotypes [a]			Molecular weight of plasmid (megadaltons)
	Cib	IcIb	Phi	
C600 (pGB19)	-	-	+	4.5
C600 (pGB40)	-	-	+++	3.6
C600 (pGB41)	-	-	+++	7.5
C600 (pGB43)	+	+	-	-
C600 (pGB55)	+	+	-	7.3

[a] Cib = production of colicin 1b

IcIb = immunity to killing by colicin 1b

Phi = abortive infection by BF23. (+ and +++ denote approximate relative resistance to BF23 infection)

[b] The approximate molecular weight of the recombinant plasmids was obtained by comparing their mobility in agarose gels relative to plasmids of known molecular weight (Fig. 6-4)

Figure 6-1: SCHEME FOR THE ISOLATION OF Tra$^-$ AND Sex$^-$ MUTANTS OF R144*drd-3*

The details of this procedure are described in Materials and Methods. Str^R = resistance to streptomycin; Km^R = resistance to kanamycin; Spc^R = resistance to spectinomycin. Failure to generate Km^R Spc^R progeny patches in replica plate mating 1 identifies possible Tra$^-$ mutants and failure to form *his trp lys* Str^R Km^R progeny in plate mating 2 identifies possible amber Tra$^-$ mutants of R144*drd-3*. The formation of *leu*$^+$ Str^R Km^R progeny in replica plate mating 3 identities putative Sex$^-$ mutants of R144*drd-3*.

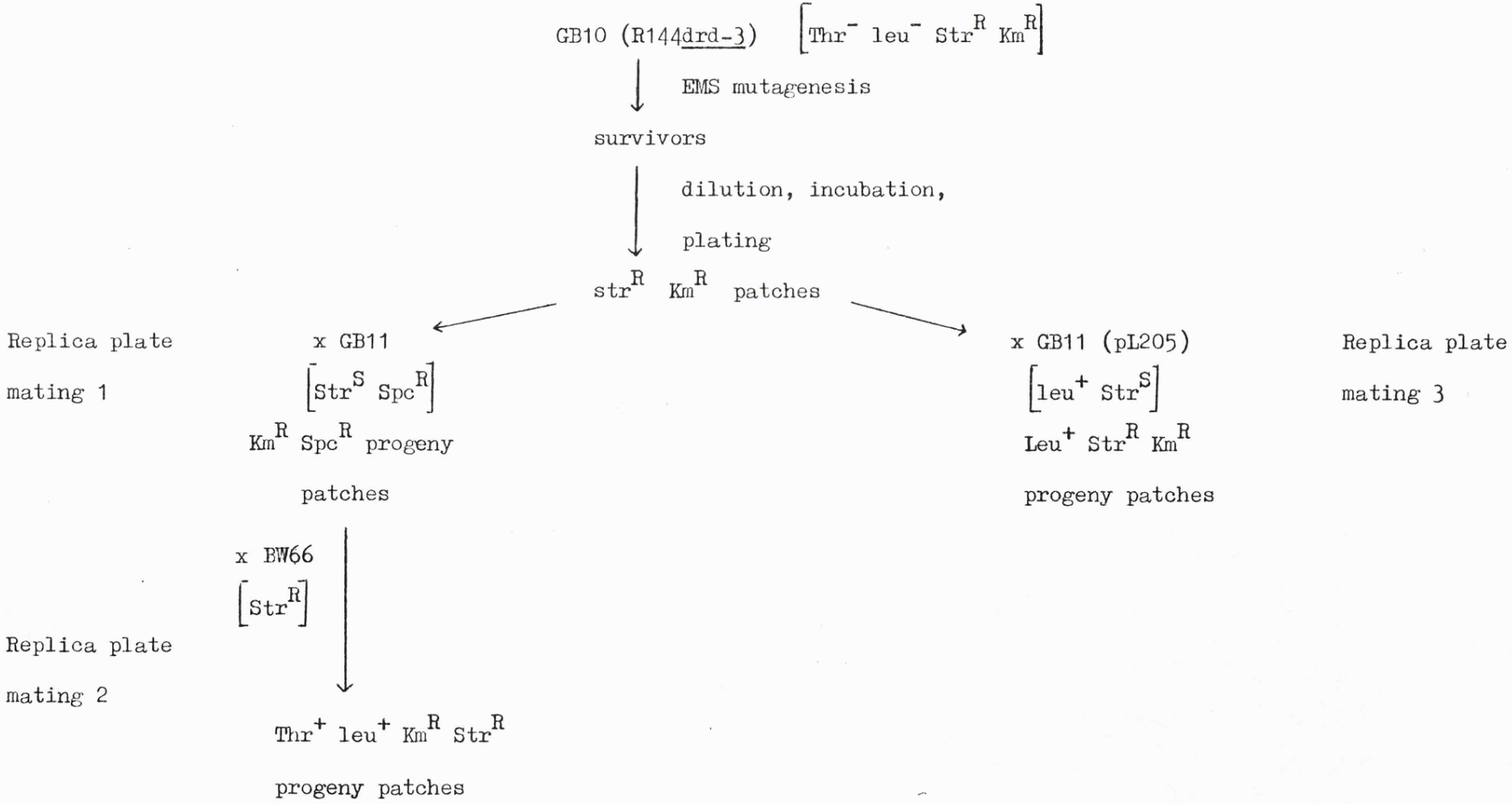

GB10 (R144drd-3) [Thr⁻ leu⁻ Str^R Km^R]
EMS mutagenesis
survivors
dilution, incubation,
plating
str^R Km^R patches
Replica plate
mating 1
x GB11
[Str^S Spc^R]
Km^R Spc^R progeny
patches
x EW66
[Str^R]
Replica plate
mating 2
Thr^+ leu^+ Km^R Str^R
progeny patches
x GB11 (pL205)
[leu^+ Str^S]
Leu^+ Str^R Km^R
progeny patches
Replica plate
mating 3

Figure 6-2: RESTRICTION ENZYME ANALYSIS OF COLIdrd-1

ColIdrd-1 DNA was cleaved with either EcoRI or PstI. EcoR1 cleaved DNA was subjected to electrophoresis through a 0.5% agarose gel (A) whilst Pst1 cleaved DNA was analysed on 0.5% (B) and 1.5% (C) gels. Each track contains 2 μg of cleaved DNA. Molecular weight size markers were obtained by digesting λ DNA with either EcoR1 or HindIII. The molecular weights of the EcoR1 and HindIII fragments of λ were taken from Thomas and Davis (1974) and Murray and Murray (1975) respectively.

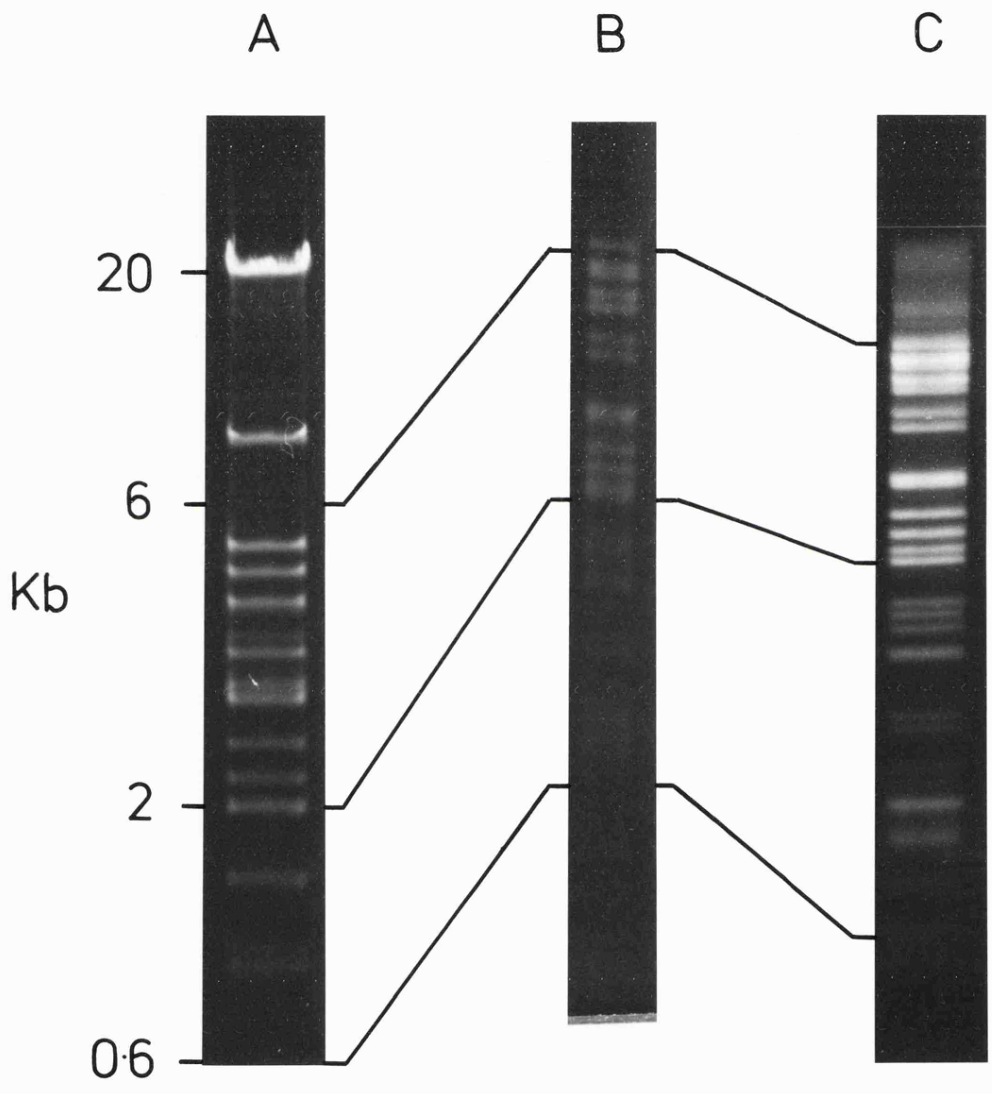

A
B
C
20
6
Kb
2
0·6

Figure 6-3: SCREENING TRANSFORMANTS FOR RECOMBINANT PLASMIDS BY A SINGLE-COLONY LYSIS PROCEDURE

One or two single colonies of each transformant were lysed in the slots of a vertical agarose gel (Eckhardt, 1978). After electrophoresis the gel was stained in ethidium bromide and photographed. Track A contains a lysate of C600 recently transformed with pBR322. Tracks B-I contain lysates of C600 containing putative recombinant plasmids.

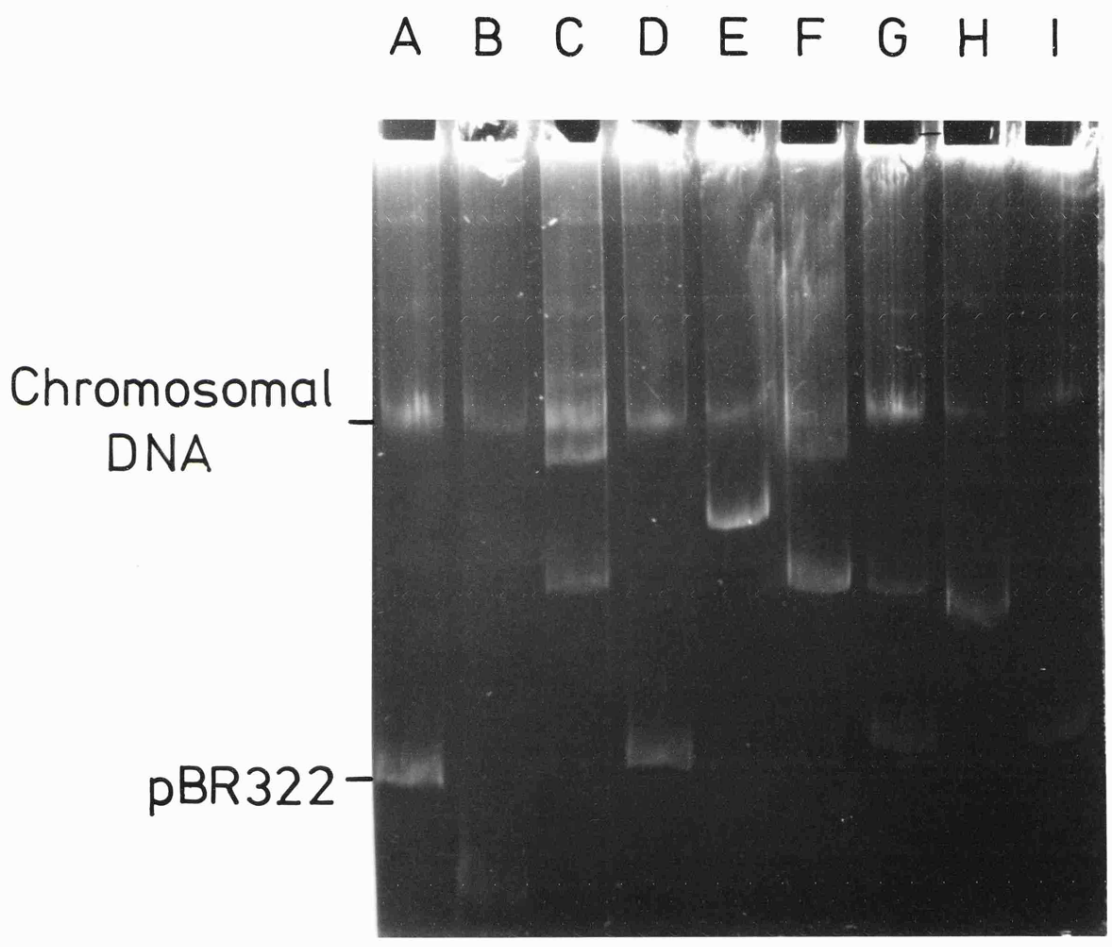
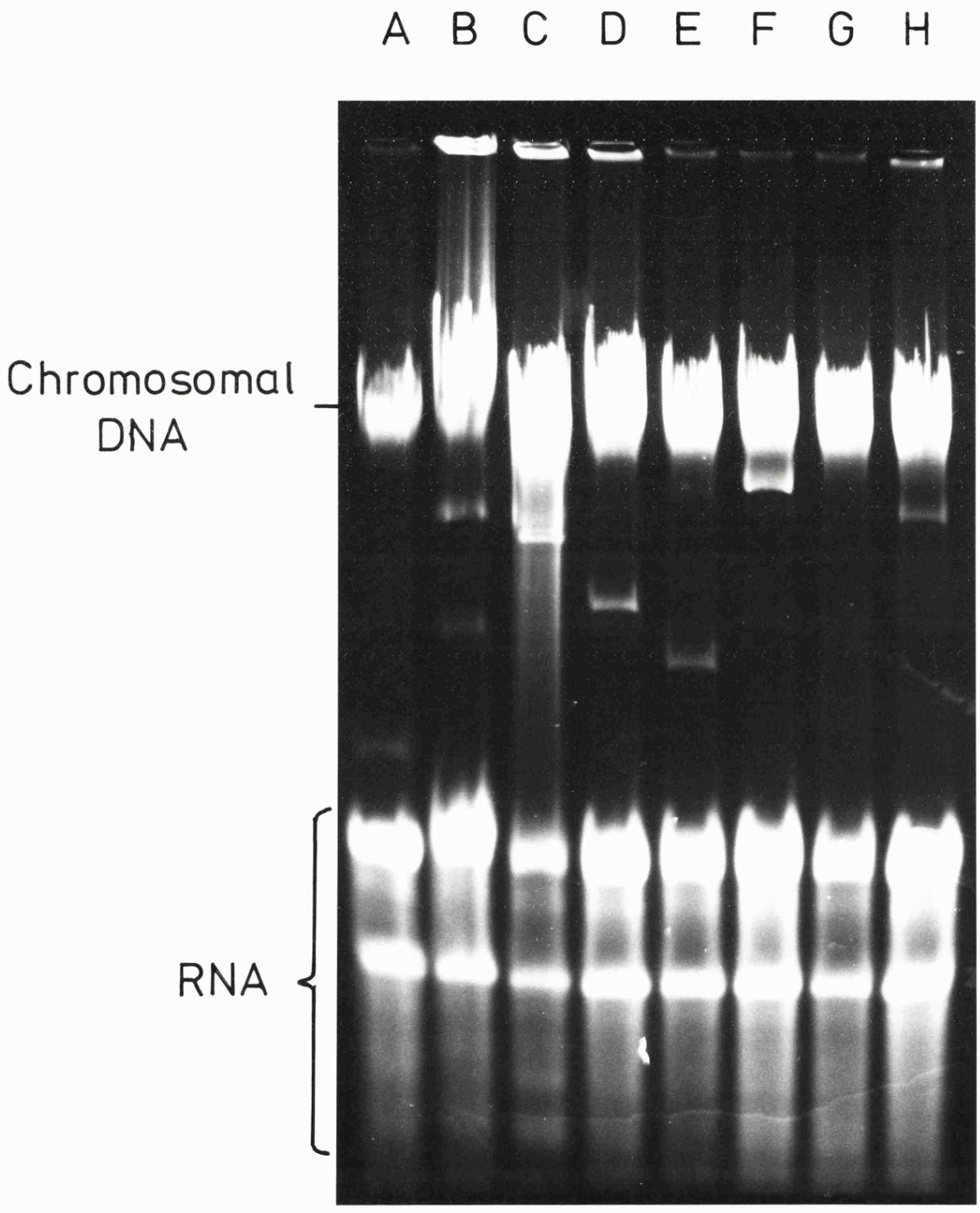

A B C D E F G H I
Chromosomal DNA
pBR322

Figure 6-4: SCREENING TRANSFORMANTS FOR RECOMBINANT PLASMIDS USING A CLEARED LYSATE PROCEDURE

Bacteria were scraped off agar plates and lysed with SDS. After a clearing spin each lysate was subjected to electrophoresis through a 0.5% agarose gel. The gel was stained with ethidium bromide and photographed. Tracks contain lysates of the following A: RR1 (pBR322); B: C600 (ColE1); C: C600 (pSF2124). D-H contain C600 (pGB19); C600 (pGB40); C600 (pGB41); C600 (pGB43); C600 (pGB55) respectively. The molecular weight of the plasmid markers were taken to be: pBR322, 2.6×10^6 (Bolivar et al., 1977); ColE1, 4.2×10^6 (Hershfield et al., 1974); pSF2124, 7.4×10^6 (So et al., 1975). The two plasmid species in track B are assumed to be open and covalently closed circular ColE1.


A B C D E F G H
Chromosomal DNA
RNA

CHAPTER 7

A REEVALUATION OF IncIα PLASMID TRANSFER

Studies of plasmid transfer have predominantly involved the archetypal sex factor, F, and research over the last decade has led to a considerable understanding of many aspects of the conjugal transfer of this plasmid. However, arguably the most important aspect of F-mediated conjugation, that of DNA transfer, remains poorly understood. In contrast, whilst most details of IncIα plasmid-mediated conjugation are obscure, the metabolism of plasmid DNA during transfer has been investigated in some detail. These studies have not only confirmed ideas obtained from research with the F plasmid, but have identified features of the conjugation process not manifested by F. Examples of these are transfer limitation (Chapter 3) and the postulated involvement of *sog* product in conjugal DNA synthesis in recipients (Chapter 5). It is therefore appropriate at this stage to reevaluate ideas on the mechanism of transfer in the light of results presented in this thesis.

At the outset of this study, ideas on the transfer of IncIα plasmids were greatly influenced by two models for the transfer of R64*drd-11* (Curtiss and Fenwick, 1975). The central feature of these models was a requirement, in the donor, for the synthesis by RNA polymerase of an untranslated species of RNA. This RNA synthesis was essential for plasmid transfer and it was postulated to be required for the initiation of synthesis of replacement strands of the plasmid in the donor. This involvement of RNA was suggested by the apparent rifampicin-sensitivity of transfer (Fenwick and Curtiss, 1973b). To accommodate this finding

the models of Curtiss and Fenwick (1975) were necessarily complex and a radical departure from the essentially simple rolling circle model proposed for F transfer by Ohki and Tomizawa (1968) and Matsubara (1968).

In chapter 4, I have argued that the apparent rifampicin-sensitivity of IncIα plasmid transfer may reflect an unexpected side effect of rifampicin on DNA metabolism in bacteria that contain IncIα plasmids. Treatment of such bacteria with the drug results in an extensive disruption of both chromosomal and conjugal DNA synthesis, the molecular basis of which is unclear. I have suggested that as a result of this disruption of DNA metabolism, donors of IncIα plasmids lose their ability to transmit plasmid DNA. Implicit in this interpretation is the notion that IncIα plasmid transfer does not involve an untranslated RNA molecule made by RNA polymerase. Rejection of such a requirement allows simpler models for IncIα plasmid transfer to be considered.

(a) Events in the donor

As for F, transfer of IncIα plasmids is asymmetric and a specific strand is transferred from donor to recipient (Vapnek et al., 1971). By analogy with F, it is assumed that the transferred strand enters the recipient with the 5' terminus leading. Replacement strands are made in the donor and a complement to the transferred material is synthesised in recipients. Implicit in this scheme is the introduction of a nick into a specific strand of the plasmid. As is the case for F, this nicking presumably occurs at a specific site (oriT). The biochemistry of the nicking reaction is obscure but the events during the mobilisation of ColE1 may provide a clue. A strand-specific, site-specific nicking event has been implicated in this process (Warren et al., 1978). In this case nicking is thought to be mediated by the relaxation complex proteins.

Since ColI is known to undergo relaxation (Clewell and Helinski, 1970b) perhaps nicking at the origin of ColI transfer involves the relaxation complex of ColI.

Transfer of IncIα plasmids does not appear to be by a classical rolling circle mode. There is a delay between transfer of one strand of an IncIα plasmid and the transmission of its replacement, a process that requires synthesis of new protein (Fenwick and Curtiss, 1973b; see also Chapter 4). Moreover, transfer involves transmission of unit or near unit lengths of plasmid DNA (Fenwick and Curtiss, 1973a,b; see also Chapter 3). How unit lengths of plasmid DNA are generated is unclear. They would be generated if the presumed 3'OH group, created by nicking at oriT is unavailable as a primer terminus for a DNA polymerase. If this were the case the newly synthesised plasmid DNA would not be covalently attached to the preexisting strand, providing a natural break in transfer. Alternatively, unit lengths of plasmid DNA may result from a nicking event at the regenerated oriT : covalent extension of the 3' terminus of the original nicked strand would regenerate the oriT sequence which may be susceptible to a second nicking event.

Perhaps the most poorly understood aspect of IncIα plasmid transfer is the synthesis in donor bacteria of DNA to replace the transferred material. Conjugal DNA synthesis in the donor occurs concomitantly with transfer, the amount of DNA made in the donor equalling the amount synthesised in the recipient (Wilkins and Hollom, 1974). Which of the three bacterial DNA polymerases mediates this synthesis is not known, but it seems likely to be DNA polymerase III.

Donor conjugal DNA synthesis is independent of the activity of RNA polymerase and dnaB product (see Chapter 4). The independence of dnaB product rules out the possibility that primase, the dnaG gene product,

is involved in initiating this synthesis in a system analogous to that operating during conversion of ØX174 viral DNA to the duplex form. Conjugal DNA synthesis in the donor may involve as primer terminus the presumed 3'OH group created by nicking at oriT. If this is correct then a second nicking event at the regenerated oriT sequence is required to generate unit lengths of plasmid DNA. Alternatively synthesis of plasmid DNA in the donor may involve primase, as in the conversion of G4 viral DNA to the duplex form. It is interesting to speculate that conjugal synthesis of plasmid DNA in the donor might be initiated by sog product.

It is not known whether the topology of the plasmid influences the efficiency of transfer and conjugal DNA synthesis in the donor. Transfer from and conjugal DNA synthesis in donors of R64drd-11 is markedly reduced by nalidixic acid (Fenwick and Curtiss, 1973c), a drug that inhibits DNA gyrase (Gellert et al., 1977; Sugino et al., 1977), implicating this enzyme in some aspect of the transfer process. These findings may suggest that transfer involves a molecule with some superhelicity.

(b) Events in the recipient

In the recipient a complement to the transferred strand is synthesised by DNA polymerase III (Wilkins and Hollom, 1974) in a reaction that is independent of dnaB product, RNA polymerase (Wilkins and Hollom, 1974; see also Chapters 3 and 4) and primase (Chapter 5). Since DNA polymerases require a 3'OH primer terminus upon which to initiate the synthesis of DNA, I have proposed (Chapter 5) that the necessary primer is made by sog product. This product, thought to be encoded by a transfer gene, may synthesise primers in the donor on the strands destined for transfer. Alternatively, the protein itself may be transferred to the recipient where it promotes primer synthesis. The cellular site and conformation of

the template for conjugal DNA synthesis in the recipient is not known. The transferred DNA may circularise prior to complementary strand synthesis or vice versa.

One of the most interesting and unsolved aspects of ColI transfer is the mechanism which generates circular molecules from the transferred unit lengths of plasmid DNA. This process does not require transcription of the immigrant DNA (Chapter 3). Circularisation may be mediated by a protein analogous in function to the cisA protein of phage ØX174 (Eisenberg et al., 1977). Nicking at oriT in the donor may be associated with the complexing of the putative cisA - protein analogue (perhaps a relaxation complex protein) to the 5' terminus of the nicked strand. Transfer of the DNA protein complex may allow the direct joining of the 5' and 3' termini of the transferred strand in the recipient. Similar models have been proposed for the transfer of Flac (Kingsman and Willetts, 1978) and for the mobilisation of ColE1 (Dougan et al., 1978; Staudenbauer, 1978). If circularisation is achieved in this manner then this provides another example of the transfer of specific proteins during conjugation. Alternatively, circularisation may be achieved as described in Fig. 3-5a, involving the formation of a hairpin structure at the 3' terminus of the transferred strand. This model lacks appeal because of its inherent complexity.

Plasmid expression in the newly infected recipients has several consequences. Plasmid products are involved in limiting further transfer (Chapter 3), causing active breakdown of mating aggregates (Achtman, 1977) and surface exclusion (Meynell, 1969). Continued expression of plasmid genes converts newly infected recipients into active donors of the plasmid.

The molecular mechanisms of several features of IncI$_\alpha$ plasmid transfer remain obscure. Of particular interest for further study are the

phenomenon of transfer limitation and the postulated involvement of a plasmid-specified enzyme (the product of *sog*), in the priming of conjugal DNA synthesis in recipients. These features are particularly interesting because they are not manifest in matings involving F-like plasmids and they may prove to be unique to Iα plasmid-mediated conjugation. Possible approaches to the further analysis of transfer limitation have been considered in Chapter 3. For the remainder of this discussion I will concern myself with *sog* product and possible approaches to its further characterisation.

The product of *sog* has been postulated to act as a DNA primase during conjugation (Chapter 5), a classification that is based on its ability to suppress the temperature sensitivity of *dnaG* mutants. Furthermore, it was postulated that *sog* product is the IncIα plasmid-specified DNA primase identified by Lanka *et al*. (1979). This is plausible because production of the DNA primase and ability to suppress *dnaG* mutations show the same plasmid specificity (Wilkins, 1975; Lanka *et al*., 1979; Sasakawa and Yoshikawa, 1978; Lanka, E., personal communication). Moreover, suppression of *dnaG3* mutations and high relative amounts of the novel primase tend to be observed only when plasmid genes involved in conjugation are expressed constitutively (Wilkins, 1975; Sasakawa and Yoshikawa, 1978; Lanka *et al*., 1979).

However several observations appear to conflict with the idea that *sog* product is the DNA primase described by Lanka *et al*. (1979). First, the relative amount of the primase does not correlate with the extent to which the plasmids cause suppression of *dnaG* mutations. For example, ColI*drd-1* suppresses *dnaG* mutations much more efficiently than R64*drd-11* (Wilkins, 1975) but R64*drd-11* directs the synthesis of more than twice the amount of DNA primase than ColI*drd-1* (Lanka *et al*., 1979). Secondly,

Sasakawa and Yoshikawa (1978) have claimed that IncI$_{\alpha}$ plasmid-mediated suppression of dnaG mutants, measured by the ability of bacteria to form colonies at high temperature, is dependent on recA gene product. Thus they suggested that suppression stems from the recA dependent induction of SOS repair functions following thermal inactivation of dnaG product. Co-operation between an induced host product and an unidentified plasmid protein was envisaged to lead to functional restoration of dnaG product by some unspecified interaction. Implicit in this hypothesis is that sog product is not a DNA primase. This requirement for recA product for suppression contrasts with the findings of Wilkins (1975) who claimed that suppression, at least at the level of DNA synthesis was recA independent.

These conflicting reports have recently been reconciled by Newley and Wilkins (unpublished results). They found that, in the absence of recA product, ColIdrd-1 can suppress efficiently the temperature sensitivity of DNA synthesis in a dnaG3 mutant. However the recA product was required for efficient formation of colonies. Newley and Wilkins have therefore proposed that DNA made under suppressing conditions contains lesions that must be repaired in a recA-dependent process as a prerequisite for colony formation. These findings are consistent therefore with sog product being a DNA primase.

Perhaps the strongest argument that the DNA primase identified by Lanka et al. (1979) may not be sog product comes from an analysis of the activity of purified DNA primase in in vitro DNA replication systems. Lanka et al. (1979) observed that whilst the purified primase could substitute for dnaB, dnaC and dnaG products in the formation of the replicative form of ØX174 DNA, it could not substitute for the requirement for dnaG product in the replication of ColE1 in the in vitro system developed by Staudenbauer (1976). However it is possible that the

purification procedure for the DNA primase removed protein factors (perhaps plasmid-encoded) that are essential for the DNA primase to initiate DNA synthesis on duplex DNA.

Another seemingly paradoxical situation exists regarding the requirement for dnaB product during suppression. Since dnaB product appears to be intimately involved in promoting the activity of dnaG product in bacterial DNA replication (McMacken et al., 1977b), it might be expected that if sog product is indeed a DNA primase then IncIα plasmids should also be able to suppress the temperature sensitivity of dnaB mutations. However a number of studies have failed to show evidence of such suppression (Fenwick and Curtiss, 1973a,b,c; Wilkins and Hollom, 1974; see also Fig. 4-4). Lack of suppression of dnaB mutations may reflect an involvement of dnaB product in promoting the activity of sog product during replication of duplex DNA. Alternatively the activity of sog product may be independent of dnaB protein but dnaB product may be required for maintaining the integrity of the complex of replication enzymes. One possibility is that thermal inactivation of dnaB product may alter the conformation of the complex such that DNA polymerase III is lost from it.

Recent reports by Wang and Iyer (1977, 1978) have suggested that plasmids from a number of compatibility groups (including IncIα) can suppress the temperature sensitivity of dnaB mutations. However suppression of dnaB mutations lacks plasmid specificity (Wang and Iyer, 1977) in contrast to suppression of dnaG mutations (Wilkins, 1975; Sasakawa and Yoshikawa, 1978). Furthermore, unlike suppression of dnaG mutations, expression of transfer genes is not a prerequisite for suppression of dnaB mutations. These observations suggest that suppression of dnaB and dnaG mutations are unrelated phenomena.

With regard to further research it is imperative to establish whether or not the product of *sog* is the plasmid-encoded primase identified by Lanka *et al*. (1979). Examination of this possible relationship is greatly facilitated by the availability of a recombinant plasmid (pLG211) that contains an *Eco*RI fragment of Col*Idrd-1* inserted into the chloramphenicol resistance gene of pBR325 (Bolivar, 1978). This plasmid suppresses the temperature sensitivity of *dnaG3* mutations (Beddoes and Wilkins, unpublished data). The critical question is does this plasmid direct the synthesis of the DNA primase?

Further studies to analyse the role of *sog* product in conjugal DNA synthesis will be facilitated by the isolation of a *sog*$^-$ derivative of Col*Idrd-1*. Studies described in Chapter 6 suggest that the generation of such a mutant by conventional, chemical mutagenesis will be difficult. Chemical mutagenesis, *in vivo*, is made even less appealing by the fact that mutations in genes other than *sog* can give rise to plasmids that will be phenotypically Sog$^-$. For example, the mutants might contain a reversion of the *drd-1* mutation or a polar Tra$^-$ mutation. These problems might be avoided by the isolation of a *sog*$^-$ derivative of the Sog$^+$ recombinant plasmid, pLG211, following *in vitro* mutagenesis with hydroxylamine (Humphreys *et al*., 1976). Defined *sog*$^-$ mutations may then be introduced into Col*Idrd-1* by homogenotisation (Miller, 1972; Kennedy *et al*., 1977). Col*Idrd-1* plasmids mutant in *sog* could then be used in experiments to measure DNA transfer and conjugal DNA synthesis. One could seek answers to a number of important questions. For example, is a *sog*$^-$ plasmid phenotypically Tra$^+$ or do donors transmit plasmid DNA to recipients where synthesis of complementary strands is defective? Can RNA polymerase or *dnaG* product substitute for *sog* product?

It was proposed in Chapter 5 that sog product may be transferred from donors to recipients. Little is known about the transfer of proteins during conjugation and this subject warrants detailed analysis. The studies of Silver (1966) suggested that very little transfer of donor protein occurred during F-mediated mating. However, Silver did not examine whether a limited number of specific proteins were transferred to the recipient. To test the specific possibility that sog product is transferred during mating, protein transfer might be examined in a system essentially identical to that used for measuring DNA transfer. This would involve donors labelled with $[^{35}S]$-methionine and UV-irradiated recipients treated with rifampicin and streptomycin. Such treatments would limit protein synthesis in the recipients and the antibiotics would also enhance DNA transfer (Chapter 3). Conditions which enhance plasmid transfer might be expected to increase the amount of protein transfer. Following removal of donors by addition of phage T6 and extensive washing, analysis of lysates of recipients by SDS polyacrylamide gel electrophoresis and autoradiography (fluorography) might reveal proteins that are transferred from donors during mating. The resolution of this method would depend greatly upon the efficiency of removal of proteins released from the lysed donors. Results obtained by Silver (1966) using a similar approach suggest that this may not be an insurmountable problem. An analysis of the possible transfer of sog product would be helped by the availability of a ColIdrd-1 plasmid mutant in sog. Such an approach, apart from providing information about sog product, may also indicate that transfer of specific proteins is an important feature of bacterial conjugation.

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ABSTRACT

STUDIES ON THE TRANSFER OF IncIα PLASMIDS IN ESCHERICHIA COLI K-12

G.J. Boulnois

The studies concern the mechanism that primes the synthesis of DNA complementary to the strand of DNA that is transferred during IncIα plasmid-mediated conjugation. This DNA synthesis was found to be independent of the two defined priming enzymes of the host bacterium, RNA polymerase and primase. It is proposed that the synthesis is initiated by a novel priming enzyme which is specified by the plasmid and supplied by the donor parent.

It was found that rifampicin, an inhibitor of RNA polymerase, has complex effects on mating bacteria. Rifampicin treatment of recipients enhanced plasmid transfer from drug-resistant donors, implying that the amount of DNA transferred is normally limited by the synthesis of a plasmid-specified product made in newly infected recipients. Enhanced transfer occurring in rifampicin-treated matings has been exploited to search for intermediates of transfer. The results show that the transferred material, isolated from recipients, is the same size as the parental plasmid.

In previous studies it was claimed that transfer of IncIα plasmids was inhibited by rifampicin. It is shown that rifampicin-treatment of donors allowed transfer to proceed normally for about 10 min when it stopped abruptly. Cessation of transfer correlated with an abrupt disruption of conjugal DNA synthesis in donor cells. The inhibitory effect of rifampicin on IncIα plasmid transfer may result from a side effect of the drug on DNA metabolism, rather than a direct result of inhibiting RNA polymerase. Previous models for IncIα plasmid transfer, involving a requirement for rifampicin-sensitive synthesis of an untranslated RNA molecule in the donor, have been reevaluated.

In an attempt to understand the molecular bases of these phenomena, a genetic analysis of IncIα plasmids was initiated.