PLASMID ENCODED DNA PRIMASES.

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Plasmid ColIb-P9 of the Iα incompatibility group encodes a DNA primase that acts in conjugal transfer of the plasmid and can substitute for mutant dnaG gene product in the vegetative replication of the Escherichia coli chromosome. The relevant genetic determinant (sog) has been cloned into a multicopy vector plasmid. Prototype IncB plasmid R16 also suppresses host dnaG mutations. The equivalent gene(s) (pri) of R16 have been cloned into plasmid pBR325 and shown by Southern transfer hybridisation to be different from the ColIb-P9 primase gene(s). The cloned fragment carrying the pri determinant encodes two polypeptides with apparent molecular weights 240,000 and 180,000 which can initiate DNA synthesis in vitro on single stranded phage M13 template, but which are antigenically distinct from ColIb-P9 primase. The cloned primase genes were used as probes in colony hybridisation screening of strains carrying plasmids of the IncI complex and IncB group, which specify serologically similar conjugation pili. Plasmids R64drd11, R144drd3 (IncIα), R621a (IncIγ), RIP72 and R864a (IncB) contain nucleotide sequences honologous with the cloned ColIb-P9 sog gene(s). Plasmids R805a (IncIζ), R724 (IncIδ), TP125 and pLG101 (IncB) showed sequence homology with the R16 pri determinant.

R387 (IncK) is also a member of the I-complex and encodes a primase which is genetically and serologically distinct from ColIb-P9 primase, but which is genetically similar to R16 primase. The gene(s) has been cloned into plasmid pBR328 and the fragment carrying the pri determinant encodes two polypeptides of 270,000 and 200,000 daltons.

The hybridisation and dnaG suppression screens were extended to include representatives of all of the E.coli incompatibility groups. R40a (IncC) and RA3 (IncU) were shown to suppress the dnaG3 mutation and to encode DNA primases. The IncC primase encoded by R40a is genetically and antigenically distinct from ColIb-P9 primase.

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CHAPTER ONE

1) PLASMIDS:GENERAL INTRODUCTION.

Bacterial plasmids are a diverse group of double stranded DNA molecules, in the form of supercoiled covalently closed circles (although recently linear DNA plasmids have been identified in Streptomyces sp. (Hayakawa et al.,1979; Hirochika and Sakaguchi, 1982)), which can be stably maintained without being linked to the chromosome (Rownd et al.,1966). Plasmids range in size from 1kb to over 300kb, ie about 0.025% to 7.5% of the Escherichia coli chromosome. Originally discovered in the Enterobacteriaceae, plasmids have been identified in many bacterial species such as, Bacillus megaterium (Carlton and Helinski, 1969), Clostridium perfringens (Brefort et al.,1977), Cyanobacteria (Hondel et al.,1979), Haemophilus spp. (Mann and Rao, 1979), Halobacterium halobium (Weidinger et al.,1979), Pasteurella multocida (Silver et al.,1979), Pseudomonas spp. (Chakrabarty, 1976), and Streptococcus spp. (Clewell, 1981), and in fungi (Gubbins et al.,1977; Collins et al.,1981).

The first major groups of plasmids, apart from F which was discovered due to its ability to mobilise the Escherichia coli chromosome during conjugation (Hayes, 1953; Jacob and Wollman, 1961), were identified by their ability

to transmit multiple antibiotic resistances from one host to another (Mitsuhashi et al.,1960; Watanabe, 1963; Datta, 1965). Since then plasmids have been isolated which express a wide variety of genes although many plasmids appear to be cryptic and no phenotype can be assigned to them. The readily observable phenotype of the majority of plasmids has led to the classification of plasmids into groups, R-plasmids, which confer resistance to one or more antibacterial compounds (Davies and Smith, 1978), Col plasmids, which code for antibacterial proteins called colicins (Hardy et al.,1973; Hughes et al.,1978), degradative plasmids, which carry genes that encode a variety of catabolic enzymes (Chakrabarty, 1976) and virulence plasmids, which increase the pathogenicity of their bacterial hosts (Elwell and Shipley, 1980).

Another system for classifying plasmids is by the criteria of self-transmissibilty, size and copy number: the large, low copy number conjugative plasmids, such as F and ColIb-P9, which are able to transfer their own DNA from one cell to another; the small, high copy number mobilisable plasmids, such as ColE1, which require a conjugative plasmid for transfer of their DNA from one host to another; and the non-mobilisable plasmids, which cannot be mobilised by any conjugational system (Meynell et al.,1968). There is some doubt as to whether non-mobilisable plasmids exist in the

wild and all of the well characterised non-mobilisable plasmids, such as pBR322 (Bolivar et al.,1977), are laboratory constructs.

1,1) Conjugative plasmids.

The conjugative plasmids were originally grouped into two sub-groups; fi+ plasmids, which inhibited the fertility of F and encoded F-like pili, and the fi- plasmids, which did not inhibit F fertility and did not encode F-like pili (Watanabe et al.,1964). Two plasmids are said to be incompatible if they are not maintained together in a cell without selective pressure for both plasmids, while two compatible plasmids are able to coexist in the same cell in the absence of selective pressure (Watanabe et al.,1964). It was originally observed that members of both the fi+ and fi- group were compatible with F and with members of the other class, but that within each class pairs of plasmids were incompatible (Watanabe et al.,1964; Meynell et al.,1968). However it was soon clear, as more plasmids were identified, that the members of the fi- group were not all mutually incompatible and that the fi- group could be split up into sub-groups of mutually incompatible plasmids (Hedges and Datta, 1971). Since then the number of so called incompatibility groups has increased, but most conjugative plasmids which can be tested in E.coli are members of one of

the following incompatibility groups, IncB, C, D, E, Fl, Fll, Flll, FlV, Hl, H2, Iα, Iγ, Iδ, Iζ, J, K, M, N, P, T, U, V, W, X and Com9 (Datta, 1979; Bukhari et al.,1977) (for representative plasmids see table 2.1). Similar classification systems exist for the plasmids of the Pseudomonas spp. and the Staphylococcus spp. (Bukhari et al.,1977;Iordanescu and Surdeanu, 1980).

Plasmids of the same incompatibility group have been shown to have similar patterns of restriction endonuclease generated fragments (Chabbert et al.,1979). DNA-DNA hybridisation and heteroduplex experiments with members of IncB, C, Fll, I-complex, H, M, N, and W have shown that in general there is more than 70% DNA homology between plasmids of the same incompatibility group and as little as 1% homology between plasmids of different incompatibility groups (Guerry and Falkow, 1971; Grindley et al.,1973; Sharp et al.,1973; Falkow et al.,1974; Roussel and Chabbert, 1978; Gorai et al.,1979). Plasmid encoded pili have been identified for members of most of the incompatibility groups and there is a strong correlation between members of one Inc group or complex and their type of conjugative pili (Bradley, 1980a; Bradley, 1980b;Bradley, 1981). These observations support the use of plasmid incompatibility as a classification method which reflects a great deal of genetic similarity between mutually

incompatible plasmids.

1,2) Non-conjugative plasmids.

Large non-conjugative plasmids fall into the same incompatibility groups as conjugative plasmids, presumably because they are derived from conjugative plasmids which have lost their ability to transfer their DNA (Datta and Hedges, 1973; Anderson et al.,1977). Naturally occurring small non-conjugative plasmids also express incompatibility functions, but they do not fall into the groups so far identified for the conjugative plasmids (Datta, 1979). At present there is only one Inc group, designated IncQ, into which non-conjugative plasmids have been grouped (Grinter and Barth, 1976). Plasmids in this incompatibilty group also show a great deal of DNA sequence homology (Barth and Grinter, 1974). There are sole representatives of several more groups which have not yet been given a letter (Smith et al.,1974; Barth et al.,1978b).

Thus to summarise the previous paragraphs, the plasmids of the Enterobacteriaceae can be grouped by their conjugative properties and their incompatibility relationships, which presumably reflect within one group a significant genetic similarity in their transfer and maintenance genes. Other properties of the plasmids, such as

antibiotic resistances, virulence determinants etc, are not characteristic of any one incompatibility group (Bukhari et al.,1977).

2) CHROMOSOMAL DNA REPLICATION.

Plasmids replicate independently of the chromosome, but they are dependent on at least some of the host proteins for their replication. A short summary of the replication of the bacterial chromosome at this point will provide a background to the specific plasmid properties which are to be discussed later. For useful reviews of DNA replication see Wickner (1978), Kornberg (1980), and Ogawa and Okazaki (1980).

Chromosomal DNA replication is initiated at a defined chromosomal locus (the origin of replication), and it proceeds bidirectionally to the terminus (Cairns, 1963; Masters and Broda, 1971; Prescott and Kuempel, 1972). Replication is semi-conservative (Meselson and Stahl, 1958) and proceeds at the same rate on both sides of the replication fork. DNA polymerases can only add nucleotides to a free 3'-OH group and hence polymerisation can only occur in 5'-3' direction (Kornberg, 1980). It has been proposed that as a consequence of this asymmetric reaction DNA synthesis is discontinuous on the lagging 3'-5' strand

(Okazaki et al.,1968).It is commonly assumed that synthesis on the 5'-3' strand is continuous, however this is may also be discontinuous (Ogawa and Okazaki, 1980). Thus on the lagging strand, at least, replication must be continually reinitiated. For a review of the evidence for discontinuous DNA replication see Ogawa and Okazaki (1980). Overall DNA replication can be divided into three stages; initiation at the origin of replication, elongation of the polynucleotide chains, and termination.

2,1) Initiation of replication.

The origin of replication of the Escherichia coli chromosome, oriC, has been mapped to within a kilobase (Marsh and Worcel, 1977) at 82.8 minutes. This region of the chromosome has many short inverted repeat sequences which may be involved in the promotion of initiation of replication (Sugimoto et al.,1979; Meijer et al.,1979). An order of events at the initiation of chromosome replication has been proposed, the dnaA reaction occurs, RNA polymerase is required either coincidently with, or after the dnaA reaction, and then the dnaC protein acts (Projan and Wechsler, 1981; Fuller et al.,1981). It has been proposed that the dnaC protein is absolutely required for the reconstitution of the replisome and hence initiation of rounds of replication (Nusslein-Crystalla et al.,1982).

Other gene products have also been proposed to be involved in the initiation steps, dnaB product (Zyskind and Smith, 1977), dnaI product (Beyersmann et al., 1974), and the dnaP product (Wada and Yura, 1974).

2,2) Elongation.

The discontinuous mode of replication, on at least one strand, necessitates the synthesis of a large number of RNA primers which DNA polymerase III requires to initiate polymerisation (Ogawa and Okazaki, 1980; see figure 1.1). To generate a binding site for the primer synthesising complex a helicase unwinds the DNA (Yarranton and Gefter, 1979) presumably separating the strands and single stranded binding protein stabilises the single stranded regions (Meyer et al., 1979). A prepriming complex of several proteins (see figure 1.2), including the hexameric dnaB gene product binds to the DNA and promotes the binding of the dnaG gene product, the primase, to the DNA (McMaken et al.,1977). It has been proposed that once the prepriming complex has bound to the single stranded DNA it is able to travel along the DNA in a 5'-3' direction and to promote multiple primer initiations before leaving the DNA (McMacken et al.,1977; Arai and Kornberg, 1979). The dnaG primase can polymerise both ribonucleoside and deoxyribonucleoside triphosphates to variable chain-length oligonucleotides

Figure 1.1) E.coli chromosome replication fork.

A diagramatic model of the replication fork of the E.coli chromosome, showing the major enzymes and proteins involved in the elongation steps of DNA replication (after Kornberg, 1980). DNA binding protein binds to the single stranded DNA generated by the action of the helix unwinding enzymes. On the lagging strand the dnaG primase binds to the DNA catalysed by the prepriming complex (see figure 1.2). After the synthesis of the RNA primer DNA polymerase III holoenzyme synthesises the DNA until it reaches an obstruction, usually another primer. DNA polymerase I replaces the RNA primer with DNA and ligase fills the single nucleotide gap.

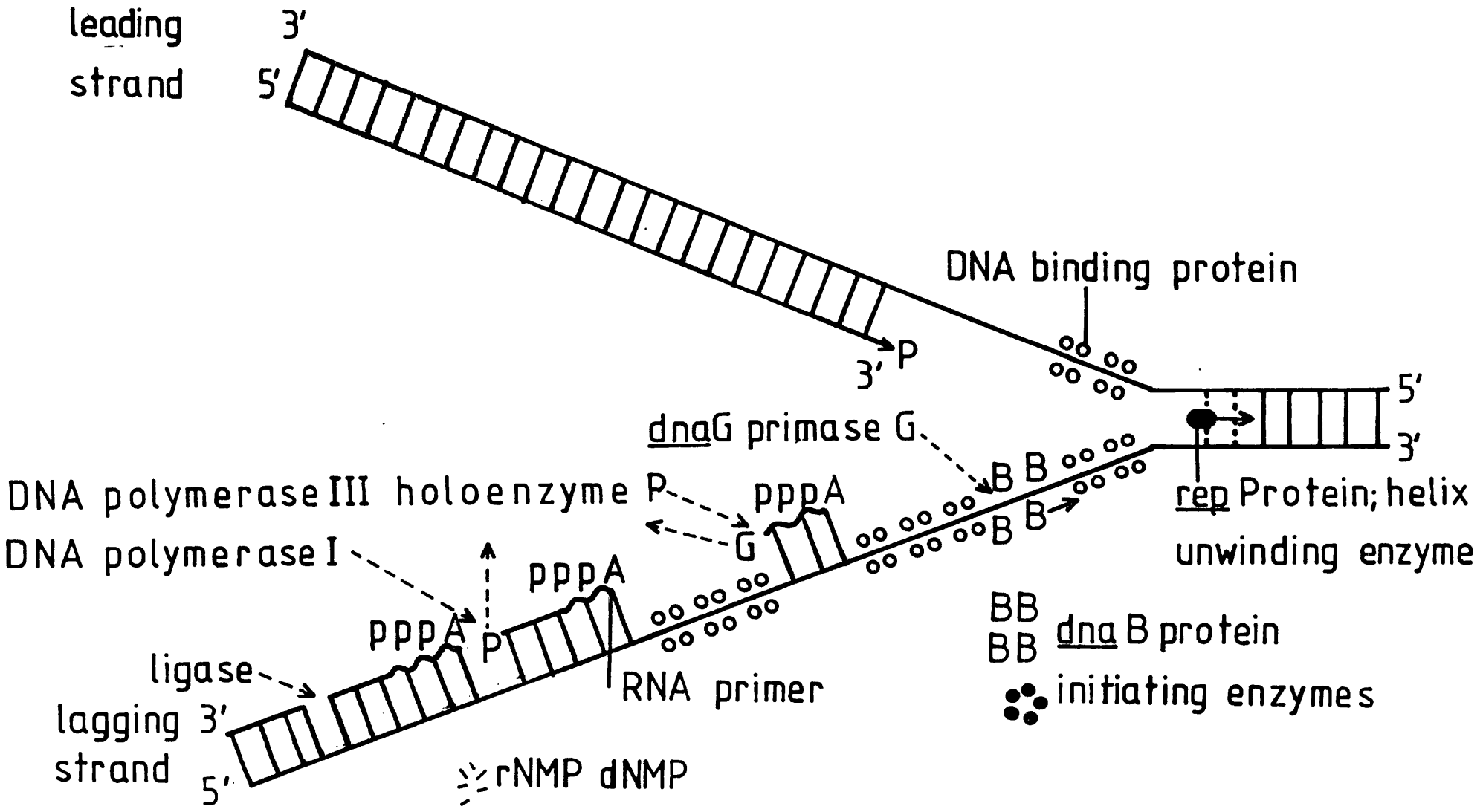
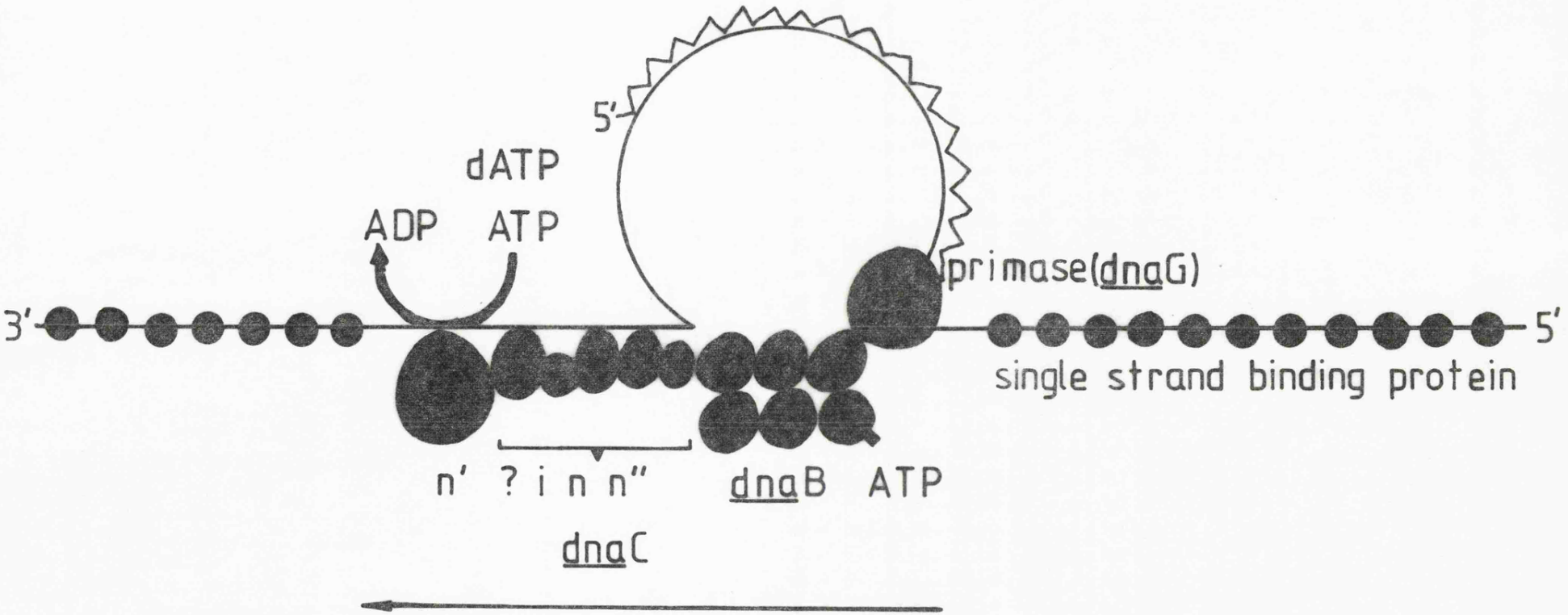

leading strand
3'
5'
3'
P
DNA binding protein
5'
3'
dnaG primase G
B B
B B
rep Protein; helix unwinding enzyme
DNA polymerase III holoenzyme
P
pppA
G
DNA polymerase I
pppA
pppA
P
ligase
RNA primer
lagging 3' strand
5'
rNMP dNMP
BB
BB
dna B protein
initiating enzymes

Figure 1.2) Priming complex.

A model of the prepriming and priming complex involved in the synthesis of short RNA primers required for discontinuous replication of the lagging strand of the *E. coli* chromosome. n, n', n'' and i are proteins involved in the prepriming complex and the association of the *dnaB* protein with the DNA. The *dnaG* protein joins the complex when this association has taken place and then synthesises the primer. The priming complex travels along the DNA from 5'-3' to facilitate multiple priming events (Kornberg, 1980).


5'
dATP
ADP ATP
primase(dnaG)
3'
5'
single strand binding protein
n' ? i n n"
dnaB ATP
dnaC

(Wickner et al.,1973; Wickner, 1977). The primer synthesised by dnaG primase has a free 3'-OH group which is used by DNA polymerase III holoenzyme to attach the first deoxyribonucleotide (Wickner, 1976; Wickner, 1977). Once DNA polymerase III has started synthesis this multimeric enzyme (Wickner and Kornberg, 1973; McHenry and Kornberg, 1977; McHenry and Crow, 1979) continues until it reaches the next primer. Excision of the primer and the filling in of the gaps created is thought to be performed by the 5'-3' exonuclease and 5'-3' polymerase activities of DNA polymerase I. The gaps between the newly synthesised DNA polymers are probably ligated by DNA ligase (Kornberg, 1980; see figure 1.1).

2,3) Termination of replication.

The Escherichia coli chromosome has a defined termination region mapping at 27-43 minutes, opposite the origin of replication, which appears to be preceeded by regions which inhibit the replication forks (Louarn et al., 1977; Kuempel and Duerr, 1978). However the termination of replication has not been studied in much detail and the involvement of chromosome encoded protein products is unknown (Kornberg, 1980).

3) PLASMID REPLICATION.

3,1) ColE1 plasmid replication.

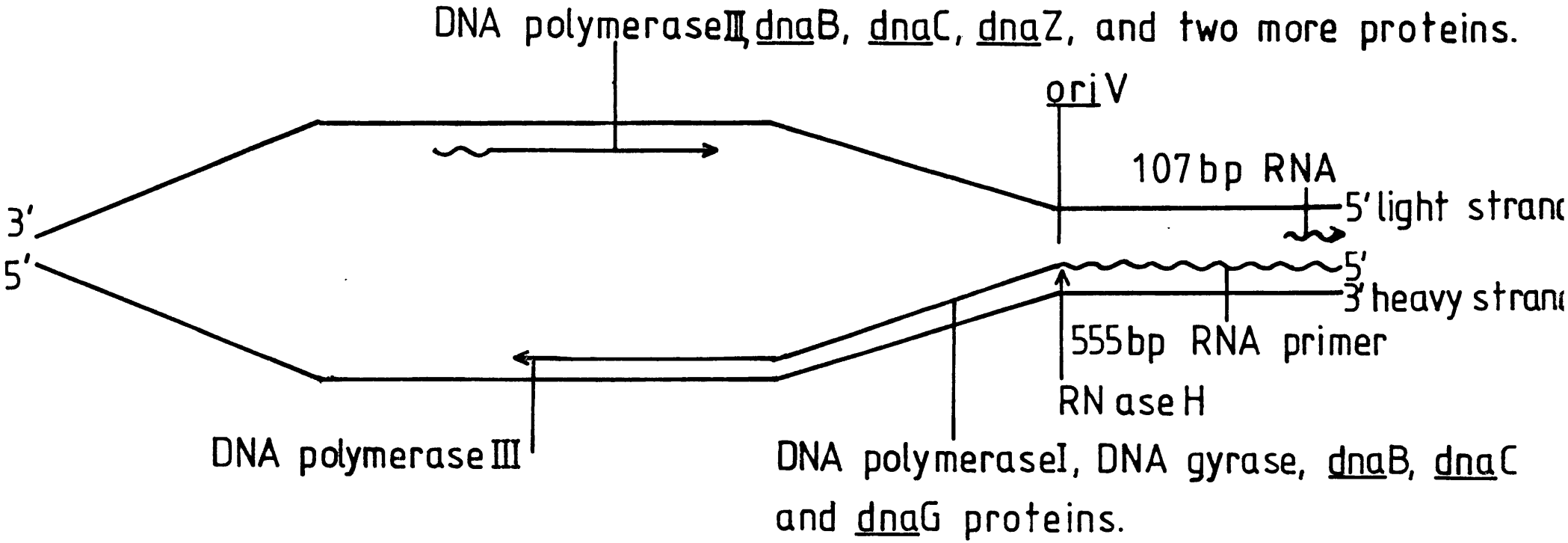
ColE1 is an example of plasmids which do not require plasmid encoded proteins (Donoghue and Sharp, 1978), or even protein synthesis for replication, (Clewell and Helinski, 1972), such as P15A (Goebel and Schroen, 1973), CloDF13 (Veltkamp et al.,1974), and ColK (Staudenbauer, 1978). ColE1 has a unique origin from which replication proceeds unidirectionally (Tomizawa et al., 1974). Initiation of ColE1 replication requires the transcription of the plasmid near the origin (oriV) by the host DNA-dependant RNA polymerase (Staudenbauer, 1976a; Itoh and Tomizawa, 1978). The RNA molecule hybridises to the heavy strand DNA and is accurately processed, probably by RNaseH (also host specified) to generate a 555 base primer RNA with a free 3'-OH group (Itoh and Tomizawa, 1978; Hillenbrand and Staudenbauer, 1982). The synthesis of this primer may be controlled by the synthesis of another RNA molecule, which is transcribed from the DNA in the opposite direction to the primer. The DNA encoding this RNA molecule is contained within that for the primer at its 5' end (Nijkamp and Veltkamp, 1981; see figure 1.3).

Once the primer has been synthesised

Figure 1.3) A model for the initiation of ColE1 replication.

A long RNA primer is synthesised by RNA polymerase and processed at oriV by RNaseH to 555bp. Extension of this primer requires DNA gyrase, dnaB, dnaC and dnaG proteins as well as DNA polymerase I. After the first 500bp only DNA polymeraseIII is required. Replication of the light strand requires dnaB, dnaC, dnaZ and two more proteins in addition to DNA polymerase III.

A model for the intiation of ColEI replication.



polymerisation appears to occur in two distinct steps. The first five hundred nucleotides of the light strand are synthesised by DNA polymerase I and require DNA gyrase and the dnaB, dnaC, and dnaG gene products (Staudenbauer, 1976b; Staudenbauer et al.,1979), then DNA polymerase III takes over. The heavy strand is synthesised discontiuously requiring in addition dnaC, dnaB, and dnaZ gene products and at least two more replication proteins (Sakakibara, 1978; Staudenbauer et al.,1978). Elongation of ColE1 is probably identical with the chromosome elongation mechanism.

Vegetative replication of ColE1 proceeds through a theta type structure (the Cairns mode of replication) where the unreplicated portion remains supercoiled (Fuke and Inselburg, 1972; Lovett et al.,1974). To generate two indepedent daughter ColE1 molecules the two parental strands separate before the replication fork reaches the termination of replication (Sakakibara and Tomizawa, 1974), for ColE1 this is the same as the origin of replication (Staudenbauer, 1978). This separation is probably brought about by the nick-unwind-rejoin activity of DNA gyrase (Gellert et al.,1977).

3,2) Replication of large plasmids.

One group of related plasmids, R1 (Kollek et al

.,1978; Diaz et al., 1981), R100-1, R12, R222, NR1 (Miki et al.,1980), and R6-5 (Timmis et al.,1978) have been studied extensively and the origins of replication of R100, R12, and R1, at least, are essentially identical (Armstrong et al.,1981). All are replicated unidirectionally from an origin of replication which apears to be located on the distal side of a transcription unit that encodes a small 11.5kd protein (Kollek et al.,1978) which is possibly involved in copy number control (Armstrong et al.,1981). A larger 33kd protein encoded in this region (repA gene product) may also be required for the replication of R100 (Armstrong et al.,1981). An RNA transcript from this region, which is very similar in all IncFII plasmids, is, thought to control the incompatibility and copy number functions, perhaps by affecting initiation of replication (Rownd et al.,1981). It has been demonstrated that the copA and copB genes code for functions which inhibit replication (Molin et al.,1981) and repA expression (Light and Molin, 1981). It is possible that plasmid replication control occurs at the level of repA expression (Light and Molin, 1981). However the mechanism of the initiation of replication remains unclear as does its relationship to copy number control and incompatibility (Kline et al.,1981; Eichenlaub et al.,1981).

R6K (IncX) is an unusual, large, multicopy (10-15 copies per chromosome) antibiotic resistance plasmid (38kb)

(Kontomichalou et al.,1970), which replicates in a "sequentially asymmetrically bidirectional" mode (Crosa et al., 1976), in which replication is initiated first in one direction and after the replication fork has reached the termination site the same origin initiates a second replication fork which moves in the opposite direction. In fact three origins of replication have been identified for R6K although only two are thought to be functional in the initiation of replication (Shafferman et al.,1981).

A 35kd trans-acting protein (the product of the pir gene) which is involved in the initiation of replication has been identified (Kolter et al., 1978). The origin sequence which is located proximal to the pir gene promoter contains seven tandem direct repeats of 22bp which are nearly identical in sequence; an eighth repeat overlaps the pir gene promoter sequence (Shafferman et al.,1981). It has been proposed that these repeats may constitute operator -like binding sites for the pir protein, which might then be acting as an autorepressor and initiator protein simultaneously (Shafferman et al.,1981). Thus the pir protein may have a functional role in the synthesis of the primer during the initiation of total plasmid DNA replication. However the pir protein does not regulate the initiation of plasmid DNA replication.

RK2 (IncP) is a 56.4kb low copy number plasmid (Meyer et al.,1977) which replicates unidirectionally from a fixed origin (Meyer and Helinski, 1977). RK2 carries two initiator genes, trfA and trfB, which encode trans-acting proteins required for the initiation of plasmid replication. However unlike the equivalent determinants of the F-like plasmids these genes do not map adjacent to the origin of replication (Kolter et al.,1978; Thomas et al.,1980). The products of the trfA and trfB genes may be able to complement replication deficiencies of temperature sensitive mutants of RP1, a closely related IncP plasmid (Thomas, 1981).

Very little is known about the initiation of replication of the other groups of large plasmids.

3,3) Incompatibility and segregation.

Initiation of plasmid replication requiring a trans-acting protein would lead to random replication of the plasmids of the same or similar type within a cell (Gustafsson et al.,1978). If two plasmids with closely related replication systems are present in the same cell segregation of the plasmids would inevitably occur as a consequence of the random initiation of replication in the plasmid population, hence the phenomenon of incompatibility

(Pritchard and Grover, 1981). Indeed the cop (control of copy number, presumably by control of rounds of replication) and inc (incompatibility) functions of F have been shown to map at almost identical positions (Rownd et al.,1981; Kline et al.,1981).

In order to maintain a plasmid in a population of cells at least one copy must be present in each daughter cell at cell division. This segregation of plasmids can also give rise to the phenomenon of incompatibility, and the genes responsible for this partition are designated par (Nordstrom et al.,1981). The method by which ordered segregation is brought about has not been elucidated, but several models have been proposed; the plasmids associate with specific binding sites on the membrane (Jacob et al.,1963; Timmis, 1979), or they associate with specific binding sites on the nucleoid chromosome DNA complex. Association of plasmid DNA with chromosome has certainly been demonstrated (Miller et al.,1978). In these models incompatibility is due to similar plasmids competing for a limited number of binding sites, ensuring ordered segregation of the bound plasmids and random segregation of the unbound plasmids. It has been proposed that incompatibility is the result of two independant functions, replication control and partition (Nordstrom et al.,1981). For more detailed discussion of the models for

incompatibility see the reviews of Pritchard (1978), Timmis (1979), and Pritchard and Grover (1981).

4) CONJUGATION.

Conjugation is a process by which plasmid DNA molecules are transferred from one cell to another. All of the studied conjugation systems (of IncF,I,N, and P plasmids) show three common features: an extracellular organelle called a pilus, which is essential for the recognition and mating pair (or aggregate) formation with potential donors (Achtman, 1975); a special system for conjugal DNA replication (which differs from the vegetative DNA replication) and transfer of the plasmid DNA; and "surface exclusion" proteins in the cell envelope, which prevent the cell from acting as a recipient to another donor cell carrying the same plasmid. Despite their similarities the conjugation systems of the IncF, I, N, and P plasmids are phenotypically distinct (Bradley, 1980a). The sex-pilus specific bacteriophages, such as If1, f1, PR64FS, M13, fd, etc, generally adsorb only to the pili determined by members of a few incompatibility groups (Bradley, 1981). The systems are genetically distinct as mutations in the tra genes of one incompatibility group of plasmids cannot generally be

complemented by plasmids from another incompatibility group (Cooke et al.,1970; Willetts, 1970).

4,1) F-like plasmid conjugation systems.

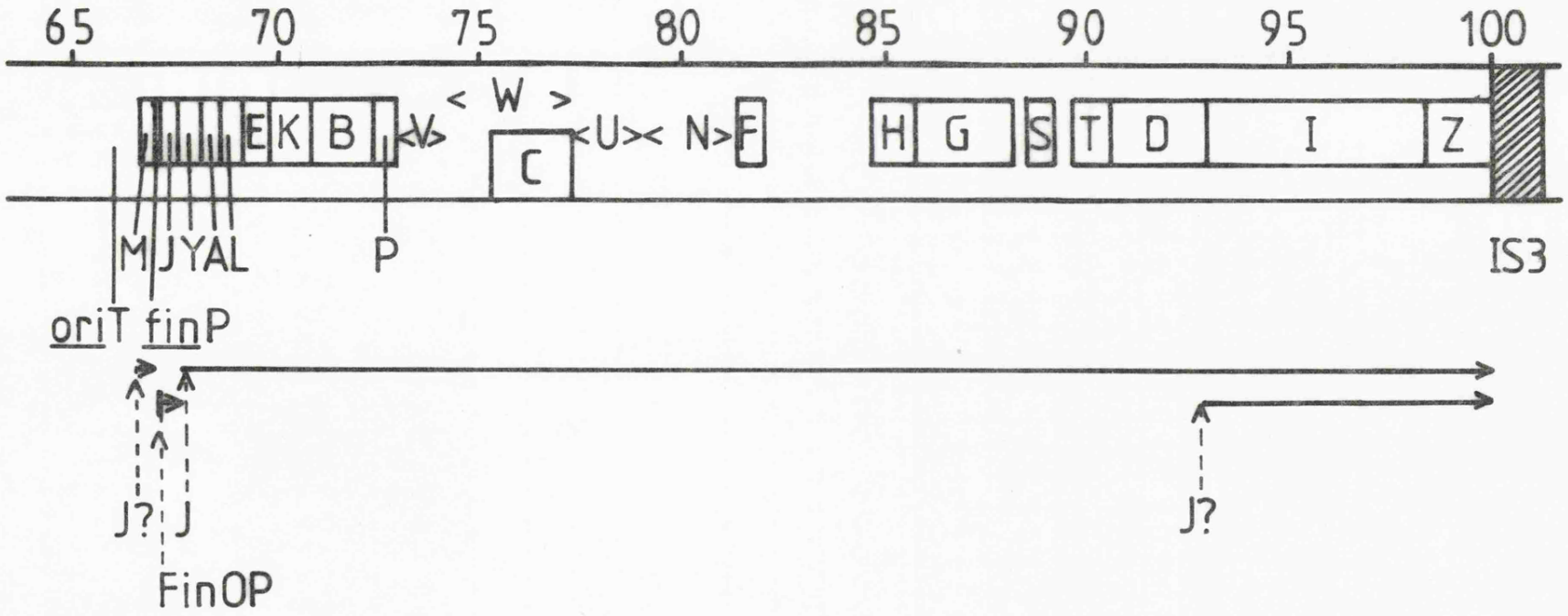
The F conjugation system is the best studied and very similar F-like conjugation systems are encoded by a large number of naturally occuring plasmids of IncF1, F11, F111, and F1V groups (Willetts and Skurray, 1980; Manning and Morelli, 1982). The pili encoded by these plasmids all adsorb F-specific bacteriophages, fd, f1, M13, etc, and most tra gene mutations can be complemented by members of the other plasmid subgroups (Willetts and Skurray, 1980). The four operons of the conjugational system of F occupy about a third of the plasmid DNA and contain at least nineteen contiguous genes. The gene products of traA, L, E, K, B, V, W, C, U, F, H, and G are required for pilus formation. The gene products of traN and traG are required for the stabilisation of mating pairs and the gene products of traM, Y, G, D, I, and Z are concerned with the conjugal DNA metabolism. The expression of most, if not all, of the tra genes is controlled by the gene product of traJ (Willetts and Skurray, 1980; see figure 1.4).

4,2) F pilus structure and function.

Figure 1.4) Map of the F conjugation region.

A map of the conjugation region of F after Willetts and Skurray (1980) and Willetts (1981). The scale is in kb with the boundary between IS3 and traZ being taken as 0kb and the size of plasmid F is taken as 100kb. Each letter represents one tra gene and the boxes indicate the extent of the genes. Where the extent of the genes is not known the approximate size is indicated by arrows. Tra operon transcription and the regulatory systems are drawn in below the map.

Map of the F conjugation region.



Cells carrying repressed F-like plasmids have one to three flexible F pili (Curtiss et al.,1969) of up to 20um in length (Lawn et al.,1967). The pili are phosphate-carbohydrate-protein complexes with a single polypeptide subunit, pilin, of 11-12kd (Armstrong et al.,1980). The subunits are thought to form a hollow cylinder 8nm in diameter with a 2nm axial hole (Folkhard et al.,1979). Sex pili from all F-like plasmids are biochemically similar and cells carrying two F-like plasmids assemble both subunit types into a single pilus (Lawn et al.,1971). In fact all twelve of the traY-Z gene products involved in pilus biosynthesis are interchangeable among F-like plasmids (Willetts and Skurray, 1980). The traE and traG gene products are inner membrane proteins, whereas the traJ, A, L, K, and B gene products are associated with the outer membrane of minicells (Kennedy et al.,1977). The traA gene product has been identified as pilin (Minkley et al.,1976) and presumably the products of the other pilus related tra genes are required for pilin modification, subunit assembly and outgrowth/retraction mechanisms.

The precise role of the pilus is not understood (Manning and Achtman, 1979), however pili are certainly involved in the early stages of mating pair formation (Achtman, 1975). Once initial contact has been established the extended pilus may act as a DNA transfer bridge (Ou,

1978). However there is normally a rapid conversion of pilus-wall to wall-wall contact, perhaps by pilus retraction (Curtiss, 1969), and most or all DNA transfer may occur in the absence of extended pili (Ou, 1978).

4,3) Conjugal DNA metabolism.

Conjugal DNA transfer and donor conjugal DNA synthesis are probably initiated at a nick in a specific DNA sequence, known as oriT, on the transferred strand (Reeves and Willetts, 1974; Willetts and Skurray, 1980). The site specific endonuclease responsible for this nick is thought to be the product of the traY and traZ genes, the first and last genes in the traY-Z operon (Everett and Willetts, 1980). It has been suggested that when mating pair formation occurs the traM and traI gene products displace the traY,Z endonuclease from oriT thereby triggering donor conjugal DNA synthesis and DNA transfer (Everett and Willetts, 1980). A specific pre-existing strand of plasmid DNA is then transferred in unit lengths into the recipient cell with its 5' end leading (Ohki and Tomizawa, 1968). Transfer can occur in the absence of conjugal DNA synthesis in the donor, however it is thought that conjugal DNA synthesis in the donor generally occurs concomitant with transfer (Kingsman and Willetts, 1978). Conjugal DNA synthesis in the donor cell probably occurs by the rolling circle mode (Gilbert and

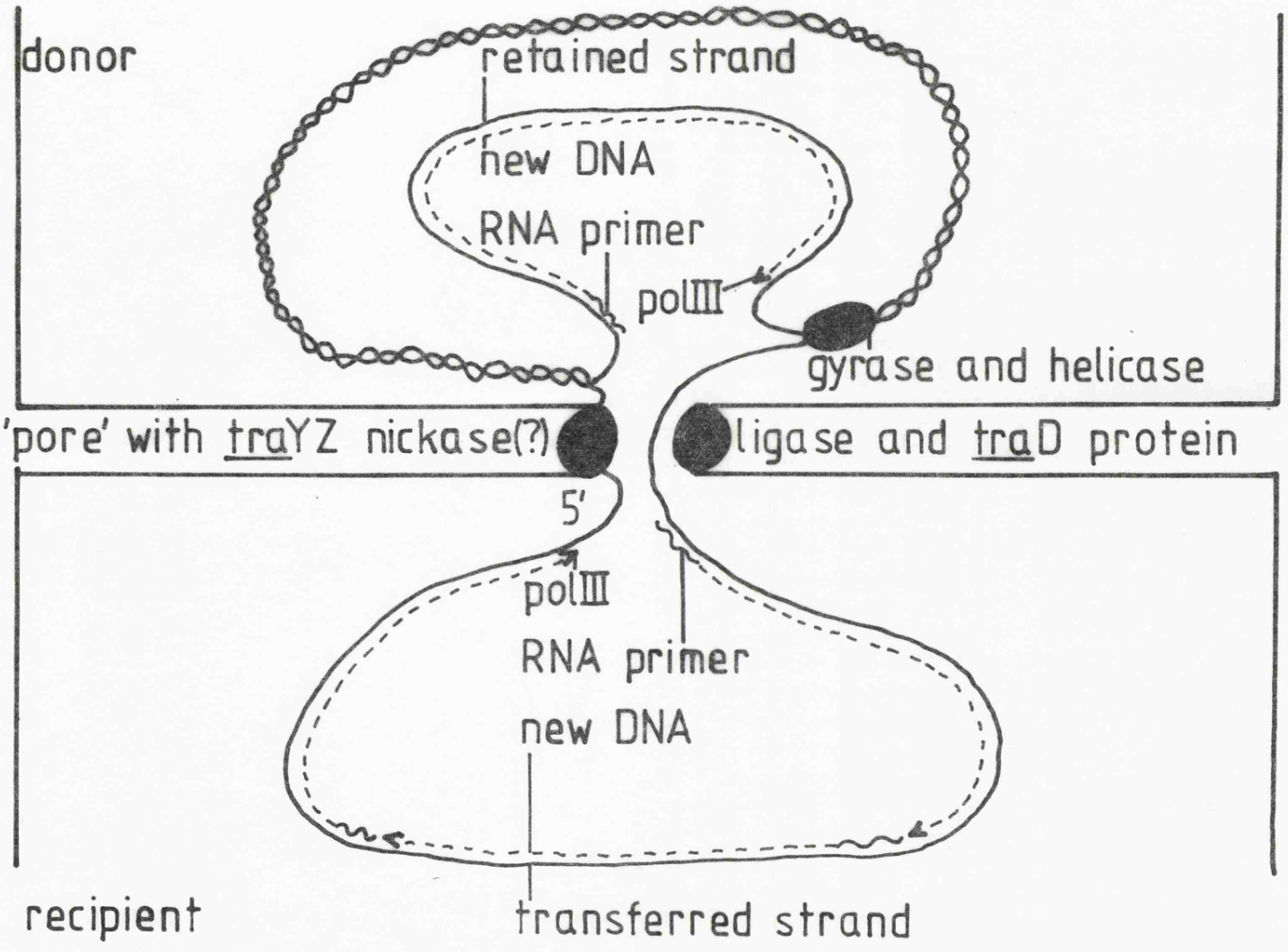
Dressler, 1968) rather than the Cairns mode of replication. The 3' terminus generated by the presumptive nick cannot prime conjugal DNA synthesis in the donor and an untranslated RNA primer is proposed (Willetts, 1977; see figure 1.5).

It has also been proposed that both the 3' and 5' termini of the transferred strand are linked to protein(s) located within the fused membranes of the donor and recipient (Kingsman and Willetts, 1978). The 5' linkage might conserve the energy of the cleaved phosphodiester bond allowing this energy to be used for the religation of the 5' end to the 3' end (Guiney and Helinski, 1975; Kingsman and Willetts, 1978). It is not known whether synthesis of the complementary strand occurs before or after recircularisation, but recircularisation does not require expression of any plasmid tra genes in the recipient cell (Hiraga and Saitoh, 1975). There is some evidence that complementary strand synthesis and recircularisation occur while the plasmid molecule is still bound to the membrane (Falkow et al.,1971). Recipient conjugal DNA synthesis is expected to be discontinuous and in F-like plasmids may be primed either by dnaG primase plus the dnaB gene product, or by RNA polymerase. The elongation is carried out by DNA polymerase III (Wilkins and Hollom, 1974; see figure 1.5).

Figure 1.5) A model for F conjugation.

The plasmid is shown in the process of transferring a single strand and synthesising replacement strands in the donor and recipient. However these processes may not necessarily occur concomitantly. The traYZ nickase may be involved in the nicking of the plasmid DNA at the origin of transfer and the transferred strand may also be religated by the action of the traYZ proteins. The primers required for conjugal DNA synthesis in the recipient are synthesised by RNA polymerase or dnaG primase (Willetts, 1981).

A model for F conjugation.



4,4) Active disaggregation and surface exclusion.

When DNA transfer is complete the expression of the plasmid genes in the recipient leads to active disaggregation of the mating bacteria and the establishment of surface exclusion (Willetts and Skurray, 1980). The plasmid gene(s) or gene product(s) responsible for the disaggregation have not been identified, but it has been suggested that the plasmid directs the synthesis of enzymes which modify the cell envelope and render mating aggregates unstable (Achtman, 1975).

A bacterium which carries an F plasmid is unable to act as an efficient recipient in matings with bacteria carrying the same or related plasmids (Sheehy *et al.*,1972). This phenomenon is known as "surface, or entry, exclusion" and is not a result of the F pili. Two *tra* gene products, *traS* and *traT*, probably jointly mediate surface exclusion. The *traT* gene product is thought to primarily inhibit stable mating aggregate formation and the *traS* gene product to limit the DNA transfer by affecting triggering of conjugal DNA metabolism (Achtman *et al.*,1980).

4,5) Regulation of transfer.

The transfer genes of F are constitutively

expressed and hence it is derepressed for transfer, while in contrast most F-like plasmids are repressed for transfer (Meynell *et al.*,1968). The expression of the *tra* operons is controlled by the products of the two structural genes of the FinOP fertility inhibition system: the *finO* gene product is relatively non-specific in the F complex; but several plasmid subgroup specific alleles of the *finP* gene have been identified (Willetts and Skurray, 1980). F is *finO finP+*, hence the constitutive expression of its transfer genes. The transcription of *traJ* is reduced by the FinOP inhibitor (the two gene products act in concert) and hence negative control of the expression of the positively regulating *traJ* gene product prevents expression of the *traY-Z* operon (Willetts, 1977). The product of the *traJ* gene possibly also positively regulates the transcription of the *tra M* gene and *tra I-Z* operon (see figure 1.4).

Although the transfer systems of the F-like plasmids are generally repressed (Meynell *et al.*,1968), these plasmids are able to spread infectiously through a bacterial population (Broda, 1975). When a recipient recieves a plasmid the FinOP system takes up to six hours to be fully expressed thus the plasmids are transiently derepressed and the cells function as efficient donors (Cullum *et al.*,1978).

4,6) IncI Plasmid transfer.

There is a great deal of evidence that the transfer systems of the plasmids of the F and I incompatibility groups are phenotypically and genetically distinct. Pili specified by the I-complex plasmids do not adsorb F-pilus specific phages and F pili do not adsorb I-pilus specific phages (Meynell and Lawn, 1968). F and I pili do not cross react serologically (Lawn and Meynell, 1970; Bradley, 1980a) and are structurally distinct (Lawn et al.,1967). Transfer deficient mutants of the IncIα plasmid R64drd11 are not complemented by Flac and several Flac tra- mutants are not complemented by R64drd11 (Cooke et al.,1970; Willetts, 1970). However it has been shown that R64drd11 is transferred in a very similar way to the F-like plasmids (Vapnek et al.,1971). A single strand of R64drd11 DNA of unit length (Boulnois and Wilkins, 1978) is transferred 5'-3' asymmetrically from a unique origin of transfer, the oriT (Fenwick and Curtiss, 1973a,b). More recent experiments have shown that ColIb-P9 (IncIα) is transferred in a similar way (Boulnois, 1980).

A supercoiled circular DNA-protein relaxation complex has been identified for ColIb-P9 (Clewell and Helinski, 1970) and there is evidence that a similar structure observed for ColE1 is involved in plasmid transfer

by generating a specific single strand nick in the DNA (Staudenbauer, 1978). Thus unit lengths of plasmid DNA for transfer may be generated in one of two ways; the presumed 3'-OH group generated by the nicking event at the regenerated oriT may be unavailable as a primer for a DNA polymerase, or alternatively a nicking event at the regenerated oriT releases the displaced strand (Boulnois et al.,1979). After the transfer of one strand of an IncIα plasmid there is a delay before its replacement strand is transmitted (Fenwick and Curtiss, 1973b). However donor conjugal DNA synthesis occurs concomitantly with transfer and the synthesis of a new protein(s) (which requires double stranded DNA) is thought to be required for the transfer of the next strand (Fenwick and Curtiss, 1973b; Boulnois et al., 1979). The ColIb-P9 gene products involved in events in the donor have not yet been identified and the mechanism of donor conjugal DNA synthesis and transfer is not understood (see figure 1.6).

Complementary strand synthesis in the recipient requires a primer, because the transferred DNA is a single strand. In the absence of dnaG primase and RNA polymerase synthesis of the complementary plasmid strand still occurs (Boulnois and Wilkins, 1979). This DNA synthesis is thought to be primed by primers synthesised by a novel plasmid encoded primase (see section 6). Elongation of the new DNA

Figure 1.6) A model for ColIb-P9 conjugation.

A) The relaxation protein(s) binds to the plasmid at the origin of transfer (oriT).

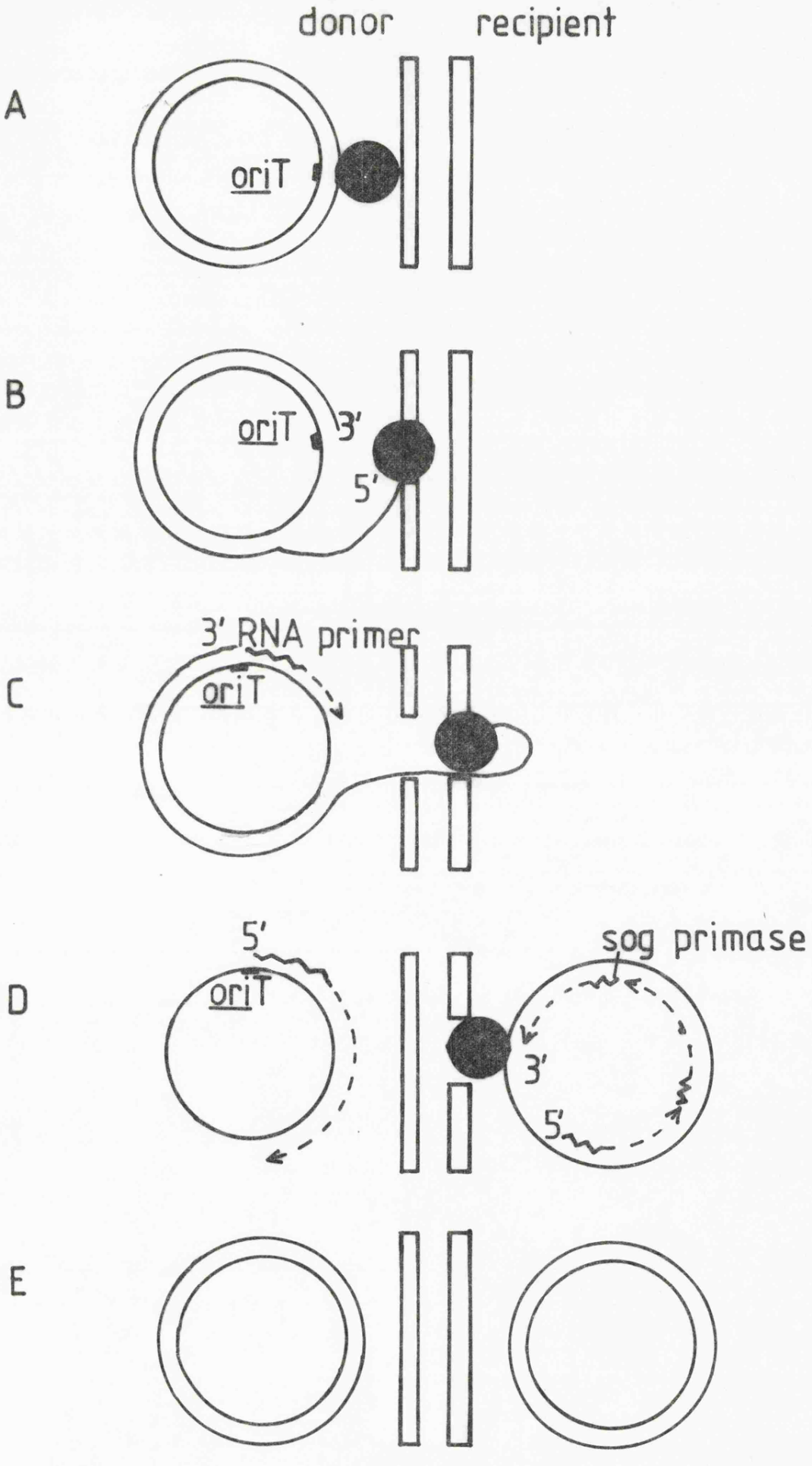
B) The relaxation protein(s) nicks the plasmid at oriT to generate a single strand break.

C) The transferred strand is carried across into the recipient as a relaxation complex or with the plasmid primase bound or both. An RNA primer is synthesised on the nontransferred strand, by dnaG primase or possibly by plasmid primase.

D) Conjugal DNA synthesis in the donor continues with DNA polymerase III. In the recipient the complementary strand synthesis is primed by RNA primers synthesised by plasmid primase (sog). DNA polymerase III continues the elongation.

E) Conjugation and DNA synthesis is now complete and both cells have intact copies of the plasmid.

A model for ColIb-P9 conjugation.



strand is carried out by DNA polymerase III (Wilkins and Hollom, 1974). The conversion of transferred ColIb-P9 DNA into covalently closed circular monomeric molecules does not require the synthesis of plasmid specified products in the recipient (Boulnois and Wilkins, 1978; see figure 1.6).

4,7) Conjugational systems of other plasmids.

The transfer genes of RP4 (IncP) have been mapped and the minimum of five complementation groups (some of which contain more than one phenotypic class) fall into two separate regions on either side of the kanamycin resistance gene (Barth and Grinter, 1977; Barth et al.,1978a). A further tra region, tra3, involved in surface exclusion has also been identified (Barth, 1979; see figure 1.7). Thus RP4 appears to have three tra regions rather than one as in plasmid F (Willetts, 1980). A similar result has also been found for RK2, a plasmid which is probably identical to RP4 (Meyer and Shapiro, 1980). A DNA protein relaxation complex has been identified for RK2 and the site-specific nick is thought to be in the region of the proposed origin of transfer (Guiney and Helinski, 1979).

The regions encoding conjugal transfer genes on the related IncP plasmid (R751) have also been mapped and two tra regions are proposed (Meyer and Shapiro, 1980). The

Figure 1.7) Location of the known genes of RP4.

Redrawn from the maps of Barth *et al*. (1978) and Lanka and Barth (1981). The distances are in kb from the unique *Eco*RI site of RP4.

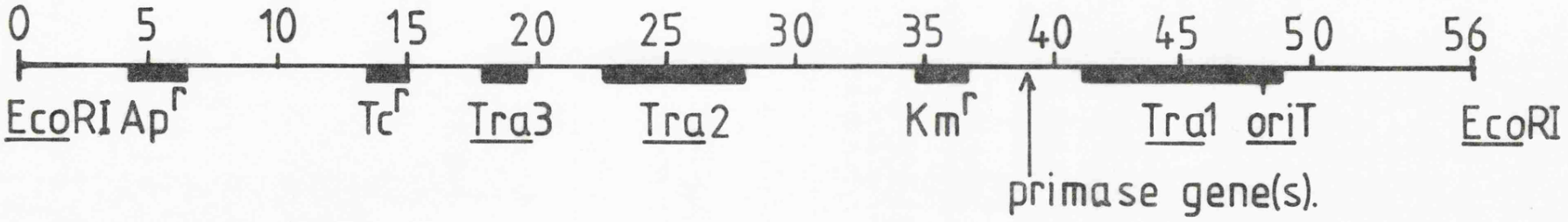
*Tra*1, 2 and 3- the approximate extent of the three regions of transfer genes mapped for RP4.

oriT- the origin of transfer, relaxation site.

Ap, Tc and Km- the approximate extent of the ampicillin, tetracycline and kanamycin resistance genes.

The primase gene(s) are located between the kanamycin resistance genes and *tra*1.

Location of the known genes of RP4.



tra regions of RK2 and R751 are trans-complementing suggesting that both of the plasmids carry closely related transfer systems (Meyer and Shapiro, 1980). The tra genes of an IncP-10 plasmid (R91-5) have also been mapped and all ten tra cistrons fall in one of the two proposed tra regions. Tral contains the genes for conjugal DNA metabolism and tra2 the genes for pilus synthesis and function (Moore and Krisnapillai, 1982a,b).

Two relaxation nick sites have been located on R6K close to the oriV sites and implicated in conjugal transfer (Nordheim et al.,1980). It is proposed that the recipient cell receives a single plasmid DNA strand which has been primed at its proximal end by a short stretch of complementary DNA synthesised, from the oriV to the oriT/nic site, in the donor by the host replication enzymes (Nordheim et al.,1980; see figure 1.8).

4,8) Mobilisable plasmids.

ColE1 is a non-conjugative plasmid which can be mobilised with a high efficiency by F and most F-like plasmids (Reeves and Willetts, 1974), and also by plasmids of IncI, P, and W (Warren et al.,1979). ColE1 forms a relaxation complex of three proteins and a supercoiled plasmid molecule (Lovett and Helinski, 1975). The plasmid

Figure 1.8) A model for R6K conjugation.

A) Initiation events at the origin of vegetative replication (oriV) result in the synthesis of a short DNA sequence from oriV to oriT. This complementary strand primer is synthesised on the strand which is nicked during relaxation.

B) The template strand is nicked and a relaxation protein is covalently linked to the 5'-terminus of the nicked strand.

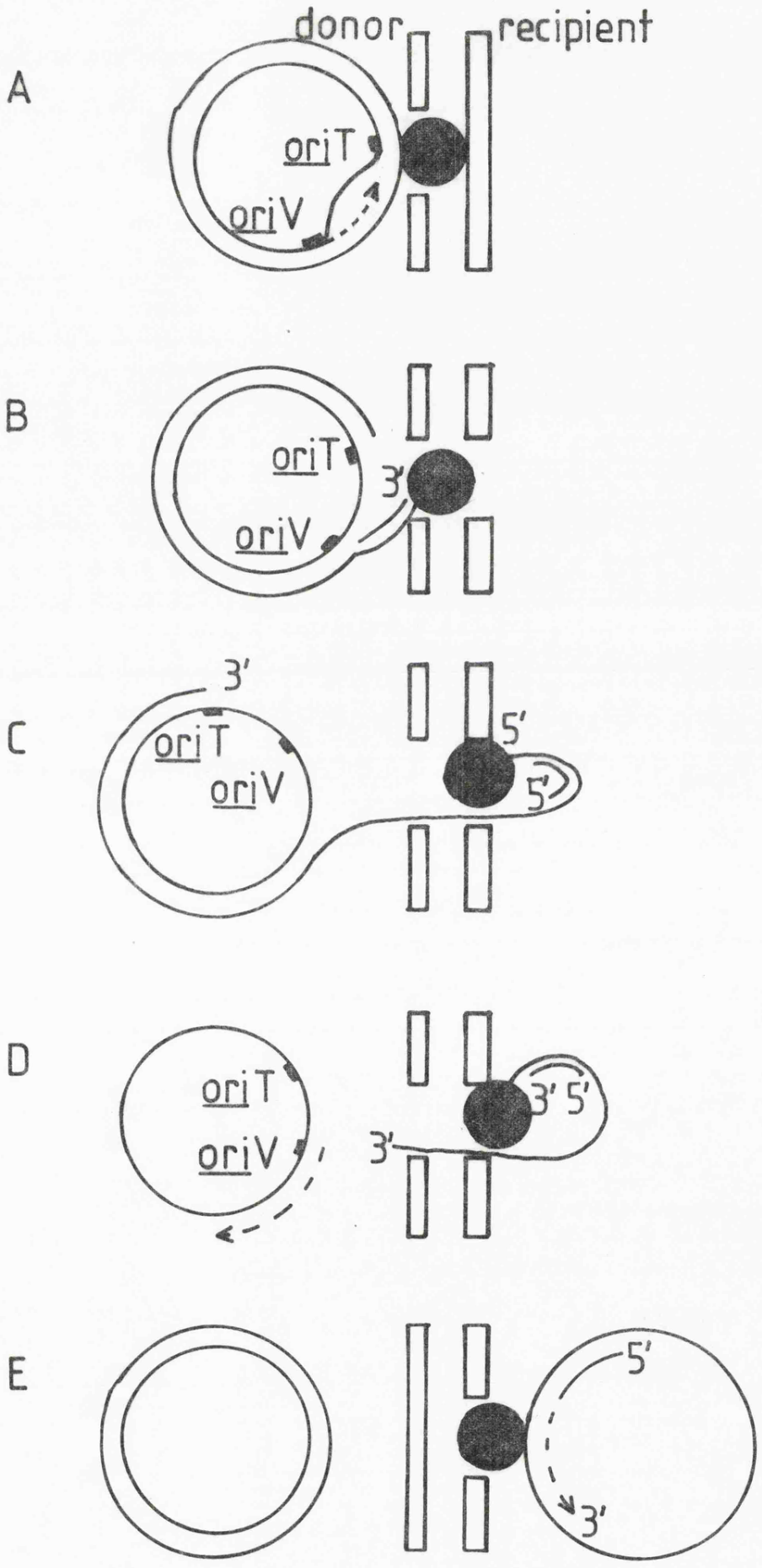
C) The 5'-terminus of the nicked strand plus the associated relaxation protein(s) and complementary strand primer are transported through the two cell envelopes.

D) The remainder of the strand is transferred. Replacement strand synthesis starts from the origin of vegetative replication in the donor.

E) The 3' and 5' ends of the transferred strand are rejoined by the relaxation protein(s). Synthesis of a DNA strand complementary to the transferred strand then occurs by extension of the complementary strand primer.

DNA transfer is represented as a simple peeling off of the nicked strand, rather than involving a rolling-circle type intermediate. Redrawn from Nordheim et al. (1980).

A model for R6K conjugation.



molecule in the relaxation complex is nicked in the presence of sodium dodecyl sulphate, probably at oriT (Warren et al.,1978). A 60kd protein becomes covalently bound to the DNA at the 5' terminus of the DNA (Guiney and Helinski, 1975). ColE1 mutants which do not form a relaxation complex are non-mobilisable (mob-) (Dougan and Sherratt, 1977). It has been proposed that the 60kd protein and the nicked ColE1 DNA are an intermediate in the nicking-religation steps required for conjugal DNA transfer (Staudenbauer, 1978). ColE1 transfer also requires all of the F tra gene products except traM, I, and Z (traY has not yet been tested) (Willetts, 1981). The ColE1 mobilisation genes may carry out the nicking, triggering, and recircularisation steps while the F tra gene products carry out the other steps.

ColE1 mobilisation does not require covalent union with the conjugative plasmid (Reeves and Willetts, 1974), and this is true of all mobilisable plasmids such as pSC101 and RSF1010 (Nordheim et al.,1980) which carry their own oriT and can utilise the conjugational systems of a wide variety of plasmids (Willetts, 1981). Non-mobilisable plasmids, such as the laboratory construct pBR328, do not have an oriT and can only be mobilised via a covalent interaction with the mobilising plasmid (Willetts et al.,1981).

5) PLASMID ENCODED ENZYMES INVOLVED IN DNA METABOLISM.

5,1) Plasmid encoded nucleases and recombination enzymes.

In 1965/6 it was reported that strains of Salmonella typhimurium carrying the IncIα plasmid ColIb-P9 were more resitant to u.v. light and had an increased u.v.-induced mutation rate (Howarth, 1965; Howarth, 1966). Since then a similar u.v. protection activity has been reported for many plasmids, mainly from IncN and the I-complex (including R16, IncB) (Molina et al.,1979; Chernin and Mikoyan, 1981). Such plasmids are also able to repair single stranded gaps in DNA by an unknown mechanism, which may involve a stimulation of the host repair systems rather than a direct action by a plasmid encoded product (Chernin and Mikoyan, 1981). It has also been reported that some plasmids increase the sensitivity of their host to u.v. light. This increased sensitivity has been attributed to a plasmid encoded nuclease which degrades the damaged DNA (Alper et al.,1972).

Plasmid R46 carries one gene, uvp, which is responsible for the increased u.v. induced mutagenesis and protection against u.v. killing (Mortelmans and Stocker, 1979). R46 also increases the spontaneous mutation rate of its host (Baburdi and Monti-Bragadin, 1977) and it is

hypothesised that R46 encodes a constitutive repair system that cooperates with the inducible S.O.S. repair system (Chernin and Mikoyan, 1981).

Recently it has been shown that R1-19 encodes a novel ATP-dependent exonuclease activity (Chernin and Ovadis, 1980). It is speculated that this plasmid product, probably an exonucleaseV-like nuclease, plays a regulatory role in several repair pathways of the host (Chernin and Mikoyan, 1981).

F has been shown to carry genes, *ferA* and *ferB*, which are involved in *recA*-independent excision of transposons Tn*5* and Tn*10* from the chromosomal or plasmid DNA and *recA* dependent recombination between two IS*3* elements (Hopkins *et al.*,1980). The product of *ferA* is thought to be a site specific nuclease which excises IS*3* DNA and stimulates recombination (Miller and Cohen, 1980).

5,2) Plasmid encoded DNA replication enzymes.

The use of *E.coli* mutants with temperature sensitive defects in essential DNA replication genes has proved a fruitful method of demonstrating the presence of plasmid encoded enzymes involved in DNA replication.

Mutations in E.coli dnaA gene allow residual synthesis of DNA but are defective in initiation of rounds of DNA replication; thus when the current round of replication is completed no further rounds of replication are initiated (Kuempel, 1969; Wechsler and Gross, 1971). Many plasmids are able to integratively suppress the DnaA- phenotype, presumably by providing the chromosome with new initiator and replicator loci. The replication of such cointegrates acquires many of the features typical of plasmid replication (Sotomura and Yoshikawa, 1975). So far the list of plasmids includes, F and the F-like plasmids (Wechsler and Gross, 1971; Nishimura et al.,1973), R483 and R144 (IncIα, Datta and Barth, 1976), R6K and R485 (IncX, Sotomura and Yoshikawa, 1975; Hochmannova et al.,1982), RP1 (IncP), and ColE1 (Sasakawa and Yoshikawa, 1980). Bacteriophages P1 and P2 (Lindahl et al.,1971), which can replicate in a stringent fashion as plasmid-like molecules under lysogenic conditions, can also suppress the dnaA mutation by integration (Chesney and Scott, 1978). The integrative suppression of some dnaC mutations by F plasmids has also been reported (Beyersmann et al.,1974).

Some E.coli dnaB mutants show virtually immediate shutdown of DNA synthesis at 42 °C, while others exhibit a considerable residual DNA synthesis (Wechsler and Gross, 1971). The product of the dnaB gene is involved in the

promotion of primer synthesis by the dnaG primase (see figures 1.1 and 1.2). Presumably some mutants allow residual priming while others totally cease to be effective in promoting the binding of dnaG primase to the DNA. However dnaB mutants defective in DNA initiation have also been isolated (Zyskind and Smith, 1977).

Bacteriophage P1 encodes a dnaB analogue (D'Ari et al.,1975) which is synthesised at repressed levels in lysogens carrying the wild type phage. The phage P1 analogue of the dnaB protein has been isolated and characterised (Lanka et al.,1978; Schuster et al.,1978; Touati-Schwartz, 1979). In 1977 Wang and Iyer proposed that the majority of wildtype conjugative plasmids have the ability to suppress, or enhance, the temperature sensitivity of dnaB mutations of E.coli K-12. They suggested that there was an interaction between a plasmid specified product and the host dnaB protein (Wang and Iyer, 1977). In a further study Wang and Iyer chose to look at plasmids R64drd11 and R144drd3 (IncIα), R386 (IncF1), RP4 (IncP), and R621a (IncIɣ) in more detail. From the indirect evidence that the plasmids did not encode an amber suppressor but that they were able to rescue dnaB266, which has an amber mutation in the dnaB gene, Wang and Iyer again proposed that these plasmids carry ban genes (dnaB analogue) (Wang and Iyer, 1978). However there is no physical evidence for ban genes or proteins associated with

any of these plasmids.

The product of the dnaE gene of E.coli K-12 is a subunit of DNA polymerase III holo-enzyme and hence complete inactivation of the gene product will lead to a complete and immediate halt to DNA synthesis (Wechsler and Gross, 1971). The suppression of a temperature sensitive mutation in dnaE by R6K (IncX) has been reported (Chernin and Mikoyan, 1981). However the precise mechanism of this suppression is unknown. R205 of S.typhimurium (McPhee, 1974) and pMG2 of P.aeruginosa (Lehrbach et al.,1977) may encode a DNA polymerase I activity. However there is some debate as to whether the plasmids encode an analogue of DNA polymerase I or not (Chernin and Mikoyan, 1981).

6) PLASMID ENCODED dnaG ANALOGUES.

6,1) ColIb-P9 encoded DNA primase.

The product of the dnaG gene of E.coli K-12 is the DNA primase responsible for the synthesis of the primers required for the discontinuous synthesis of the lagging strand during DNA replication. The dnaG primase is a single polypeptide of 60kd and is present in 50-100 copies per cell (Rowen and Kornberg, 1978; also see section 2).

In 1975 Wilkins published the first observations that plasmids were able to suppress the temperature sensitive dnaG3 mutation of E.coli K-12. ColIb-P9drd1, R144drd3, and R64drd11 (IncIα), which are all mutants derepressed for transfer functions, were shown to suppress the dnaG3 mutation much more efficiently than the wildtype plasmids, while three F-like derepressed conjugative plasmids, F101, R100drd1, and R1drd16 were not able to suppress the dnaG3 mutation at all (Wilkins, 1975). Wilkins proposed that the suppression of the dnaG3 mutation was due to a protein synthesised from a gene in the tra operon, or operons, of IncIα plasmids. He also proposed that the product of the plasmid gene could substitute for the bacterial dnaG gene product during DNA replication (Wilkins, 1975).

Almost three years later Sasakawa and Yoshikawa published the results of a more extensive survey of plasmid incompatibility groups (Sasakawa and Yoshikawa, 1978). They confirmed that R64drd11 suppressed the dnaG3 mutation and that IncF plasmids did not. They also showed that representatives of IncM, S, W, N, H, T, and X did not suppress the dnaG3 mutation. An IncJ plasmid, R391, and R621a, an IncIδ plasmid closely related to the IncIα plasmids, were also able to suppress the dnaG3 mutation. R621a was studied in more detail and the efficiency of

suppression was correlated with the level of derepression of pilus synthesis (and presumably the transfer genes or tra operons). Sasakawa and Yoshikawa (1978) proposed three requirements for the complete expression of suppressibility; an authentic derepressed mutation of the conjugal fertility system, a further mutation leading to a far higher degree of derepression of this system, and an intact host recA function.

In 1979 Lanka and co-workers demonstrated that cell-free extracts of strains containing IncIα plasmids R64drd11, R144drd3, and ColIb-P9drd1 were able to prime rifampicin resistant DNA synthesis on single stranded DNA phages ØX174, fd, and G4. The priming activity of extracts of strains containing repressed R64, R144, and ColIb-P9 was very much less than that of extracts of strains containing the derepressed mutants. They were unable to detect any priming activity in extracts of strains containing R1drd16 or R100drd1, both derepressed IncF plasmids (Lanka et al.,1979). They purified the proteins responsible for the priming activity and the purified enzyme extracts consisted mainly of two major polypeptides of apparent molecular weight 180,000 and 140,000. The R64drd11 encoded DNA primase was able to substitute in vitro for the functions of E.coli dnaB, dnaC and dnaG proteins for ØX174 replication, of E.coli RNA polymerase for fd replication and of E.coli dnaG

protein for G4 replication. The enzyme required all four ribonucleoside triposphates and co-operated specifically with DNA polymerase III (Lanka et al.,1979).

The following models for plasmid encoded primase involvement in DNA replication were proposed by Lanka et al. (1979). The enzyme could be cotransferred with the DNA from the donor to the recipient cell, where it synthesises the primer(s). Alternatively, it could generate the primer(s) in the donor, and the transferred strand would arrive in the recipient cell complete with primers attached.

Also in 1979 Boulnois and Wilkins observed that synthesis of DNA complementary to the transferred strands of R144 (IncIα) still occurs when the dnaG primase and RNA polymerase are inactive in both donors and recipients (Boulnois and Wilkins, 1979). They proposed that the enzyme responsible for synthesising the primers was supplied by the donors and was also responsible for the suppression of the dnaG phenotype.

In order to study this phenomenon more closely Boulnois and Wilkins cloned the soq (suppression of dnaG) function of ColIb-P9 onto a multicopy plasmid vector (pBR325) to generate a plasmid, pLG215, containing a 7.9kb EcoRI generated fragment of the parent plasmid (Wilkins et

al.,1981). Crude cell extracts of strains of E.coli containing this recombinant plasmid, which suppressed the dnaG3 mutation of E.coli K-12, were able to prime DNA synthesis on single stranded phage fd template (Wilkins et al.,1981). The cloned fragment codes for two polypeptides of apparent molecular weights 240,000 and 180,000. However due to the instability of the large polypeptides a strain carrying a plasmid with a temperature induced deletion of the 7.9kb EcoRI fragment, pLG214, which synthesised two polypeptides of reduced size, apparent molecular weights 87,000 and 42,000, was used as the source of protein to characterise the enzyme. This plasmid also suppressed the dnaG3 mutation and extracts were able to prime DNA synthesis in vitro. The priming activity of the crude cell extracts was correlated with the presence of the 87,000 dalton protein and purified protein was able to prime DNA synthesis on single stranded phage fd template (Wilkins et al.,1981). Antiserum raised against this protein inhibited the activity of the wild-type primase, but did not inhibit DNA synthesis on viral strands of phages ØX174 and G4 in an in vitro assay with an extract of dna+ cells. Thus the ColIb-P9, or soq, primase is not antigenically related to the E.coli dnaG primase (Wilkins et al.,1981). The antiserum also inhibited the activity of primases specified by two other IncIα plasmids, R144drd3 and R64drd11, and the IncIγ plasmid R621a. Thus it was assumed that all four plasmids encode

similar or identical primases (Wilkins et al.,1981).

In vitro the primase directs the synthesis of a primer of more than ten bases, but can utilise d(3')GTTTTTTT(5') or even d(3')GT(5'), but not d(3')TG(5'), as templates. The primer always has a cytidine or cytidine 5'-monophosphate at the 5' terminus, unlike the dnaG primase generated primer which has an adenosine nucleotide. In vitro the primer can be utilized by purified preparations of phage T7 DNA polymerase, phage T4 DNA polymerase, E.coli DNA polymerase I and E.coli DNA polymerase III (E.Lanka, private communication). ColIb-P9drd1 and R64drd11 cannot suppress the temperature sensitivity of DNA replication in dnaB70 (Boulnois et al.,1979) and dnaB43 mutants (Fenwick and Curtiss, 1973a), contrary to the report for R64 by Wang and Iyer (1977). This implies that plasmid primase cannot bypass the requirement for dnaB protein in chromosomal replication. However R64drd11 primase acts independently of the dnaB protein in the priming reaction of single-stranded DNA phage ØX174, while dnaG primase requires dnaB protein to prime DNA synthesis on single-stranded DNA of phage ØX174 (E.Lanka, private communication).

The phenonenom of suppression of the dnaG3 mutation by the IncIα plasmids is presumably due to the substitution of the sog primase for the E.coli dnaG primase

in the discontinuous synthesis of the bacterial chromosome, and any primase dependant event that may occur at the origin of DNA replication. The results of the experiments with dnaB mutants indicate that the sog primase does not require dnaB protein for priming DNA synthesis, but that it cannot substitute for the dnaB protein in chromosomal replication. The plasmid primase is able to substitute for the dnaG primase in the replication of phage DNA and of ColE1 replicons in vivo (E.Lanka, private communication). Thus the IncIα plasmid encoded DNA primases are able to synthesise primers and initiate DNA synthesis on a large variety of templates and in several different environments.

In wild type E.coli K-12 the dnaG primase primes host chromosomal and plasmid discontinuous DNA replication (L.Chatfield, personal communication). Hence the plasmid enzyme is not generally utilised for chromosomal replication and is not essential for plasmid replication. However the synthesis of the enzyme appears to be related to the level of derepression of the conjugational genes of the plasmid. Chatfield and Wilkins (personal communication) have shown that sog- mutants of ColIb-P9drd1 are transferred less efficiently than sog+ ColIb-P9drd1 plasmids. However if the recipient strain contains pLG215 the transfer efficiency of sog- ColIb-P9drd1 is increased, presumably due to more efficient complementary strand synthesis in the recipient

when high levels of sog primase are present (L.Chatfield, personal communication). For sog+ ColIb-P9drd1 the primase is synthesised in the donor since expression of the plasmid genes is not required for the formation of covalently closed circular molecules in the recipient (L.Chatfield, personal communication). However the actual site of primer synthesis has not yet been identified. One hypothesis is that the primase is synthesised in the donor and attached to the transferred strand at the site of oriT and that the primase synthesises the primers in the recipient (see figure 1.6).

The importance of primer synthesis for the IncIα plasmids may be that it enables the establishment of the plasmids in a wide variety of bacterial species other than E.coli, although this has not yet been convincingly demonstrated (Datta and Hedges, 1972b). Only the 240kd polypeptide has primase activity and the function of the 180kd polypeptide is obscure, although approximately equal amounts of both polypeptides are synthesised. It is possible that the molecules have another as yet unidentified function.

6,2) RP4 plasmid encoded DNA primase.

In 1980 it was reported that R68.45 (IncP) was able to suppress the dnaG3 mutation of E.coli (Ludwig and

Johansen, 1980). At the end of 1981 the results of a large scale survey of plasmids for primase activity was published (Lanka and Barth.,1981). The majority of the plasmids proved negative but some were positive including R16drd4 (IncB), R64drd11, R112, R144drd3, and ColIb-P9 (IncIα), R621a (IncIγ), TP114drp1 (IncI2), R387 (IncK), R446b and R831 (IncM), and RP4 (IncP) (Lanka and Barth, 1981).

Lanka and Barth chose the RP4 plasmid primase as the most amenable to study and they mapped the location of the RP4 primase adjacent to the tral region of the plasmid. In fact the primase gene(s) may be part of the tral region (see figure 1.7). A primase overproducing mutant of RP4 was used as a source of protein to purify the enzyme. They purified a polypeptide of 118Kd which primed complementary strand synthesis on phages ØX174, G4, fd, and If1 single-stranded DNA templates, with no requirements for dnaB or dnaC gene products. Like sog primase, but unlike dnaG primase, the enzyme can only utilise ribonucleotide triphosphates (Lanka and Barth, 1981). RP4 is unable to suppress the dnaG3 mutation of E.coli although its pilus synthesis is constitutive and the plasmid is effectively derepressed for transfer. However an overproducing mutant of RP4 (RP4-4B) and a clone of the primase genes (pJF108) do suppress the dnaG3 mutation (E.Lanka, private communication). Of the colonies that do grow at 40°C in a

dnaG strain more than 90% contain mutant plasmids and many of these contain deleted primase genes (E.Lanka, personal communication). In this respect the RP4 primase is similar to sog primase.

Antiserum raised against the 118,000 dalton primase protein also cross-reacted with an 80,000 dalton plasmid encoded protein (Lanka and Barth, 1981). The 80,000 dalton protein was also active in priming complementary strand synthesis on ϕX174 etc. It is not clear whether this sequence related protein is a processed product of the 118Kd protein, or an overlapping, but in phase, gene product, or even the product of an entirely separate gene. Thus RP4 may encode two related polypeptides with primase activity, perhaps analogous to ColIb-P9 240,000 and 180,000 dalton proteins (Wilkins et al.,1981), however only the larger of the ColIb-P9 proteins has priming activity. The activity of RP4 primase is not inhibited by antiserum raised against dnaG primase or antiserum raised against sog primase. Thus the RP4 primase represents another serologically distinct group of primases.

Lanka and Barth found that primase defective RP4 plasmids did not show a reduction in transfer efficiency compared to the wild-type in E.coli to E.coli matings, but that a significant reduction in transfer frequency was

observed for matings from E.coli into Klebsiella aerogenes, Providencia spp., Serratia marcescens (5 fold reduction), Proteus mirabilis and Salmonella typhimurium (25 fold reduction). By reverse matings it appeared that the primase correlated effect on conjugation frequency depended only on the recipient and not on the donor species. One conclusion drawn from these observations is that the primase molecules are transferred from the donor to the recipient and that the primers are synthesised in the recipient allowing the establishment of RP4 in foreign environments. Lanka and Barth also presented evidence that the primase is involved in RP4 maintenance in E.coli and S.typhimurium as well as in conjugation (Lanka and Barth.,1981).

7) Introduction to the results.

The survey of Lanka and Barth (1981) showed that most of the plasmids tested in the I-complex (with the exception of the IncIδ plasmids) encoded a DNA primase. In addition they identified two new incompatibility groups, IncM and P, from which representatives encoded DNA primases. My work has had two major objectives,the study of the different primases encoded by the members of the I-complex and their interrelationships and the screening of representatives of the other incompatibility groups for further plasmid encoded DNA primases.

CHAPTER TWO

MATERIALS AND METHODS.

1) Bacterial strains and plasmids.

The plasmids used in this work are described in table 2-1. The bacterial strains were all derivatives of Escherichia coli K-12 and are listed in table 2-2. Bacteriophage PR64FS (Coetzee et al.,1980) was obtained from B.M. Wilkins and DNA from bacteriophages M13 and fd was a gift from E. Orr.

2) Plasmid curing.

Plasmid-bearing bacterial strains were cured of derivatives of pBR325 and pBR328 by growth in the absence of antibiotic selection for twenty generations. The cultures were plated out in the absence of antibiotics and individual colonies were screened for the loss of antibiotic resistance.

3) Colicin production and sensitivity.

Colicin production was demonstrated by killing colonies with chloroform and overlaying with molten soft

Table 2.1) Plasmids used in this work.

A) Conjugative plasmids.

plasmid	Inc group	phenotype (a)	source (b)	references (c)
R16	B	Ap Sm Su Tc	P.R.C.	1
RIP72	B	Km Cib	P.R.C.	2
pLG101	B	Cib	this lab.	3
TP125	B	Cm Sm Su Tc	P.R.C.	4
R864a	B	Km	P.R.C.	5
RA1	C	Su Tc	N. Datta	6
R40a	C	Ap Km Pm Su	N. Datta	7
R57b-1	C	Cm Su	N. Datta	7
R905	D	Cm Sm Su	N. Datta	8
ColV3-K30	FI	Cva	this lab.	9
R27	H1	Tc	N. Datta	10
R478	H2	Cm Km Tc	N. Datta	11
ColIb-P9	Iα	Cib	B. Wilkins	12
ColIb-P9drd1	Iα	Cib	B. Wilkins	13
R64	Iα	Sm Tc	B. Wilkins	13
R64drd11	Iα	Sm Tc	B. Wilkins	13
R144	Iα	Km Tc Cib	N. Datta	13
R144drd3	Iα	Km Cib	B. Wilkins	13
R621a	Iδ	Tc	N. Datta	14
R724	Iδ	Cm Sm Su Tc	P.R.C.	5
R805a	Iζ	Km	P.R.C.	5
R391	J	Km Nm Hg	N. Datta P. Barth	15,16
R387	K	Cm Sm	N. Datta	17
pTM557	K	Sm Su Tc	N. Datta	
pTM559	K	Km Sm Tp	N. Datta	
pIE312-1	K	Tc	N. Datta	17
R446b	M	Sm Tc	N. Datta	18,19
R471a	M	Ap Cm Km	N. Datta	18,20
N3	N	Sm Su Tc Hg	R. Grant	21,22
RP4	P	Ap Km Nm Tc	N. Grinter	23

Rts1	T	Km	N. Datta	24,25
RA3	U	Cm Sm Su	N. Datta	26
Sa	W	Cm Km Sm Su	N. Datta	21,27
R6K	X	Ap Sm	R. Grant	28
pIP71a	Com9	Ap Cm Sm Su Tc	N. Datta	2

a) Ap-ampicillin resistance, Cib-colicin Ib synthesis, Cm-chloramphenicol resistance, Cva-colicin V synthesis, Hg-mercuric ion resistance, Km-kanamycin resistance, Nm-neomycin resistance, Pm-paramomycin resistance, Sm-streptomycin resistance, Su-sulphonamide resistance, Tc-tetracycline resistance, Tp-trimethoprim resistance.

b) P.R.C.-plasmid reference centre.

c) References:-

1) Evans et al.,1968.
2) Scavizzi, 1973.
3) Williams et al.,1978.
4) Grindley et al.,1973.
5) Datta and Olarte, 1974.
6) Aoki et al.,1971.
7) Datta and Hedges, 1974.
8) Hedges, 1975.
9) Bukhari et al.,1977.
10) Meynell and Datta, 1966.
11) Hedges et al.,1975.
12) Clewel and Helinski, 1970.
13) Meynell and Datta, 1967.
14) Falkow et al.,1974.
15) Coetzee et al.,1972.
16) Nugent, 1981.
17) Tschape and Tietze, 1980.
18) Richards and Datta, 1979.
19) Hedges et al.,1973.
20) Hedges et al.,1975.
21) Watanabe et al.,1968.
22) Ando and Arai, 1981.
23) Datta et al.,1971.
24) Terawaki et al.,1967.
25) Terawaki et al.,1981.
26) Tschape et al.,1981.
27) Hedges and Datta, 1971.
28) Kontomichalou et al.,1970.

pLG221	Km	ColIb-P9 with a Tn5 insertion in the colicin gene(s).

B) Mutants of conjugative plasmids mediating efficient suppression of the dnaG3 mutation.

Plasmid	incompatibility group	mutant of
pLG106	B	R16
pLG107	B	TP125
pLG134	C	R40a
pLG135	C	R40a
pLG136	C	R40a
pLG137	K	R387
pLG138	K	R387
pLG139	K	R387
R805adrp1	Iγ	R805a
R864adrd1	B	R864a

C) Nonconjugative plasmids.

plasmid(a)	penotype(b)	derivation	refs.(c).
pBR325	Ap Cm Tc		1,2
pBR328	Ap Cm Tc		3
pMOB45	Ap Cm		4
pLG105	Ap Tc R16pri	15.8kb EcoRI fragment from R16	5
pLG108	Ap Tc R16pri	4.1kb EcoRI-HindIII fragment	5
pLG112	Ap R16pri	4.8kb EcoRI-BamHI fragment	
pLG113	Ap Tc	15.8kb EcoRI fragment reversed	
pLG114	Ap R16pri		
pLG115	Ap R16pri	derivatives of pLG108 generated	
pLG116	Ap R16pri	by a deletion analysis of the	
pLG117	Ap	primase gene(s)	
pLG120	Ap		
pLG121	Ap		
pLG122	Ap Tc R387pri	18kb EcoRI fragment from R387	
pLG125	Ap	deletion derivatives of pLG108	
pLG126	Ap		
pLG127	Ap Tc R387pri	4.85kb EcoRI-HindIII fragment	
pLG128	Ap R387pri	6.7kb EcoRI-BamHI fragment	
pLG129	Ap	BamHI-BamHI fragment of pLG122	
pLG132	Cm	5.1kb BamHI-BamHI fragment from pLG112	
pLG133	Cm	gene(s) inserted in both orientations	
pLG214	Ap Tc sog	deletion derivative of pLG215	6,7
pLG215	Ap Tc sog	7.9kb EcoRI fragment ColIb-P9	6,7

a) vectors, pBR325- pLG105, pLG108, pLG112-pLG117, pLG120, pLG121, pLG125 and pLG126.

pBR328- pLG122, pLG127-pLG129.

pMOB45- pLG132 and pLG133.

b) Ap-ampicillin resistance, Cm-chloramphenicol resistance and Tc-tetracycline resistance.

c) References

1) Bolivar 1978.
2) Prentki et al.,1981.
3) Soberon et al.,1980.
4) Bittner and Vapnek, 1981.
5) Dalrymple et al.,1982.
6) Boulnois et al.,1982.
7) Wilkins et al.,1981.

Table 2.2) Bacterial strains.

Strain	genotype
BW86	dnaG3, leu, thyA, deoB, rpsL, Δ(chlA-uvrB), cir.
W3110	
BC1304	dnaB1304, dnaC201, arg, thy, polA1, rpsL. end

nutrient agar containing a colicin sensitive strain and incubating for six to eight hours. Clear zones around colonies identified the colicin producing bacteria.

Colicin sensitivity was demonstrated by a similar method. Colicin producing strains were streaked on to an agar plate, grown up overnight, killed with chloroform, and overlaid with the strains to be tested. Clear zones around the streaks identified the sensitivity of the test strain to the various colicins.

4) Preparation of phage PR64FS.

An overnight culture of W3110 containing plasmid pLG221 was inoculated into fresh nutrient broth to give an O.D.600 of 0.05 and the culture was grown with shaking to an O.D.600 of 0.4 (2x10-8 cells/ml). The culture was then diluted to 1x10-8 cells/ml and phage PR64FS was added to give a multiplicity of infection of 0.05, and grown with shaking until the increase in optical density ceased. The cells were then killed with ether and after five hours the culture was centrifuged (10 minutes at 10,000 rpm in a Sorvall SS34 rotor) and the supernatant retained. Polyethylene glycol 6000 (BDH Chemicals Ltd.) was added to the supernatant to a final concentration of 6% w/v and NaCl was added to a final concentration of 0.5M. The supernatant

was left overnight at 4°C, and centrifuged (10 minutes at 10,000 rpm in a Sorvall SS34 rotor) and discarded. The pellet was resuspended in phosphate buffer and the ether was removed by bubbling air through the phage suspension.

5) Phage sensitivity.

Phage sensitivity was demonstrated by streaking a suspension of bacterial cells across a dry streak of a phage suspension. A complete absence of cells, or a reduction in the density of growth, on the distal side of the phage streak indicated that the strain was sensitive to the phage.

6) Introducing plasmids into new strains.

Conjugative plasmids were introduced into the relevant strain by mating with a suitable donor strain in nutrient broth. In general donors and recipients were mixed in a ratio of 10:1 and mated for an hour before plating on medium selective for transconjugants.

Non-conjugative plasmids were introduced into the relevant strain by transforming competent cells with purified plasmid DNA. To make competent cells a rapidly growing culture of the recipients was harvested in early log. phase (5 minutes at 10,000 rpm in a Sorvall SS34), washed in

a half volume of cold 10mM CaCl, and resuspended in a twentieth volume of 100mM CaCl. To 200ul of competent cells, 300ul of 100mM CaCl containing plasmid DNA were added, and the cells were incubated on ice for 45 minutes. The cells were then heat shocked at 42°C for two minutes, 500ul of nutrient broth were added and the cells incubated at 30°C for 90 minutes to allow expression of the tetracycline resistance phenotype before plating on to selective nutrient agar plates.

7) Media and buffers.

Nutrient broth contained 13g of Nutrient Broth 'E' (London Analytical and Bacteriological Media Ltd.) per litre of distilled water and was adjusted to pH 7.0. Nutrient agar contained per litre of distilled water; 25g nutrient broth 'E', and 14.5g Davis agar. For soft nutrient agar the amount of agar was reduced to 6g.

T.Y. medium contained 10g tryptone (Oxoid), 5g yeast extract (Oxoid), and 5g NaCl (Fisons) per litre of distilled water.

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 , 4g glucose, 2g Casamino acids (Difco) and, 1mg

thiamine hydrochloride (Sigma). The last five components were added separately from stock solutions after autoclaving. Minimal medium was made up as for SGC medium except that the Casamino acids were omitted. SGC and minimal agar were made up as for the media except that the components were added, after autoclaving independantly, to autoclaved distilled water containing 15g/l Davis agar. Minimal medium and minimal agar were supplemented, when required, with 2ug/ml of the appropriate aminoacids (Sigma).

Antibiotics (Sigma) were added to molten agar (at about 50°C) and to broth as follows: streptomycin sulphate (Glaxo), 100ug/ml; tetracycline hydrochloride, 5ug/ml; ampicillin, 20ug/ml; chloramphenicol, 20ug/ml; nalidixic acid, 20ug/ml; sulfathiozole, 300ug/ml; and kanamycin, 20ug/ml.

8) Suppression of the temperature-sensitive dnaG3 mutation.

BW86 strains containing plasmids were grown up overnight in nutrient broth, supplemented with 2ug/ml thymine (Sigma), at 30°C. Serial dilutions of the cultures were plated in duplicate on nutrient agar containing the relevant antibiotics and incubated at 30°C or 40°C for 36 hours. The plating efficiency of a strain is defined as the number of colonies formed at 40°C divided by the number

formed at 30°C - for plasmid free BW86 the plating efficiency was less than 10-7. Plating efficiencies of greater than 10-7 for plasmid- bearing derivatives of BW86 indicated plasmid-mediated suppression of the dnaG3 phenotype.

9) The isolation of plasmid mutations which mediated a high efficiency of suppression of the dnaG3 mutation.

The isolation of plasmid mutants was based on a method previously described (Sasakawa and Yoshikawa, 1978). Strains of BW86 carrying plasmids were grown overnight at 30°C. Serial dilutions were plated on to nutrient agar and incubated at 40°C until colonies were clearly visible. Ten colonies were independently resuspended in phosphate buffer. The bacterial suspensions were streaked out for single colonies on nutrient agar and the plates were incubated at 40°C. A single colony from each plate was resuspended in phosphate buffer and restreaked for single colonies and again incubated at 40°C. The plasmids in the purified thermoresistant strains were then transferred by conjugation into W3110, a prototrophic dnaG+ strain, and then into BW86. The plating efficiency of the final recipient was measured (see section 8). All strains with a low plating efficiency were discarded, while strains with a high plating efficiency were retained as strains carrying plasmids with mutations

which allow the plasmid to suppress the dnaG3 mutation with high efficiency.

10) Measurement of transfer efficiency.

Exponentially growing plasmid containing strains were mixed with exponentially growing recipients, in a ratio of 1 donor: 10 recipients and incubated at 30°C for 1 hour. After interruption of mating, selection was made on medium containing the appropriate antibiotics. The index of transfer efficiency is the yield of transconjugants per 100 input donor bacteria for a one hour mating.

11) The isolation of plasmid mutants derepressed for transfer.

Exponentially growing cultures were mated in broth for 15 minutes and plated onto selective media. The recipient colonies, which had recieved plasmids, were pooled and the culture was mated into a new recipient in a short broth mating. The recipient colonies containing the plasmids were again pooled and the mating procedure was repeated two or three times. The transfer efficiencies of the recipient colonies were then tested and those with a significant increase in transfer efficiency were taken as authentic derepressed mutants.

12) Screening for plasmids.

10 ml of an overnight culture were harvested (5 minutes at 10,000 rpm in a Sorvall SS34 rotor) and the cells were resuspended in 200ul of 25% w/v sucrose- 50mM Tris (pH 8.0). 100ul of 10% w/v sodium dodecyl sulphate (SDS) was added and the cells were incubated at room temperature for 30 minutes to lyse. The samples were vortexed for two short bursts of 5 seconds and then spun in an Eppendorf microfuge for 10 minutes. The supernatant was collected and samples were analysed on horizontal 0.8% agarose gels in the absence of ethidium bromide. The samples were loaded with a fifth volume of 5xloading buffer (80mM Tris pH 6.8-glycerol 10% v/v-Bromophenol blue 0.001% w/v). The running buffer contained 25mM Tris-acetate (pH 7.7)-1mM EDTA and a voltage of 100V. was applied for 1-4 hours. The gels were stained after electrophoresis in 0.5ug/ml ethidium bromide. The DNA was visualised using a long wavelength u.v. trans-illuminator and photographs were taken using a 35mm camera with Kodak HS23 film or a land camera with type 57 or 55 large format polaroid film.

13) Large scale purification of plasmid DNA.

Plasmids were purified from exponentially growing cultures by one of several methods: chloramphenicol

sensitive derivatives of pBR325 and pBR328 were amplified by the addition of chloramphenicol (180ug/ml) and incubating, with shaking, overnight; pBR325 and pBR328 were amplified by the addition of spectinomycin (300ug/ml) and incubating, with shaking, overnight; and pMOB45 and its derivatives were amplified by incubating growing cultures at 40°C for 4 hours prior to harvesting. For large plasmids the cells were harvested at O.D.600 0.8.

The cells were harvested by centrifugation (10 minutes at 5,000 rpm in a Sorvall GS3 or GSA rotor) and the pellet was resuspended in 8ml of 25% w/v sucrose-50mM Tris (pH 8.0) per litre of culture. 0.6ml of 10mg/ml lysozyme (Sigma) was added to the resuspended cells, which were kept on ice throughout the lysis procedure. After 5 minutes 0.4ml of 250mM EDTA (pH 7.5) was added per litre of the original culture. After 20 minutes 8ml of lysis mix (50mM Tris pH 8.0-62.5mM EDTA-20% w/v Triton X-100) was blown from the pipette into the suspension of spheroplasted cells, to ensure uniform lysis, and the lysed cells were immediately centrifuged (30 minutes at 18,000 rpm in a Sorvall SS34). The supernatant was decanted into fresh tubes and mixed with CsCl (Fisons) and 5mg/ml ethidium bromide (Sigma) in the ratio of 10ml lysate: 9.7g CsCl: 0.44ml ethidium bromide. The refractive index of the CsCl solution was adjusted to 1.377 with 50mM Tris (pH 8.0) or solid CsCl as required.

Centrifugation was for 40 hours at 38,000 rpm in a Beckman 50Ti, 70Ti or 75Ti rotor, or for 12 hours at 55,000 rpm in a Beckman VTi50 or VTi65 rotor.

After centrifugation the DNA was collected by inserting a needle into the tube and withdrawing the lower plasmid band with a syringe. Ethidium bromide was extracted with CsCl saturated propan-2-ol and the CsCl was extracted by dialysis against 4xl litre lmM EDTA at 4°C for an hour each time. If necessary the DNA was precipitated by the addition of sodium acetate to a final concentration of 0.2M and 3 volumes of absolute ethanol. The DNA solution was kept at -70°C for at least an hour and centrifuged for 15 minutes at 10,000 rpm in a Sorvall HB4 rotor at -10°C, or at 4°C in an Eppendorf microfuge. The pellet was dried under vacuum and redissolved in distilled water.

14) Purification of small amounts of restrictable DNA.

To screen a large number of colonies for the nature of their recombinant plasmids one of two methods was used; the first for plasmids of which a large amount of DNA was required (Klein et al.,1980), and the second when only a small amount of DNA was required (Holmes and Quigley, 1981).

The first method required the amplification of

cultures (see section 14). The 10ml amplified cultures were harvested (10 minutes at 10,000 rpm in a Sorvall SS34 rotor) and resuspended in 500ul of 50mM Tris (pH 8.0). 50ul of lysozyme in 10mM Tris (pH 8.0) was added and the cells were incubated for 15 minutes at room temperature. 500ul of phenol mix (100g phenol (BDH Chemicals Ltd.)-100ml chloroform (Fisons)-4ml isoamylalcohol (Fisons)-0.1g 8-hydroxyquinoline (BDH Chemicals Ltd.)) was added and mixed by gentle inversion. After a 15 minute spin in an Eppendorf microfuge the aqueous layer was re-extracted with 300ul of the phenol mix by vortexing and a 5 minute spin in an Eppendorf microfuge. The phenol was extracted from the aqueous layer by three washes of 1ml of chloroform: isoamylalcohol (24:1) mix. The DNA was then precipitated with 3 volumes of absolute ethanol (see section 13), washed with 70% ethanol, dried under vacuum and resuspended in 100ul of distilled water. The DNA can transform cells and is sufficiently clean to be digested by restriction endonucleases. The yield may be as high as 25ug of DNA from a 10ml culture.

The second method does not require amplified cultures and 1ml of an overnight culture is sufficient. The culture was spun down in an Eppendorf microfuge and the cells resuspended in 25ul STET buffer (8% w/v sucrose-5% v/v Triton X-100-50mM EDTA-50mM Tris pH 8.0). 2ul of 10mg/ml

lysozyme (in 50mM Tris) was added and the cells were vortex mixed. The tubes were placed in a boiling water bath for 40 seconds and immediately centrifuged in an Eppendorf microfuge for 10 minutes. The supernatant fluid was carefully removed and an equal volume of isopropanol was added and the nucleic acids were precipitated at -70°C for 10 minutes. The nucleic acids were pelleted by a ten minute spin at 4°C in an Eppendorf microfuge. The supernatant was decanted and the pellet was resuspended in 25ul of 0.3M sodium acetate, 75ul of absolute ethanol was added and the nucleic acids reprecipitated at -70°C for 10mins and treated as in section 13.

15) Restriction enzyme digestions.

All restriction digests were carried out at a DNA concentration of 40ug/ml, and with the following buffers, the concentrations given are for the 5xbuffer stocks used to make up the reaction mixes:-

a) General buffer
30mM Tris pH 7.4
30mM $MgCl_2$
2.5mM DTT (Di-thiothreitol (Sigma))

b) Ava I (Biolabs) general buffer

c) Ava II (BRL Inc.)
100mM Tris pH 7.4
50mM $MgCl_2$
150mM NaCl

d) BamHl (BRL Inc.)
30mM Tris pH 7.4

30mM $MgCl_2$
250mM NaCl
30mM 2-mercaptoethanol (Sigma)

e) BglII (BRL Inc.)
250mM Tris pH 7.9
50mM $MgCl_2$
50mM DTT
250mM KCl

f) EcoRI (Boehringer Mannheim GMBH.)
500mM Tris pH 7.5
50mM $MgCl_2$

250mM NaCl

g) HindIII (BRL Inc.)
100mM Tris pH 8.0
35mM $MgCl_2$
2.5mM DTT
300mM NaCl

h) HpaI (BRL Inc.) general buffer.

i) KpnI (BRL Inc.) general buffer.

j) PstI (V. Wilson)
30mM Tris pH 7.4
30mM $MgCl_2$
2.5mM DTT

k) PvuII (Biolab) general buffer.

l) SalI-general buffer.

m) Sau3A (BRL Inc.)
50mM Tris pH 7.4
50mM $MgCl_2$
250mM NaCl
2.5mM DTT

n) TaqYI (BRL Inc.)
50mM Tris pH 8.4
30mM $MgCl_2$
500mM NaCl
30mM 2-mercaptoethanol

o) XbaI (BRL Inc.)
30mM Tris pH 7.4
30mM $MgCl_2$
750mM NaCl
30mM 2-mercaptoethanol

p) XhoI (BRL Inc.) XbaI buffer.

All of the restriction mixes were incubated at 37°C for an hour, except for TaqYI digestions which were incubated at 65°C for an hour. Double and triple digests were carried out at the same time, if this was possible, otherwise the digests were carried out sequentially with the conditions being changed between each digest. The enzymes were then inactivated by incubation at 65°C for 5 minutes, and a fifth volume of 5xloading buffer was added and the samples loaded on to a gel (see section 12). To size large fragments of DNA 0.5% or 0.8% w/v agarose gels were run with phage lambda DNA digested with EcoRI, HindIII or EcoRI and HindIII as standard size markers. The mobility of each fragment was measured and the length of the standards, in kilobase pairs, was plotted (on a log. scale) against mobility (on a linear scale). The sizes of the unknown fragments were then read off the graph. Generally three independent gels were used to calculate the size of the fragments generated by restriction enzyme digestion of a plasmd. To size small fragments 1%, 1.5%, or 2% w/v agarose gels were run. Phage lambda DNA digested with EcoRI and HindIII, and pBR322 DNA digested with Sau3A or TaqYI (Sutcliffe, 1978), were used as standard size markers.

16) Ligation.

Restricted vector and sample DNA were mixed in a ratio of four ends of the average sample molecule to one end of a vector molecule at a concentration of 100ug/ml DNA. The 5xligation buffer contained 66mM Tris (pH 7.5)-6.6mM MgCl -10mM DTT-0.4mM ATP. 2 units of T4 ligase was added per ug of DNA and the mixture was incubated overnight at 4°C.

17) Preparation of a restriction fragment from an agarose gel.

To prepare pure DNA of a particular restriction fragment the DNA sample was restricted with the requisite enzyme and run on a 0.8% w/v agarose gel (see section 12). Up to 20ug DNA was loaded per cm width of the gel slot. The DNA was electrophoresed at 1V/cm until adequate separation of the required band was observed. Using a scalpel the agarose slice containing the required DNA fragment was cut out of the gel and placed in a dialysis bag, with a small volume of a tenth concentration electrophoresis buffer (2.5mM Tris-acetate pH 7.7-0.1mM EDTA-0.5ug/ml ethidium bromide). The DNA was electrophoresed out of the agarose onto the diaysis bag at 200V/cm, when this was complete the current was reversed for a minute to release the DNA. The buffer was recovered from the bag and reduced to about 500ul with successive butanol extractions. The sample was then treated as in the first method of section 14, from and including the phenol extractions.

18) In vitro labelling of DNA.

To label DNA samples with 32P the method of Rigby et al. (1977) was followed. The following reagents were kept as stock solutions (at -20°C), DNA polymerase I (Boehringer Mannheim GMBH., 4 units/ul), 10xnick mix (500mM Tris pH 7.8-50mM MgCl -100mM 2-mercaptoethanol), 50uM dATP, dGTP and dTTP, nick quench mix (10mM Tris pH 7.5-10mM EDTA-0.5% SDS) and 0.1mg/ml DNase I in 10mM Tris (pH 7.5).

The DNase stock was diluted to 4ng/ml in 10mM Tris (pH 7.5) just before use and then kept at 4°C. To nick translate the DNA sample the following reaction mix was made up in an Eppendorf tube, 0.1ug DNA, 2.5ul 10xnick mix, 2ul each of 50uM dATP, dGTP and dTTP, 1ul 4ng/ml DNase, 1.5ul 4 units/ul DNA polymerase I, 1.5ul∝32P-dCTP (10uCi/ul Amersham International) and distilled water to make the final volume up to 25ul.

The reaction mixture was incubated at 15°C for an hour before 25ul of quench mix and 25ul of phenol mix (see section 14) were added to stop the reaction. The tube was centrifuged for a minute in an Eppendorf microfuge and the aqueous layer removed. 50ul of 10mM Tris (pH 7.5) was added to the phenol layer and the tube shaken and spun. The aqueous layers were pooled and 12ul of 2M sodium acetate (pH

5.6), 50ug of high molecular weight salmon sperm DNA (Sigma) and 260ul of absolute ethanol were added. The contents of the tube were mixed and the supernatant was discarded. The precipitated nucleic acids were washed, redissolved and reprecipitated. The DNA was finally redissolved in 500ul of 10mM Tris (pH 7.5) and stored at -20°C.

19) Nitrocellulose filter hybridisation.

Agarose gels containing approximately 0.1ug of separated single stranded restriction fragments of plasmid DNA per sample slot were soaked in 20xSSC (1xSSC is 0.15M NaCl-0.015M trisodium citrate) for 20 minutes and transferred to the blotting machine. A piece of nitrocellulose the size of the gel was soaked in 3xSSC for five minutes and placed on top of the gel. One piece of Whatman 3MM filter paper, cut to gel size was soaked in 3xSSC and placed on top of the nitrocellulose and three dry pieces were then placed on top of the soaked filter.Baby nappies, four layers thick, were used as the final absorbing layer and a weight on a glass plate was used to provide pressure for the blotting. The gels were left on the blotting machine overnight at 4°C. The nitrocellulose filter was removed and washed for 5 minutes in 3xSSC and blotted dry before being dried at 80°C for at least 5 hours (Southern, 1975).

For colony hybridisation screening of plasmids, single colonies of strains were streaked onto nitrocellulose filters laid on nutrient agar plates and incubated overnight. Cells were lysed directly on the filters by placing the filters successively onto Whatman 3MM filter paper soaked with 500mM NaOH for 7 minutes, and twice on 3MM paper soaked with 1M Tris (pH 7.4). The filters were air dried and baked at 80°C for at least 5 hours (Grunstein and Hogness, 1975).

The baked filters were washed at 65°C for periods of 1 hour with, successively: 3xSSC; 3xSSC containing 0.2% w/v Ficoll 400 (Pharmacia Fine Chemicals AB.)-0.2% w/v polyvinylpyrollidone (Fisons)-0.2% w/v bovine serum albumin fraction V. (Sigma); the latter solution also containing 50ug/ml low molecular weight denatured salmon sperm DNA (Sigma) and 0.1% w/v SDS.; the last solution also containing in addition 9% w/v dextran sulphate (Sigma) and ^{32}P-labelled heat denatured probe DNA. After overnight incubation at 65°C, with gentle shaking, the filters were washed thoroughly with complete hybridisation mix. Then the filters were washed several times in 0.1xSSC containing 50ug/ml denatured salmon sperm DNA and 0.1% w/v SDS for 30 minutes, until few additional counts were lost from the filters with each wash. The filters were rinsed in cold 3xSSC before

being air dried and autoradiographed using Kodak X-Omat R film and an Ilford fast tungstate intensifying screen (Jeffreys and Flavell, 1977).

The filters were washed in distilled water at 65°C to remove the hybridised probe and hence the same filters could be used several times with different probes.

20) Preparation of crude cell extracts.

Crude cell extracts were made from 50ml of exponentially growing cultures, following the method of Lanka et al. (1977). The cells were centrifuged, washed with 25mM HEPES (N-2-Hydroxyethyl piperazine-N'-2-ethanesulphonic acid, Sigma, pH 8.0)-1mM DTT-50mM KCl, and resuspended in 1ml of 25mM HEPES (pH 8.0)-5% w/v sucrose-4mM spermidine (Sigma)-100mM KCl-1mM DTT-1mM EDTA-1mg/ml lysozyme (Sigma). After incubation on ice for 30 minutes Brij58 solution was added to a final concentration of 0.5%, and the mixture left on ice for a further 40 minutes. The lysates were centrifuged for 15 minutes at 4°C in an Eppendorf microfuge. The supernatant was decanted and stored at -70°C until required.

Crude cell extracts for enzyme purification were prepared in a similar way except that the cells from a litre

culture in exponential phase were lysed in 10ml. The lysates were centrifuged for 30 minutes at 4°C in a Beckman 50Ti rotor at 30,000 rpm. The supernatant was decanted and stored at -70°C until required.

If labelled crude cell extracts were required the bacteria were grown in minimal medium, plus the required aminoacid supplements, to an O.D.450 0.8. 1.5ml aliquots were then labelled with 50uCi/ml ^{35}S-methionine (Amersham International) by incubating for 30 minutes after the addition of the methionine. The cells were then lysed in a scaled down procedure based on that outlined above. Incorparation of ^{35}S-methionine into the proteins was measured by spotting samples of the crude cell extract onto filter paper. The dried filter papers were soaked in 10% TCA and methionine at 4°C for 30 minutes. Then the filters were heated to 90°C for 10 minutes in 5% TCA and methionine. They were washed several times in 5% TCA, dried and counted in the scintillation counter. The scintillation fluid was toluene (Cambrian Chemicals) containing 33g/l 2,5-diphenyloxazole (Fisons) and 5g/l 1,4-di-2(4-methyl-5-phenyl oxazolyl)benzene (Fisons).

21) Preparation of receptor extracts.

The method of preparation of the receptor extracts

was modified from the method of Lanka et al. (1979). A litre of TY medium (containing 0.2% glucose) was inoculated from an overnight shaken culture of BC1304 and grown, at 30°C, to an O.D.600 of 1.0. The pH of the culture was maintained at greater than 6.5 by the addition of aliquots of 5M KOH. The cells were harvested by centrifugation (10 minutes at 5,000 rpm in a Sorvall GSA rotor), washed in 30mls of 25mM HEPES (pH 8.0)-50mM KCl, and 1 ml of this buffer was added per gram of wet cell paste to resuspend the cells. The suspension was frozen in liquid nitrogen and thawed slowly on ice. A fiftieth volume of 15mg/ml lysozyme in 50mM EDTA was added and the cells incubated on ice for a further 30 minutes. The cells were again frozen in liquid nitrogen and thawed over fifteen to twenty minutes. The debris was spun out in a Beckman 50Ti rotor at 30,000 rpm for 30 minutes at 4°C. The supernatant was decanted, aliquoted, frozen and then stored in liquid nitrogen. The receptor extract was never completely thawed and could be stored for at least three months.

21) Assay of DNA primase activity.

The primase activity of the crude cell extracts was assayed using the incorporation of [methyl-3H] thymidine-5'-triphosphate (1000cpm/pmol Amersham International Ltd.) into bacteriophage M13 or fd single

stranded DNA. Rifampicin (Sigma) was used to inactivate RNA polymerase, which would otherwise be able to prime DNA synthesis on these phages. The dnaG primase cannot prime DNA synthesis on these phages. Receptor extract was used as a source of polymerisation enzymes. The assays were always carried out in duplicate and all results are the mean of two concurrent experiments.

The assays were carried out using the following stock solutions (stored at -20°C), rNTP's (120uM, rCTP, rGTP, rUTP, Sigma), dNTP's (12.5uM, dATP, dCTP, dGTP, dTTP-143mM Mg -360uM NAD-360uM cAMP-285mM HEPES, Sigma) and energy (33.3mM ATP-250mM Creatine phospate-1.7 mg/ml Creatine Kinase, Sigma). The assay mixtures contained 0.5ug of single stranded phage M13 or fd DNA-1mM DTT-2.5mM spermidine-20ug/ml rifampicin-2.5-5ul receptor extract-0.75ul rNTP's-1.75ul dNTP's-1.5ul energy mix-1ul 3H-TTP and buffer (25mM HEPES pH8.0-1mM DTT-50mM KCl) to make the final reaction mix volume 25ul.

When the activity of a crude cell extract was being measured the crude cell extract was diluted with a similar extract of BW86. In this way the concentration of proteins was maintained at a constant level. Approximately 50ug of receptor extract protein was used per assay. When the energy cocktail was added to the other components the

tube was vortexed to mix the components and to start the reaction. The tubes were incubated at 30°C for an hour. The reactions were stopped by the addition of 250ul of stop mix (0.5M NaOH-0.5% w/v SDS-10% w/v sodium pyrophosphate-0.5mg/ml calf thymus DNA) and incubated at 90°C for 10 minutes before being placed on ice. 500ul of 2M TCA was added and the samples kept for a further five minutes on ice. The samples were filtered through nitrocellulose filters (Sartorius, 0.45um pores) or GF/C (Whatman), dried and counted in the scintillation counter. The scintillation fluid was as in section 20 or Fisofluor-1 (Fisons).

This procedure was slightly modified for the antiserum experiments. The antiserum was diluted, if required, in 20mM HEPES (pH 8.0)-50mM KCl and added to the crude cell extract. The antiserum and crude cell extract were incubated on ice for 10 minutes before the other components were added. The other components were added in the following order; buffer, DTT, spermidine, rifampicin and DNA mix, receptor, and finally the cocktail. The samples were then treated as described above.

22) Immunoprecipitation.

The immunoprecipitation experiments were carried

out in Eppendorf tubes in a final volume of 25ul. 5ul of neat antiserum was added to 10-15ul of 35S-methionine labelled crude cell extract (10-15ug of protein). The reaction volume was made up with 5ul of 5x immunoprecipitation buffer (250mM Tris pH 7.5-500mM NaCl-0.5% nonidet P.40, Sigma-5mM phenyl methyl sulphonyl fluoride, Sigma) and sterile distilled water. The reaction mix was incubated overnight at 4 C, then 20ul of a 10% w/v suspension of Staphylococcus aureus cells were added and the mix was incubated at room temperature for 30 minutes. The cells were pelleted in an Eppendorf microfuge and washed four times with 10 volumes of buffer (150mM NaCl-5mM EDTA-50mM Tris pH 7.4-0.1% SDS-1% sodium deoxycholate-1% Triton X-100).The pellet was resuspended in 50ul of sample buffer (see section 23) and boiled for 3-5 minutes. The unlysed cells and debris were pelleted in an Eppendorf microfuge and the supernatant was analysed by SDS polyacrylamide gel electrophoresis (see section 23).

23) Polyacrylamide gel electrophoresis.

Vertical polyacrylamide slab gels were used to separate proteins by molecular weight. The gels were made on a home made kit by mixing acrylamide solution (44% w/v acrylamide and 0.8% w/v bisacrylamide, BDH Chemicals Ltd.), water, and one of two gel buffers; A (0.75M Tris pH 8.8-0.2%

w/v SDS.), or B (0.25M Tris pH 6.8-0.2% w/v SDS.) in the correct proportions. Polymerisation was catalysed by ammonium persulphate (Biorad, stock solution 10mg/ml) and TEMED (N,N,N',N'-tetramethyl-ethylenediamine, Eastman Kodak). To vary the percentage of acrylamide in the gels the following recipies were used:-

Main gel

	5%	7%	8.5%	11%	15%
Acrylamide	3.1ml	4.3ml	5.3ml	6.8ml	9.5ml
Buffer A	13.5ml	13.5ml	13.5ml	13.5ml	13.5ml
Distilled water	9.7ml	8.5ml	7.5ml	6.5ml	3.3ml
Ammonium persulphate	0.95ml	0.95ml	0.95ml	0.95ml	0.95ml
TEMED	75ul	75ul	75ul	75ul	75ul

Stacking gel

	3%	5%
Acrylamide	1.4ml	2.4ml
Buffer B	10ml	10ml
Distilled water	8.6ml	7.6ml
Ammonium persulphate	0.48ml	0.48ml
TEMED	40ul	40ul

The main gel was poured first, to within 3cm of the top of the back plate and sprayed with 0.1% SDS. When the main gel had set the stacking gel was poured and the comb was inserted immediately. A 3% stacking gel was poured for a 5% or 7% main gel and a 5% stack was poured for an 8.5%, 11% or 15% main gel.

The samples, of up to 50ul, were boiled in sample buffer (62.5 mM Tris pH 6.8-2% w/v SDS-10% v/v glycerol-5% v/v 2-mercaptoethanol- 0.001% w/v Bromophenol blue, Fisons) for three minutes and loaded on to the gel with a Hamilton syringe. The gels were run at 10mA, constant current, until

the dye front had entered the main gel. The current was then increased to 25mA, constant current until the dye front had reached the end of the gel. The gel running buffer was 25mM Tris-192mM glycine-0.1% w/v SDS.

To stain the proteins the gel was shaken overnight in 0.5g/l Coomassie brilliant blue R-250 (Biorad), 25% v/v propan-2-ol, and 10% v/v acetic acid. To destain the gel it was shaken for several hours in 10% v/v acetic acid, and 10% v/v propan-2-ol, and finally destained in 10% v/v acetic acid.

Gels with radiolabelled samples were shaken in destain for 15 minutes, to fix the proteins, and either dried down immediately or taken through the following procedure. The gel was soaked in DMSO (di-methyl sulphoxide) for 30 minutes, and then soaked for another 30 minutes in fresh DMSO. The gel was then soaked in DMSO containing 22% w/v 2, 5-diphenyloxazole for 90 minutes. The gel was washed in distilled water for at least an hour to precipitate the PPO in the gel. The gel was then placed on a piece of stiff card and dried under vacuum for at least four hours. The dried down gels were then autoradiographed with Kodak X-Omat R film for as long as was necessary.

To size the proteins the mobility of the

polypeptides and the molecular weight standards were measured and the molecular weight (on a log. scale) was plotted against mobility (on a linear scale). The molecular weights of the unknown fragments were then read off the graph.

24) Primase purification.

Crude cell extracts were prepared from two litres of exponentially growing cells (see section 20). Approximately 25ml of extract was obtained from two litres of cells. This was diluted to 50ml, with 25mM HEPES (pH 8.0)-1mM DTT-1mM EDTA-20% glycerol, and loaded at 4°C onto a heparin Sepharose CL-6B column at 10ml an hour. The column had been preequilibriated with 25mM HEPES (pH 8.0)-1mM DTT-50mM KCl-1mM EDTA-10% glycerol. After the protein had been loaded the column was washed overnight with the same buffer. A 150ml linear gradient from 50mM KCL to 800mM KCl in 25mM HEPES (pH 8.0)-1mM DTT-1mM EDTA- 10% glycerol was run through the column at 20ml an hour. Fractions were collected every 5 minutes, approximate volume of a fraction was 1.7ml. The protein concentration and primase activity was measured for every second fraction. 50ul samples of fractions which contained primase activity were run on an acrlyamide gel. The column was washed for two hours with 1M KCl-25mM HEPES (pH 8.0)-1mM DTT-1mM EDTA-10% glycerol in

between each run.

The fractions from the first column, which contained primase activity were pooled and dialysed against 20mM KPO_4 (pH 6.8)-0.1mM EDTA-10% glycerol. The dialysed samples were loaded, at 12ml an hour, onto a 20ml column of hydroxyapatite BTG gel (Biorad). The column had been preequilibriated with this buffer and after loading the column was washed overnight with this buffer. The primase was eluted with a 150ml linear gradient from 20mM to 300mM KPO_4 at 24ml an hour. Fractions were collected every 5 minutes, approximate volume of a fraction was 2ml. The protein concentration and primase activity of every second fraction was measured. Samples of fractions which contained primase activity were run on a polyacrylamide gel. The column was washed for two hours with 1M KPO_4 (pH 6.8) in between each run.

25) Protein concentration assay.

The concentration of protein in samples was measured using the method of Bradford (1976) as modified by Spector (1978). The dye was prepared by dissolving 100mg of Coomassie brilliant blue G in 50ml of 95% ethanol. This solution was mixed with 100ml of 85% w/v phosphoric acid, diluted to a volume of 1l and filtered. The final reagent

was stable for at least two weeks at room temperature.

2ml of the dye solution was added to 100ul of the diluted sample of protein and after 2 minutes at room temperature the O.D.595 was measured with a Gilford spectrophotometer. This ratio of dye to sample is accurate in the range of 2-20ug protein in the sample. Bovine serum albumin was used to plot a standard curve.

26) Concentration of DNA.

The concentration of DNA was calculated from a standard nonogram after the O.D.260 and O.D.280 had been measured using a Unicam SP1800.

CHAPTER THREE

Suppression of the E.coli DnaG- phenotype by plasmids of the I-complex.

1) Introduction.

ColIb-P9 is the prototype IncIα plasmid and it determines the synthesis of the prototype Iα plasmid conjugative pilus. By the criteria of antisera cross reactivity (Bradley, 1980a) and phage sensitivity (Coetzee et al., 1980) all of the plasmids grouped into IncIα, due to their incompatibility relationships, encode similar pili. This grouping of plasmids is also supported by DNA-DNA hydridisation experiments (Grindley et al.,1973; Falkow et al.,1974; Chabbert et al.,1979) and the similarity of the EcoRI generated pattern of restriction fragments from a variety of IncIα plasmids (Chabbert et al.,1979). Thus the presumably closely related IncIα plasmids ColIb-P9, R64 and R144 all suppress the dnaG3 mutation (Wilkins, 1975) and encode DNA primases which are inhibited by antiserum raised against ColIb-P9 primase (Wilkins et al.,1981).

The IncIα plasmids are not the only plasmids which determine I-like pili, the members of IncIγ,Iδ,Iζ,B, and K also determine one of two types of I-like pili (Coetzee et

al.,1980; Bradley, 1980a).

IncIγ: R621a is the prototype member of this group, it determines Iα pili (Bradley, 1980a) and has approximately 53-65% DNA-DNA duplex formation with R144, an IncIα plasmid (Falkow et al.,1974; Grant et al.,1980). R621a is compatible with members of all of the other sub-groups (N.Datta, personal communication), although it has been reported to be incompatible with R144drd3 (IncIα) (Grant et al.,1980). The suppression of an E.coli dnaG3 mutation by R621a has been reported (Sasakawa and Yoshikawa, 1978) and more recently it has been demonstrated that R621a encodes a primase (Lanka and Barth, 1981). It is possible that R621a is an aberrant IncIα plasmid, with a slightly different incompatibility system but essentially identical transfer genes.

IncIδ: The IncIδ group includes plasmids previously grouped in IncI2 (N.Datta, personal communication). The pili encoded by the IncIδ plasmids are antigenically distinct from Iα pili and have been designated I2 (Bradley, 1980a). But phages If1 and PR64FS, isolated using strains carrying IncIα plasmids, can adsorb to the pili and inject their DNA into cells carrying IncIδ plasmids (Coetzee et al.,1980) and hence the pili are classified as I-like. This apparent discrepancy may be because the phages

adsorb to the tips of the pili and the antiserum molecules adsorb to the sides of the pili (Falkow et al.,1974; Bradley, 1980a; Coetzee et al.,1980). TP114 (IncIδ) has less than 6% polynucleotide sequence homology with members of IncIα (TP110 and TP102) and IncB (TP113 and TP125) (Grindley et al.,1973). However R724 (IncIδ, N.Datta, personal communication), which was once placed in IncO (now IncB) with R16, shows 96% polynucleotide sequence homology with R16 (Falkow et al.,1974). Thus R724 may well be an atypical IncIδ plasmid. The IncIδ plasmids are compatible with members of all of the other sub-groups of the I-complex (N.Datta, personal communication). TP114drpl, a mutant of TP114 which is derepressed for pilus synthesis, has been shown to encode a primase, but extracts of strains containing TP114 and other IncIδ plasmids did not have primase activity (Lanka and Barth, 1981).

IncIζ: R805a, the prototype IncIζ plasmid, determines pili which cross react with both Iα and I2 antisera (Bradley, 1980a). The pili appear to be a hybrid of the two pilus types and R805a may carry both Iα and I2 pilus genes or encode a pilus protein with antigenic determinants for both antisera. If1 and PR64FS are able to adsorb to the pili (Coetzee et al.,1980). R805a shows about 70% polynucleotide sequence homology with R144 (IncIα) and about 20% with R724 (IncIδ) (Falkow et al., 1974). R805a is

incompatible with IncIα and IncB plasmids, but not with IncIδ plasmids (N. Datta, personal communication). In their large survey of plasmids Lanka and Barth (1981) did not find primase activity in extracts of strains containing R805a.

IncB: The prototype IncB plasmid is R16, which determines Iα pili (Bradley, 1980a; Bradley, 1980b). Neither If1 nor PR64FS have been shown to adsorb to the pili of IncB plasmids (N.Datta, personal communication; Falkow et al.,1974; Coetzee et al.,1980). Recently an R16 specific phage, designated 0B, was isolated by Coetzee, but its specificity and host range are not yet known (R.Hedges, personal communication). IncB plasmids TP113 and TP125 exhibit about 20% polynucleotide sequence homology with IncIα plasmid TP102 (Grindley et al., 1973). A similar level of homology has been demonstrated between R144 (IncIα) and R16 (IncB) (Falkow et al.,1974). R16 is incompatible with IncIγ and IncIζ plasmids (N.Datta, personal communication).

IncK: The prototype IncK plasmid is R387, which determines pili of the Iα serotype (Bradley, 1980a; Bradley, 1980b). Adsorbtion of If1 or PR64FS to pili encoded by an IncK plasmid has not been demonstrated (N.Datta, personal communication). No polynucleotide sequence homology experiments have been carried out between IncK plasmids and the other I-like plasmids. Hence the gross relationship of

IncK plasmids to the other groups is not known. R387 is compatible with representatives of all of the other I-like plasmids (N.Datta, personal communication). Primase activity has been observed in extracts of strains containing R387 (Lanka and Barth, 1981).

On the basis of pilus types the plasmids of the I-complex can be divided into three groups: Iα pili, IncIα, Iγ, B and K plasmids; I2 pili IncIδ plasmids; and mixed serotype pili IncIζ plasmids. The demonstration of phage sensitivity appears to require that plasmids are derepressed for pilus synthesis. Such derepressed mutants have not been tested for IncB and K plasmids.

Experiments with plasmids of the IncF complex have shown that the maintenance genes occupy a small amount of DNA while the transfer genes can occupy more than 30kb (see figure 1.4) (Willetts and Skurray, 1980). Thus part of the large amount of polynucleotide sequence homology between plasmids of the same incompatibility group and the smaller amount between members of related sub-groups is generally expected to be in the transfer genes. The majority of the transfer genes are responsible for the synthesis and construction of the pili (Willetts and Skurray, 1980), and so since the plasmids of the I-complex encode similar pili one would expect all the plasmids to have similar transfer

genes.

The plasmid determined DNA primases of ColIb-P9, R64, R144 (IncIα), R621a (IncIδ) and RP4 (IncP) are thought to be involved in the conjugal transfer of the plasmids. Thus the primase gene(s) are probably transfer genes (Wilkins et al.,1981; Lanka and Barth, 1981) and presumably map close to the other transfer genes. In fact this has already been shown for the RP4 primase (Lanka and Barth, 1981), and may also be true for ColIb-P9 primase (see figure 3.1) (Uemura and Mizobuchi, 1982).

2) Suppression of the DnaG- phenotype.

The suppression of the DnaG- phenotype by IncIα and IncIδ plasmids and the plasmid primases encoded by IncIα plasmids have been studied in some detail. However plasmids from IncIζ, Iγ, B, and K have not been screened for the suppression of the DnaG- phenotype.

The plasmids were transferred by conjugation into BW86 and the plating efficiency of each strain was measured (see table 3.1). As previously reported the wild type plasmids ColIb-P9, R64, and R144 (IncIα) (Wilkins, 1975), and R621a (IncIδ) (Sasakawa and Yoshikawa, 1978) promoted a small but significant increase in the plating efficiency of

Figure 3.1) Maps of ColIb-P9.

Two slightly different restriction site maps of ColIb-P9 have been published. Distances are in kb from the HindIII site at the junction of HindIII fragments 2 and 3, map A, and fragments 2 and 6, map B.

A) Redrawn from Walia et al. (1982). Cib-colicinIb synthesis gene. Ibf-abortive phage infection T7 and BF23.

B) Redrawn from Uemura and Mizobuchi (1982).

C) Redrawn from Uemura and Mizobuchi (1982), showing the mapped EcoRI sites and the location of the unmapped EcoRI fragments. Sog-primase genes on EcoRI fragment 3. Rep, Inc- replication and incompatibility functions located on EcoRI fragment 2. Cib, Imm and Ibf-colicinIb synthesis and immunity genes and abortive phage infection genes on EcoRI fragments 11, 13 and 17.

Maps of ColIb-P9.

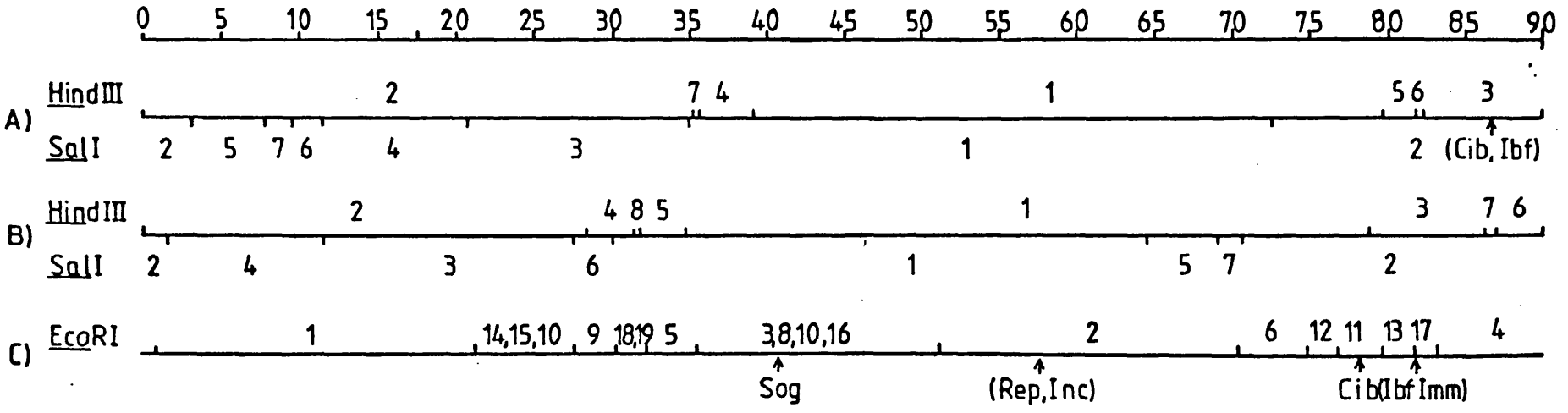


Table 3.1) Survey of I-complex plasmids for the suppression of the dnaG3 mutation and for sequences homologous with the sog primase genes.

plasmid	Inc group	plating efficiency	hybridisation of pLG215 DNA
none		<10-8	- (a)
ColIb-P9	Iα	10-6	NT (b)
ColIb-P9drd1	Iα	0.2	+ (c)
R64	Iα	10-6	NT
R64drd11	Iα	2x10-5	+
R144	Iα	10-6	NT
R144drd3	Iα	2.5x10-3	+
R621a	Iδ	5x10-5	+
R724	Iδ	10-7	-
R805a	Iζ	10-4	-
R16	B	2x10-5	-
RIP72	B	2x10-5	+
TP125	B	10-5	-
R864a	B	2x10-4	+
pLG101	B	6x10-6	-
R387	K	2.3x10-6	-

a) '-' no hybridisation of 7.9Kb sog probe
b) NT not tested
c) '+' hybridisation of 7.9Kb sog probe

BW86. The derepressed mutants ColIb-P9drd1, R64drd11, and R144drd3 promoted a much greater enhancement in the plating efficiency of BW86. The much greater enhancement of the plating efficiency by the derepressed plasmids is thought to be due to the derepression of their transfer systems.

R805a (IncIγ) R16, RIP72, TP125, R864, pLG101 (IncB) and R387 (IncK) also promoted a significant enhancement in the plating efficiency of BW86. The level of enhancement varied widely, possibly due to the differences in the level of expression of the transfer genes. The IncIδ plasmid, R724, did not significantly increase the plating efficiency of BW86.

3) Screening I-complex plasmids and RP4 for homology with the ColIb-P9 primase gene(s).

DNA-DNA hybridisation is a powerful tool for the identification of related sequences of DNA. At the level of stringency chosen for these experiments the hybridisation of the probe to the sample DNA indicates that there are stretches of 100 or so bases on the sample DNA with more than 80% sequence homology with a sequence on the probe. Thus a positive result indicates that there are regions of high homology between the probe and the sample. A negative result indicates that there are no regions of such high

homology between the probe and the sample, and that the two sequences are not closely related.

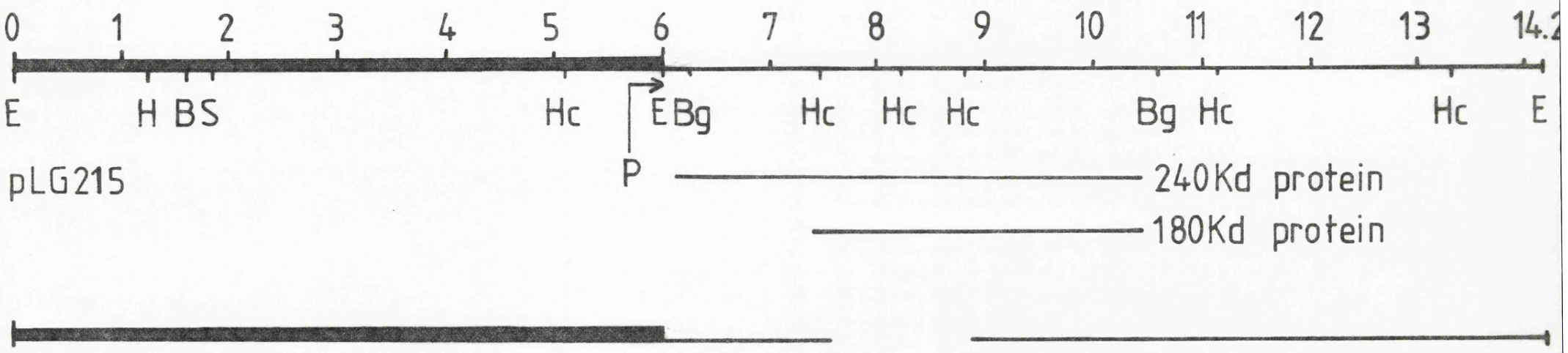
The genetic determinant (sog) of plasmid ColIb-P9drd1 responsible for the suppression of the DnaG- phenotype is carried on a 7.9kb EcoRI fragment. This fragment has been cloned into pBR325 to give recombinant plasmid pLG215 (see figure 3.2) (Wilkins et al.,1981). The sog gene(s) are said to occupy about 60% of the cloned DNA fragment and only two polypeptides have been identified as encoded by this fragment (see figure 3.2) (Wilkins et al.,1981; Boulnois et al.,1982). Thus the 7.9kb EcoRI fragment can be used as a probe for sequence homology with the sog genes.

Plasmid pLG215 DNA was treated with EcoRI endonuclease, and the two fragments generated were separated by agarose gel electrophoresis. The 7.9kb EcoRI fragment was recovered from the gel and labelled in vitro with 32P. The labelled fragment was used as a probe in hybridisation experiments with bacterial colonies harbouring plasmids of the I-complex and RP4 (IncP). As shown in figure 3.3 the probe failed to hybridise with the DNA of strain BW86 while there was a clear positive reaction with bacteria carrying pLG215 or ColIb-P9. Strains carrying R64drd11, R144drd3 (IncIα) or R621a (IncIγ) also contained sequences to which

Figure 3.2) Map of pLG214 and pLG215

Distances are in kb from one of the two EcoRI sites of pLG215. The vector (pBR325) is represented by the thick line and the insert from ColIb-P9 is represented by the thin line. The approximate extent of the deletion in pLG214 is indicated by the break in the line representing the insert. P- the chloramphenicol acetyl transferase promoter. The approximate extent of the genes encoding the 240,000 dalton and 180,000 dalton proteins is also indicated.

Map of pLG 214 and pLG215.

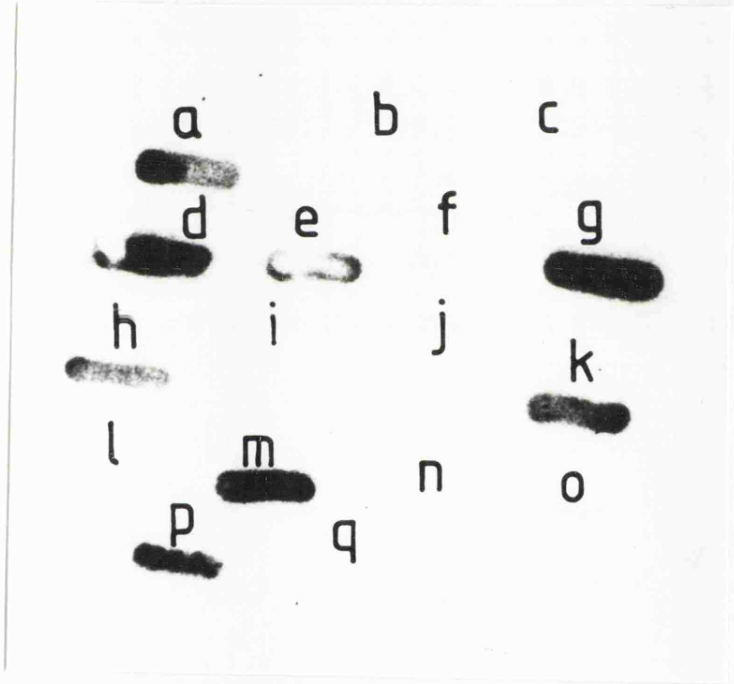


E-EcoRI H-HindIII B-BamHI S-SalI Hc-HincII Bg-BglII

Figure 3.3) Colony hybridisation screen.

Colony hybridisation screening of strain BW86 carrying plasmids of IncI, B, K and P groups for nucleotide sequences homologous with the 7.9kb insert from pLG215.

plasmid	incompatibility group	plasmid	incompatibility group
a) R64drd11	Iα	j) R805a	Iζ
b) R16	B	k) R864a	B
c) RP4	P	l) pLG101	B
d) pLG215		m) ColIb-P9drd1	Iα
e) RIP72a	B	n) BW86	
f) TP125	B	o) pLG105	
g) R144drd3	Iα	p) pLG214	
h) R621a	Iδ	q) R387	K
i) R724	Iζ		


a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q

the ColI-derived fragment could hybridise. Futhermore, strains harbouring RIP72 or R864a (IncB) also showed significant hybridisation with the probe.

However strains containing other IncB plasmids (R16, TP125, or pLG101), the IncIδ plasmid R724, the IncIζ plasmid R805a or the IncK plasmid R387 lacked sequences to which the sog gene probe could hybridise (see table 3.1). RP4 (IncP) also lacked sequences to which the sog probe could hybridise.

4) Genetic similarities among plasmids carrying the sog gene(s).

Chabbert et al.(1979) proposed a core of fifteen EcoRI generated fragments which were common to ColIb-P9, pIP186, pIP112, pIP565, and pIP111, all members of IncIα. EcoRI digests of R64 also contained thirteen of these fragments. Chabbert et al.(1979) proposed that EcoRI digests of all IncIα plasmids would have some if not most of these core fragments. It is reasonable to expect that the transfer genes would be carried on some of these core fragments.

Plasmid DNA of pLG215, ColIb-P9drd1, R64drd11, R144drd3, R621a, RIP72, and R864a was purified, restricted with either EcoRI or HindIII endonucleases, and separated by

agarose gel electrophoresis. Restriction fragments were transferred, by blotting, to nitrocellulose filters and used in filter hybridisation experiments. The 7.9kb EcoRI fragment from pLG215 was again used as the radioactive probe. For each of the six plasmids the probe DNA hybridised to a single 7.9kb EcoRI fragment (see figure 3.4). Furthermore, with the exception of the ColIb-P9 plasmid itself, each plasmid contained two HindIII fragments of approximately 13.5kb and 4.5kb to which the probe DNA hybridised. ColIb-P9drd1 had a single HindIII generated fragment of greater than 30kb with homologous sequences to the probe (see figure 3.4).

Chabbert did not publish the sizes of the common fragments, but the 7.9kb EcoRI fragment of ColIb-P9 is probably the fragment he designated number three, which was common to all of the IncIα plasmids tested (Chabbert et al.,1979). Thus it is probable that most IncIα plasmids carry the sog gene on a 7.9kb EcoRI fragment.

In figures 3.5, 3.6, and 3.7 are drawn out the EcoRI, HindIII, and BglII endonuclease generated fragment patterns of the sog+ plasmids. The 7.9kb EcoRl and 13.5kb and 4.5kb HindIII fragments are common to all of these plasmids, except for ColIb-P9drd1. The observed sizes of the ColIb-P9drd1 fragments agrees with those published in the

Figure 3.4) Filter hybridisation analysis of restriction fragments.

Filter hybridisation analysis of sog+ I-complex plasmids for homology with the cloned EcoRI fragment of pLG215. Plasmid DNA was restricted with EcoRI or HindIII as indicated. Fragment sizes are shown in kb.

	plasmid	incompatibility group
a)	pLG215	
b+h)	ColIb-P9drd1	Iα
c+i)	R64drd11	Iα
d+j)	R144drd3	Iα
e+k)	R621a	Iγ
f+l)	RIP72	B
g+m)	R864a	B

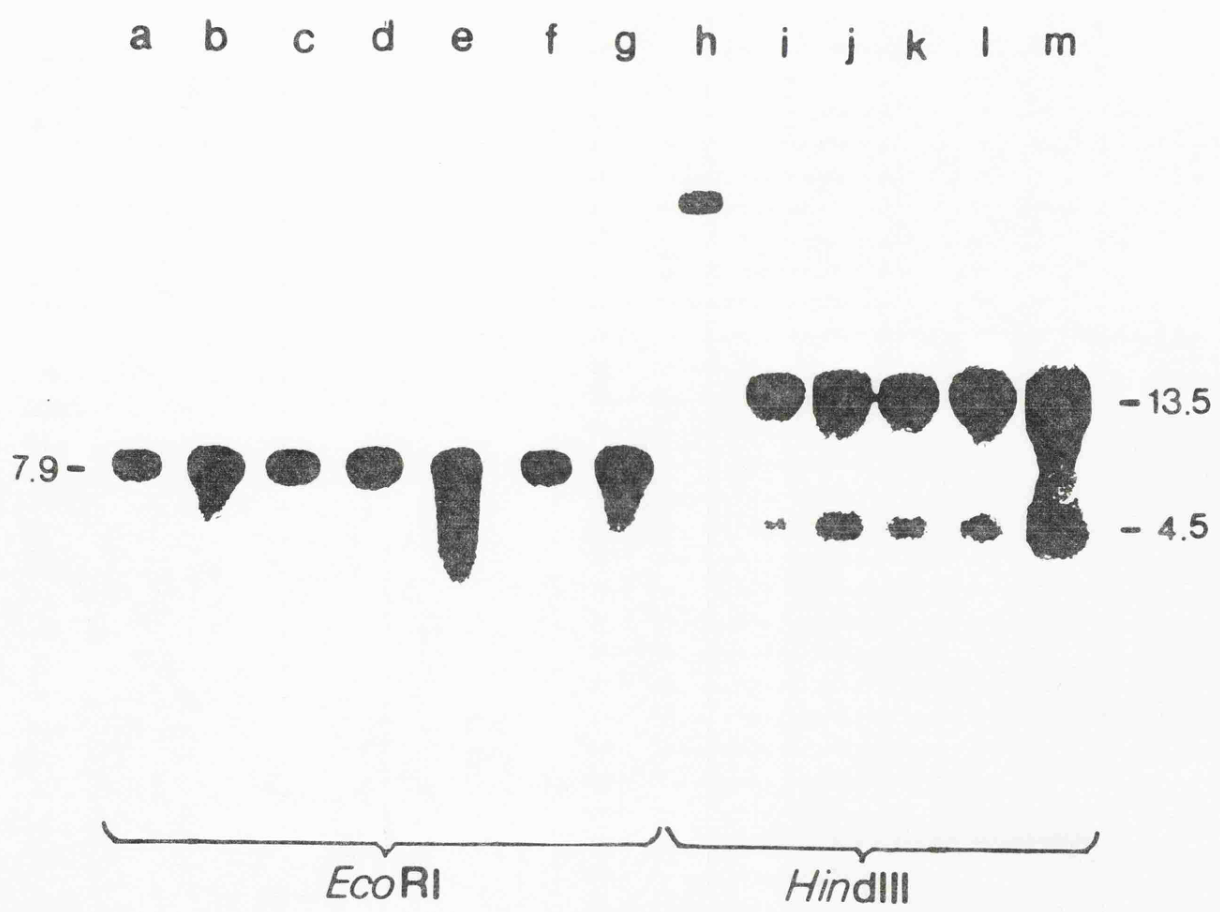

a b c d e f g h i j k l m
7.9
13.5
4.5
EcoRI
HindIII

Figure 3.5) Restriction enzyme digests:- EcoRI.

Fragment patterns for EcoRI digests of purified DNA from IncI and B group plasmids. 'x' the 7.9kb EcoRI fragment which carries the sog genes. All positions are the mean of atleast three experiments.

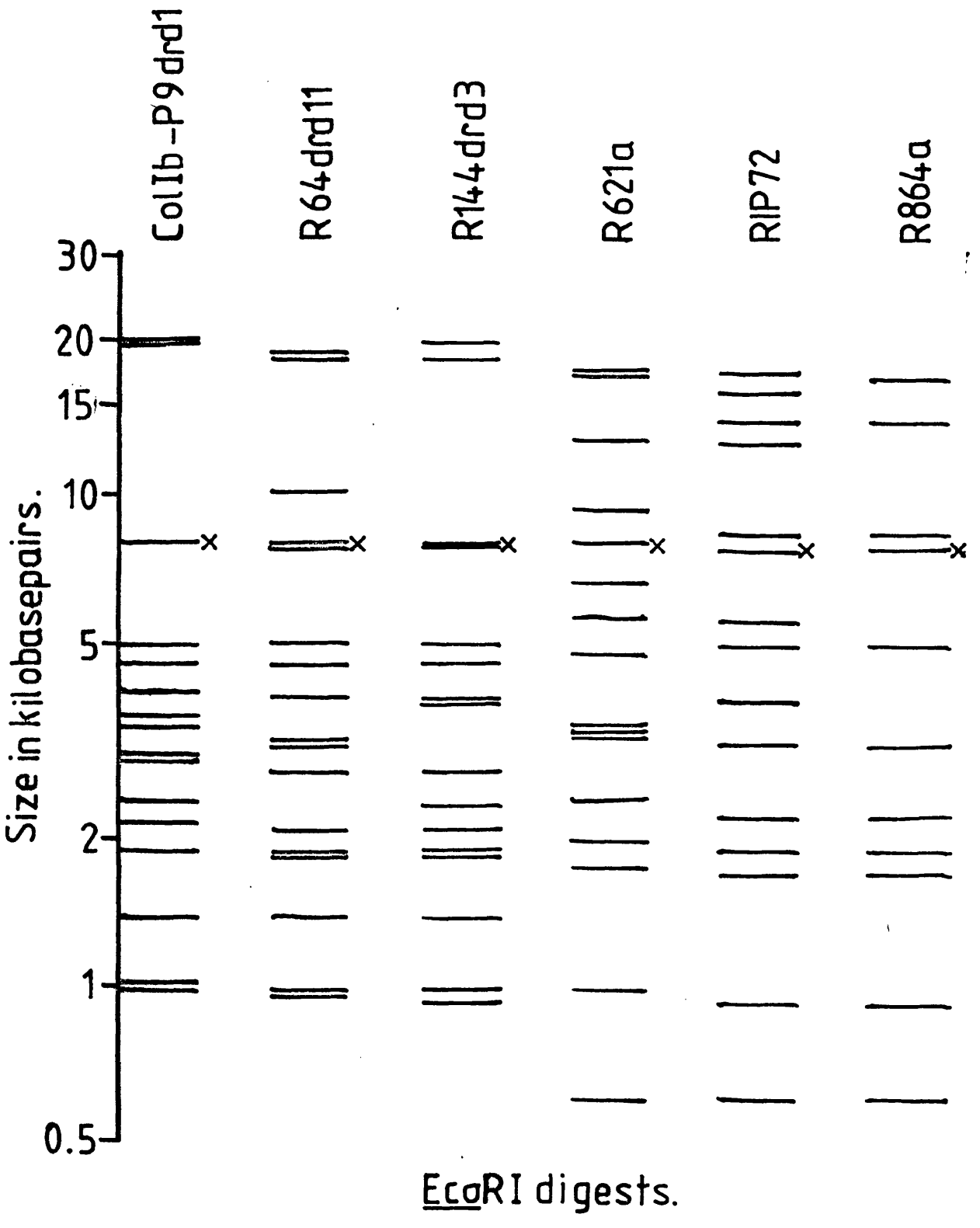

ColIb-P9drd1
R64drd11
R144drd3
R621a
RIP72
R864a
30
20
15
10
5
2
1
0.5
Size in kilobasepairs.
EcoRI digests.

Figure 3.6) Restriction enzyme digests:- HindIII.

Fragment patterns for HindIII digests of purified DNA from IncI and B group plasmids. 'x' the HindIII fragments to which the 7.9kb EcoRI sog gene probe hybridised. All positions are the mean of at least three experiments.

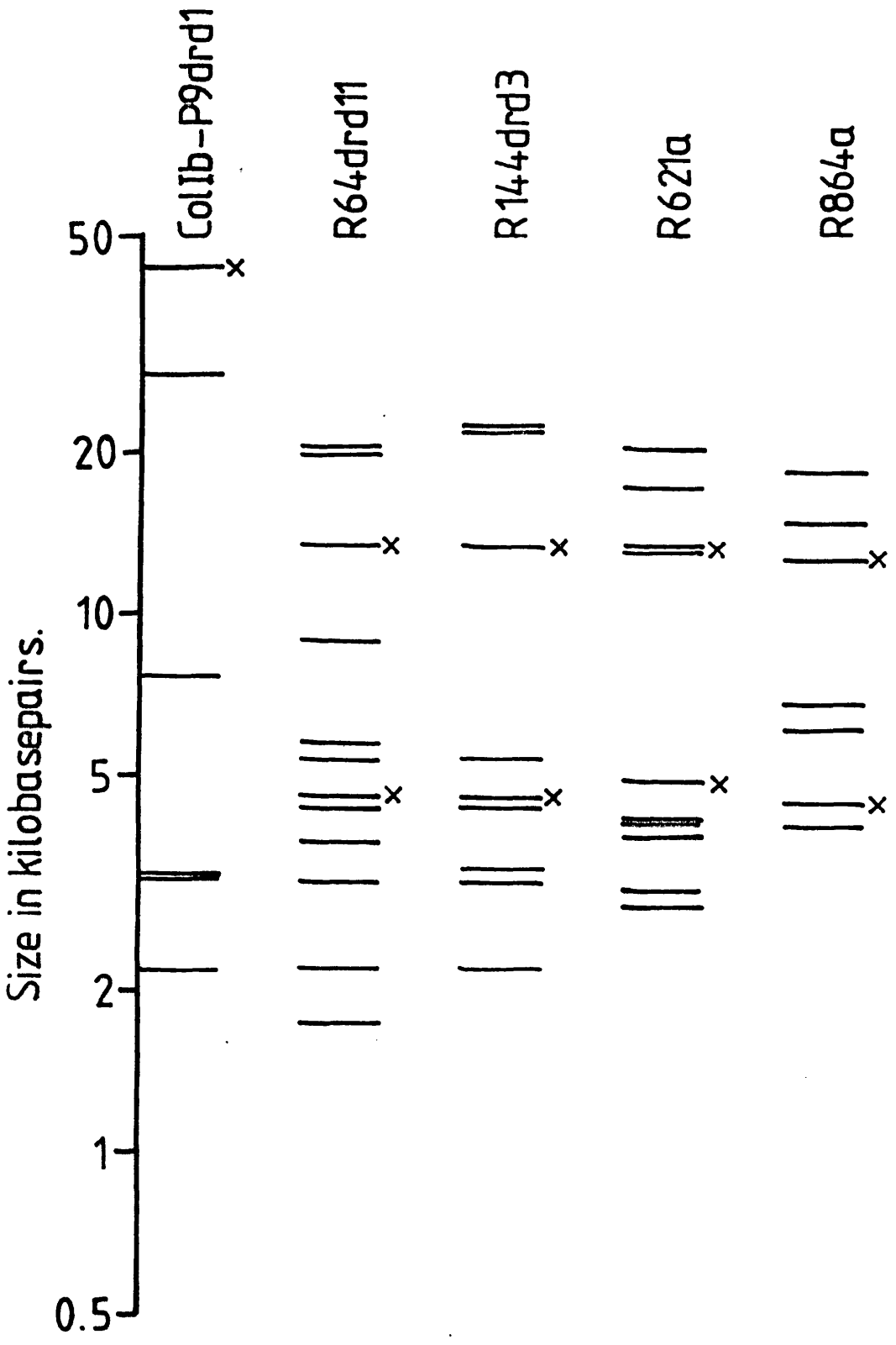
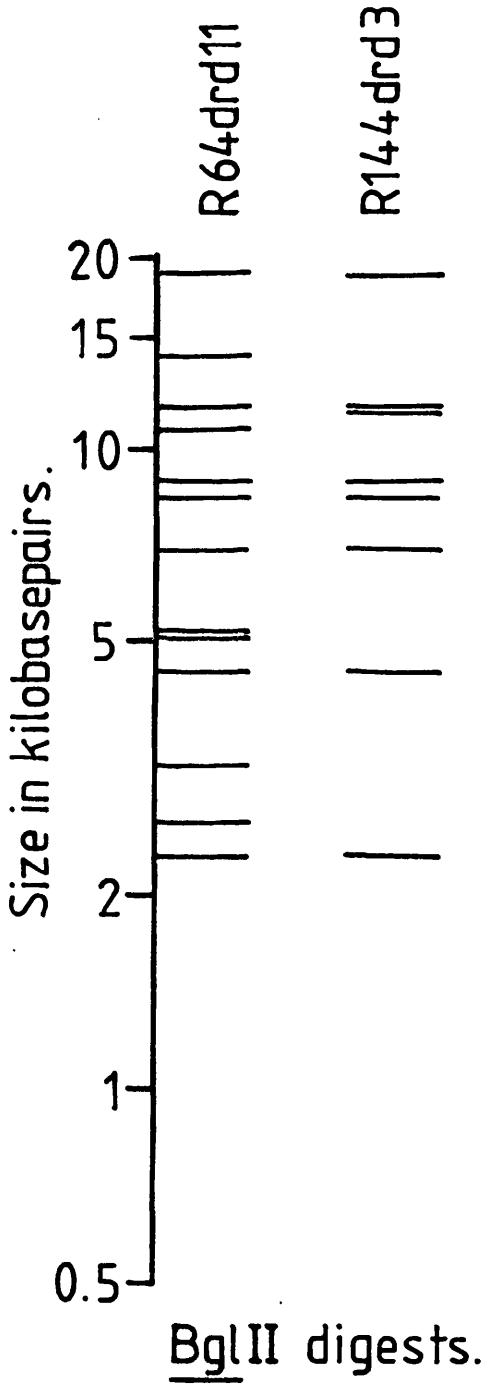

ColIb-P9drd1
R64drd11
R144drd3
R621a
R864a
50
20
10
5
2
1
0.5
Size in kilobasepairs.
Hind III digests.

Figure 3.7) Restriction enzyme digests:- BglII.

Fragment patterns for BglII digests of purified DNA from R144drd3 and R64drd11 (IncIα).


R64drd11
R144drd3
20
15
10
5
2
1
0.5
Size in kilobasepairs.
BglII digests.

two most recent reports in the literature (Uemura and Mizobuchi, 1982; Walia et al.,1982), but not with the results published in an earlier paper (Skorupska et al.,1979). It has also been previously reported that there was no HindIII site in the 7.9kb EcoRI fragment of pLG215 (Boulnois et al.,1982). R64drd11 and R144drd3 have fairly similar EcoRI, HindIII, and BglII endonuclease generated fragment patterns and may well be closely related. The HindIII pattern for ColIb-P9 is not similar to any of the other plasmids and it may be an atypical sog+ plasmid.

R621a, RIP72, and R864a, if they have IncIα plasmid core fragments at all, lack some of the typical features of IncIα plasmids. RIP72 appears to have an equivalent EcoRI fragment for all of the EcoRI fragments of R864a. The two plasmids were isolated independantly and well separated geographically, but are obviously closely related. Comparison of the restriction fragments of R864a with those of R16 or other IncB plasmids does not show any overall core set of fragments for IncB plasmids (see figure 4.2).

5) Regulatory similarities among plasmids carrying the sog gene(s).

The extent to which the plating efficiency and DNA synthesis, at the restrictive temperature, is enhanced by

the presence of IncIα and Iδ plasmids is dependent on the level of derepression of plasmid transfer functions (Wilkins, 1975; Sasakawa and Yoshikawa, 1978; Lanka and Barth, 1981). Mutants of R864a conferring thermoresistance on BW86 were isolated. All such mutants were also derepressed for pilus synthesis, as shown by the sensitvity of strains containing these plasmids to PR64FS, and derepressed for plasmid transfer. One example of these plasmids, designated R864a*drd1*, conferred a plating efficiency of 0.2 on its *dnaG* host and had a transfer efficiency over four orders of magnitude greater than R864a (see table 3.2). When crude cell extracts of strains containing R864a and R864a*drd1* were assayed for primase activity both were active but the extract from a strain containing R864a*drd1* was about a hundred fold more active. R144 and a derepressed mutant R144*drd3* behaved in a similar way (see table 3.2).

Selection of IncIα and Iδ plasmids with mutations for efficient suppression of the *dnaG3* mutation always selects for overproducing mutants. The simplest mutation which leads to overproduction appears to be in the controlling regions of the transfer genes.

6) Immunological relationship of R864a primase to sog primase.

Table 3.2) Comparison of transfer efficiency, plating efficiency and primase activity of repressed and derepressed mutants of R864a and R144.

Plasmid	Transfer efficiency(a)	Plating efficiency	Primase activity(b)
R864a	7.1x10-4	2x10-4	0.07
R864a<u>drd1</u>	34.3	0.2	6
R144	2.7x10-3	10-6	0.17
R144<u>drd3</u>	62.5	2.5x10-3	16

a) transconjugants per 100 donors in 1 hour at 30°C.
b) pmoles 3H-TMP incorporated per ug protein.

Although R64drd11 and R144drd3 primases are inhibited by ColIb-P9 sog primase antiserum the presence of the 240,000 dalton and 180,000 dalton proteins encoded by the sog gene(s) has only been shown in extracts of strains containing ColIb-P9drd1 and pLG215 (Wilkins et al.,1981). In fact the published characterisation of the R64drd11 primase describes the primase as two polypeptides with apparent molecular weights of 180,000 and 140,000 (Lanka et al.,1979). The primases purified from pLG215 and R64drd11 containing strains also appeared to have different sedimentation coefficients in glycerol density gradients. However the genetic and antigenic similarities between the two systems required the resolving of this discrepancy in protein characterisation.

Crude cell extracts of strains containing R64, R64drd11, R144, R144drd3, R864a, R864adrd1, and pLG215 were run on SDS polyacrylamide gels (see figure 3.8). No large protein was observable in extracts of strains carrying the wild type plasmids, but a polypeptide of apparent molecular weight of 220,000, and of the same electrophoretic mobility as the '240,000' dalton polypeptide encoded by pLG215, was observed in extracts of strains carrying R864adrd1 as well as R64drd11 and R144drd3. The '180,000' dalton polypeptide is hidden under the β' subunit of RNA polymerase in this

Figure 3.8)

SDS polyacrylamide gel electrophoresis of crude cell extracts of plasmid bearing BW86 derivatives. Samples containing up to 500ug of protein were analysed on a 7% gel with a 3% stacking gel and stained with Coomassie blue. Myosin has an estimated molecular weight of 212,000, β' subunit of E.coli RNA polymerase has an estimated molecular weight of 165,000 and β subunit has an estimated molecular weight of 155,000.

- sog primase
pLG215
BW86
R864a drd1
R864a
R144 drd3
R144
R64 drd11
R64
myosin -
RNA polymerase β'=
β=

system and thus has an apparent molecular weight of 165,000. The primase activity of all of these extracts was completely inhibited by antiserum raised against the primase products of pLG214 (see table 3.3) providing further evidence that R64, R144, ColIb-P9 and R864a encode essentially identical primases (Dalrymple and Williams, in press).

7) Summary.

The results presented in this chapter confirm the previously published expectations that R64 and R144 encode a primase which is genetically and antigenically identical with the sog primase of ColIb-P9. It is also reasonable to assume that most, if not all, IncIα plasmids will have a 7.9kb EcoRI fragment and encode a sog primase on this fragment, which is one of the IncIα plasmid core fragments proposed by Chabbert et al.(1979). The IncIα plasmids encode one type of pilus and probably one type of primase, thus they may well have essentially identical transfer genes. The sog primase gene appears to form part of a operon of transfer genes and IncIα may have only one transfer operon. All of the evidence gathered so far points to the IncIα plasmids being a closely related group of plasmids. R621a (IncIδ) also encodes a primase which is genetically similar to sog primase and it may well be an anomolous IncIα plasmid.

Table 3.3) The effect of sog primase antiserum on the priming ability of crude cell extracts of plasmid carrying strains of BW86.

luCi of 3H-thymidine was used per assay with 5ul of crude cell extract and 2.5ul of a 1:10 dilution of sog primase antiserum or pre-immune antiserum.

plasmid	ug of protein in crude cell extract	no antiserum cpm.	sog antiserum cpm.	pre-immune antiserum cpm.
pLG214	0.16	22,800	1,500	N.D.(a)
	1.6	59,000	1,800	64,480
	16	97,000	32,000	N.D.
R144	51	10,800	4,600	9,000
R144drd3	0.48	9,500	2,000	8,800
	4.8	31,000	2,500	20,700
R64drd11	0.6	6,000	2,900	5,500
	6	21,500	2,100	13,200
	60	63,500	4,400	52,400
R864a	66.75	9,200	2,200	9,500
R864adrd1	0.29	5,600	2,700	4,500
	2.9	17,300	2,900	22,600
	29	44,600	3,100	43,100

a) N.D. not determined

The sog primase has been reported to be antigenically distinct from the RP4 (IncP) primase and the chromosomally encoded E.coli dnaG primase (Lanka and Barth, 1981). The evidence presented here shows that the sog primase is also genetically distinct from the RP4 primase and the chromosomally encoded E.coli dnaG primase.

The IncIγ, IncB, and IncK plasmids suppressed the DnaG- phenotype, but only RIP72 and R864a (IncB) showed significant sequence homology with the sog probe. The sog primase is therefor not restricted to plasmids of the IncIα group. R864a and RIP72 appear to be closely related to each other and must presumably share at least some of the IncIα plasmid transfer genes. The R864a plasmid primase appears to be genetically and antigenically identical to the sog primase. A polypeptide of identical apparent molecular weight to the sog primase is encoded by R864a and this polypeptide is synthesised in much larger amounts by the derepressed plasmid, R864adrd1. The expression of the primase gene(s) of R864a appears to be linked to the derepression of the transfer genes in the same way as the expression of the IncIα and IncIδ primase genes are linked to derepression of their transfer genes. These results suggest that R864a has a very similar transfer system to the IncIα plasmids.

Strains containing R864a<u>drd1</u> are sensitive to PR64FS, thus the pilus encoded by at least some of the IncB plasmids is sensitive to PR64FS.

CHAPTER FOUR.

Cloning of the R16 determinant responsible for the suppression of the DnaG- phenotype.

1) Introduction.

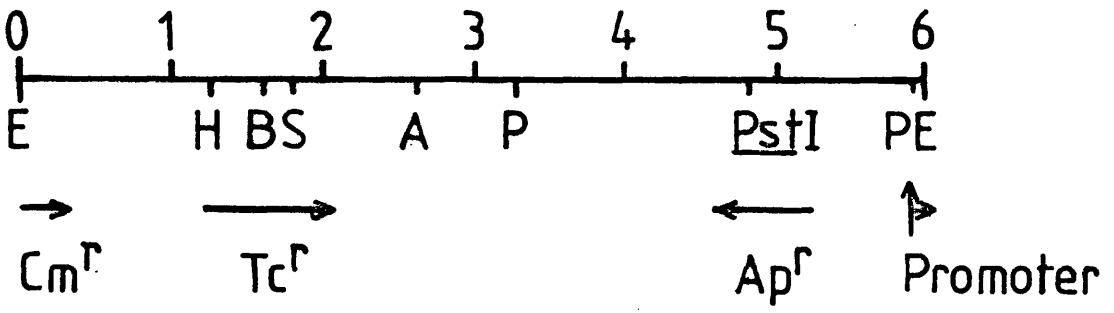
In the previous chapter I showed that all of the plasmids tested, with the possible exception of R724, suppressed the *dnaG3* mutation. Thus the I-complex plasmids can be divided into two groups on the basis of the type of *dnaG* suppression system: those with ColIb-P9 *sog* gene(s) (ColIb-P9, R64, R144, R621a, RIP72 and R864a) and those which suppress the *dnaG3* mutation but do not have sequences homologous with the *sog* gene(s) (R16, TP125, pLG101, R805a, R724, and R387).

R16 is often considered to be the prototype IncB plasmid, yet it is different from the *sog+* IncB plasmids RIP72 and R864a. In order to investigate the suppression of the DnaG- phenotype by R16 in more detail it was decided to clone the determinant responsible for the suppression of the *dnaG3* mutation into the multicopy vector plasmid, pBR325 (see figure 4.1). Expression of many genes, such as the *sog* primase gene(s) of ColIb-P9, is repressed and only very small numbers of protein molecules are synthesised. Due to

Figure 4.1) Map of pBR325.

The distances are in kb from the unique EcoRI site of pBR325. Cm, Tc and Ap – the chloramphenicol, tetracycline and ampicillin resistance genes. The promoter drives transcription of the chloramphenicol acetyl transferase gene in the direction indicated.

Map of pBR325.



E-EcoRI H-HindIII B-BamHI S-SalI

A-AvaI P-PvuII

the high copy numbers of many cloning vectors, and the powerful promoters carried by them, large amounts of gene products may be synthesised from a cloned gene. The large amounts of products makes identification and purification of the gene products much easier. A further advantage is that, again due to the high copy number of the vector, large amounts of the DNA encoding for the gene product are readily purified away from the rest of the cellular DNA. Such purified DNA is essential for restriction endonuclease analysis of the genes and to provide clean probes for hybridisation studies.

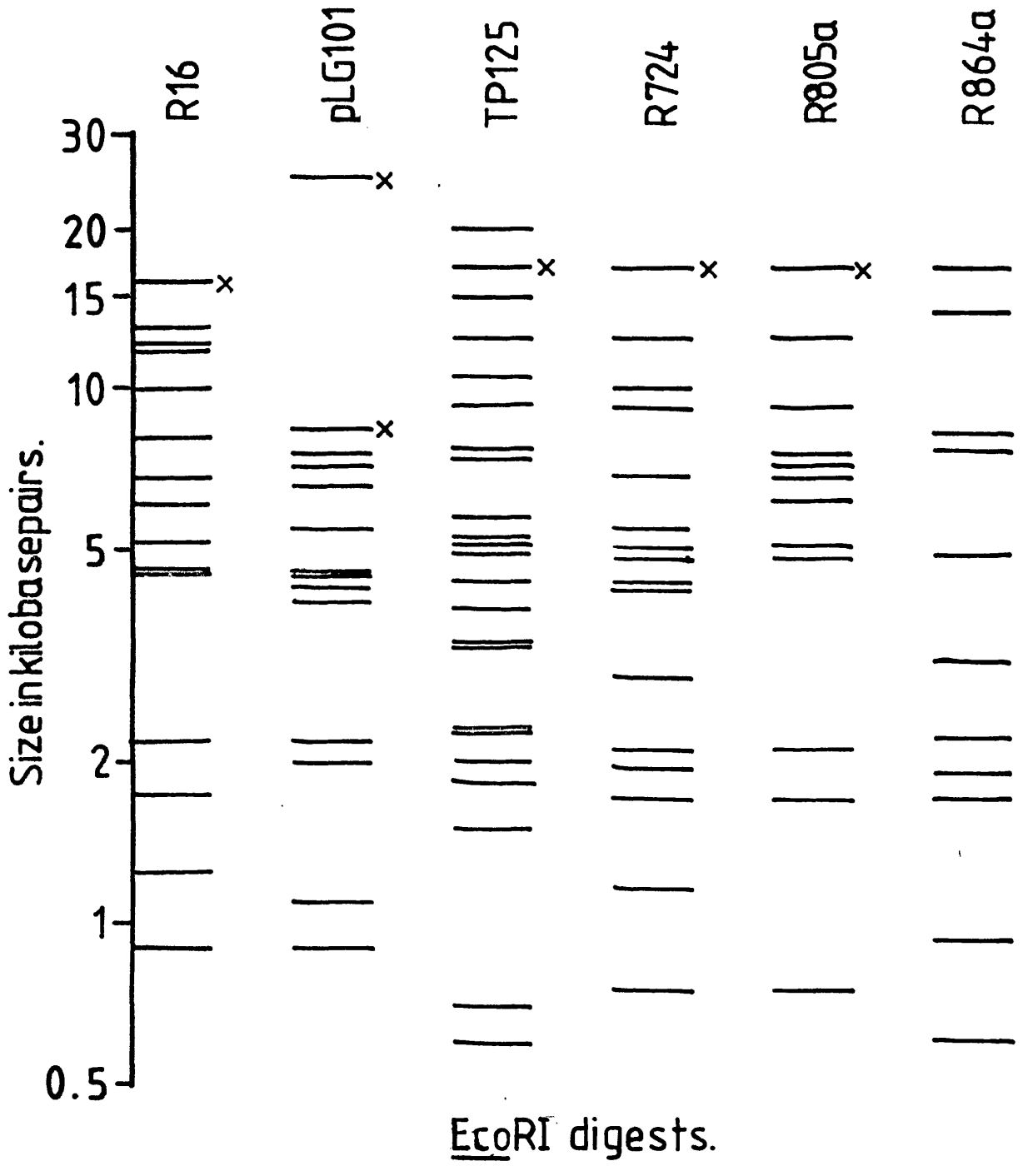
2) The cloning.

pBR325 was chosen as the vector for these experiments because it is a small multicopy plasmid which has been well characterised. pBR325 carries a strong promoter, just upstream of a single EcoRI site, which can drive a high level of transcription of the succeding inserted DNA.

Endonuclease EcoRI cleaves purified R16 DNA into fifteen major fragments (see figure 4.2). EcoRl cleaved R16 and pBR325 were mixed at a molar ratio of 4:1, ligated, and used to transform a competent culture of BW86. Ampicillin resistance at 30°C was used to select for transformants. The

Figure 4.2) Restriction enzyme digests:- EcoRI.

Fragment patterns of EcoRI digests of purified DNA from IncI and B plasmids. 'x' R16, the EcoRI fragment which carries the determinant responsible for the suppression of the dnaG mutation. 'x' others, the EcoRI fragment(s) to which the 15.8kb EcoRI fragment from R16 hybridises.

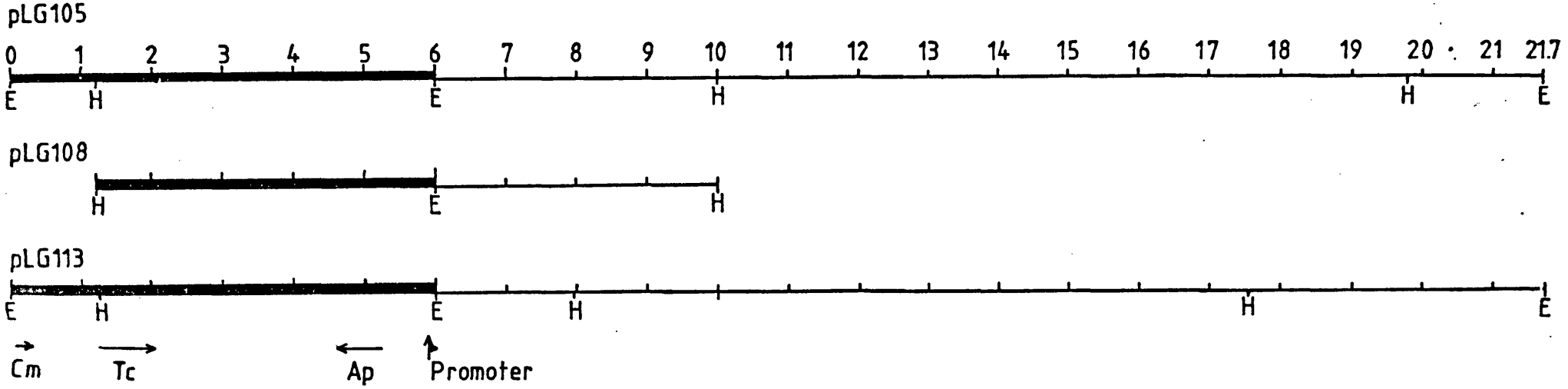

R16
pLG101
TP125
R724
R805a
R864a
30
20
15
10
5
2
1
0.5
Size in kilobasepairs.
EcoRI digests.

transformants were screened for insertional inactivation of the chloramphenicol resistance determinant, and sensitive clones were tested for their ability to form colonies at 40°C. One transformant had a plating efficiency of approximately 1; this strain carried a plasmid, designated pLG105, comprising a 15.8kb fragment of R16 ligated into the EcoRI cleavage site of pBR325 (see figure 4.3). Cured derivatives of this transformant were fully temperature sensitive. When BW86 was transformed with purified pLG105, transformants acquired thermoresistance. These results confirm that plasmid pLG105 carries the genetic determinant of R16 responsible for the suppression of the DnaG- phenotype of BW86.

Agarose gel electrophoretic analysis of single and double EcoRI and HindIII endonuclease digests of pLG105 yielded the restriction map shown in figure 4.3. In order to reduce the size of the cloned R16 fragment, while still retaining the capacity to suppress the dnaG3 mutation, pLG105 was subcloned. pLG105 DNA digested with EcoRI and HindIII was mixed with HindIII digested pBR325, ligated, and transformed into BW86 with selection at 30°C for ampicillin resistance. This mixture would allow the recloning of all three EcoRI and HindIII double digest generated fragments of the 15.8kb insert. Transformants which were chloramphenicol or tetracycline sensitive, or sensitive to both drugs were

Figure 4.3) Maps of pLG105, pLG108 and pLG113.

The distances are in kb from one of the two EcoRI sites. The vector (pBR325) is represented by the thick line and the insert by the thin line. Cm, Tc and Ap, are the chloramphenicol, tetracycline and ampicillin resistance genes. The promoter, P, drives transcription in the direction indicated. The insert in pLG113 is inverted with respect to pLG105 and pLG108.



E-EcoRI H-HindIII

further tested for their ability to form colonies at 40°C. All the transformants which showed a high efficiency of plating at 40°C carried a recombinant plasmid with the 4.1kb EcoRI-HindIII fragment of the 15.8kb insert of pLG105 cloned into a deletion derivative of pBR325 (see figure 4.3). One example of these plasmids, which conferred a plating efficiency of 10-3 on BW86, was designated pLG108. Cured derivatives of this transformant regained temperature sensitivity of colony formation. BW86 transformed with purified pLG108 DNA showed thermoresistance of the same level as the original transformant.

These observations confirm that the 4.1kb EcoRI-HindIII fragment derived from R16 and cloned into pLG105 and recloned into pLG108 carries the genetic information for the suppression of the primase deficiency of strain BW86. However it is not clear why the absolute levels of dnaG suppression observed for strains carrying plasmids pLG105 and pLG108 are different.

3) R16, pLG105 and pLG108 encode a DNA primase.

The priming activity of crude cell extracts of strains containing R16, pBR325, pLG105, and pLG108 were measured (see table 4.1). Extracts of R16, pLG105 and pLG108 containing strains were able to prime DNA replication in the

Table 4.1) Plating efficiency and priming activity of R16 and the cloned R16 primase genes.

plasmid	plating efficiency	priming activity (a)
none	<10-7	- (b)
pBR325	<10-7	-
pLG105	1	75
pLG108	10-3	60
pLG113	<10-7	-
R16	2x10-5	0.9

a) pmoles of 3H-TMP incorporated per ug of protein.
b) '-' no detectable primase activity.

in vitro primase assay system. Extracts from pBR325 containing strains were unable to prime significant DNA replication, and pBR325 cannot, of course, suppress the dnaG3 mutation. The enhanced suppression of the dnaG mutation and the high priming activity in extracts of bacteria carrying the cloned fragments is presumably due, at least in part, to the increased gene copy number. The copy number of genes cloned into pBR325 could be at least ten times as high as in strains carrying R16, which probably has a copy number of one to three.

If the 15.8kb EcoRI insert in pLG105 is inverted with respect to the vector the resulting recombinant, pLG113 (see figure 4.3), cannot supress the dnaG3 mutation. Crude cell extracts of strains containing pLG113 cannot prime DNA synthesis in the in vitro assay (see table 4.1). Thus the primase genes probably do not have their own promoter contained on the cloned 15.8kb EcoRI fragment. The transcriptional promoter for CAT probably drives transcription of the cloned primase genes.

4) Immunological relationship of R16 primase to sog primase.

To determine the antigenic relationship of the R16 primase to the sog primase, the effect of antiserum raised against the 87,000 dalton polypeptide encoded by pLG214 (see

figure 3.2) was investigated (see figure 4.4 and table 4.2). The antiserum (kindly provided by E.Lanka) did decrease the priming activity of crude cell extracts of strains containing pLG105 or R16. This inhibition of priming activity was greater than than that observed with pre-immune serum (kindly provided by T.Harrison). However at equivalent concentrations of proteins, and presumably of the two primases, the level of inhibition of activity was at least an order of magnitude less for extracts of pLG105 than for extracts of pLG214 containing strains. Thus the R16 primase is inhibited by sog primase antiserum, but not as efficiently as sog primase is inhibited. This may reflect some similarities bewteen the enzymes, but not large regions of homology. This observation predicts that antiserum raised against R16 primase would inhibit sog primase activity to a small degree. However no antiserum against R16 primase is yet available.

5) Screening for plasmid sequences homologous with R16 primase gene(s).

I investigated the occurrence of the R16 primase gene(s) using pLG105 and pLG108 DNA as the probes in colony hybridisation screening of strains carrying I-complex and IncP plasmids (see figure 4.5). Using the level of stringency which permitted no interaction of radioactive

Figure 4.4) The effect of ColIb-P9 sog primase antiserum on the primase activity of pLG105.

Crude cell extracts of strain BW86 carrying pLG214 or pLG105 were diluted as indicated with identical extracts of plasmid free BW86 and assayed for primase activity. The overall height of each histogram represents the total activity in each dilution. The black area of each histogram indicates the primase activity in the presence of antiserum. Mixtures of 2.5ul of crude cell extract (16 to 18ug total protein at each dilution) and 2ul of a 1:10 dilution of antiserum were incubated on ice for 10 minutes before assaying.

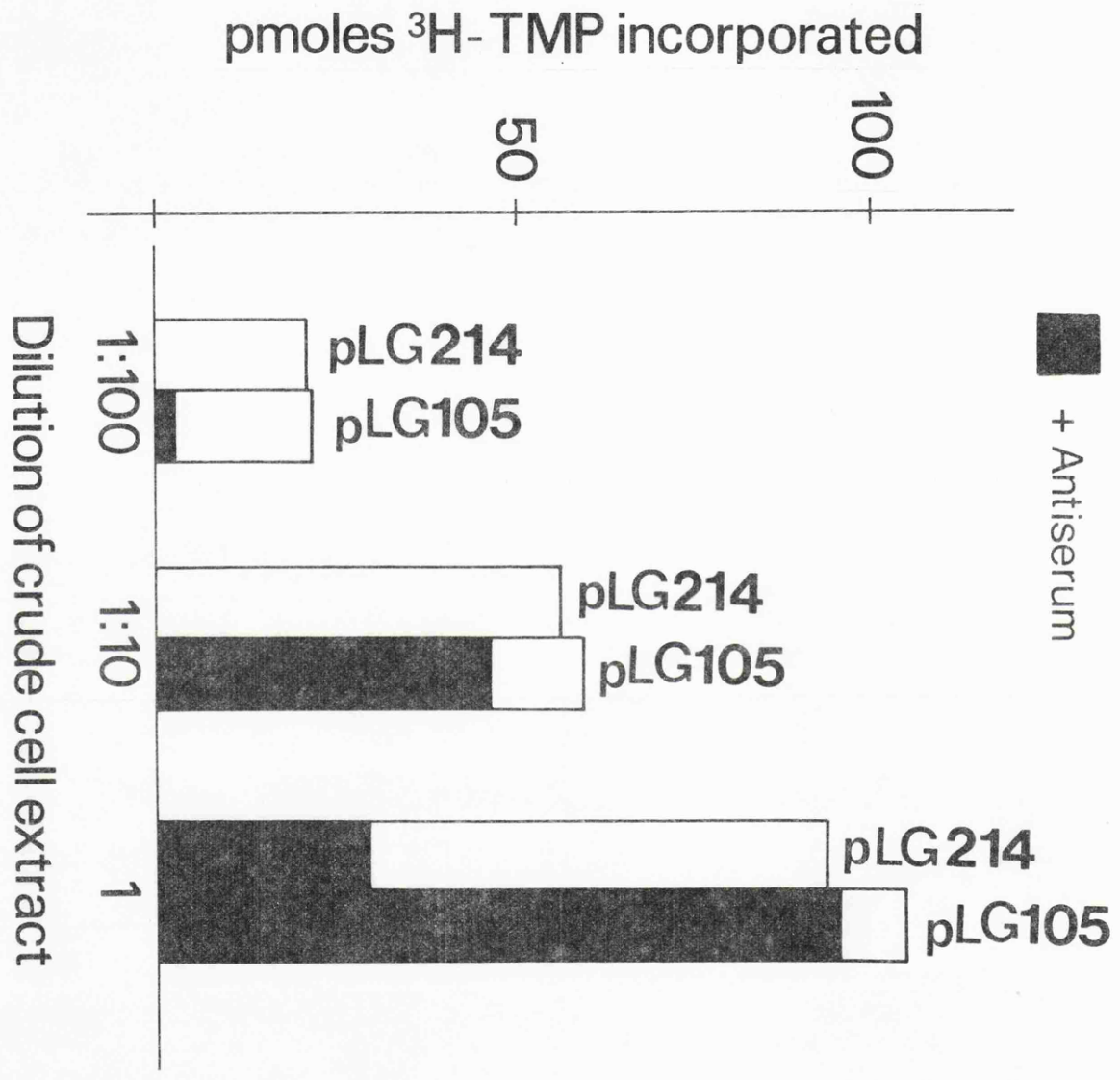

pmoles ³H-TMP incorporated
50
100
Dilution of crude cell extract
1:100
1:10
1
pLG214
pLG105
pLG214
pLG105
pLG214
pLG105
+ Antiserum

Table 4.2) The effect of sog primase antiserum on the priming ability of crude cell extracts of plasmid carrying strains of BW86.

luCi of 3H-thymidine was used per assay with 5ul of crude cell extract and 2.5ul of a 1:10 dilution of sog primase antiserum or pre-immune antiserum.

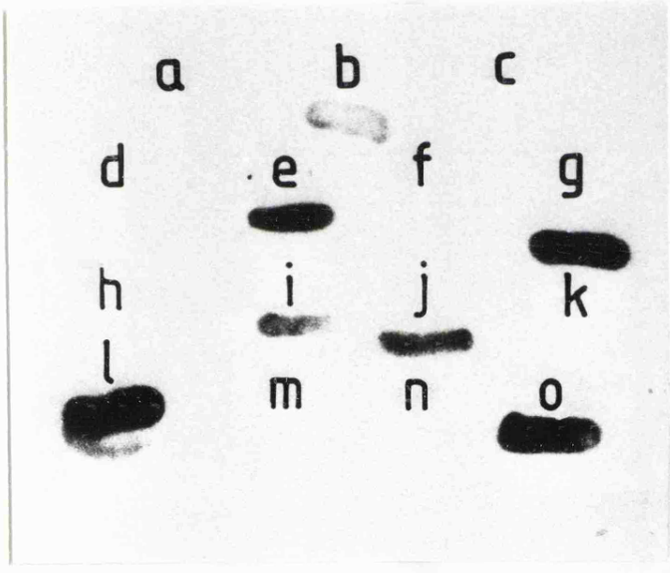
Plasmid	ug of protein in crude cell extract.	no antiserum cpm.	sog antiserum cpm.	pre-immune antiserum cpm.
pLG214	0.16	22,800	1,500	N.D. (a)
	1.6	59,000	1,800	64,500
	16	97,000	32,000	N.D.
pLG105	0.185	23,300	4,500	N.D.
	1.85	62,500	48,200	63,000
	18.5	107,900	97,700	N.D.

a) N.D. not determined.

Figure 4.5) Colony hybridisation.

Colony hybridisation of BW86 strains carrying IncI, B, K and P group plasmids. pLG108 DNA labelled with ^{32}P was the probe.

plasmid	incompatibility group	plasmid	incompatibility group
a) R64drd11	Iα	i) R724	Iδ
b) R387	K	j) R805a	Iζ
c) RP4	P	k) R864a	B
d) RIP72	B	l) pLG101	B
e) TP125	B	m) ColIb-P9drd1	Iα
f) R144drd3	Iα	n) BW86	
g) R16	B	o) pLG105	
h) R621a	Iγ		


a
b
c
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f
g
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j
k
l
m
n
o

vector plasmid pBR325 with DNA of any of the strains tested, labelled pLG108 DNA hybridised strongly with DNA of a strain carrying R16 or the probe plasmid itself, but not at all with the plasmid free host strain. In addition, positive interaction with pLG108 DNA was observed in the case of plasmids TP125, pLG101, R724, R805a, and R387. Futhermore, plasmids having sequences homologous with the ColIb-P9 sog region of pLG215 did not show significant homology with the R16-derived clones pLG105 and pLG108. Also RP4 (IncP) did not show significant homology.

The R16 primase gene(s) and 11kb of extra DNA showed no significant homolgy with the DNA of the sog+ plasmids. Thus not only are the primase genes genetically distinct, but DNA in the vicinity of the gene(s) is not significantly homologous with DNA of the IncIα plasmids or with R621a, RIP72 and R864a. Evidence from RP4 and to a lesser extent ColIb-P9 suggests that the primases may be adjacent to the transfer genes. If this is true for the R16 primase these results suggest that there are major differences between the transfer genes of R16 and R864a.

6) Genetic similarity among plasmids carrying the R16-like primase gene(s).

Plasmid DNA from pLG105, R16, TP125, pLG101,

R805a, R724, and R387 was purified by caesium chloride/ethidium bromide density gradient centrifugation. The DNA was restricted with either EcoRI or HindIII restriction endonucleases and separated by agarose gel electrophoresis (see figures 4.2 and 4.6). Restriction fragments were transferred to nitrocellulose filters by Southern blotting. The filters were used in filter hybridisation experiments with the R16-derived inserts from pLG105 or pLG108 as radioactive probes (see figures 4.7 and 4.9).

When pLG105 was used to probe R16 EcoRI digests it hybridised to a fragment of about 16kb, as expected this was the same size as the probe. When pLG105 was used to probe R16 HindIII digests it hybridised to three HindIII fragments, of approximate sizes 23kb, 17kb and 10kb (see figure 4.7). The strongest reactions were with the 10kb and 23kb HindIII fragments. The 10kb fragment must be the HindIII fragment which is completely contained within the 15.8kb EcoRI fragment in pLG105. When pLG108 DNA was used to probe R16 HindIII digests it hybridised to the 23kb HindIII fragment. Thus the 4.1kb EcoRI-HindIII fragment of pLG108 is presumably also carried on the 23kb HindIII fragment of R16. The 17Kb HindIII fragment, which is only weakly hybridised to by the pLG105 probe and not at all by the pLG108 probe, presumably carries the 2kb HindIII-EcoRI fragment. From this

Figure 4.6) Restriction enzyme digests:- HindIII.

Fragment patterns of purified plasmid DNA of IncI and B group plasmids. All sizes are the mean of at least three experiments.'x' R16, the HindIII fragments to which the 15.8kb EcoRI fragment from pLG105 hybridises.

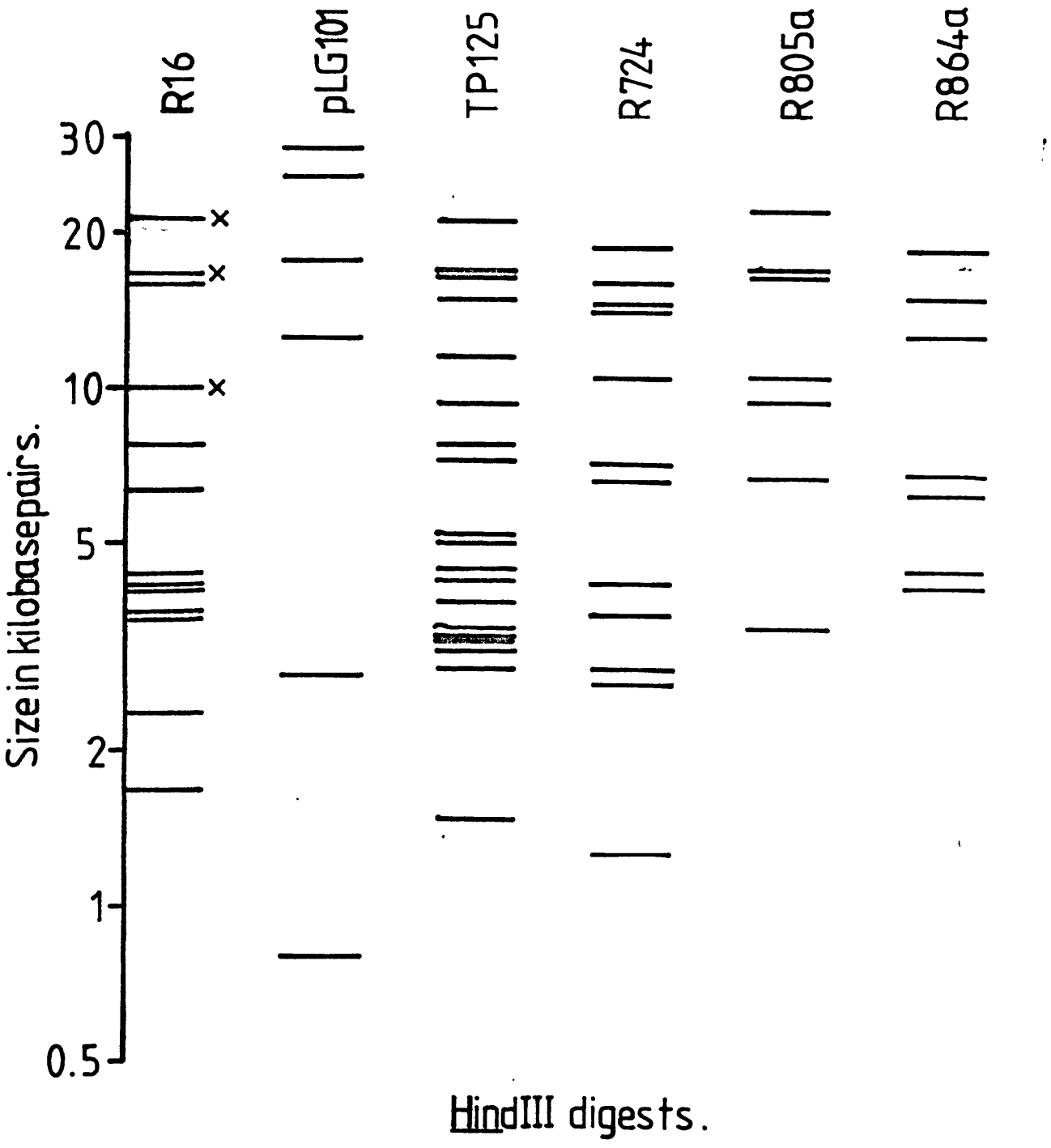

R16
pLG101
TP125
R724
R805a
R864a
30
20
10
5
2
1
0.5
Size in kilobasepairs.
HindIII digests.

Figure 4.7)

Hybridisation of 32P-labelled pLG105 and pLG108 DNA to EcoRI and HindIII digests of R16 and pLG105 DNA. Fragment sizes are shown in kb.

EcoRI
EcoRI
HindIII
EcoRI
HindIII
R16
pLG105
R16
R16
R16
23
16
10
pLG 105
pLG108
probe

data a map of the HindIII and EcoRI sites of R16 in the vicinity of the primase gene can be drawn (see figure 4.8). From my work R16 is about 105kb long, thus the region covered by this map is approximately half of R16.

There are no easily recognisable large scale similarities between the EcoRI or HindIII endonuclease generated restriction patterns among the plasmids to which the R16 probe hybridised (see figures 4.2 and 4.6). However the R16 derived probe hybridised to a 17kb EcoRI generated fragment of TP125, R724 and R805a and to 23.6kb and 7.4kb EcoRI fragments from pLG101. Thus at least one core EcoRI fragment can be proposed for some of these plasmids, the primase gene carrying fragment. This survey could easily be extended to HindIII digests of these plasmids and to other related plasmids (see figure 4.9).

7) R16-type dnaG suppression, primase activity and expression of plasmid transfer functions.

The expression of the sog primase appears to be co-ordinately linked to the expression of the transfer genes and selection for mutants of R864a suppressing the dnaG3 mutation with a high efficiency always leads to the isolation of primase overproducing mutants. RP4 is a derepressed plasmid and as yet no experiments have been

Figure 4.8)

Map of R16 in the vicinity of the primase genes compiled from the results of the hybridisation experiments. The EcoRI site closest to the primase genes was taken as 0 and distances are in kb from this site.

Map of R16 in the vicinity of the primase gene(s).

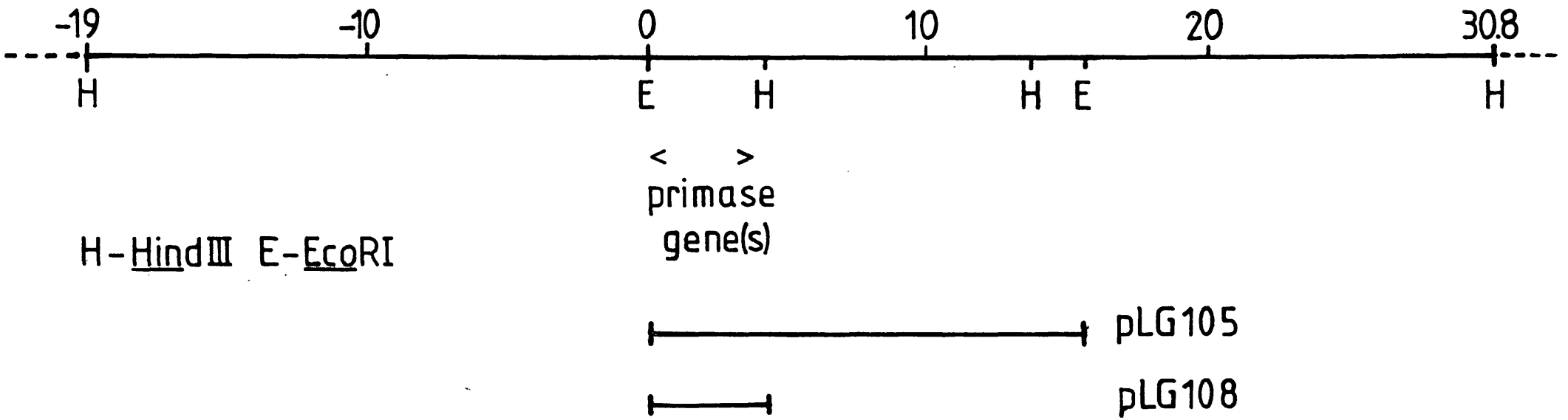
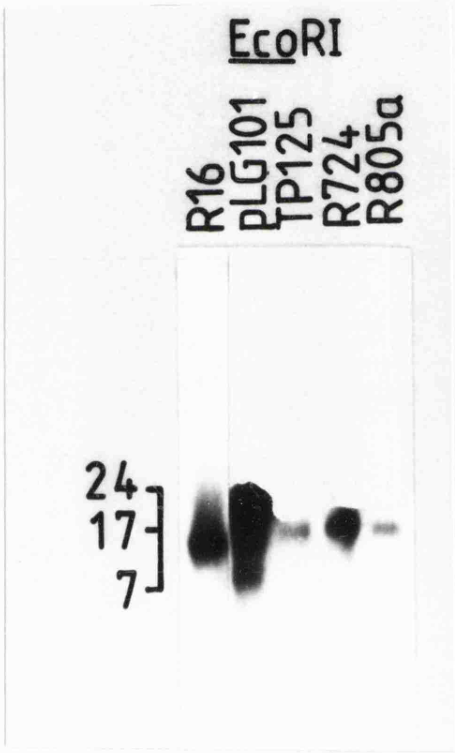


Figure 4.9)

Hybridisation of 32P-labelled pLG105 DNA to EcoRI digests of IncI and B group plasmids. Fragment sizes are shown in kb.


EcoRI
R16
pLG101
TP125
R724
R805a
24
17
7

carried out which demonstrate a relationship between the expression of the RP4 primase gene(s) and the transfer genes. I have investigated the correlation between the level of suppression of the dnaG3 mutation, the primase activity of crude cell extracts, and the regulatory state of the plasmid transfer functions in plasmids of the R16 type. Plasmids carrying mutations enabling them to suppress the dnaG3 mutation with high efficiency were purified as described in materials and methods.

For plasmid R805a, mutants mediating efficient suppression of the dnaG3 mutation were all found to be derepressed for pilus synthesis and to support detectable plaque formation by phage PR64FS. However these mutants were not derepressed for conjugal transfer and thus one of these mutants was designated R805adrpl. Extracts from strains containing R805a had no detectable priming activity (see table 4.2), but extracts from strains containing R805adrpl had a detectable priming activity. I was unable to isolate mutants of R805a authentically derepressed for conjugal transfer.

Mutants of R16 (pLG106) and TP125 (pLG107) which conferred thermoresistance on BW86 failed to act as more efficient donors in conjugation. Strains containing these plasmids were not sensitive to phage PR64FS, and crude cell

Table 4.3) Transfer efficiencies, plating efficiencies and primase activities of plasmids and mutants carrying sequences homologous with the R16 primase genes.

plasmid	transfer (a) efficiency	plating efficiency	primase activity(b)
none		<10-7	-(c)
R16	7x10-3	2x10-5	0.9
R16drd4	8x10-3	2x10-5	1
pLG106	7.8x10-3	1	0.6
TP125	8x10-3	10-5	-
pLG107	7x10-3	1	-
R805a	2.6x10-3	10-4	-
R805adrp1	1.6x10-3	1	0.5
R724	N.D. (d)	10-7	-

a) transconjugants per hundred donors in 1 hour at 30 C.
b) pmoles 3H-TMP incorporated per ug protein.
c) '-' no detectable primase activity.
d) N.D. not determined.

extracts did not contain any more priming activity than the wild type plasmids. A derepressed mutant of R16, R16drd4 (supplied by P. Barth) did not suppress dnaG more efficiently than our example of R16, it was not a more efficient donor in conjugation, and crude cell extracts did not have any more priming activity than R16. I was not able to isolate mutants of R16 or TP125 authentically derepressed for conjugal transfer. I was not able to isolate mutants of R724 which confered thermoresistance on BW86, or to detect any priming activity in crude cell extracts of strains containing R724.

The phenomenon of the suppression of the DnaG- phenotype by plasmid encoded primases is not fully understood. It is thought that the plasmid encoded primase may directly substitute in the primasome for the defective dnaG primase. This is a more complex situation than its presumed normal role in the priming of DNA synthesis on single stranded plasmid DNA, which does not require a primasome. In the in vitro phage replication assay the plasmid primase also does not require a primasome. In chromosomal replication changes in the binding sites of the primase may be very important to increase the efficiency and/or specificity of priming. Such changes in the binding efficiency may not significantly alter the primase activity as measured in the much simpler in vitro phage replication

system. These observations may explain the apparent discrepancies between plating efficiencies and primase activities of the mutant plasmids.

8) Summary.

R16 carries a gene or genes which are responsible for the suppression of the DnaG- phenotype. The product of these gene(s) is a DNA primase active in an identical assay system to that used for the sog and RP4 primases. The designation pri has been suggested for the genetic determinant of R16 primase (Dalrymple et al.,1982). The R16 primase has been shown to be genetically distinct from sog primase, RP4 primase and chromosomally encoded E. coli dnaG primase and antigenically distinct from the sog primase. The primase gene(s) have been cloned onto a multicopy vector, pBR325, and are contained on a 15.8kb EcoRI fragment of R16. This fragment can be reduced to a 4.1kb EcoRI-HindIII fragment and suppression of dnaG and primase activity remain high in strains containing pLG108. Strains carrying the cloned gene(s) contain about a hundred times the priming activity of strains carrying R16 because of the high copy number of the vector and the strong transcriptional promoter.

All of the I-complex plasmids were hybridised to

by either the sog primase gene(s) or the pri (R16 primase) gene(s). Thus the plasmids can be divided into two groups; those which carry sog gene(s), and those which carry pri gene(s). The plasmids R724, TP125 and R805a all have one EcoRI fragment to which the 15.8kb primase gene probe hybridises, although at 17kb this is slightly larger than the EcoRI fragment from R16. Thus like the sog EcoRI fragment the EcoRI sites in the vicinity of the primase gene appear to be common to a group of presumably closely related plasmids.

The evidence that TP125 encodes a primase is that it suppresses the dnaG3 mutation and carries sequences on a 17kb EcoRI fragment to which the R16 probe hybridises. The only evidence that R724 encodes a primase is that it carries sequences on a 17kb EcoRI fragment to which the R16 probe hybridises. R805a and pLG101 have both been shown to encode primases by dnaG suppression, primase activity of crude cell extracts and the hybridisation of the pri probe.

R387 (IncK) also suppressed the dnaG3 mutation and the R16 probe hybridised to the DNA, albeit fairly weakly. The R387 system is discussed in more detail in chapter six.

The control of the suppression of the dnaG mutation by the R16-like plasmids is much more confused than

that for the sog+ plasmids. Efficient suppressors may or may not also have slightly derepressed transfer systems, but plasmids with derepressed transfer systems will probably have an increased efficiency of dnaG suppression. The uncloned systems show no correlation, except for R805a, between dnaG suppression and priming activity of crude cell extracts. Thus unlike the sog+ plasmids selection for efficient suppressors probably selects mutants with altered primase binding affinities, rather than overproducing mutants. This may be because these plasmids have a more complex control system for the expression of their transfer genes.

CHAPTER FIVE.

Characterisation of the R16 DNA primase.

1) Introduction.

The primase gene(s) of ColIb-P9 encode two antigenically related polypeptides of apparent molecular weights 240,000 and 180,000 (Wilkins *et al.*,1981; Boulnois *et al.*,1982). In work described earlier in this thesis the sizes of the two polypeptides were estimated as 220,000 and 165,000 daltons (see section 3.6), and I will refer to them by these sizes in this section (Dalrymple and Williams, in press). The smaller polypeptide is encoded by DNA specifying the C-terminal region of the larger protein (Boulnois *et al.*,1982). The polypeptides may be separate translation products from a single transcript of the *sog* gene(s). Although both polypeptides are made in approximately equal amounts only the larger polypeptide appears to have primase activity. Thus the active site, or one of the active sites, is encoded in the first quarter of the gene. Plasmids such as pLG214 (see introduction, section 6.1), which contain a large deletion of the *sog* primase genes still synthesize two antigenically related polypeptides of 87,000 and 42,000 daltons (Boulnois *et al.*,1982)(see figure 3.2).

RP4 encodes a primase with an apparent molecular weight of 118,000 and an antigenically related poypeptide of apparent molecular weight 80,000 co- purifies with the larger polypeptide. Both of these polypepetides are thought to have primase activity (Lanka and Barth, 1981).

2) Mapping of the primase gene(s).

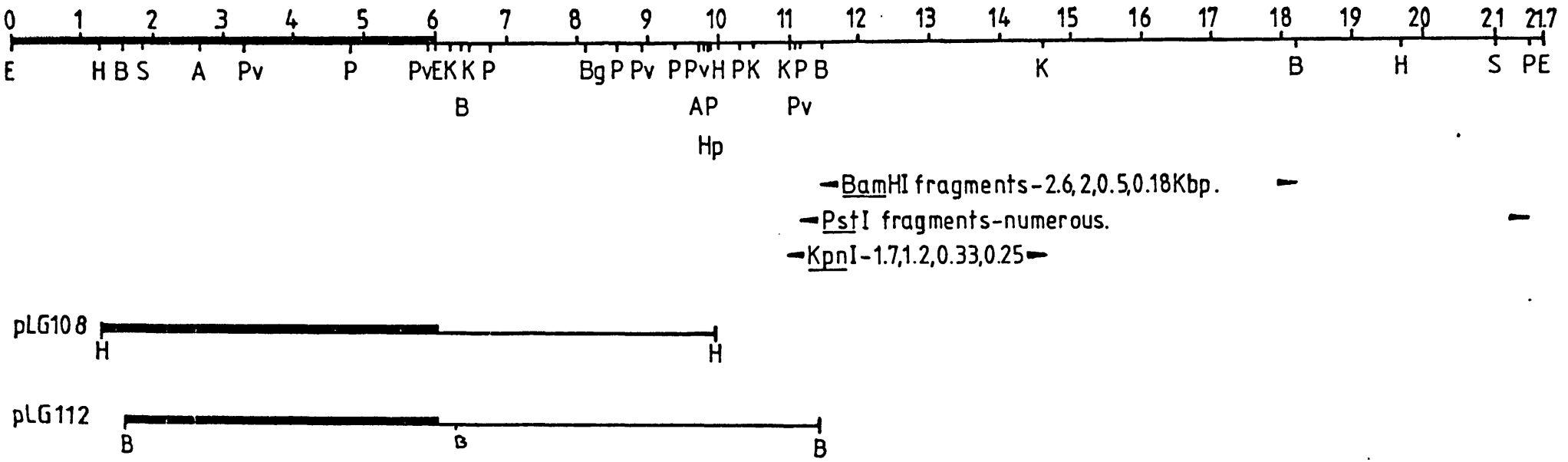
Mapping of some of the restriction enzyme sites of the cloned fragment is essential before a genetic characterisation of the cloned system can be commenced, because the rationale of further experiments may depend on the nature of the restriction enzyme sites present on the insert and the vector and their distribution along the DNA.

Using some of the restriction endonucleases listed in chapter two, section 15, detailed maps of pLG105 and pLG108 (see figure 5.1) were constructed. These maps confirm that pLG108 carries the section of the insert in pLG105 which is proximal to the transcriptional promoter of the chloramphenicol acetyl transferase gene carried by the vector. The region between the first HindIII site and the second BamHI site of the 15.8kb EcoRI fragment was mapped using a subclone of pLG105, designated pLG112, containing the EcoRI-BamHI site 2 fragment proximal to the promoter (see figure 5.1). The published positions of restriction

Figure 5.1) Detailed physical map of pLG105 showing the extent of pLG108 and pLG112.

Distances are in kb from one of the two EcoRI sites present on pLG105. The vector (pBR325) is represented by the thick line and the extent of the inserted EcoRI fragment by the thin line. The PvuII sites beyond the site at 11.1 kb (pLG105) have not been determined. The exact order of the PvuII, AvaI, HpaI and PstI sites between 9.7 and 10kb (pLG105) has not been determined. There is one site for XhoI on the insert in pLG105, but there are no sites on pLG108 or pLG112. There are no sites for XbaI on the insert or vector DNA for pLG105, pLG108 and pLG112.

Detailed map of pLG105



E-EcoRI H-HindIII B-BamHI S-SalI A-AvaI Pv-PvuII P-PstI K-KpnI Bg-BglII H-HpaI

enzyme cutting sites for pBR325 were used in the construction of the maps (Bolivar, 1978; Prentki et al.,1981).

3) Generation of deletion derivatives of pLG108.

The approximate extent of the primase gene(s) on the cloned fragment had been established by the construction of pLG108. In order to determine more precisely the extent of the gene(s), and to aid in the identification of the gene products, a deletion analysis of the EcoRI-HindIII fragment carried by pLG108 was undertaken.

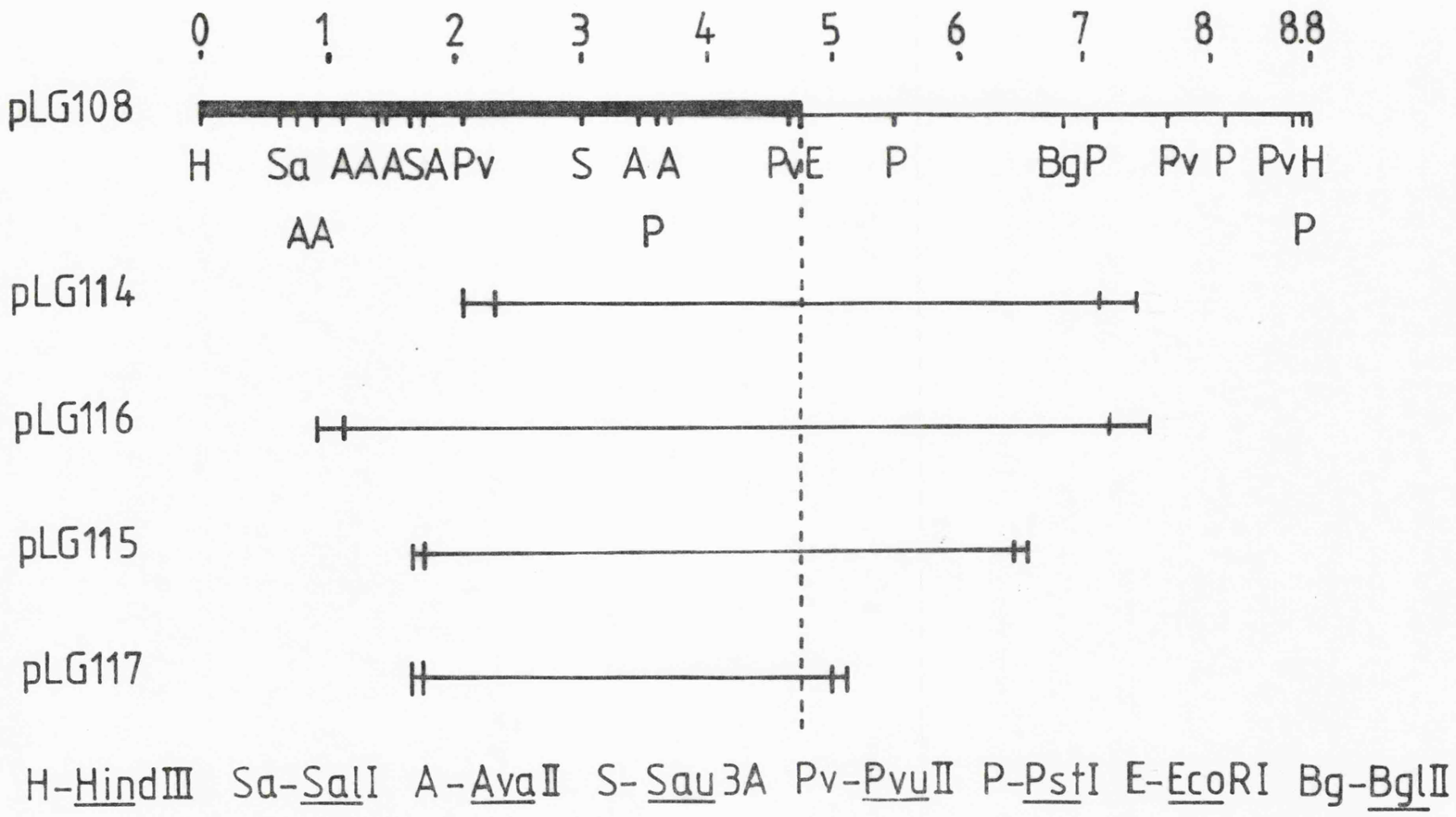
Plasmid pLG108 DNA was first cleaved at the unique SalI site in the tetracycline resistance genes of the pBR325 moiety (see figure 5.2). This generated a linear molecule which was used to transform BW86. Transformation by linear DNA occurs with about 1% of the efficiency for circular DNA. Many of the recircularised plasmids have deletions extending from the restriction site where the plasmid was originally linearised. The mechanism by which this occurs is not understood (Soberon et al.,1980). Recircularised derivatives of pLG108 having suffered deletions would have an inactivated tetracycline resistance system.

Transformants were initially selected for

Figure 5.2) Maps of deletion derivatives of pLG108.

Distances are in kb from the unique HindIII site of pLG108. The thick line shows the extent of the vector (pBR325) DNA and the thin line the extent of the inserted EcoRI-HindIII fragment. For the deletion derivatives the line indicates the extent of the DNA from pLG108 which they still retain. The errors in the positioning of the ends of the molecule are indicated by the short vertical lines.

Maps of deletion derivatives of pLG108.



ampicillin resistance (at 30°C) and were then screened for tetracycline sensitivity (at 30°C). Deletions could occur in both directions from the SalI site, but only those plasmids also missing the unique HindIII site would have deletions which could extend into the primase gene(s). Plasmid DNA was made from 55 ampicillin resistant, tetracycline sensitive transformants using the phenol mini plasmid preparation method. The plasmid DNAs were screened for the HindIII endonuclease site and twelve plasmids were missing the HindIII site. Large amounts of DNA was prepared from these plasmids by caesium chloride/ethidium bromide density gradient centrifugation to allow a more detailed mapping to be carried out.

The extent of the deletions present in these derivatives of pLG108 were then mapped using the detailed restriction maps of pLG108 (see figure 5.1). The closely spaced Sau3A and AvaII sites were used to map the deletions in the pBR325 moiety to within half a kb (see figure 5.2). Plasmids pLG120 and pLG121 were not linearised by EcoRI and hence the whole insert had been deleted. Plasmids pLG114 and pLG116 had suffered similar small deletions, of about 1.5kb, into the cloned fragment. They had therefore retained about 2.5kb of the insert. Plasmid pLG115 had retained about 1.7kb of the insert. Plasmid pLG117 had approximately 0.45kb of the insert remaining (see figure 5.2).

In order to study the extent of the gene at the end of the insert directly adjacent to the EcoRI site the DNA between the EcoRI site and the first BamHI site was removed by the following strategy. Insertion of the BamHI-BamHI fragment of pLG112 (see figure 5.1) into the BamHI site of vector pMOB45 (Bittner and Vapnek, 1981) generated two plasmids, designated pLG132 and pLG133 (see figure 5.3). These plasmids have the 5.1kb BamHI fragment inserted in both possible orientations. Thus pLG132 and pLG133 effectively have the first 0.4kb of the insert from the EcoRI-BamHI site2 deleted.

4) Characterisation of the deleted plasmids.

The plating efficiences of the deletion derivatives of pLG108 and pLG132 and pLG133 were measured (table 5.1). BW86 containing pLG114 has a higher plating efficiency than pLG108. Whereas BW86 containing pLG115 or pLG116 has a similar plating efficiency to strains containing pLG108. Crude cell extracts of all three strains have a high level of primase activity. These results show that while crude cell extracts of strains containing plasmids such as pLG105, pLG108, pLG114 and pLG115 have very similar priming activity in vitro they have a significantly different suppression activity in vivo. This may be due to

Figure 5.3) Map of vector pMOB45 and derivatives, pLG132 and pLG133.

Distances are in kb from one of the two SmaI sites of pMOB45. Cm and Tc, chloramphenicol and tetracycline resistance genes. oriV, origin of vegetative replication. cop, copy number control. rep, replication control.

pLG132 carries the 5.1kb BamHI fragment from pLG112 in the correct orientation for transcription to be driven by the promoter of the tetracycline resistance genes.

pLG133 carries the 5.1kb BamHI fragment in the reverse orientation.

Map of vector pMOB45 and derivatives, pLG132 and pLG133.

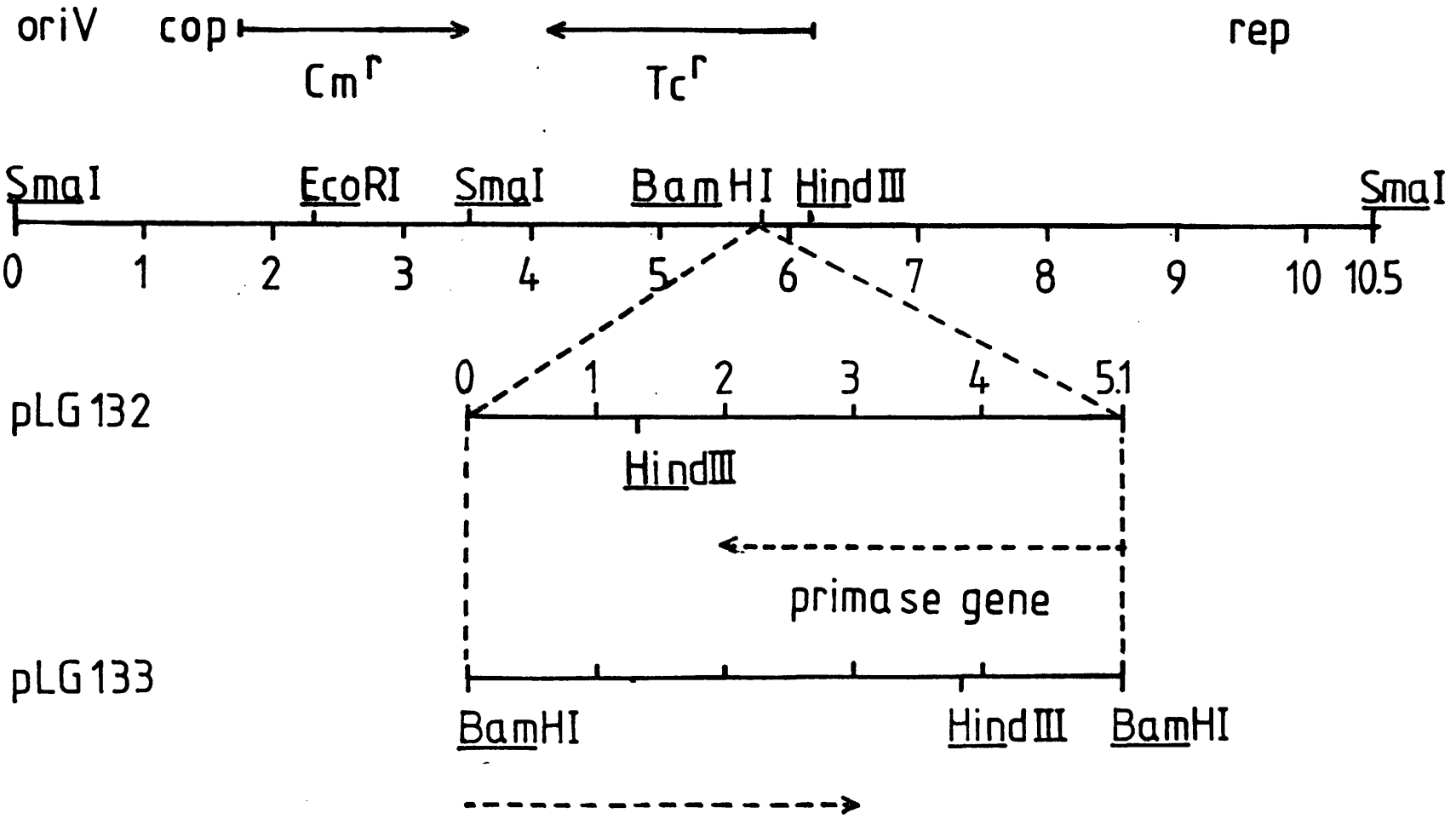


Table 5.1) Plating efficiencies and primase activities of deletion mutants of pLG108.

Plasmid	plating efficiency	primase activity (a)
none	<10-7	- (b)
pLG108	10-3	60
pLG112	1	N.D. (c)
pLG114	1	65
pLG115	10-3	95
pLG116	10-3	66
pLG117	<10-7	-
pLG120	<10-7	-
pLG121	<10-7	-
pLG132	<10-7	-
pLG133	<10-7	-

a) pmoles 3H-TMP incorporated per ug of protein.
b) '-' no detectable primase activity.
c) N.D. not determined.

altered DNA binding, or priming efficiency, caused by the synthesis of altered proteins. pLG117 confers a negligible increase in plating efficiency on BW86 and crude cell extracts of strains containing pLG117 have no detectable priming activity. Presumably the large deletion has totally inactivated the gene or only a very truncated and inactive primase is synthesised.

Neither pLG132 nor pLG133 suppress the dnaG3 mutation or encode for measurable primase activity (see table 5.1). The validity of measuring the suppression of the dnaG3 mutation by pLG132 and pLG133 is questionable. pMOB45 is a thermosensitive vector and at high temperature uncontrolled plasmid replication and protein synthesis occurs. However this is not lethal and one could expect to see some viable colonies of BW86 at 40°C if large amounts of active primase were synthesised from the plasmids. Thus either no effective promoter of transcription or translation is present on the vector DNA or the expression of the primase genes requires at least some of the DNA between the EcoRI site and the first BamHI site on the insert.

5) Protein products of the recombinant plasmids.

The proteins encoded by the plasmids were analysed by labelling the protein products of a coupled in vitro

transcription/translation system with 35S-methionine and electrophoresing the products on SDS-polyacrylamide gels (see figures 5.4 and 5.5). The autoradiographs show the characteristic products of pBR325 and the ladder of polypeptides up the gel whose synthesis was directed by the inserts in pLG105, pLG108, pLG112, pLG114, pLG115 and pLG116. This ladder of polypeptides is probably due to premature termination, at preferred sites, during the transcription or translation steps of the Zubay system. The laddering cannot be due to a series of multimeric protein structures as either a very small monomer would be involved or several differently sized components. The pattern of polypeptides is very similar for the products of the different plasmids and the deletion mutants retain essentially identical patterns below the largest polypeptide synthesised. A similar result was observed by G. Boulnois (unpublished data) for the products of the cloned sog primase. This laddering may be a characteristic of such large polpeptides and the Zubay system may not be able to cope with large gene products due to a depletion of one or more substrates, or the lack of stabilisation of partially completed molecules (Zubay and Chambers, 1969).

pLG105, pLG108, and pLG112 appear to encode two large polypeptides of apparent molecular weights 215,000 and 170,000. pLG114 and pLG116 encode one large polypeptide of

Figure 5.4)

Autoradiograph of the 35S-methionine labelled protein products of an *in vitro* coupled transcription/translation system run on an 11% SDS polyacrylamide gel. Purified plasmid DNA was used to initiate the reactions. The experiment was carried out by J. Pratt.

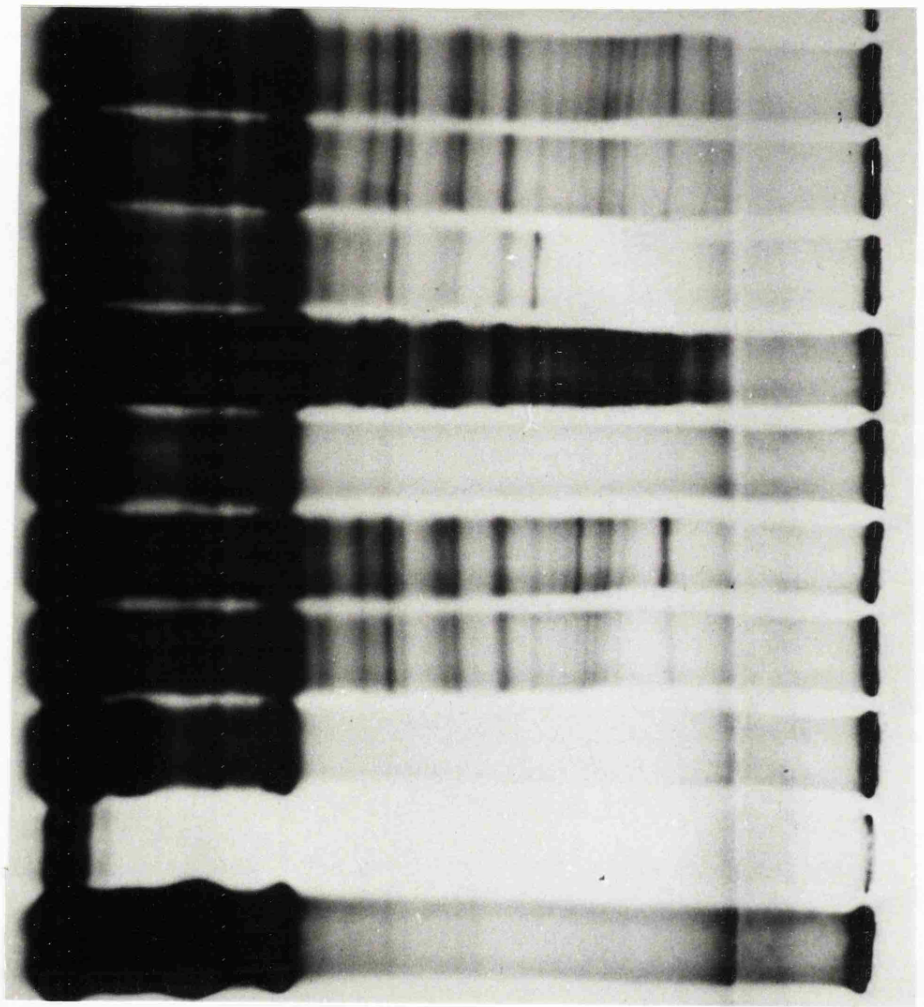
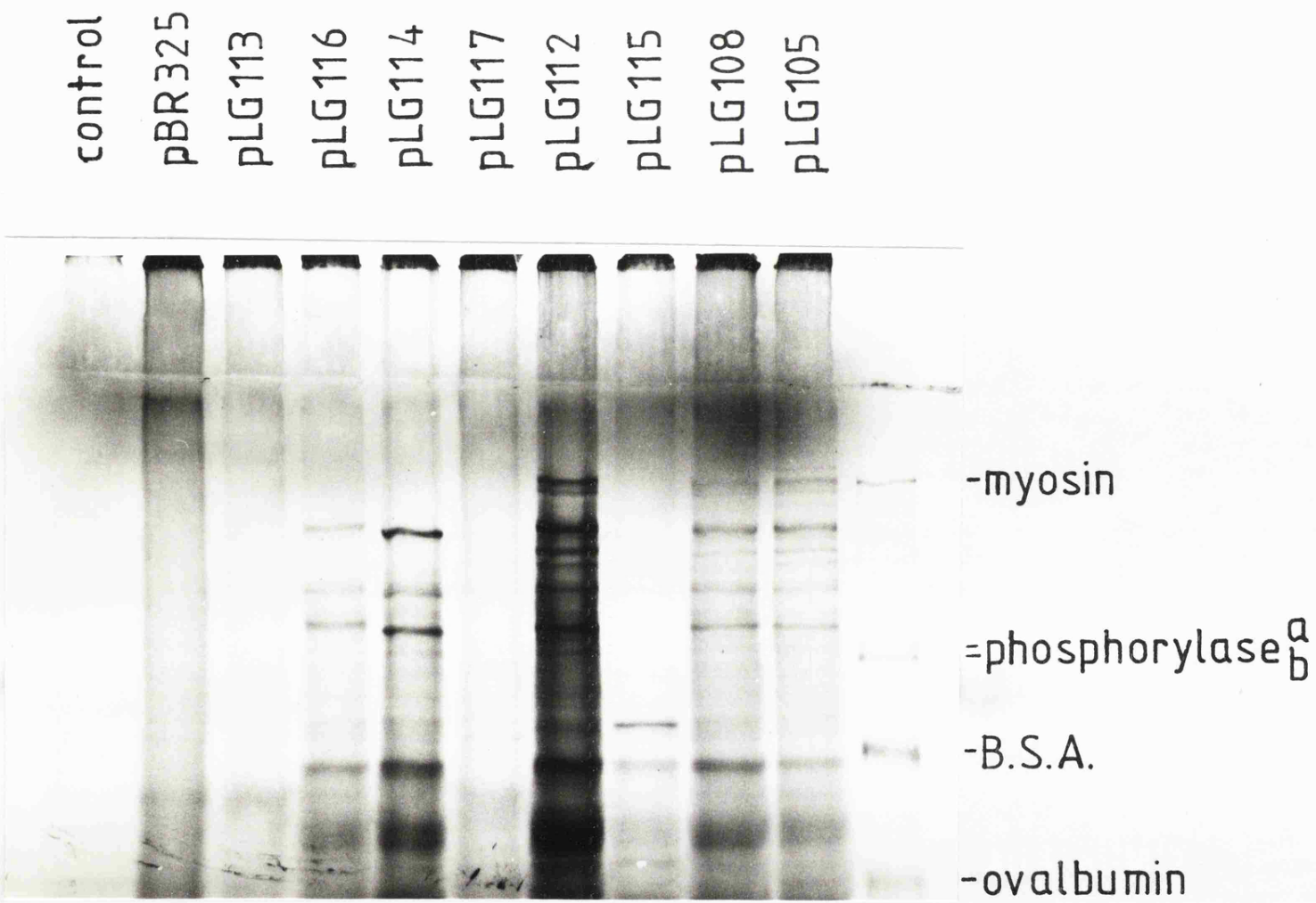

pLG105
pLG108
pLG115
pLG112
pLG117
pLG114
pLG116
pLG120
control
pBR325

Figure 5.5)

Autoradiograph of the 35S-methionine labelled protein products of an in vitro coupled transcription/translation system run on a 5% SDS polyacrylamide gel. Purified plasmid DNA was used to initiate the reactions. The experiment was carried out by J. Pratt. Myosin has an estimated molecular weight of 212,000, phosphorylase 'a' has an estimated molecular weight of 100,000, phosphorylase 'b' has an estimated molecular weight of 94,000, B.S.A. has an estimated molecular weight of 69,000 and ovalbumin has an estimated molecular weight of 43,000.


control
pBR325
pLG113
pLG116
pLG114
pLG117
pLG112
pLG115
pLG108
pLG105
-myosin
=phosphorylase a/b
-B.S.A.
-ovalbumin

apparent molecular weight 170,000, although the pLG114 encoded polypeptide may be slightly smaller. pLG115 encodes one polypeptide of apparent molecular weight 76,000. pLG117 has no visible product larger than the proteins encoded by the vector. pLG113 also does not encode a visible product larger than the proteins encoded by the vector.

The larger proteins are also visible in crude cell extracts of plasmid containing strains run on SDS polyacrylamide gels (see figure 5.6). The larger protein has an apparent molecular weight of 240,000, somewhat larger than the apparent molecular weight of 215,000 estimated for the product of the Zubay system. This discrepancy may be because the largest polypeptide is made in very small amounts in the Zubay system and hence is not visible, or that the products of the Zubay system run differently on gels from the in vivo products. The smaller polypeptide has an apparent molecular weight of 175,000, which is similar to the size of 170,000 calculated from the products of the Zubay system. The presence of the 170,000 dalton polypeptide in the products of the Zubay sytem correlates exactly with the presence or absence of the 175,000 dalton polypeptide in the crude cell extracts. The sog primase, which is also shown on the gel, has an apparent molecular weight of 220,000 and 165,000 and thus is slightly smaller than the R16 primase. pLG124 and pLG125 are further deletion

Figure 5.6)

Coomassie blue stained protein profiles of crude cell extracts of plasmid containing derivatives of BW86 run on a 7% SDS-polyacrylamide gel. Samples contained up to 500ug of protein. Myosin has an estimated molecular weight of 212,000, E.coli RNA polymerase subunits β', β, σ and α have estimated molecular weights of 165,000, 155,000, 90,000 and 39,000 respectively.

myosin-
β'
β
γ-
α-
BW86
pLG221
pLG215
pLG105
pLG108
BW86
pLG114
pLG115
pLG116
pLG124
pLG125
-R16 primase?
-sog primase
-R16 primase?

derivatives of pLG108. pLG125 is essentially identical to pLG114 and pLG124 has a deletion of about 300 base pairs into the insert, but has an identical phenotype to pLG108.

The 240,000 dalton polypeptide appears to be present in significantly larger amounts than the 175,000 dalton polypeptide in strains containing pLG105 and pLG108. In contrast the equivalent sog primase polypeptides are thought to be synthesised in approximately equal numbers.

In addition to the two polypeptides of apparent molecular weights 240,000 and 175,000 there is a band on the gel for a polypeptide of apparent molecular weight 190,000 which increases in intensity when pLG105 or pLG108 are present in the strain from which the extract was made. A similar increase in intensity is not visible when pLG215 is present in the strain. It is possible that a third polypeptide product, of 190,000 daltons, is synthesised from the cloned genes.

6) Correlation of polypeptides with DNA.

The commonly accepted figure for amount of protein per kb of DNA is 35,000 daltons. Thus proteins of apparent molecular weights of 240,000 and 175,000 would be encoded by approximately 6.9kb and 5.1kb of DNA respectively, which is

about 75% of the 15.8kb EcoRI insert of pLG105. However both polypeptides are encoded on the 4.1kb EcoRI-HindIII insert of pLG108. For the expected ratio of 35,000 daltons of protein per kb of DNA virtually all of the coding capacity of pLG108 would be required to encode for the 240,000 dalton polypeptide. Even assuming that the proteins are encoded by overlapping genes about 65,000 daltons of protein appears to be encoded per kb. The truncated polypeptides also appear to be encoded by similarly small amounts of DNA. Hence there is a linear relationship between extent of deletion and amount of polypeptide lost as a consequence of the deletion. This is good evidence that the 240,000 polypeptide is a single molecule and not just a very denaturation resistant multimeric protein. A similarly large amount of protein encoded per kb of DNA has been observed for the sog primase (Boulnois et al.,1982). These apparently very large primase molecules may behave anomolously on SDS polyacrylamide gels due to an unusual tertiary structure or amino acid composition. However there is no obvious explanation for this large discrepancy in coding capacity. In contrast the RP4 primase appears to encoded by much more DNA than is required.

7) The proteins are probably not fusion products.

The large discrepancy in ratio of protein to DNA

could be due the fusion of the primase gene product to a vector gene product. However polypeptides of the same apparent molecular weight, 240,000, are synthesised by pLG105, pLG108 and pLG112. These plasmids have from less than one kb to over ten kb of DNA distal to the apparent end of the primase gene. Thus fusion at the C-terminal end of the protein is unlikely, although small differences in molecular weight would not be easily detectable with such large polypeptides. It is unlikely that in each case almost identical lengths of protein have been fused to the C-terminus of the primase molecule. However if only a small fragment of extra protein is fused to the primase moeity it would not significantly affect the high ratio of protein per kb of DNA.

Between the chloramphenicol acetyl transferase promoter and the EcoRI site there is a translational start and codons for part of the N-terminus of chloramphenicol acetyl transferase. It is possible that genes cloned directly adjacent to the EcoRI site may be expressed due to the synthesis of a fusion product consisting of the N-terminus region of chloramphenicol acetyl transferase and the C-terminus of the protein encoded by the inserted DNA.

Antiserum raised against pBR325 encoded chloramphenicol acetyl transferase (kindly provided by

W.Shaw), did not inhibit the priming activity of strains containing pLG105. The antiserum was shown to precipitate chloramphenicol acetyl transferase protein in immunoprecipitation experiments. Thus either there is no fusion product between the primase moeity and a truncated N-terminus of chloramphenicol acetyl transferase synthesised or the binding of the antiserum does not significantly inhibit primase activity. Again such a fusion, if it were present, would not significantly reduce the ratio of protein per kb of DNA.

8) The polypeptides may be encoded by overlapping genes.

The constraints of space which have been discussed above suggest that the 240,000 dalton and 180,000 dalton polypeptides may be the products of overlapping genes. A deletion of 1.5kb into the primase genes, such as suffered by pLG116, still allows the synthesis of a 175,000 dalton polypeptide. This product appears to be identical in size to the 175,000 dalton polypeptide encoded by pLG105 and pLG108. Thus it is tempting to imagine that it is an identical gene product and not a truncated 240,000 dalton polypeptide as observed in the equivalent sog primase gene deletion. However small differences in size would be difficult to detect with such large polypeptides. No new polypeptide of about 110,000 daltons is obvious on the autoradiograph of

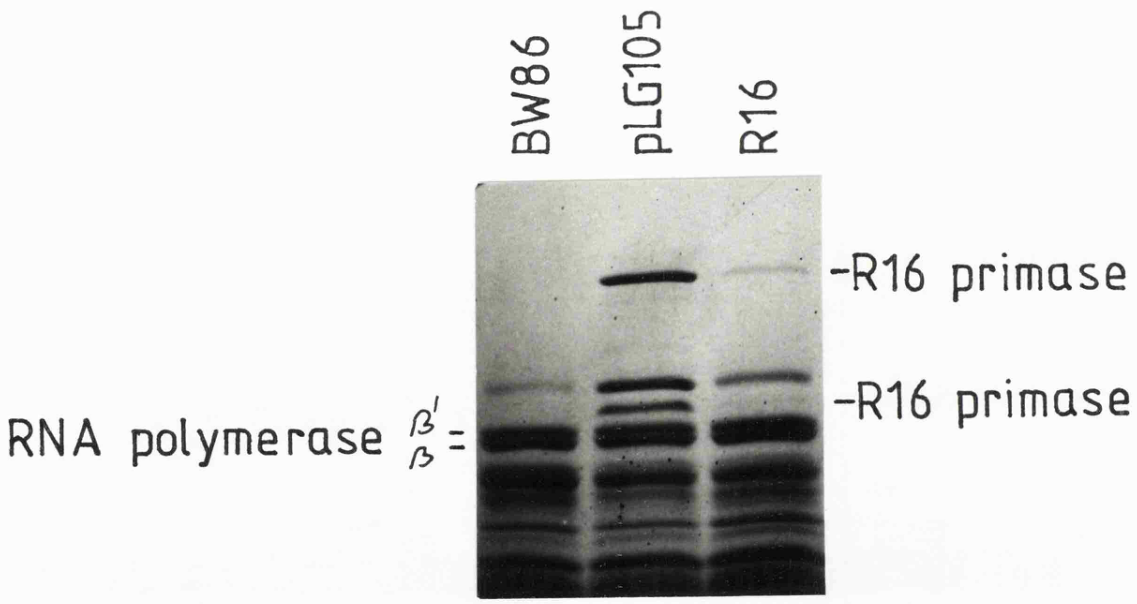
the products of the Zubay system, although it could be obscured by a premature termination product already present in the sample. Further experiments are required to unequivocally determine the organisation of the overlapping genes, if they are overlapping genes. Minicells or maxicells systems may give a cleaner profile of products and allow one to see if a further smaller product is encoded by pLG116. Another explanation of these results is that the smaller polypeptides are processed products of the large polypeptide. Deletion of part of the gene could then leave only polypeptides of the same length due to processing.

9) R16 encodes the 240,000 and 175,000 dalton polypeptides.

Futher evidence that the large polypeptides with apparent molecular weights of 240,000 and 175,000 are not fusion products is provided by the observation that wild type R16 encodes polypeptides of apparently identical size (see figure 5.7). The polypeptides can only be observed in crude cell extracts of R16 containing strains when over 500ug of protein is loaded per slot. This severely overloads the gel with small proteins and hence only the larger proteins are shown on the photograph. The difference in intensity of the 240,000 dalton band in extracts of pLG105 and R16 containing strains is approximately a hundred fold. This agrees with the hundred fold difference in the priming

Figure 5.7)

Coomassie blue stained protein profiles of crude cell extracts of plasmid containing derivatives of BW86 run on a 5% SDS-poyacrylamide gel. Samples contained up to 500ug of protein. E.coli RNA polymerase subunits β' and β have estimated molecular weights of 165,000 and 155,000 respectively.


BW86
pLG105
R16
-R16 primase
-R16 primase
RNA polymerase β' =
β

activity of the crude cell extracts (see table 4.1). Also in extracts of strains containing R16 the 240,000 dalton polypeptide appears to be present in larger amounts than the 175,000 dalton polypeptide.

An increase in the intensity of the 190,000 dalton polypeptide band can also be seen in extracts of both pLG105 and R16 containing strains. In between the 240,000 dalton band and the 190,000 dalton band two new bands can just be distinguished. These may be breakdown products of the large protein or prematurely terminated gene products.

10) Partial purification of the R16 primase.

A variety of purification procedures have been used to purify plasmid encoded primases (Lanka et al.,1979;Wilkins et al.,1981;Lanka and Barth, 1981). The first plasmid encoded DNA primase to be purified was from crude cell extracts of strains containing R64drd11 (Lanka et al.,1979). A 10,000 fold increase in specific activity was achieved and polypeptides of apparent molecular weight 180,000 and 140,000 were purified. A problem of breakdown of the large polypeptides was encountered during the purification of sog primase from extracts of strains containing pLG215. A plasmid, pLG214, which carried a large deletion and synthesised truncated primase molecules was

used as the source of primase protein (Wilkins et al.,1981).

It was decided to use a heparin Sepharose CL-6B column as a crude purification step for the R16 primase encoded by pLG105. The DNA primases are obviously DNA binding proteins and heparin Sepharose is an affinity column for such proteins. Linear gradients of increasing salt concentration elute the proteins from the heparin Sepharose in order of their affinity for DNA. Those proteins which bind strongly to DNA require high salt concentrations to be eluted from the column. For sog primase and RP4 primase NaCl was used to elute the proteins from the column. Primase activity eluted in a single peak at 450-500mM NaCl (Wilkins et al.,1981;Lanka and Barth, 1981).

50ml of crude cell extract from a strain of BW86 containing pLG105 was loaded onto a heparin Sepharose CL-6B column. The bound proteins were eluted with a linear gradient of KCl, from 50mM to 800mM (see figure 5.8). The first major protein peak eluted at about 170mM KCl, followed by a second peak eluting at 250-400mM KCl. The primase activity eluted as a single peak at 375mM KCl (fraction 52). Samples of fractions were run on an SDS polyacrylamide gel (see figure 5.9). The large pLG105 encoded polypeptide of apparent molecular weight eluted concomitantly with primase activity. However the 175,000 dalton polypeptide was not

Figure 5.8)

Profile of the elution of bound pLG105 encoded R16 primase from a heparin Sepharose CL-6B column with a linear gradient from 50mM to 800mM KCl (-). The protein concentration (+) and primase activity (x) are plotted against fraction number. A 150ml gradient was used and 1.7ml fractions were collected.

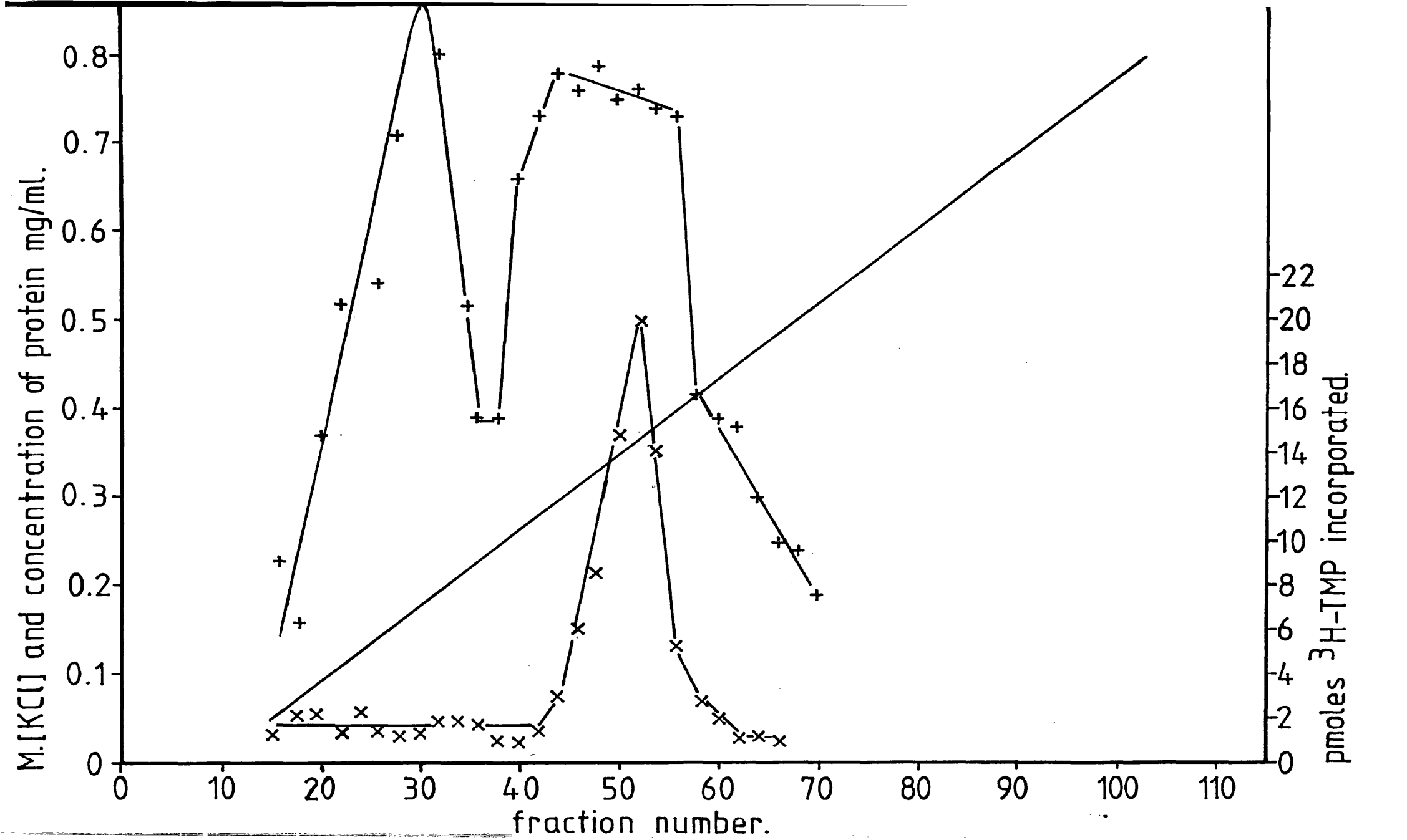
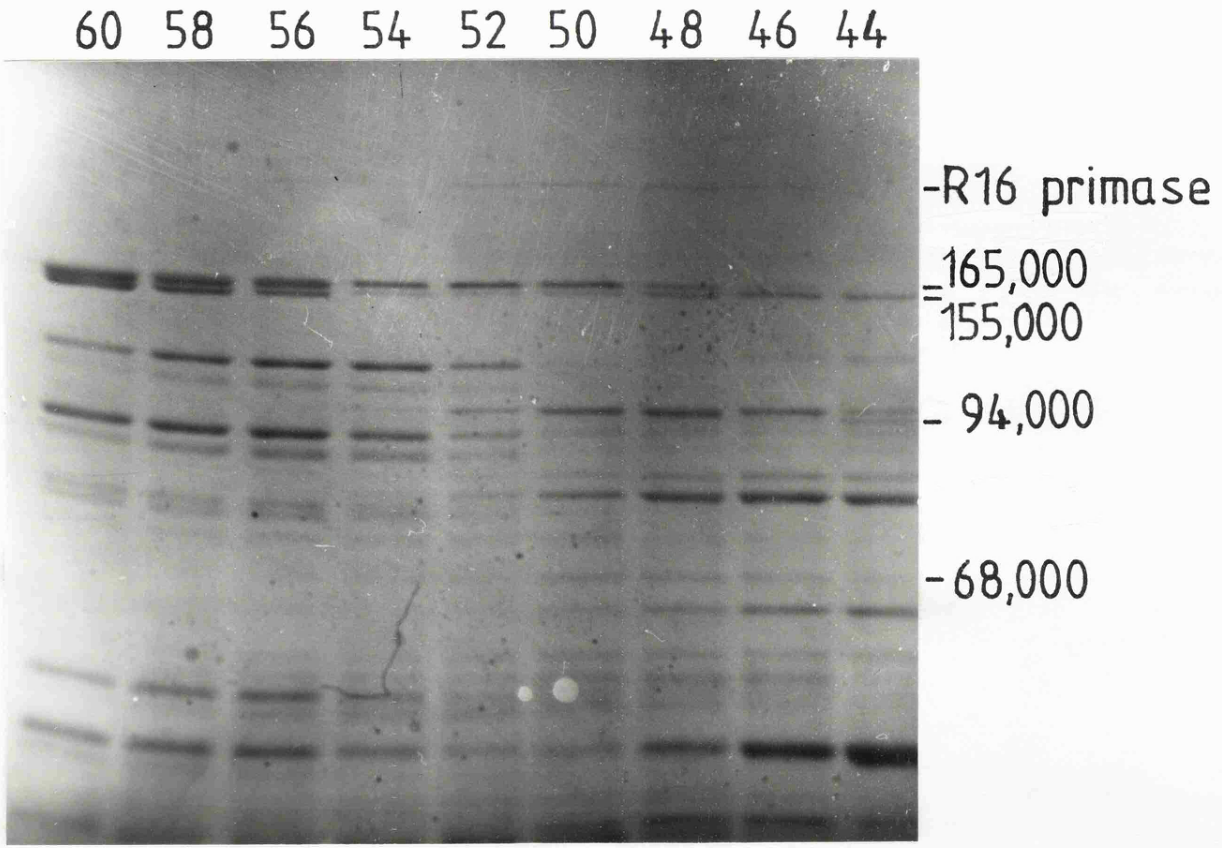

M.[KCl] and concentration of protein mg/ml.
pmoles ^{3}H-TMP incorporated.
fraction number.
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
2
4
6
8
10
12
14
16
18
20
22
0
10
20
30
40
50
60
70
80
90
100
110

Figure 5.9)

Coomassie blue stained protein profiles of 50ul samples of fractions of the eluant of the heparin Sepharose CL-6B column (see figure 5.8) and run on a 7% SDS polyacrylamide gel.


60 58 56 54 52 50 48 46 44
-R16 primase
165,000
155,000
94,000
68,000

visible on the gels, this may be because it is present in smaller amounts than the 240,000 polypeptide in the original crude cell extract. The 240,000 dalton polypeptide itself is only just visible on the gel.

The R16 primase elutes from the column at a lower salt concentration than RP4 and sog primases which are both genetically and almost certainly antigenically distinct from each other and R16 primase. Thus the R16 primase may have a lower affinity for DNA than RP4 and sog primases. However as the experiments were carried out in different laboratories a direct comparison may not be advisable.

11) Summary.

The 15.8kb EcoRI fragment cloned from R16 appears to encode only two major polypeptides, of apparent molecular weights 240,000 and 175,000. These two polypeptides are still encoded when only 4.1kb of the insert remains. Hence the genes on the other 11.7kb of the fragment are not translated in the in vitro transcription/translation system. This may be due to the absence of suitable transcription promoters on the insert. It would be of interest to know what other genes are adjacent to the primase genes, presumably further transfer genes.

A series of deletion mutants of pLG108 were generated to help in the identification of the gene products. The size of the deletions corresponds fairly well with the size of the products, assuming 60-70,000 daltons of protein per kilo base of DNA. A similar figure has been observed for sog primase (Boulnois et al.,1982), but this is somewhat greater than the usually quoted figure of 35,000 daltons of protein per kb of DNA. Like the 220,000 and 165,000 dalton sog primase polypeptides the two R16 polypeptides appear to be encoded by the same stretch of DNA. However the limited evidence indicates that if two products are encoded by overlapping genes then they probably have a common N-terminus rather than the common C-terminus observed for the sog gene products. This observation predicts that both of the pLG105 encoded polypeptides would have primase activity. These polypeptides are also synthesised from R16, but in much smaller amounts than from pLG105. Thus it is probable that the products synthesised from the cloned genes are identical to the products synthesised from R16.

The primase protein can be truncated to a third of its original size and still retain activity, ColIb-P9 primase (Boulnois et al.,1982) and RP4 primase (E. Lanka, personal comminication) can also be truncated in a similar way.

Primase activity and the large polypeptide of apparent molecular weight 240,000 coelute from a heparin Sepharose CL-6B affinity column. This provides further evidence that this protein is the R16 encoded DNA primase.

CHAPTER SIX

Cloning and characterisation of the R387 primase.

1) Introduction.

In chapter three I showed that the IncK plasmid R387 can suppress the dnaG3 mutation of BW86. In chapter four I showed that the 4.1Kb HindIII-EcoRI fragment from R16, cloned into pLG108, hybridised to R387 DNA, albeit weakly. Thus R387 appears to carry some sequences which are homologous with DNA in the vicinity of the R16 primase genes.

A study of IncK plasmids has been carried out (Tschape and Tietze, 1980) and the molecular and genetic properties of these plasmids demonstrate a phylogenetic relatedness. Thus although EcoRI digest patterns of the members of IncK were different a number of common fragments were identified. The proposed core fragments are 18, 15.3, 13.8, 12.6, 9.75, and 8.1kb (Tschape and Tietze, 1980). R387, the reference plasmid, contains all of these fragments except the 13.8 and 8.1kb fragments. Apart from this paper the IncK plasmids have not been studied in much detail.

In order to study the R387 suppression system in

more detail it was decided to clone the determinant into a multicopy cloning vector.

2) The cloning.

pBR328, which is a deletion derivative of pBR325 and retains all the features of pBR325 with 1.1Kb less DNA, was chosen as the vector for these experiments (see figure 6.1) (Soberon et al.,1980). Endonuclease EcoRI cleaves purified R387 DNA into seven major fragments. EcoRI cleaved R387 and pBR328 DNA were mixed in a ratio of 4:1, ligated and used to transform a competent culture of BW86. Ampicillin resistance at 30°C was used to select for transformants. The transformants were screened for insertional inactivation of the chloramphenicol resistance gene, and sensitive clones were tested for their ability to form colonies at 40°C. One transformant had a plating efficiency of approximately 2.5x10-2; this plasmid carried a plasmid, designated pLG122, with an 18Kb EcoRI fragment ligated into the EcoRI cleavage site of pBR328 (see figure 6.2). Cured derivatives of this transformant were fully temperature sensitive. When BW86 was transformed with purified pLG122 DNA transformants aquired thermoresistance. These results confirm that plasmid pLG122 carries the genetic determinant of R387 responsible for the suppression of the DnaG- phenotype of BW86.

Figure 6.1) Map of pBR328.

The distances are in kb from the unique EcoRI site of pBR328. Cm, Tc and Ap are the chloramphenicol, tetracycline and ampicillin resistance genes. The promoter drives transcription of the chlorampenicol acetyl transferase gene in the direction indicated.

Map of pBR328

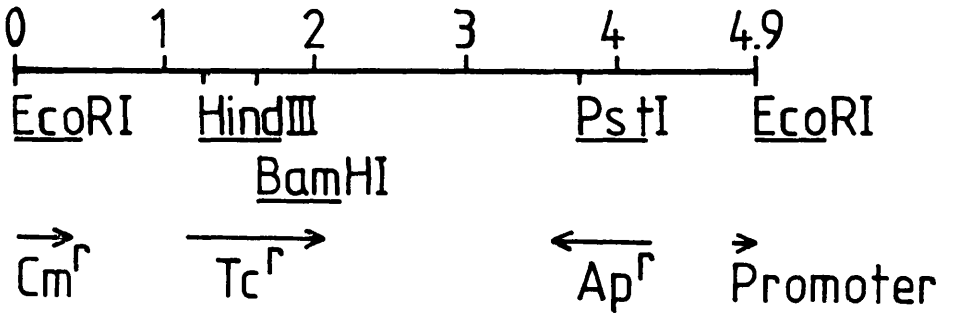
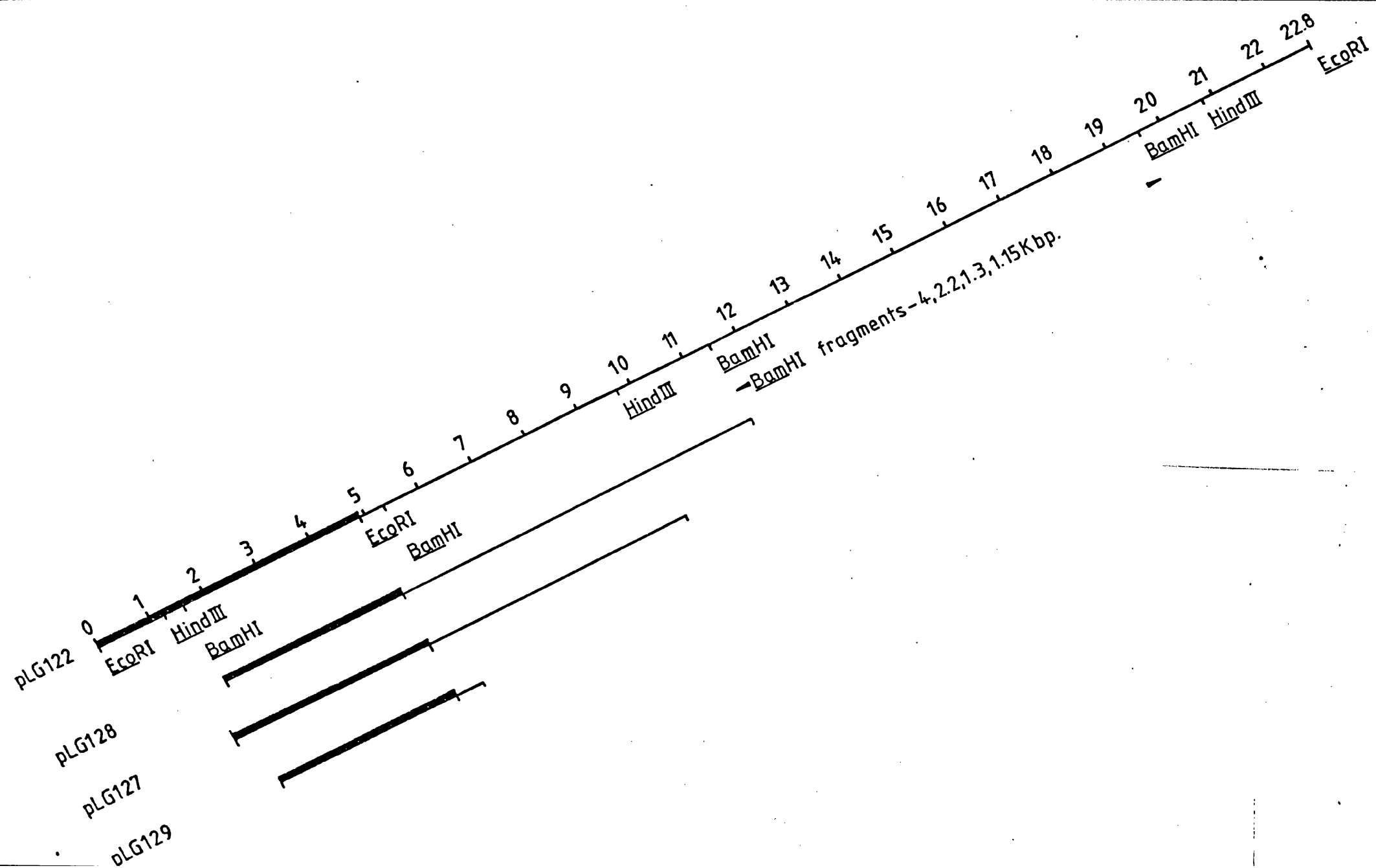


Figure 6.2) Maps of pLG122, pLG127, pLG128 and pLG129.

The distances are in kb from one of the two EcoRI sites on pLG122. The vector (pBR328) is represented by a thick line and the insert by a thin line. 'P'- promoter for the chloramphenicol acetyl transferase gene. pLG127, pLG128 and pLG129 are aligned with the corresponding section of pLG122.


0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
22.8
EcoRI
BamHI
HindIII
BamHI
BamHI
HindIII
EcoRI
BamHI fragments-4,2.2,1.3,1.15Kbp.
pLG122
pLG128
pLG127
pLG129

Agarose gel electrophoretic analysis of single, double and triple EcoRI, HindIII and BamHI digests yielded the restriction map shown in figure 6.2. It was immediately apparent that the insert in pLG122 was very similar to the insert in pLG105, but with slightly larger distances between the sites (see figure 6.3). This is not unexpected due to the hybridisation of the R16 fragment to R387 DNA. In order to reduce the cloned R387 fragment, while still retaining the capacity to suppress the dnaG3 mutation, pLG122 was subcloned using two strategies.

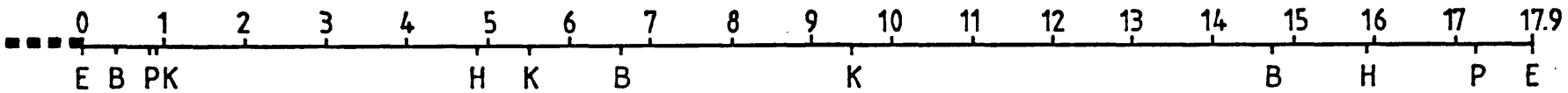
pLG122 DNA was digested with HindIII, ligated and transformed into BW86 with selection at 30°C for ampicillin resistance. All the transformants were chloramphenicol sensitive and formed colonies at 40°C. They all carried a recombinant plasmid with the 4.85 EcoRI-HindIII fragment of the 18Kb insert cloned into a deletion derivative of pBR328 (see figure 6.2). One example of these plasmids, which conferred a plating efficiency of 1 on BW86 was designated pLG127. Cured derivatives of this transformant regained temperature sensitivity of colony formation. BW86 transformed with purified pLG127 DNA showed thermoresistance of the same level as the original transformant.

The second strategy was to partially digest pLG122

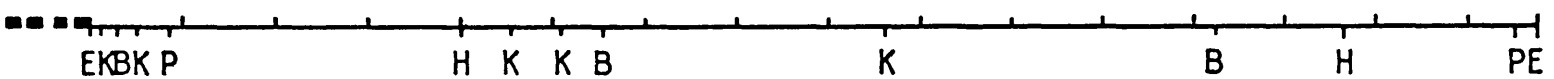
Figure 6.3) Comparison between the inserts carried by pLG122 and pLG105.

The inserted fragments from pLG122 and pLG105 are aligned such that the junction between the vector and the insert at the EcoRI site proximal to the chloramphenicol acetyl transferase promoter are directly equivalent. The distances are in kb from this EcoRI site. The thick dashed line represents the vector DNA (pBR325 or pBR328) and the thin line the insert DNA. Not all of the PstI, KpnI and BamHI sites have been mapped.

pLG122



pLG105



E-EcoRI K-KpnI B-BamHI P-PstI H-HindIII

DNA with BamHI to generate a family of molecules. The partially digested DNA was ligated and transformed into BW86 with selection at 30°C for ampicillin resistance. This approach would allow the recloning of any of the BamHI fragments. Transformants which were chloramphenicol and tetracycline sensitive were further tested for their ability to form colonies at 40°C. Three out of a hundred transformants conferred a plating efficiency of one on BW86. They all carried a recombinant plasmid with a 6.7kb EcoRI-BamHI (site 2) fragment ligated to the BamHI-EcoRI fragment of pLG122 which included the majority of the pBR328 DNA (see figure 6.2).

Another plasmid was identified in the transformants from the BamHI partial digest. These transformants were chloramphenicol and tetracycline sensitive and contained only one BamHI site. One such plasmid which did not suppress the dnaG3 mutation of BW86 was designated pLG129 (see figure 6.2).

3) The gene product is a primase.

The genetic similarities between the R16 and R387 suppression systems suggest that R387 encodes a primase which is very similar to the R16 primase.

The priming activity of crude cell extracts of strains containing R387, pTM559, pTM557, pIE312-1 (IncK), pLG122, pLG127, pLG128, pLG129 and pBR328 was measured (see table 6.1). Extracts of cells containing the wild type repressed IncK plasmids were unable to prime detectable DNA replication in the *in vitro* primase assay system. However all of the IncK plasmids were able to suppress the *dnaG3* mutation of BW86. Extracts of pLG122, pLG127 and pLG128 containing strains were able prime DNA replication in the assay, presumably due to the increased copy number of the cloned genes. Extracts of strains containing pLG129 could not prime detectable DNA replication. *Sog* and R16 primases seem to have similar efficiencies of suppression of the *dnaG* mutation and similar levels of activity in the primase assay for roughly similar numbers of molecules. The R387 primase may be less active, although the cloned genes may synthesise fewer primase molecules. Therefore the R387 determinant responsible for the suppression of the *dnaG3* mutation is a primase gene which is presumably expressed at undetectable levels in the wild type plasmid.

4) The immunological relationship of R387 primase to *sog* primase.

To determine the antigenic relationship of the R387 primase to the *sog* primase, the effect of antiserum

Table 6.1) Plating efficiencies and primase activities of IncK plasmids and the cloned R387 primase gene(s).

Plasmid	Plating efficiency	Primase activity (a)
none	<10-7	-(b)
pBR328	<10-7	-
R387	2.3x10-6	-
pTM557	1.1x10-5	-
pTM559	1x10-5	-
pIE312-1	3.5x10-6	-
pLG122	2.5x10-4	13.5
pLG127	1	N.D.(c)
pLG128	1	18
pLG129	<10-7	-

a) pmoles 3H-TMP incorporated per ug protein.
b) '-' no detectable primase activity
c) N.D. not determined.

raised against the 87,000 dalton polypeptide encoded by pLG214 (see figure 3.2) was investigated (see table 6.2 and figure 6.4). The antiserum (kindly provided by E. Lanka) did decrease the priming activity of crude cell extracts of strains containing pLG122. This inhibition of primase activity was greater than that observed with pre-immune serum (kindly provided by T.Harrison). However the inhibition was as at least an order of magnitude less than for extracts of pLG214 containing strains. Thus the R387 primase is inhibited but not entirely inhibited by sog primase antiserum. The R387 primase may share some antigenic sites with the sog primase. It is probable that all three primases are related and that the IncB and IncK primases are closely related. In order to demonstrate this relationship antiserum raised against the purified IncB and/or IncK primases would be required. These observations predict that antiserum raised against R16 primase would almost totally inhibit R387 primase and vice versa.

5) R387 dnaG suppression, primase activity and expression of plasmid transfer functions.

The expression of the sog primase genes appears to be coordinately linked to the expression of the transfer genes. The R16-like primase appears to have a more complex relationship between expression of primase and transfer

Figure 6.4) The effect of ColIb-P9 sog primase antiserum on the primase activity of pLG122.

Crude cell extracts of strain BW86 carrying pLG214 or pLG122 were diluted as indicated with identical extracts of plasmid free BW86 and assayed for primase activity. The overall height of each histogram represents the total activity in each dilution. The black area of each histogram represents the total activity in the presence of antiserum. Mixtures of 2.5ul of crude cell extract (16 to 18ug of protein in each dilution) and 2ul of a 1:10 dilution of antiserum were incubated on ice for 10 minutes before assaying.

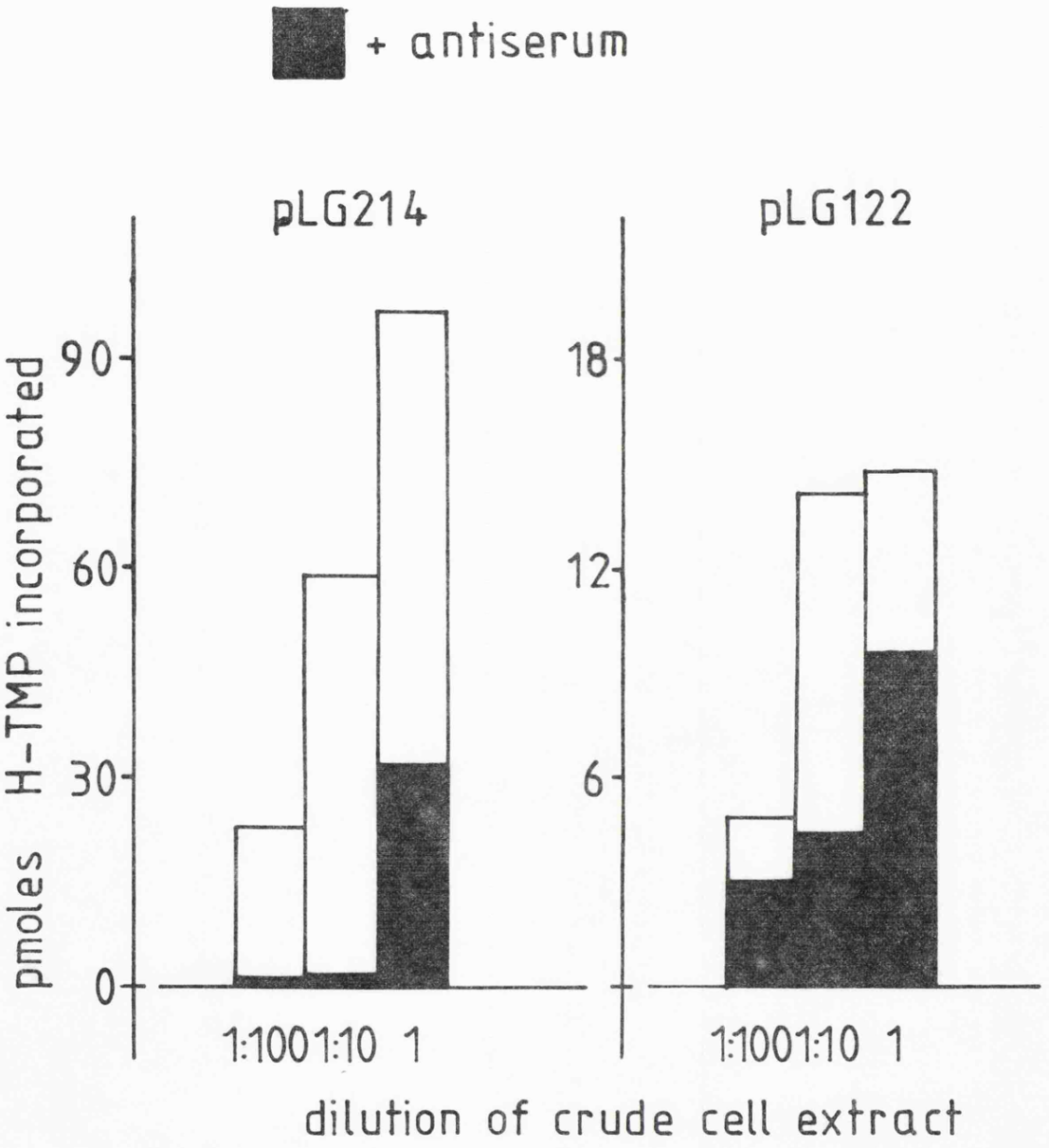

+ antiserum
pLG214
pLG122
pmoles H-TMP incorporated
90
60
30
0
18
12
6
1:100
1:10
1
1:100
1:10
1
dilution of crude cell extract

Table 6.2) The effect of sog primase antiserum on the priming ability of crude cell extracts of BW86 carrying pLG122.

luCi of 3H-thymidine was used per assay with 5ul of crude cell extract and 2.5ul of a 1:10 dilution of sog primase antiserum or pre-immune antiserum.

plasmid	ug crude cell extract per assay	no antiserum cpm.	sog antiserum cpm.	pre-immune antiserum cpm.
pLG214	0.16	22,800	1,500	N.D.(a)
	1.6	59,000	1,800	64,480
	16	97,000	32,000	N.D.
pLG122	0.36	4,900	3,000	4,500
	3.6	14,200	4,400	9,250
	36	14.900	9,600	11,600

a) N.D. not determined.

genes.

IncK plasmids carrying mutations enabling them to suppress the dnaG3 mutation with high efficiency were isolated. For plasmid R387 three such mutant plasmids (plating efficiency approximately 1) were isolated and designated pLG137, pLG138 and pLG139. These three plasmids did not have a significantly increased transfer efficiency and extracts of strains containing these plasmids did not have detectable priming activity (see table 6.3). Presumably the selection of R387 mutants which efficiently suppress the dnaG mutation selects for primases with increased priming efficiency on the chromosome rather than for the over production of primase molecules. I was unable to isolate an authentic derepressed mutant of R387.

6) Restriction enzyme analysis of pLG122 and comparison with pLG105.

pLG122 was mapped in more detail with a selection of endonucleases and compared with pLG105 (see figure 6.3). The EcoRI and HindIII sites are directly analogous between the two fragments, although more widely separated on pLG122. PstI digests of pLG105 and pLG122 also produce very similar patterns of fragments. Some of the BamHI sites have similar relative positions while the overall pattern of fragments is

Table 6.3) Transfer efficiencies, plating efficiencies and primase activities of mutants of R387.

plasmid	transfer efficiency	plating efficiency	primase activity(a)
R387	4.3x10-5	2.3x10-6	-(b)
pLG137	4x10-5	1	-
pLG138	4.5x10-5	1	-
pLG139	5x10-5	1	-

a) pmoles 3H-TMP incorporated per ug protein.
b) '-' no detectable activity.

somewhat dissimilar. When the data and the results of similar experiments carried out with pLG108 and pLG127 are compiled to form a detailed restriction map of pLG122 (see figure 6.3) it can be seen that the order of restriction sites is essentially very similar. This confirms the expectation from the hybridisation experiments that the R387 primase determinant is very similar to the R16 primase determinant.

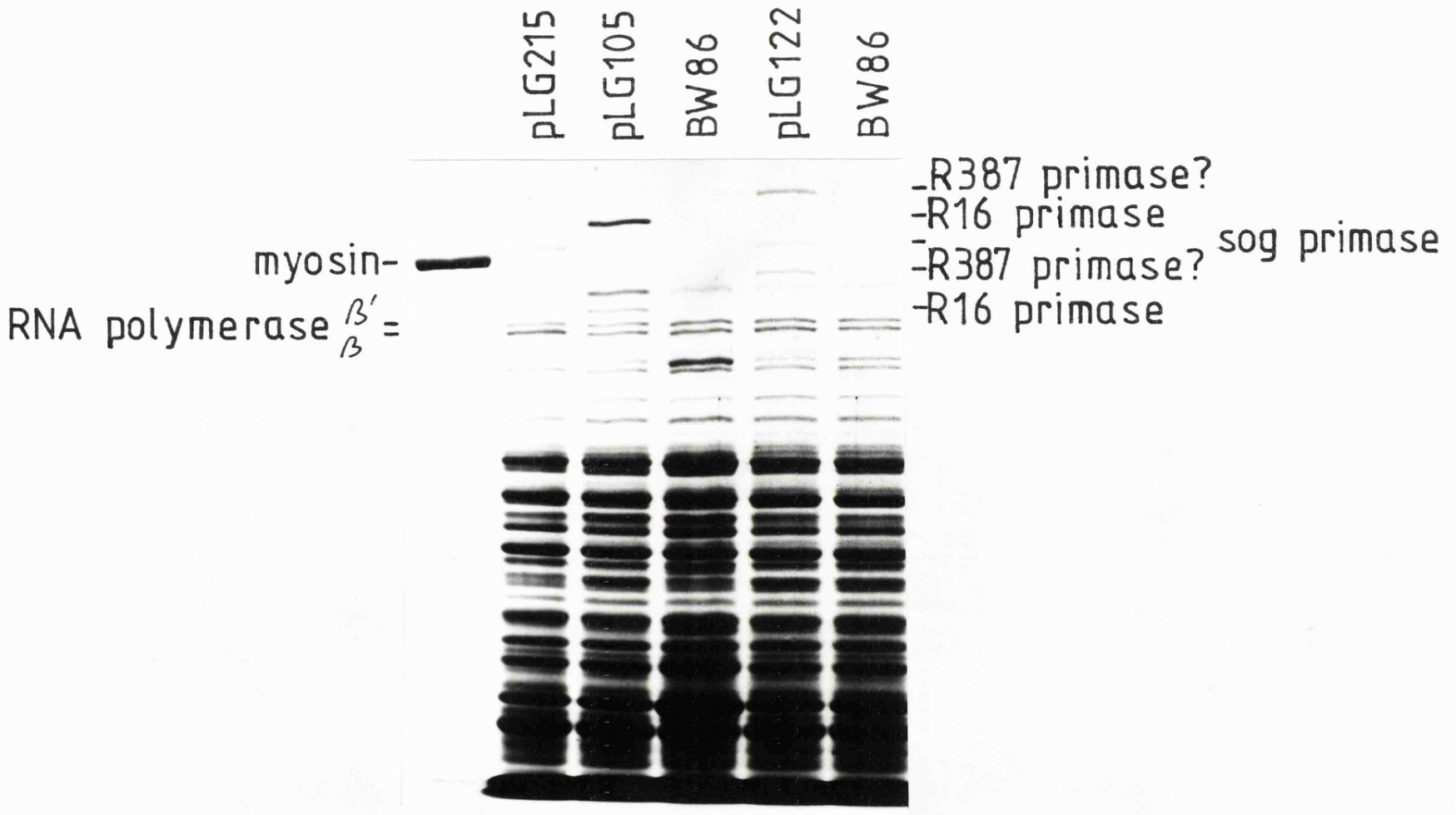
7) Protein products of the recombinant plasmids.

The proteins encoded by the plasmids were analysed by labelling the protein products in an *in vitro* transcription/translation system and electrophoresing the products on SDS polyacrylamide gels. The autoradiographs showed the characteristic products of pBR328 and the ladder of polypeptides encoded by the inserts of pLG122, pLG127 and pLG128 (see chapter 5, section 6). pLG122, pLG127 and pLG128 appear to encode two high molecular weight polypeptides. pLG129 does not appear to encode any products not encoded by pBR328, as is to be expected due to the very small insert which pLG129 carries.

The proteins are also visible in crude cell extracts of plasmid containing strains run on SDS polyacrylamide gels (see figure 6.5). Two major bands,

Figure 6.5)

Coomassie blue stained protein profiles of crude cell extracts of plasmid containing derivatives of BW86 run on a 5% SDS polyacrylamide gel. Samples contained up to 500ug of protein. Myosin has an estimated molecular weight of 212,000 and E.coli RNA polymerase β' and β subunits have estimated molecular weights of 165,000 and 155,000 respectively.


pLG215
pLG105
BW86
pLG122
BW86
myosin-
RNA polymerase β' =
β
R387 primase?
R16 primase
sog primase
R387 primase?
R16 primase

polypeptides with apparent molecular weights of 270,000 and 200,000, and a minor band, a polypeptide with an apparent molecular weight of 220,000, appear to be encoded by R387. Identically sized polypeptides are also present in extracts of strains containing pLG127 and pLG128. The largest band is also the most prominent, as was previously noted for the products of pLG105. Considering the genetic similarities between the R16 and R387 primase genes it is tempting to consider that the 270,000 and 200,000 dalton polypeptides encoded by pLG122 are analogous with the 240,000 and 175,000 dalton polypepetides encoded by pLG105. Perhaps part of the extra 0.75kb between the EcoRI and first HindIII site is located in the primase gene.

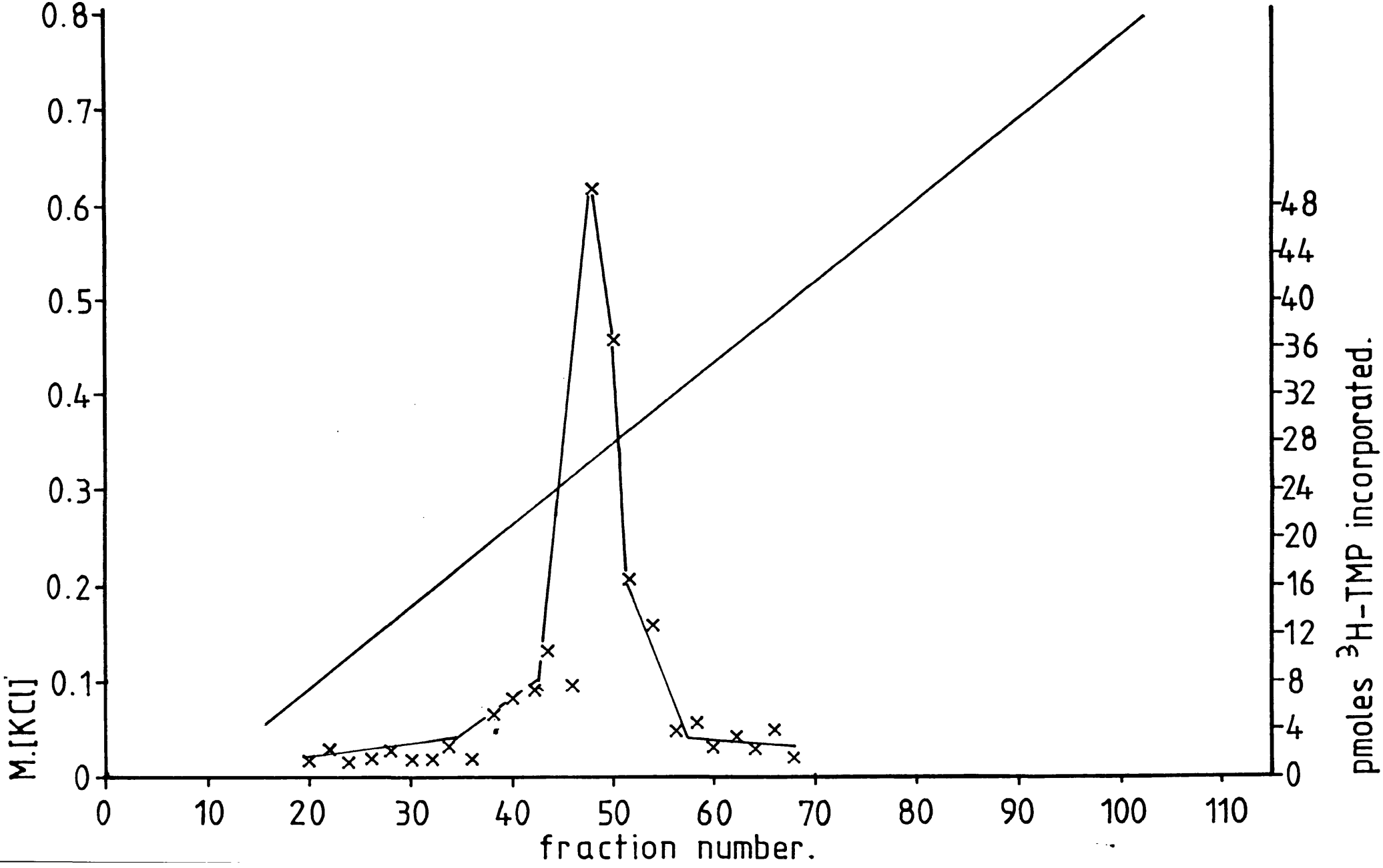
The same considerations of amount of protein per kb of DNA and the possibility of overlapping genes or processed polypeptides, which apply to pLG105, also apply to pLG122.

8) Partial purification of the primase.

50ml of crude cell extract from a strain of BW86 carrying pLG128 was loaded on to a heparin Sepharose CL-6B column. The proteins were eluted with a linear KCl gradient from 50mM to 800mM (see figure 6.6). The primase activity eluted as one peak at 330mM KCl (fraction 48). Neither the

Figure 6.6)

Profile of the elution of bound, pLG122 encoded, R387 primase from a heparin Sepharose CL-6B column with a linear gradient from 50mM to 800mM KCl (-). The primase activity (x) is plotted against fraction number. A 150ml gradient was used and 1.7ml fractions were collected.


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
M.[KCl]
0
10
20
30
40
50
60
70
80
90
100
110
fraction number.
48
44
40
36
32
28
24
20
16
12
8
4
0
pmoles ³H-TMP incorporated.

270,000 nor the 200,000 dalton proteins were visible when samples of eluant fractions with primase activity were run on SDS polyacrylamide gels. This may be because insufficient protein was loaded onto the gel or due to the breakdown of the very large primase molecules.

The R387 primase encoded by pLG122 elutes at a lower concentration of KCl than R16 primase encoded by pLG105 and presumably has a lower affinity for DNA. The two primases differ in size and presumably have a certain amount of sequence divergence and could have significantly different DNA binding sites.

9) Summary.

R387 carries a gene or genes which are responsible for the suppression of the DnaG- phenotype. The genes have been cloned onto a multicopy vector plasmid, pBR328, and are contained on an 18kb EcoRI fragment of R387. This fragment can be reduced to a 4.85kb EcoRI-HindIII fragment and the suppression of the dnaG3 mutation remains high in strains containing pLG127. Primase activity cannot be detected in strains carrying the wildtype IncK plasmids, but is readily detectable in strains carrying the cloned primase genes. The product of these gene(s) is a DNA primase active in an identical assay system to that used in the identification of

the sog and RP4 primases.

The 18kb EcoRI fragment which carries the R387 primase genes is one of the proposed core EcoRI fragments of the IncK plasmids (Tschape and Teitze, 1980). It is possible that this fragment carries primase genes in many of the IncK plasmids. So far all incK plasmids tested suppress the dnaG3 mutation of BW86, and hence presumably encode an R387-like primase, although this has not yet been shown.

There appear to be three large polypeptides of apparent molecular weights 270,000, 220,000 and 200,000 which are encoded in the region of the primase genes of pLG122. However it is not known which of these proteins is the primase and none are visible in fractions of eluant with primase activity. Breakdown of the primase during purification of the sog primase encoded by pLG215 was a major problem (Wilkins et al.,1981) and perhaps breakdown of the very large primase molecules may be occurring during purification of the pLG122 encoded primases.

The R387 primase is genetically very similar to the R16 primase, but it is genetically and antigenically distinct from sog primase. It probably also genetically and antigenically distinct from RP4 primase and E.coli chromosomally encoded dnaG primase.

CHAPTER SEVEN.

The search for more primases.

1) Introduction.

In the search for plasmid encoded DNA primases two approaches have been taken; screening representative plasmids of the different incompatibility groups for the suppression of the dnaG3 mutation, and screening for primase activity in crude cell extracts. The first approach was used by Sasakawa and Yoshikawa (1978), who screened members of some but not all E.coli plasmid incompatibility groups. They found only two more plasmids (than found by Wilkins, 1976), R621a (IncIg) and R391 (IncJ), which suppressed the DnaG- phenotype. Lanka and Barth (1981) screened members of virtually all incompatibility groups for primase activity. Extracts of strains containing R16drd4 (IncB), members of IncIα and IncIγ, TP114drp1 (IncI2), R387 (IncK), R446b and R831b (IncM) and RP4 (IncP) had priming activity. They found no primase activity in crude cell extracts of strains containing members of IncC, D, F-complex, H-complex, Iδ, Iζ, J, N, Q, T, V, W, X and Y. With the exception of RP4, Lanka and Barth (1981) did not screen plasmids for the suppression of the DnaG- phenotype. Wildtype RP4, which is derepressed for transfer, does not suppress the DnaG- phenotype but does

encode a DNA primase.

The suppression of the DnaG- phenotype by plasmids has so far been shown to be due to the substitution of a plasmid encoded primase for the host's inactive dnaG primase. However the RP4 primase is an example of primases which are detectable in the in vitro assay but which cannot substitute efficiently for dnaG primase.

I decided to combine these two approaches, and to use hybridisation screening with the sog and R16 primase probes, in order to study the distribution of plasmid encoded primases in the E.coli plasmid incompatibility groups.

2) Screen for the suppression of the dnaG3 mutation.

All of the plasmids were transferred to BW86 by conjugation and the plating efficiency of each strain was measured (see table 7.1). R40a (IncC), R391 (IncJ) and RA3 (IncU) suppressed the dnaG3 mutation while the other plasmids tested, including R446b (IncM) and RP4 (IncP), did not suppress the dnaG3 mutation. R446b appears to be a further example of plasmids like RP4 which encode a primase but do not suppress the dnaG3 mutation. The replication of Rts1 is temperature sensitive and hence the suppression of

Table 7.1) Plating efficiencies of representative plasmids of the enteric bacteria incompatibility groups.

Plasmid	Inc group	plating efficiency
R40a	C	2x10-6
R905	D	1x10-7
ColV-K30	FI	<10-7
R27	H1	<10-7
R478	H2	<10-7
R391	J	1.5x10-4
R446b	M	5x10-8
R471a	M	2x10-8
N3	N	1x10-7
RP4	P	1.4x10-7
Rts1	T	temperature sensitive
RA3	U	3.5x10-6
S-a	W	8x10-8
R6K	X	4.5x10-8
pIP71a	Com9	4x10-8

the dnaG3 mutation cannot be measured.

3) Hybridisation screen with primase gene probes.

Labelled pLG215 and pLG108 DNA was used to screen colonies of the same plasmid containing BW86 strains (see table 7.1), lysed in situ on nitrocellulose paper. Under the stringency used vector DNA did not hybridise to the samples. The probes only hybridised to the positive controls and R391, none of the other plasmids appeared to carry sequences homologous to sequences on either the sog or R16 primase probes. RA3 was not screened with either probe.

4) Characterisation of the R40a determinant responsible for the suppression of the DnaG- phenotype.

IncC is a group of fairly diverse plasmids which includes plasmids previously grouped into IncA and IncA-C. The IncC plasmids are also members of IncP-3 of the plasmid incompatibility system of Pseudomonas spp. The plasmids have a wide host range including, P. aeruginosa, S. typhimurium, K. pneumoniae, Providence spp., S. marcescens, P. stuartii and E. coli.

The wild type IncC plasmids tested suppress the dnaG3 mutation but primase activity is not detectable in

extracts of strains containing these plasmids (see table 7.2). A similar result was also found by Lanka and Barth (1981). It is not obvious why RA1, which is repressed for pilus synthesis, suppresses the dnaG3 mutation so efficiently but does not have detectable primase activity in crude cell extracts. It is possible that RA1 encodes a mutant primase which can prime chromosomal DNA replication more efficiently.

Two approaches were taken to characterise the determinant of R40a responsible for the suppression of the dnaG3 mutation. An unsuccessful attempt was made to clone the gene(s) using the strategy outlined for R16 and R387 (see chapter 4 section 2). Mutants of R40a mediating high levels of suppression of the dnaG3 mutation were isolated and three were designated pLG134, pLG135 and pLG136. Crude cell extracts of all three had detectable primase activity (see table 7.3) but were not significantly derepressed for transfer (ie. less than a 1000 fold higer transfer efficiency). We do not have an authentic derepressed mutant of R40a to compare with these mutants. The level of pilus synthesis by pLG134, pLG135 and pLG136 has not been studied. However increase in primase synthesis is not coordinate with derepression of the transfer system. These mutant plasmids may have a slightly higher level of transcription of the transfer genes or perhaps only the transcription of the

Table 7.2) Plating efficiencies and primase activities of IncC, J, P and U plasmids.

plasmid	Inc group	plating efficiency	primase activity(a)
RA1	C	0.75	- (b)
R40a	C	2x10-6	-
R57b-1	C	8x10-5	-
R391	J	1.5x10-4	-
RP4	P	1.4x10-7	3.25
RA3	U	3.5x10-6	1.3

a) pmoles 3H-TMP incorporated per ug protein.
b) '-' not detected.

Table 7.3) Transfer efficiencies, plating efficiencies and primase activities of mutants of R40a.

Plasmid	Inc group	transfer efficiency	plating efficiency	primase activity (a)
R40a	C	6.7x10-5	2x10-6	-(b)
pLG134	C	9x10-4	1	0.71
pLG135	C	2x10-4	1	1.8
pLG136	C	2.1x10-4	1	1.0

a) pmoles 3H-TMP incorporated per ug protein.
b) '-' no detectable primase activity.

primase genes has been significantly increased. The mutants may, like similar mutants of R16 and R387, encode primases with an increased DNA priming efficiency but which unlike the mutant primases encoded by pLG106 and pLG107 show an increase in activity in the in vitro primase assay.

The primase encoded by pLG134 was tested for inhibition by sog primase antiserum (see table 7.4). There was no observable inhibition of the priming activity of crude cell extracts of strains containing pLG134. Under the same conditions the primases encoded by pLG105 and pLG122 are inhibited by a small but significant amount (see figures 4.4 and 6.4). However the crude cell extracts of pLG134 containing strains have only a low level of activity compared to the activity of crude cell extracts of pLG105 containing strains. Thus the primase encoded by pLG134 has no antigenic sites at all in common with sog primase and R40a primase is antigenically as well as genetically totally distinct from sog primase.

5) Partial purification of the R40a primase.

Strain BW86 containing pLG135 was chosen as the source of R40a primase for the partial purification of the protein. 50ml of crude cell extract was loaded onto a heparin Sepharose CL-6B column (see materials and methods).

Table 7.4) The effect of sog primase antiserum on the priming ability of crude cell extracts of BW86 carrying pLG134.

luCi of 3H-thymidine was used per assay with 5ul of crude cell extract and 2.5ul of a 1:10 dilution of sog primase antiserum or pre-immune antiserum.

Plasmid	ug of protein in crude cell extract	no antiserum cpm.	sog antiserum cpm.	pre-immune antiserum cpm.
pLG214	0.16	22,800	1,500	N.D.(a)
	1.6	59,000	1,800	64,000
	16	97,000	32,000	N.D.
pLG134	3.2	4,200	4,200	4,400
	32	44,900	44,200	45,800

a) N.D.- not determined.

The bound proteins were eluted with a linear KCl gradient from 50mM to 800mM (see figure 7.1). The primase activity eluted as a major peak at 320mM KCl (fraction 48) and a minor peak at 220mM KCl (fraction 36). Samples of fractions containing primase activity were run on an SDS polyacrylamide gel (see figure 7.2). There is an obviuos major band which peaks in intensity in fraction 48. This polypeptide has an apparent molecular weight of 100,000, but a band of similar size also peaks in fraction 48 of the eluant from the pLG105 column (see figure 5.9). However there is a minor band which peaks in fractions 46-48 with an apparent molecular weight of 135,000. A similar band is not visible in the eluant from the pLG105 column (see figure 5.9).

Fractions 34 to 56 were pooled and after suitable treatment (see materials and methods) were loaded on to a Hydroxyapatite column. The bound proteins were eluted with a linear phosphate gradient from 20mM to 300mM. Primase activity eluted as two peaks, a minor peak at 150mM phosphate (fraction 48) and a major peak at 190mM phosphate (fraction 58, see figure 7.3). Samples of fractions containing primase activity were run on an SDS polyacrylamide gel (see figure 7.4). A band of apparent molecular weight 115,000 peaks in fraction 58. However the band at 135,000 observed on the previous gel is no longer

Figure 7.1)

Profile of the elution of bound, pLG135 encoded, R40a primase from a heparin Sepharose CL-6B column with alinear gradient from 50mM to 800mM KCl (-). The primase activity (x) is plotted against fraction number. A 150ml gradient was used and 1.7ml fractions were collected. 'pLG135'- fractions from the pLG135 column, 'pLG105'- fractions from the pLG105 column.

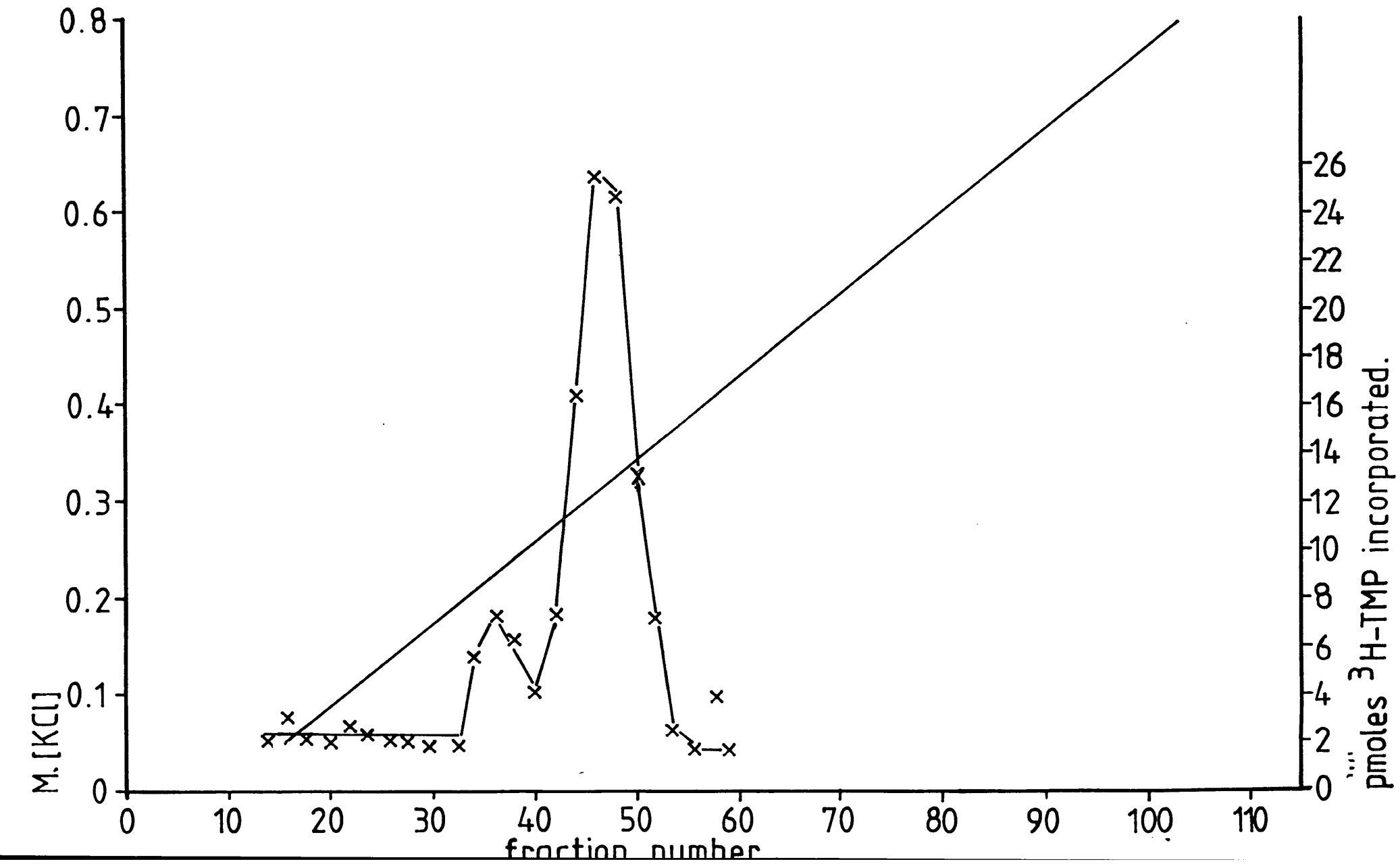

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
M. [KCl]
0
10
20
30
40
50
60
70
80
90
100
110
fraction number
26
24
22
20
18
16
14
12
10
8
6
4
2
0
pmoles ^{3}H-TMP incorporated.

Figure 7.2)

Coomassie blue stained protein profiles of fractions from the eluant of the heparin Sepharose CL-6B column (see figure 7.1) and run on an 11% SDS polyacrylamide gel. Myosin has an estimated molecular weight of 212,000 and E.coli RNA polymerase β' and β subunits have estimated molecular weights of 165,000 and 155,000 respectively. The estimated molecular weights of phosphorylase 'a' subunit, B.S.A. and pyruvate kinase are, 100,000, 68,000 and 57,000 respectively.

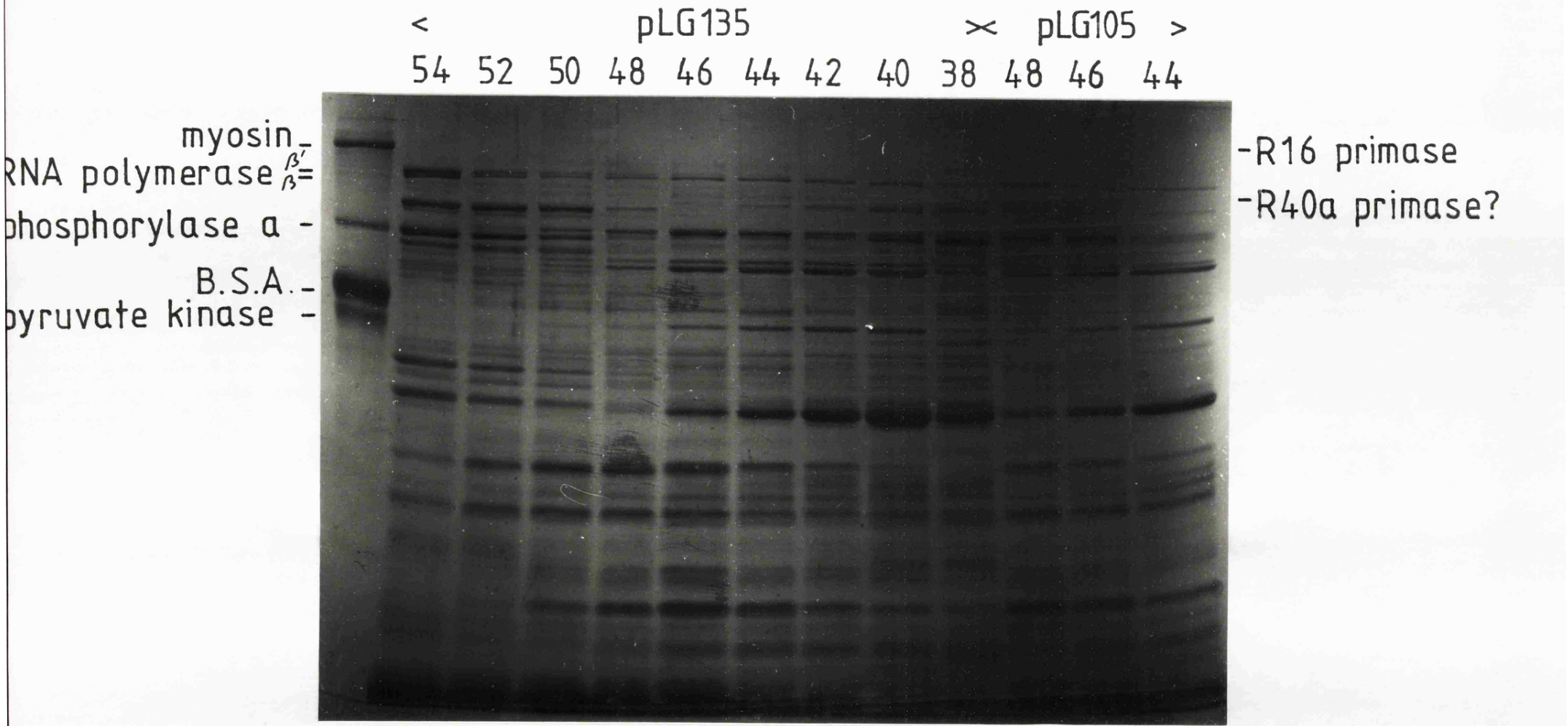

< pLG135 × pLG105 >
54 52 50 48 46 44 42 40 38 48 46 44
myosin
RNA polymerase β'
β
phosphorylase a
B.S.A.
pyruvate kinase
R16 primase
R40a primase?

Figure 7.3)

Profile of the elution of bound, pLG135 encoded, R40a primase from a Hydroxyapatite column with a linear gradient from 20mM to 300mM phosphate (-). The primase activity (x) is plotted against fraction number. A 150ml gradient was used and 2ml fractions were collected.

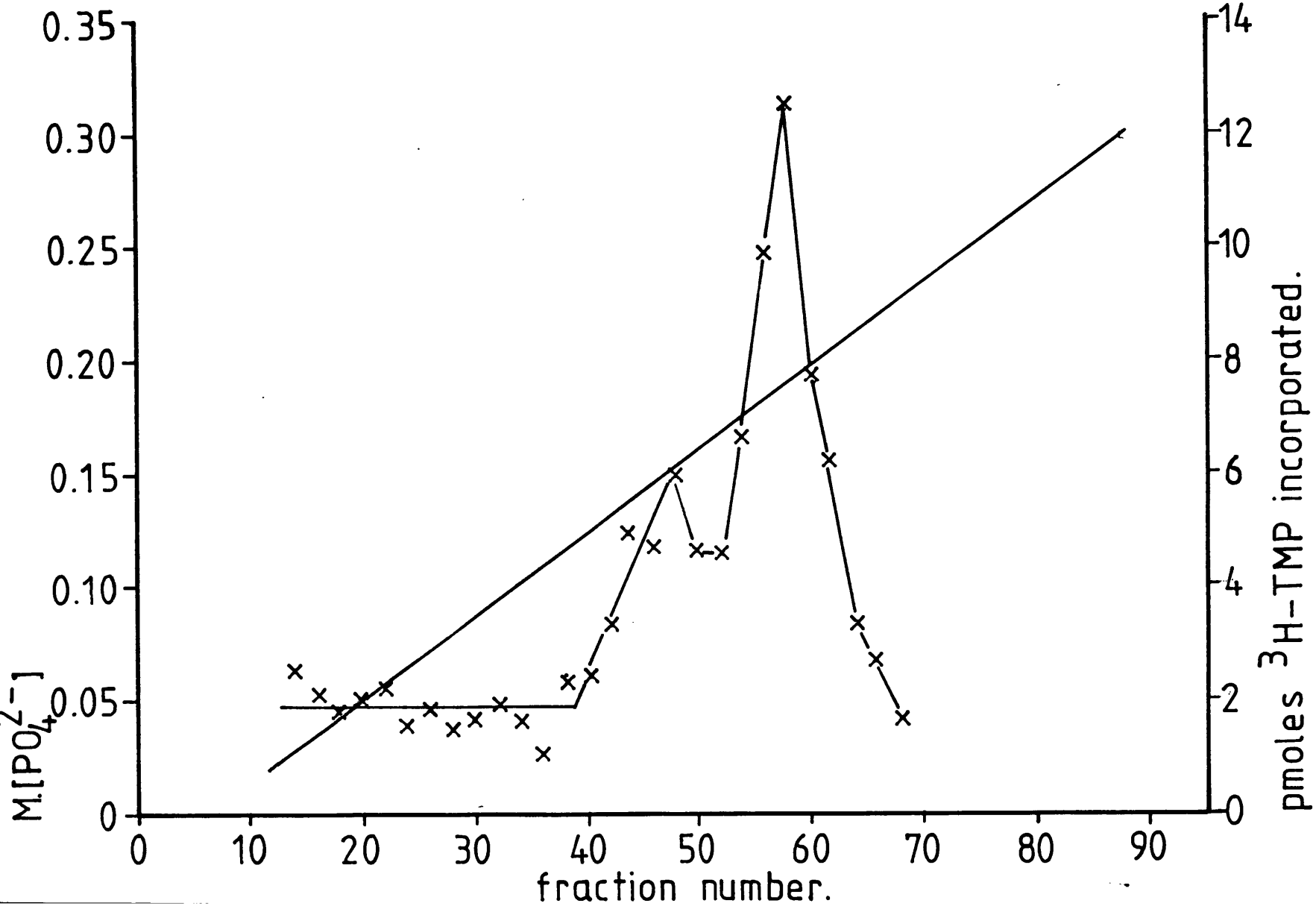
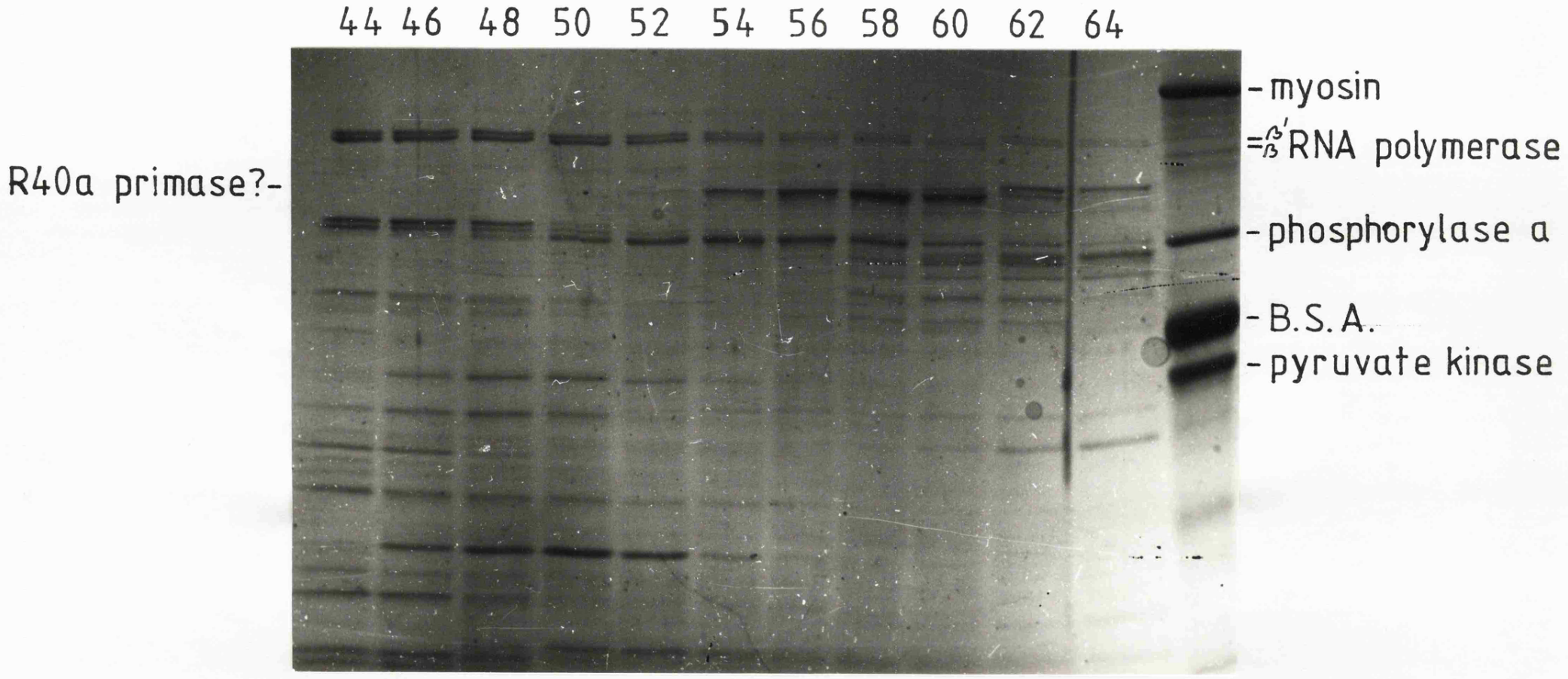

0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
M.[PO4 2-]
14
12
10
8
6
4
2
0
pmoles 3H-TMP incorporated.
0
10
20
30
40
50
60
70
80
90
fraction number.

Figure 7.4)

Coomassie blue stained protein profiles of fractions from the eluant of the Hydroxyapatite column (see figure 7.3) and run on an 11% SDS polyacrylamide gel. Myosin has an estimated molecular weight of 212,000 and E.coli RNA polymerase β' and β subunits have estimated molecular weights of 165,000 and 155,000 respectively. The estimated molecular weights of phosphorylase 'a' subunit, B.S.A. and pyruvate kinase are, 100,000, 68,000 and 57,000 respectively.


44 46 48 50 52 54 56 58 60 62 64
R40a primase?-
-myosin
=β′ RNA polymerase
-phosphorylase a
-B.S.A.
-pyruvate kinase

visible. Neither band is visible above the background when crude cell extracts of pLG135 are run on SDS polacrylamide gels.

The presence of a major and a minor peak of primase activity in the eluant from both columns suggests that there are two populations of primase molecules present in the crude cell extracts. Two distinct primases with different DNA binding efficiencies may be encoded by R40a, or one molecule may a derivative of the other, processed such that the two molecules have different DNA binding efficiencies. It is also possible that the selection of mutants of R40a with a high plating efficiency selected for a mutant plasmid and that this does not reflect the true nature of the R40a primase.

6) Characterisation of the RA3 primase.

The IncU plasmids are a diverse group isolated from Proteus morganii, E.coli, Salmonella dublin and Citrobacter freundii. Six plasmids have been characterised, they range in size from 27-56kb and have no common antibiotic resistance patterns or restriction fragment patterns (Tschape et al.,1981). Wildtype RA3, the prototype IncU plasmid, which has constitutive expression of its pili (Bradley, 1980a) , suppresses the dnaG3 mutation and primase

activity is detectable in extracts of RA3 containing strains (see table 7.2). No other groups have studied RA3 and no further work has been carried out in my laboratory.

7) Characterisation of the R391 determinant responsible for the suppression of the DnaG- phenotype.

The IncJ plasmids have been mainly isolated from Proteus and Providence and R391 is the prototype plasmid. R391, which has repressed pilus synthesis (Bradley, 1980a), is able to suppress the dnaG3 mutation, but extracts of strains containing R391 do not have primase activity (see table 7.2). The suppression of the DnaG- phenotype by R391 has also been reported by Sasakawa and Yoshikawa (1978) and the lack of primase activity by Lanka and Barth (1981). R391 carried sequences to which the probe for the R16 primase genes could hybridise. DNA was purified from BW86 containing R391 and restricted with EcoRI or HindIII, transferred to nitrocellulose filters and hybridised with pLG105 DNA. The probe DNA hydibridised to exactly the same sized fragments in the EcoRI and HindIII digests of R391 as it hybridised to for the equivalent digests of R16. Thus R391 appears to encode an identical primase to R16.

However it has been reported that it is not possible to purify plasmid DNA from E.coli strains carrying

R391 (Nugent, 1981). Another example of R391 was therefore obtained from P.Barth. This plasmid suppresses the dnaG3 mutation at almost exactly the same level as the example of R391 obtained from N.Datta and no primase activity can be detected in crude extracts of strains containing this plasmid. However so far no further experiments have been carried out to resolve whether the plasmid in the original strain is in fact R391.

8) Summary.

All of the IncC plasmids tested suppress the dnaG3 mutation and another primase, which is genetically and antigenically distinct from the sog primase, has been identified as encoded by R40a. The IncC plasmids are a fairly diverse group of plasmids but a plasmid encoded primase may be common to all members of the group. The R40a primase is also genetically distinct from the R16 primase and probably from RP4 primase. Levels of primase which are undetectable in the in vitro primase assay can substitute for dnaG primase, whereas the RP4 primase would have to be present in very large amounts to substitute for dnaG primase. Thus the R40a primase is probably also distinct from the RP4 primase.

RA3 (IncU) encodes a primase but I do not know

anything about the genetic or antigenic relationship of the RA3 primase to the other primases.

R391 (IncJ) suppresses the dnaG3 mutation but no primase has so far been identified as encoded by R391. There is some evidence that R391 may encode an R16-like primase but the disrepancies described in section 8 have yet to be cleared up.

So far plasmid encoded DNA primases have been identified from representatives of IncC, the I-complex, IncM, IncP and IncU. The other incompatibility groups of the enteric bacteria have now been screened by a variety of methods so it is perhaps unlikely that many plasmid encoded primases remain undetected in these groups of plasmids.

CHAPTER EIGHT.

DISCUSSION.

Plasmids are autonomously replicating molecules of DNA and are generally physically independent of the host chromosome. They are able to control the initiation of their own replication and hence their copy number. However, unlike many bacteriophages, plasmids appear to encode no, or few, proteins required for the biochemical reactions of DNA replication. In order to spread through populations of cells large conjugative plasmids encode transfer systems. Plasmid transfer involves a different mode of DNA replication from vegetative replication and a general model of conjugal DNA replication is presented below (see also introduction section 4). At the receipt of a signal, perhaps pilus-wall contact or wall-wall contact, the plasmid DNA is nicked at oriT in one strand by a plasmid encoded protein. This single strand nick is strand specific and releases a free 5' end which leads the transferred strand into the recipient. This free 5' end may be stabilised by a protein, perhaps analogous with the covalently attached protein of the ColE1 relaxation complex. Replacement strand synthesis in the donor probably occurs concomitantly with the transfer of the single strand and using the host replication system. The plasmid molecule is recircularised and new DNA synthesised.

Single stranded DNA and free ends are less stable than circular double stranded DNA. So in order to succesfully establish the transferred plasimd in the new recipient recircularisation and DNA synthesis must occur as quickly as possible. If DNA synthesis occurs before recircularisation multiple initiation of polymerisation is required due to the unidirectional polymerisation reactions of E.coli DNA polymerase III. Thus synthesis of primers on the transferred strand may be an important step in the establishment of the plasmid in a new recipient.

ColIb-P9 and RP4 have been shown to encode their own DNA primases, which are genetically and antigenically distinct from E.coli dnaG primase (Wilkins et al.,1981;Lanka and Barth, 1981). The function of the plasmid encoded primase appears to be to efficiently prime DNA replication in the recipient and hence to promote the conversion of the single stranded molecule to a double stranded molecule. The DNA replication systems of different bacteria are obviously very different and plasmids with wide host ranges are therefore able to establish themselves in a wide variety of genetic backgrounds. It is tempting to suppose that plasmids encoding their own primase have a wide host range due to their efficient establishment in alien environments where the host primase may not be able to efficiently recognize

the priming sites on the plasmid. Unfortunately for this hypothesis, while RP4 has a wide host range, ColIb-P9 is by no means a wide host range plasmid. Of course encoding a primase is probably not the only prerequisite for a wide host range. Other proteins involved in establishment of the plasmid after transfer or a series of relatively host specific maintenance systems may also be required.

It has been proposed (Chatfield et al.,in press) that the ColIb-P9 primase also synthesises the primer for replacement strand synthesis in the donor. This priming reaction, like the priming of DNA synthesis in the recipient, also occurs on single stranded DNA and could help to ensure the maintenance of the plasmid after it has donated a strand in conjugation. However replacement strand synthesis of F, at least in dnaB donors, is primed by a primer synthesised by RNA polymerase (Kingsman and Willetts, 1978). There may be at least two distinct mechanisms by which the synthesis of the replacement strand is primed.

The observation that primase defective RP4 plasmids are unstable in E.coli suggests that the plasmid encoded primase can prime DNA replication on double stranded DNA in the presence of the E.coli dnaG primase. It is possible that E.coli dnaG primase may synthesise primers very inefficiently on the plasmid.

The model for primer synthesis on R6K (IncX) does not require a plasmid encoded DNA primase, and it is proposed that the primer is synthesised in the donor by the host DNA replication system (Nordheim et al.,1981) (see figure 1.8). The experiments of Lanka and Barth (1981) show that R6K does not encode a primase detectable in the primase assay under the conditions used. Thus presumably a plasmid encoded primase is not the only method utilised by plasmids to prime DNA synthesis on the transferred single strand.

ColE1 does not encode a DNA primase and for mobilisation it relies on conjugative plasmids already present in the cell. These mobilisable plasmids could be primed by the host in a similar way to R6K (Nordheim et al., 1980) or by a primase encoded by the mobilising plasmid (Lanka and Barth, 1981) or in the recipient by the recipient priming system.

The molecules encoded by the three distinct primase genes of the IncI complex are all very large: sog primase, 220,000 (active) and 165,000 (inactive) daltons (Dalrymple and Williams, in press); R16 primase, 240,000 (active) and 175,000 (active?) daltons and R387 primase, 260,000 and 190,000 daltons. These molecules appear to be encoded by overlapping genes, but the smaller polypeptides

could be processed products of the larger polypepetides, at least for R16 and R387. The primase encoded by RP4 (IncP) is a polypeptide of apparent molecular weight 118,000. A second antiserum cross-reacting polypeptide of about 80,000 daltons has also been identified. This polypeptide may be a processed product of the larger protein or the product of a second in phase translational start within the primase gene or the product of a separate gene. Unlike the smaller, 165,000, ColIb-P9 encoded polypeptide the 80,000 dalton RP4 polypeptide is thought to have primase activity. Thus the primases encoded by RP4 and the plasmids of I- complex are very much larger than the dnaG primase which is a single polypeptide of apparent molecular weight 60,000.

All of the plasmid encoded DNA primases studied in detail appear to have two polypeptides associated with the primase activity. The I-complex primases appear to be encoded by overlapping genes and the RP4 primase may be organised in a similar way. The normal function of the dnaG primase is to synthesise primers on chromosomal DNA in cooperation with the primasome. The normal function of the plasmid encoded primase is probably to synthesise primers on single stranded plasmid DNA without additional proteins.

The R16 primase molecules can be truncated to approximately a third of their original length and still

retain primase activity and the ability to suppress the dnaG3 mutation. Thus the active sites of the R16 primase are present on the third of the molecule proximal to the N-terminus. This suggests that the rest of the polypeptide must have some function other than synthesising primers. The smaller polypeptides encoded by R16 and R387 are present in smaller numbers than the larger polypeptides. If the smaller polypeptides are active primases, why are two differently sized primases required? and if the smaller polypeptides are not active, what function do they fufill? It is possible that the primase has plasmid specific functions, such as the recognition of specific priming sites, or the recognition of regions involved in the transfer of the primase from the donor to the recipient. The primase may perhaps be involved in stabilising the DNA during transfer. It has been reported that sog primase can be truncated in a similar way and that the smaller and inactive sog primase polypeptide is present in the cell in similar amounts to the active polypeptide (Wilkins et al.,1981). It has recently been proposed that the sequence encoding the ColIb-P9 DNA primase is fused to another transfer gene (Chatfield et al.,in press).

The results of the hybridisation screen of the plasmids which carry the ColIb-P9 primase gene(s) suggests that the DNA in the vicinity of the primase genes is conserved. A similar observation was made with regard to the

plasmids which encode R16 primase. R16 (IncB) and R387 (IncK), which encode pili that are similar, if not identical, with those encoded by the sog+ plasmids, encode primases which are genetically distinct from sog primase. R16 and R387 encode primases which are genetically similar, but not identical, to each other. However there appears to be a small amount of antigenic similarity between the R16 and R387 primases and the sog primase. Together with the other data presented in chapters 4, 5 and 6 it is probable that at least the sog primase and R16 primase represent distinct, but related classes of primases associated with the I-like transfer system. The R387 primase is closely related to R16 primase, but may also represent a distinct class of primases.

All members of the I-complex probably encode a DNA primase and presumably a primase is an integral part of the I-like transfer system. All the IncIα plasmids tested encode sog primase, shown by the immunological, hybridisation and protein data presented chapter 3, and probably have very similar maintenance and transfer genes (Dalrymple et al.,1982). R621a (IncIδ) encodes a genetically similar primase, also shown by the hybridisation of the sog primase probe to an equivalent EcoRI restriction fragment, and may well be an anomolous IncIα plasmid. The IncB plasmids can be split into two groups; those which encode a sog primase,

shown by the immunological, hybridisation and protein data (Dalrymple et al.,1982; Dalrymple and Williams, in press), and perhaps an IncIα-like transfer system (RIP72 and R864a), and those which encode R16-like primases, shown by the hybridisation of the R16 primase gene(s) probe to their DNA (chapter 4), and perhaps an R16-like transfer system (R16, TP125 and pLG101).

The IncIδ plasmid R724 probably carries some genetic information for an R16-like primase, as shown by hybridisation, although there is no evidence that it is expressed. R724 is very closely related to R16, unlike the other IncIδ plasmids, and may well be an anomolous IncB plasmid (Falkow et al.,1974). Thus TP114, which encodes a primase of an unknown type, should perhaps be taken as the prototype IncIδ plasmid. The IncIζ plasmid, R805a, also encodes a primase which is genetically similar to the R16 primase, as shown by the hybridisation of the R16 primase probe. However the pilus serotype and incompatibility relationships of R805a make it difficult to classify. The IncK plasmid R387 encodes a primase which is genetically distinct from sog primase and fairly similar to the R16 primase, shown by the hybridization and restriction site data presented in chapters 3, 4 and 6. Thus the transfer systems of the IncK plasmids are probably more closely related to R16 (IncB) than to ColIb-P9 (IncIα).

Selection of sog+ mutant plasmids with a high efficiency of suppression of the dnaG3 mutation apparently selects for primase overproducing mutants. This increased number of primase molecules is associated with a derepression of the transfer genes of the plasmid (Wilkins, 1975; Sasakawa and Yoshikawa, 1978; Dalrymple and Williams, in press). Thus the gene(s) encoding the primase appear to under the same control as the transfer genes and are presumably part of the transfer operon or operons of the plasmids. Similar mutants of R16 and R387 pri+ plasmids, with a high efficiency of suppression of the dnaG3 mutation, do not appear to synthesise any more molecules of the primase, or to have derepressed transfer systems. Thus the control of the transfer genes of these plasmids is probably more complex than for the sog+ plasmids. Perhaps the genes are organised into several operons or more than one operon is required for the derepression of all of the transfer functions. This may also explain why derepressed mutants of these plasmids are apparently difficult to isolate. Thus the sog+ and pri+ plasmids may have a fundamentally different organisation of their transfer genes.

Table 8.1 summarises the types of maintenance and transfer systems of the IncI complex plasmids. Such a mixing of incompatibility and transfer phenotypes has been shown

Table 8.1) Summary of the incompatibility, pilus and primase groups of the plasmids of the I-complex.

plasmid	Inc group	pilus serotype	primase gene(s)	also incompatible with
ColIb-P9	Iα	Iα	Iα	
R64	Iα	Iα	Iα	
R144	Iα	Iα	Iα	
R621a	Iγ	Iα	Iα	B+Iα?
R724	Iδ	?	B	
TP114	Iδ	I2	+?	
R805a	Iζ	I2+Iα	B	B+Iα
R16	B	Iε	B	Iχ+Iζ
TP125	B	Iα?	B	
pLG101	B	Iα?	B	
R864a	B	Iα?	Iα	
RIP72	B	Iα?	Iα	
R387	K	Iα	K (B?)	

for members of the IncF complex (Willetts and Skurray, 1980). Thus presumably recombination between related plasmids can occur with a high frequency. There are many discrepancies in the published incompatibility data and a new study of the incompatibility is required, perhaps by the cloning of the incompatibility determinants. A detailed study of the transfer genes of ColIb-P9, R16, R387 and a representative IncIα plasmid is also required. From these studies a much clearer picture of the relationships of the the I-complex plasmids could be built up.

RP4 is the only IncP plasmid which has been studied in detail with regard to a plasmid encoded primase. The IncP plasmids, and the other plasmids which naturally occur in Pseudomonas spp., may well be very different in their maintenance and transfer functions from those plasmids which naturally occur in the enteric bacteria. The IncI complex primases and RP4 primase may have many biochemical and functional similarities but they are antigenically (Lanka and Barth, 1981) and genetically quite distinct classes of plasmid encoded primases.

All of the known E.coli incompatibility groups and some of the Pseudomonas spp. incompatibility groups have been screened for plasmid encoded DNA primases (see table 8.2) (Lanka and Barth, 1981). There is so far no evidence of

Table 8.2) Summary of plasmid encoded DNA primases by incompatibility group.

incompatibility group		suppress DnaG-	primase
E.coli	P.aeruginosa	phenotype	demonstrated
B		+	+
C	P-3	+	+
D		- (a)	-
F		-	-
H		-	-
I		+	+
J		+	- ?
K		+	+
M		-	+
N		-	-
P	P-1	-(+?)	+
Q	P-4	N.D. (b)	-
T		-	-
U		+	+
V		N.D.	-
W		-	-
X		-	-
Y		N.D.	-
Com9		-	-

a) '-' not detected.
b) N.D. not determined.

a primase being encoded by members of IncD,F-complex,H-complex,N,Q, T,V,W,X,Y,Z and Com9. While plasmid encoded primases have been identified for members of IncC, M, P, U and the I-complex (see table 8.2).

I have shown that R40a (IncC) encodes a primase which is genetically and antigenically distinct from the I-complex primases. All the IncC plasmids tested suppress the dnaG3 mutation of E.coli and presumably encode DNA primases, although this has not been shown. The relationship of the R40a primase to the RP4 primase has not been studied, but as both are Pseudomonas spp. plasmids there may be some similarities. However R40a can suppress the E.coli dnaG3 mutation, unlike RP4. The IncM plasmids also encode a primase (Lanka and Barth, 1981) which is genetically distinct from the I-complex primases, shown by the lack of hybridisation of the ColIb-P9 and R16 primase genes probes to R446b. Wild type R446b and R471a do not suppress the dnaG3 mutation. IncM plasmids have mainly been isolated from the enteric bacteria and thus there is no reason to suspect that the IncM primase would be related to the RP4 primase, which at a similar level of in vitro activity also does not suppress the dnaG3 mutation. The IncU plasmids were isolated from enteric bacteria and I have shown that RA3 suppresses the dnaG3 mutation and encodes a primase. So far there is no genetic or antigenic data about the RA3 primase. However the

IncU plasmids may encode yet another distinct plasmid DNA primase.

All the members of IncIα, B, C, K and M screened appear to encode a primase, thus presumably certain transfer systems are generally associated with a DNA primase and others are not. There appears to be no correlation between the occurence of plasmid encoded primases and the host range of the plasmids. Thus possession of a plasmid encoded primase is not an indication of host range.

The Pseudomonas spp. incompatibility groups, with the exception of IncP-1, P-2 and P-3, and Staphylococcus aureus plasmid incompatibility groups have not been screened for the suppression of the dnaG mutation. These plasmids are not stable in E.coli so at present screening for suppression of the DnaG- phenotype is impossible. Equivalent mutants are not available for other species, however it would be interesting to study the suppression of DNA primase defective mutants of Pseudomonas aeruginosa or Staphylococcus aureus. The screening systems only detect plasmid encoded primases which: are able to substitute for E.coli dnaG primase in temperature sensitive dnaG mutants; or are able to prime DNA synthesis on single stranded DNA phage templates, under the assay conditions used; or plasmids with sequences in common with sog primase, R16

primase or R387 primase. The RP4 primase genes have been cloned (E. Lanka, personal communication) and in our laboratory we are at present trying to clone the primase genes carried on R40a and RA3. Such clones will increase the range of hybridisation probes available.

The suppression of the DnaG- phenotype may require a specific interaction between the host encoded primasome and the plasmid encoded primase, which could occur with a varying efficiency. Sog primase, R16 primase and R387 primase substitue fairly efficiently for dnaG primase when compared with RP4 primase, but the dnaG primase is a more efficient primer of DNA replication. However an equivalent mutation in Pseudomonas aeruginosa, for example, may well be very efficiently suppressed by the RP4 encoded primase.

In the in vitro assay the incorporation of nucleotides into phage DNA is measured. The initiation of the phage replication may be by the synthesis of random primers or the synthesis of a specific primer near the origin of replication. No proteins other than the primase are involved in the synthesis of the primers on this template. In the assay system used by Lanka and Barth (1981) and in the work reported in this thesis the conditions were roughly optimised for the sog primase. Under the conditions of the in vitro assay system purified R64 sog primase

appears to be about 100 times as active as purified RP4 primase and dnaG primase. An alteration in the assay conditions may well change the relative amounts of primase activity and/or allow the detection of new primases. The use of single stranded plasmid DNA as a template may also allow the detection of new primases.

A plasmid DNA primase is obviously not an essential feature of plasmid transfer systems as primase defective mutants are still transfer proficient, albeit with a reduced efficiency, and many conjugative plasmids do not appear to encode a primase at all. These plasmids may rely on the recipient host priming system for priming DNA synthesis, or they may be primed by a mechanism similar to that proposed for R6K (Nordheim et al.,1980).

Other plasmid encoded DNA replication proteins.

Many plasmids can suppress the dnaA mutation of E.coli by integration. The chromosome appears to replicate under the control of the plasmid and probably no true analogue of the dnaA protein is synthesised. Hence suppression of the dnaA mutation is probably a property of all replicons able to integrate into the chromosome. The mechanism of the suppression of the dnaB mutation by

plasmids has not yet been clarified and the search for a dnaB analogue is still continuing with R100 (IncFII) (V.Iyer personal communication). It is probable that the results of many of the dnaB suppression experiments are not due to a true plasmid encoded dnaB analogue. However a possible role of a dnaB analogue could be to alter the primasome and primase binding such that a more efficient or more specific synthesis of primers occurrs. A role for a dnaB analogue in plasmids encoding a primase could be to make vegetative replication more efficient.

The suppression of the DnaE- phenotype by a plasmid has only been reported for R6K and it is not known whetheran analogue of DNA polymerase III is encoded by the plasmid. Interestingly this plasmid is one which probably does not encode a primase and utilises host proteins for the synthesis of the primer. It may be that efficient conjugal DNA synthesis is ensured by having a plasmid encoded polymerase.

Future experiments and work in progress.

The purification of the R16, R387 and R40a primases should be completed and antiserum raised against the three primases. This will allow a more detailed study of the interrelationships of these primases to be made. The

detailed comparison between the R16 and R387 primase gene(s) should be completed, perhaps ultimately by the sequencing of the genes. The investigation into the organisation of the R16 and R387 primase gene(s), to determine whether they encode two similar proteins on overlapping genes or if the smaller polypeptides are processed products of the larger, should be completed.

Using the cloned R387 primase gene(s) as a hybridisation probe a screen of the IncK plasmids could made to confirm whether or not they encode primases of the R387 type. TP114 and other IncIδ plasmids should be studied in more detail and screened with the sog and R16 primase gene(s) probes. Thus the nature of the primase associated with the IncIδ plasmids would be determined.

The cloning of the R40a and RA3 primase genes should be completed. The clones would allow further hydridisation screens to be undertaken and hence the distinct groups of plasmid primases can be identified. The other IncC plasmids, which suppress the dnaG3 mutation, could be screened for sequences homologous with the R40a primase gene(s). A more detailed study of the control of the expression of the primase and the organisation of the transfer genes of the IncC plasmids should also be undertaken.

More work is required with R391 to clear up the discrepancies in the data and to identify the probable primase encoded by R391.

The aim of the work presented in this thesis and the experiments outlined above is to study the distribution and interrelationships of the primases encoded by members of the different incompatibility groups. This complements the studies of the role of the primase in plasmid transfer and, perhaps, maintenance.

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PLASMID ENCODED DNA PRIMASES.

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Plasmid ColIb-P9 of the Iα incompatibility group encodes a DNA primase that acts in conjugal transfer of the plasmid and can substitute for mutant dnaG gene product in the vegetative replication of the Escherichia coli chromosome. The relevant genetic determinant (sog) has been cloned into a multicopy vector plasmid. Prototype IncB plasmid R16 also suppresses host dnaG mutations. The equivalent gene(s) (pri) of R16 have been cloned into plasmid pBR325 and shown by Southern transfer hybridisation to be different from the ColIb-P9 primase gene(s). The cloned fragment carrying the pri determinant encodes two polypeptides with apparent molecular weights 240,000 and 180,000 which can initiate DNA synthesis in vitro on single stranded phage M13 template, but which are antigenically distinct from ColIb-P9 primase. The cloned primase genes were used as probes in colony hybridisation screening of strains carrying plasmids of the IncI complex and IncB group, which specify serologically similar conjugation pili. Plasmids R64drd11, R144drd3 (IncIα), R621a (IncIγ), RIP72 and R864a (IncB) contain nucleotide sequences honologous with the cloned ColIb-P9 sog gene(s). Plasmids R805a (IncIζ), R724 (IncIδ), TP125 and pLG101 (IncB) showed sequence homology with the R16 pri determinant.

R387 (IncK) is also a member of the I-complex and encodes a primase which is genetically and serologically distinct from ColIb-P9 primase, but which is genetically similar to R16 primase. The gene(s) has been cloned into plasmid pBR328 and the fragment carrying the pri determinant encodes two polypeptides of 270,000 and 200,000 daltons.

The hybridisation and dnaG suppression screens were extended to include representatives of all of the E.coli incompatibility groups. R40a (IncC) and RA3 (IncU) were shown to suppress the dnaG3 mutation and to encode DNA primases. The IncC primase encoded by R40a is genetically and antigenically distinct from ColIb-P9 primase.