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**Determinants of the *E. coli* Polymerase I- independent and
Polymerase I-dependent pathways of replication of an
Incompatibility N group plasmid replicon**

by

Hong-Yeoul Kim, B.Sc., M.Sc

**A thesis submitted to the Faculty of
Graduate Studies and Research in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy**

Department of Biology

Carleton University

Ottawa, Ontario

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replicon"**

submitted by

Hong-Yeoul Kim

in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

Chairman, Department of Biology

Thesis Supervisor

External Examiner

Carleton University

Abstract

The 2053 bp basic replicon of the broad host-range, IncN group plasmid pCU1 shows a complexity that was not previously suspected. In addition to replicating in *E. coli* by a Polymerase I-independent and a Polymerase I-dependent mechanism, the independent mechanism can use an origin called *oriB* in one set of circumstances and another origin called *oriS* when *oriB* cannot be utilized and a family of regulatory iterons is absent. The organization of the *oriB-oriS* region suggests that they may share some sequence features for optimum function. At least the *oriB* mechanism is conserved among IncN group plasmids. Attention is drawn to structural similarities between the *oriB-oriS* region and the origin region of *ori-2* of plasmid F, and *oriV* of pGSH500 and another IncN plasmid, pR. The *trans*-acting functions of a replication protein of pCU1 are able to rescue the origin *ori-2* of F even though these two plasmids are not in the same compatibility group. The RepE protein of F was unable to rescue *oriB* of the pCU1 replicon. Two promoters were identified and their sequence shown to have similarity to known $\sigma^{32}(P_2)$ and $\sigma^{54}(P_3)$ promoters. A third promoter P_1 is expected to be also present. Although it has not been possible to determine which of the promoters function in different circumstances, evidence is presented for the functioning of P_2 . It is suggested that the third promoter P_3 may have a function in other hosts.

In addition to *oriB* and *oriS*, a third origin called *oriV* exists. This origin that is nested within the basic replicon is part of a replicon that is Polymerase I-dependent and uniquely also PolIII-dependent. This is the first report of a replicon that is PolIII-dependent. Cointegrates of this replicon and a pUC replicon were unexpectedly also PolIII-dependent.

Some chromosomal functions of *E. coli* required for the different pathways were identified. It was also shown that the host-range of derivatives using the different pathways can be distinguished. Thus, these studies have begun to provide a window on the molecular basis of broad host-range in this group of plasmids.

The truly major discoveries that have altered the face of medicine - for example, x-rays, penicillin, recombinant DNA - have all come from the pursuit of curiosity about nature without relevance to medicine. The same can be said of great industrial inventions, which were haphazard at the outset and only later recognized for their commercial value. No matter how counter-intuitive it may seem - to the scientist as well as the lay man - the most sure and cost-effective route to discovery is through the creative activity of the scientist or inventor rather than the pursuit of defined goal.

From -science is great, but scientists are still people

Arthur Kornberg, 1992

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퇴계선생의시조를
 밑내쓰다

To my family (Hye-Ryun, Seong-Hoo, Dae-Whan), with love

and

To my parents, with gratitude

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It was late spring in Boston, 1989. My wife and I received one important phone call from Seattle. During a short conversation, Dr. Iyer responded positively to my application to the doctoral program at Carleton. Oh! it was the beginning of my lucky year.

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APPENDIX I	Banerjee <i>et al.</i> reprint

List of Abbreviation

>	greater than
<	lesser than
ATP	Adenosine triphosphate
Ap	Ampicillin
<i>ard</i>	antirestriction gene(s)
bp	base pair
°C	temperature in degrees Celsius
<i>ccd</i>	coupled cell division gene(s)
Cm	Chloramphenicol
cm	centimeter
<i>cop</i>	copy number control gene(s)
Ci	Curie(s)
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
ddNTP	dideoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
<i>dnaA</i>	DNA biosynthesis, initiation gene
<i>dnaB</i>	DNA biosynthesis, chain elongation gene
<i>dnaC</i>	DNA biosynthesis, elongation and initiation
<i>dnaG</i>	DNA biosynthesis, primase

<i>dnaJ</i>	DNA biosynthesis, heat-shock gene
<i>dnaK</i>	DNA biosynthesis, heat-shock gene
<i>dnaT</i>	subunit of DNA polymerase III
dH ₂ O	distilled water
ds	double-stranded
EDTA	Ethylenediamine tetra-acetic acid
EtBr	Ethidium bromide
Fig.	Figure
fip	the fertility inhibition of IncP plasmids
<i>g</i>	acceleration due to gravity
gm	gram(s)
<i>grpE</i>	phage λ replication, heat-shock response
<i>gyrB</i>	DNA gyrase subunit B
Hg	mercury
<i>himA</i>	integration host factor α -subunit
<i>hip</i>	integration host factor β -subunit
hr	hour(s)
<i>hupA</i>	HU protein subunit A
<i>hupB</i>	HU protein subunit B
IHF	Integrational Host Factor
Inc	incompatibility
IS	Insertion sequence
IPTG	Isopropyl- β -D-thiogalactopyranoside

Km	Kanamycin
kb	kilobase pair(s)
kDa	kilodalton
<i>kik</i>	killing gene(s) of <i>Klebsiella</i>
<i>kil</i>	killing gene(s) of <i>E. coli</i>
<i>kor</i>	overriding killing gene(s)
kV	kilovolt(s)
LA	Luria agar
LB	Luria broth
M	molar
mA	milliampere
mg	milligram(s)
ml	milliliter(s)
mM	millimolar
mV	millivolt(s)
min	minute(s)
-	absence of a phenotype
mRNA	messenger RNA
muc	mutability gene(s) by UV and chemicals
Mr	molecular mass in daltons
ng	nanogram(s)
nm	nanometer(s)

<i>nuc</i>	production of an endonuclease of unknown function
ORF	open reading frame
O.D.	optical density
<i>oriT</i>	origin of conjugative transfer
<i>oriV</i>	origin of vegetative replication
<i>palA</i>	palindromic sequence gene
<i>pcn</i>	plasmid copy number control gene(s)
%	percentage
PEG	Polyethylene glycol
<i>polA</i>	<i>E. coli</i> DNA polymerase I gene
<i>polB</i>	<i>E. coli</i> DNA polymerase II gene
<i>polC</i>	<i>E. coli</i> DNA polymerase III gene
<i>priA</i>	primase A
<i>priB</i>	primase B
<i>priC</i>	primase C
+	presence of a phenotype
psi	pounds per square inch
RCR	rolling circle replication
<i>rep</i>	replication gene(s)
<i>r</i>	resistance
RNA	Ribonucleic acid
<i>rop</i>	repressor of RNA II transcription in ColE1

*	sensitivity
SDS	Sodium dodecyl sulfate
sec	second(s)
Sm	Streptomycin
sop	stability of plasmid
sp.	species
Spc	Spectinomycin
ss	single-stranded
SSB	single-stranded binding protein
ssi	single-stranded initiation sequences
Sul	Sulfonamide
ts	temperature-sensitive
Tc	Tetracycline
TE	Tris-HCl, EDTA buffer
Te	tellurite
TAE	Tris-HCl, sodium acetate, EDTA buffer, pH7.4
ter	termination gene(s)
tra	conjugal transfer gene(s)
µg	microgram(s)
µl	microliter(s)
UV	Ultra-violet light
V	Volts
v/v	volume per unit volume

w/v	weight per unit volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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Chapter I

Introduction and a Review of the Relevant Literature

Many species of bacteria contain stable genetic elements separate from their chromosomes and called plasmids. With few exceptions, they are covalently-closed and supercoiled double-stranded DNA molecules. Their replication mechanisms can be independent of that of the chromosome. Thus, a plasmid is a molecule which can be stably inherited, controlling its own maintenance without being linked to the chromosome. The replication and stable maintenance functions define the plasmid as a replicon which contains an autonomous unit of replication. Therefore, bacterial plasmids have proven to be not only interesting objects of study in themselves but also to be useful as model systems for the study of the molecular genetics and biochemistry of replication and stable intracellular maintenance of extrachromosomal DNA elements.

In Gram-negative Eubacteria that are closely related to *E. coli*, about 30 different incompatibility groups of plasmids have been defined using intracellular incompatibility tests (Couturier *et al.*, 1988). Since incompatibility is closely associated with plasmid replication or distribution, this implies differences in replication or maintenance-specificity among different plasmid groups and possible differences in mechanisms.

The incompatibility group N plasmids (i.e., pCU1) has either been isolated from diverse species of Gram-negative bacteria (Jacob *et al.*, 1977) (Table 1.1) or they have been shown to replicate and to be maintained under a selective condition when introduced into diverse species (reviewed in Iyer, 1989). A relatively broad host-range is therefore one of their interesting attributes. In

Gram-negative Eubacteria, plasmids of the incompatibility groups P (RK2, RP4, R751), W (pSa, pR388, pR7K), Q (RSF1010, R1162, R310, R750), and probably B (pMU720) have also been shown to have different degrees of promiscuity.

Recently, some plasmids of Gram-positive bacteria have also been shown to replicate in different bacterial species (Gruss and Ehrlich, 1989., Janniere et al., 1990., Janniere *et al.*, 1993). In none of these instances is the molecular basis of promiscuity understood. Furthermore, and although in a general sense, replication modes (in both plasmids and other replicons) can be grouped into different patterns (i.e., theta, rolling circle) (Kornberg and Baker, 1992) there are significant and interesting differences within the plasmid groups showing each pattern. The basis of such differences are again not well-understood.

In recent years, much information on the replicon regions and the replication and stability determinants of some plasmids has accumulated as they have been used as model systems to explore replication mechanisms in general among the Eubacteria. From this vast literature, particular plasmid replicons have been selected for review either because our understanding of them both at genetic and biochemical levels is well-advanced (*in vitro* systems have been developed and information is beginning to become available on how identified determinants participate in replication and its regulation) or because one can detect similarities between some aspects of their structure and function to that of the pCU1 replicon. Accordingly, the plasmid replicons that have been selected for review are those of :
(a) the narrow host-range plasmids F and P1 because like pCU1 they can replicate

Table 1.1 Example of IncN plasmids isolated from *E. coli* and other enteric bacteria*

Plasmid	Original Host	Reference
N3	<i>Shigella</i> sp.	Datta and Hedges (1971)
R15	<i>P. vulgaris</i>	Falkow et al (1974)
R46	<i>S. typhimurium</i>	Clewell and Franke (1974)
R269N	<i>Sh. sonnei</i>	Bannister (1969)
R348N	<i>E. coli</i>	Bannister (1970)
R390	<i>P. rettgeri</i>	Coetzee et al (1972)
R393	<i>P. mirabilis</i>	Hedges (1975)
R400	<i>Proteus</i> sp.	Coetzee et al (1972)
R448	<i>P. morganii</i>	Hedges et al (1973)
R497	<i>Providencia</i>	Hedges (1974)
R728	<i>Providencia</i>	Hedges (1974)
R48	<i>S. typhimurium</i>	Drabble and Stocker (1968)
R199	<i>S. typhi</i>	Bannister (1969)
R893	<i>K. aerogenes</i>	Matthew and Hedges (1976)
pIP113	<i>S. panama</i>	Chabbert et al (1972)

* For additional bacterial species included in the host-range of this groups of plasmids - see section 1.13.

in the absence of *E. coli* DNA polymerase I and as will be shown here have structural similarities to pCU1.

(b) the R plasmid RK2 as an example of a replicon that has a very broad-host range.

(c) plasmids R6K and pGSH500 which like pCU1 have a moderately broad host-range and in the case of R6K also has three origins of replication as does pCU1 (Banerjee *et al.*, 1992).

(d) ColE1, as the best-studied replicon that is dependent on Polymerase I. The known roles of DNA polymerases of *E. coli* is first reviewed briefly before considering each of the above plasmids. After this, information on the properties and overall structure of all known plasmids of the group N will be reviewed followed by a review of the origin, properties and structure of pCU1, the subject of this thesis.

1.1 A brief overview of plasmid replicons in relation to DNA polymerases

As the basic replicon of pCU1 has been shown to be capable of using pathways of replication that are independent or dependent on Polymerase I of *E. coli*, a brief consideration of the role of DNA polymerases in replication is relevant and potentially useful.

In *E. coli*, there are three DNA polymerases, PolI, PolII and PolIII. The regions on the chromosome that specify them have been called respectively, *polA*, *polB* and *polC*. Each polymerase is either a single large

Table 1.2 Properties of polymerases I,II, and III of *E. coli* (adapted from Kornberg and Baker, 1992)

a; + and - represent the presence and absence, respectively, of the property listed.

b; A primed single strand is a long single strand with a short length of complementary strand annealed to it.

SSB = single-strand binding protein.

c; Nucleotides polymerized at 37°C / min / molecule of enzyme.

d; Sizes of the α , ϵ , and θ subunits.

e; Also known as *dnaE*, the gene for the large (α) subunit

	polI ^a	polII ^a	polIII
Functions			
Polymerization: 5'→3'	+	+	+
Exonuclease: 3'→5'	+	+	+
Exonuclease: 5'→3'	+	-	-
Pyrophosphorolysis and PPi exchange	+		+
Template primer			
Intact duplex	-	-	-
Primed single strands ^b	+	-	-
Stimulation by SSB	-	+	-
Nicked duplex [poly d(AT)]	+	-	-
General			
Size (kDa)	103	90	130,27.5, 10 ^d
Turnover number, ^c estimated	600	30	9000
Structural genes	<i>polA</i>	<i>polB</i>	<i>polC</i> ^e
Conditional lethal mutant	yes	no	yes

polypeptide with multiple functions or a complex of several polypeptides, each of which is specified by different genes (see Table 1.2). This is particularly apparent in the case of PolIII, known to be the major and essential protein complex for chromosome replication (however, see section 1.11 on the *pcbA1* mutation). The PolIII protein has ten subunits, the α subunit being the subunit with polymerizing activity. For the *E. coli* chromosome, PolI has two secondary roles. These two roles are the removal of the RNA primer portion from the RNA-DNA hybrid intermediate of replication (so-called "Okazaki" fragment) and the editing of mispaired bases (so-called "proof-reading" function). In spite of this, *polA* null mutants including a *polA* deletion mutant of *E. coli* exist and are viable. This implies that at least the first of the above functions can be taken over by the other DNA polymerases, most likely PolIII.

Available information indicates that in Gram-negative Eubacteria, most plasmids replicate as circular structures which when visualized in an electronmicroscope resemble the Greek letter θ (theta). Therefore, the term theta (θ) replication has been used to describe this pattern. On the other hand, for most plasmids isolated so far from Gram-positive Eubacteria, electronmicroscopy of replicating DNA molecules reveal circular molecules with double-stranded tails. This pattern is referred to as the rolling circle replication (RCR). The apparent relationship between the pattern and the Gram reaction is not absolute. There are Gram negative bacterial plasmids

that use the RCR mode and Gram-positive bacterial plasmids that use the theta mode.

Plasmids of Gram-negative bacteria that use the θ mode can again be divided into two groups which are most conveniently distinguished by their requirement for PolI. For a number of plasmids (F, R1, P1, pSC101, R6K, RK2) (Fig. 1.1). PolI is not essential, as in the case of the bacterial chromosome. All of these plasmid replicons encode positively-acting proteins called Rep proteins which are each specific for their own origins of replication (For the *E. coli* chromosome, an analogous protein is the DnaA protein, the product of the *dnaA* gene). Although there are variations between plasmids, in general, these plasmid replicons contain iterons, *dnaA* boxes (binding site for the DnaA protein), IHF site, (a site for binding a protein known to bend DNA and called Integration Host Factor protein - see section 1.12) and a region that is relatively rich in A:T sequences and where initial strand separation (melting) is shown or believed to occur as part of the replication-initiation process. The plasmids of the above group require the host DnaA protein. This and the above observation on the proximity of rep-binding site(s) to DnaA-binding sites at the origin, has suggested that the role of the Rep protein is to bind to their cognate sequences and along with the DnaA protein-binding, to melt the DNA at a specific location on the circle. For this to occur, the DNA strand must be locally unwound, a feat accomplished by an ATP-dependent enzyme called a helicase. These

Fig. 1.1 Proposed model of replication initiation events at the origin of the plasmid R1.

Proposed model of replication initiation events at the origin (oriR, shown stipled white and black) of the plasmid R1. The black circles shown as A are DnaA proteins of *E. coli* and the open circles the R1-specified RepA protein. The wavy lines within the opened template DNA are RNA which is extended by synthesized DNA shown as arrows or solid lines to the RNA (Adapted from Kornberg and Baker, 1992).

events allow access of several replicating proteins to the origin region and the formation of a complex called a preprimosome composed of six proteins called respectively PriA, PriB, PriC, DnaB, DnaC and DnaT. The combination of this complex with the DnaG protein (an RNA polymerizing enzyme called primase) results in the formation of the primosome and the initiation of RNA synthesis. The synthesized RNA is called a primer RNA. DNA PolIII can add deoxyribonucleotides to the 3' end of the primer, thus, synthesizing a precursor fragment (Okazaki fragment). Synthesis occurs up to the primer of the preceding fragment, at which point PolI normally replaces PolIII to replace RNA with DNA (with the help of DNA ligase). The events described above occur in a region where initiation is started *de novo* as in the case of the *E. coli* chromosome.

1.2 F plasmid

1.2.1 The F plasmid and its structure

The F plasmid is the prototype sex factor of *E. coli*. It is a large (94.5 kb), conjugative, low copy number, *polA1* independent plasmid (Lane, 1981) which has been used as a standard replicon for the classification of IncFI group plasmids. By using *EcoRI*, the whole plasmid DNA was resolved into 19 fragments (Figure 1.2, top). Among them, three fragments showed autonomous replication in *E. coli* (i.e., f7, f5, f12). They are also called

Fig. 1.2 The replicon map of the F plasmid (Skurray *et al.*, 1976).

The numbers inside the plasmid refer to the F kb coordinates, whereas the numbers outside indicate the fragments generated by *EcoRI* digestion. The expanded regions show the fragments (f5, f7 and f12) containing the replicons of F (repFIA, repFIB and repFIC) (Bergquist *et al.*, 1986). repFIC in F is incomplete and is interrupted by the insertion of Tn1000 (Guyer, 1978). The abbreviation R1 indicates *EcoRI* restriction sites.

repFIB, repFIA, and repFIC, respectively (Bergquist *et al.*, 1986., Figure 1.2 top). These three replicons have been proposed to contribute to the stability of F (Nordström and Austin, 1989). RepFIA (primary replicon, f5) and RepFIB (secondary replicon, f7) have been further investigated. Although the secondary replicon FIB plays only a subsidiary role in F replication, sequences homologous to them appear to be important for the maintenance of other plasmids in the IncFI family. For example, FIB showed incompatibility (*incE*) to the enterotoxigenic plasmid P307 (Picken *et al.*, 1984) and other IncFI group plasmids, pCG86 (Picken *et al.*, 1984), R386, pHH507, R453, and pColV3-K30 (Bergquist *et al.*, 1985, 1986., Perez-Casal *et al.*, 1984). Recently, the *incE* region from pColV-H247 (Kim *et al.*, 1988) and similar regions were found to be present in P307 (Gibbs *et al.*, 1993). Interestingly, even though the FIB by itself is unstable in *E. coli* (Gardner *et al.*, 1985), similar regions from other IncFI plasmids were stably maintained as mini-plasmids in *E. coli*. The reason for this is unknown.

The RepFIA replicon has been studied much more intensively (Kline, 1985). Interestingly, a replicon region of the IncHI group plasmid R27 has great similarity to FIA. Coul and Bergquist (1988) have suggested that all the elements of R27 RepFIA regions are functionally equivalent to their F plasmid counterparts. In addition, the sequences of the third replicon of the F plasmid, RepFIC, showed homology to large plasmids that came from enteroinvasive *E. coli* (Silva *et al.*, 1988). The RepFIC replicon has been

observed to be widely distributed in *Salmonella* strains from the pre-antibiotic era.

In the best-studied FIA replicon, there are two different origins of replication, *oriV* (also known as *ori-1*) and *oriS* (also known as *ori-2*) (Eichenlaub *et al.*, 1977., Manis and Kline, 1977). The *oriV* (*ori-1*) region, could be deleted without the impairment of autonomous replication while *oriS* (*ori-2*) could not (Manis and Kline, 1977). Therefore, *oriS* was identified as part of the basic mini-F replicon (Murotsu *et al.*, 1981). These two origins showed different modes of replication *oriV* (*ori-1*) being bidirectional and *oriS* (*ori-2*), unidirectional. In one study, the *ori-2* initiated replication was only observed when *ori-1* was deleted from mini-F (Eichenlaub *et al.*, 1981). However, experimental evidence showed that the blocking of replication from *ori-1* did not affect the *ori-2* initiated replication (Eichenlaub and Wehlmann, 1980). Despite these observations, the *ori-2* region of the F plasmid is currently accepted as the favoured origin (Kline, 1988., Kline *et al.*, 1992., Kawasaki *et al.*, 1991). The molecular organization of RepFIA (2.25 kb) is illustrated in Figure 1.3. The *ori-2* region consists of two DnaA boxes and a 90 % A-T rich 50 bp sequence followed by four 19 bp iterons (Figure 1.3). These iterons are responsible for incompatibility properties of *ori-2* and are designated *incB* (Tsutsui *et al.*, 1983). Next to the *ori-2* sequences, is the open reading frame (*repE*). The *repE* gene was shown to encode a 29 kDa protein which is the initiator for replication of the mini-F

Fig. 1.3 Comparison of mini-F and P1 replicon organization.

The shaded arrows represent open reading frames for proteins that have been identified, black boxes represent segments that can exert incompatibility. Small arrows indicate arrangements of the 19 bp repeat sequences, small numbers represent numbers of nucleotides between repeats. The distance between *oriR* and *oriL* is 10.2 kb. *ccd*, control of cell division; *inc*, incompatibility determinants; *ori*, replication origins, of which each plasmid has several; P1-*oriR* and F-*ori2* are the origins that have been studied with respect to regulation, and P1-*par* (partitioning) and F-*sop* (stability of plasmid) are the determinants of partitioning. *sopC* thought to be the F partitioning site, corresponds to F-*incD* and to P1-*incB*. P1-*repA* and F-*rep* are the initiator protein coding sequence. *f5* is an open reading frame of unknown function. (From Novick, 1987).

plasmid (Murotsu *et al.*, 1984). Five 19 bp iterons (*incC*), similar to those in *incB* are also found downstream of *repE*. These are oriented in the opposite orientation to the *ori-2* iterons and are involved in incompatibility and copy number control (Tsutsui *et al.*, 1983). The initiator binding site of *ori-2* has been identified (Masson and Ray, 1986., Kawasaki *et al.*, 1992).

The host factors required for F replication have been studied in detail. The host encoded gene *dnaA* (Kline *et al.*, 1986), *dnaB* (Goebel and Schremppf, 1972) *dnaC* (Van Brunt *et al.*, 1977, Zeuthen and Pato, 1971), and *dnaE* (Thompson and Broda, 1973) are essential for F replication initiation. Recently, Yura and his colleagues observed that a possible interaction between *dnaK*, *dnaJ* and *grpE*-determined proteins enhanced initiation from *ori-2* (Kawasaki *et al.*, 1991, 1992).

1.2.2 The models for plasmid replication control.

In general, three models have been suggested for F plasmid replication control.

i) ***The initiation-titration model*** : It has been proposed that the iterons titrate a positive acting element(s) that is essential for replication (Tsutsui *et al.*, 1983). The positive factor was postulated to correspond to the RepE protein whereas the titrating repeats correspond to the *incC/copB* and *ori-2* (*incB*) region - see Fig 1.3 B (Tsutsui *et al.*, 1983, Kline and Trawick, 1983). Bergquist and co-workers observed that the *copA* mutations, caused by

Fig. 1.4 Models for control of chromosomal and plasmid DNA replication.

A. The autoregulatory model control of Sompayrac and Maaløe (1973).

In this model, P1 is the autorepressor and P2 is the initiator. P1 and P2 are transcribed from the same open reading frame. Transcription is regulated only by P1 binding to the promoter-operator region of P1 (black box). B.

Model for the control of mini-F replication involving RepE (E) and a putative 9 kDa protein. Transcription of both proteins is regulated from the same promoter, that of RepE (black box). In this model, the 9 kDa protein is the promoter repressor and RepE the initiator of DNA replication (Kline, 1985; Trawick and Kline, 1985). C. The same model as in (B.), though RepE is both the repressor (monomer unit) and the initiator (dimer unit). This monomer/dimer relationship is a symbolic, not a quantitative representation of the irreversible transition from repressor to initiator. The (-) represent iterons, which are the binding sites for RepE. *oriS* is an alternative designation for *ori-2*. *copA* is an alternative designation for the replication domain with copy number involvement. *copB* represents the domain containing the iterons associated with plasmid copy number control. The dashed lines represent mRNA transcription (From Kline, 1985., Trawick and Kline, 1985).

insertion of Tn3 into *repE*, led to loss of copy number control (Bergquist *et al.*, 1981., Seelke *et al.*, 1982). Also, these mutations prevented suppression of the *repE* promoter. It was therefore, suggested that RepE may be an autorepressor as well as being essential for mini-F replication (Trawick and Kline, 1985). This possibility was supported by the demonstration of binding of RepE to *incB* and *incC* and by the autoregulatory properties of *repE* *in vitro* (Bex *et al.*, 1986., Rokeach *et al.*, 1985). Since this model emphasized the RepE binding site and the concentration of free RepE in the cell, there was a need to postulate a steady-state equilibrium between free and bound RepE. However, it is possible that RepE can be binding both to *ori-2* and the *copB* loci at the same time. This could make it impossible to keep the equilibrium between free RepE and RepE binding site(s). Therefore, this would result in another round of replication with consequent sequestration of RepE, to set a new steady-state equilibrium. Eventually, these events cannot avoid run-way replication rather than controlled replication. Because of this possibility, Trawick and Kline (1985) proposed two possible variations to the titration model, based on the autorepressor/initiator operon model of Sompayrac and Maaløe (1973) (Figure 1.4.A).

ii) **Independent initiator-repressor model:** As shown in Figure 1.4.B, there are two smaller open reading frames within *repE* that encode two 9 kDa polypeptides (Murotsu *et al.*, 1981). Thus, one of these polypeptides may be expected to operate as an autorepressor. However, this remains a tentative

model because there is no direct evidence for the expression of these smaller polypeptides.

iii) ***The two-stage molecular model:*** Finally, Kline and his colleagues proposed the existence of two type of RepE protein (Figure 1.4.C). They speculated that RepE is translated in its repressor form (E) and is converted irreversibly to the initiator form (EE). Thus, the formation of EE is dependent on both the E concentration and on the rate constant for its conversion to E. Hence, the binding of EE to the binding sites has no effect on the synthesis of E with corresponding conversion to EE. However, there is no direct evidence for the existence of two forms of the RepE protein although two naturally occurring proteolytic forms of RepE have been identified (Kline *et al.*, 1992).

1.3 P1 plasmid

1.3.1 P1 plasmid and its structure.

Plasmid P1 is a large (90 kb) single-copy plasmid. Despite the low copy number, it is maintained stably. Its replication, and maintenance functions must therefore be regulated tightly (Austin and Abeles, 1983., Davis and Austin, 1988., Capage and Scott, 1983).

As shown in Figure 1.3 the mini-replicon (about 2.1 kb) of P1

Fig. 1.5 Constructional organization of P1 replicon origin, *oriR*.

The boxes represent the five 7 bp imperfect repeats containing GATC adenine methylation sites (GATC motif absent in second box). Open arrows are potential DnaA boxes. Shaded arrows are 19 bp RepA-binding repeats. Vertical shading marks the location of the 39 bp GC-rich sequence. The upper part of the diagram represents the minimal origin (Chattoraj *et al.*, 1985), whereas the lower part shows associated sequences, including the rightward set of DnaA boxes and the start of *repA* gene (Brendler *et al.*, 1991).

contains all requirements for its replication. It contains a 250 bp *cis*-acting origin, *oriR* (*incC*), the gene encoding the 32 kDa *repA* protein and a copy-number control locus, *incA* (Chattoraj *et al.*, 1985a). Chattoraj and his colleagues observed that the *oriR* region is sufficient for replication when RepA is supplied *in trans* (Chattoraj *et al.*, 1985b). The structural organization of *oriR* is shown in Figures 1.3 and 1.5 (Novick, 1987., Brendler *et al.*, 1991). It contains two DnaA boxes, but only one of these DnaA boxes is required for *oriR* function (Abeles *et al.*, 1990). Next to these DnaA boxes are five tandem repeats of 7 bp, which contain GATC sites. Origin function required dam methylation both *in vivo* and *in vitro* (Abeles and Austin, 1987). Downstream from these methylation sites is a 39 bp GC-rich region, which is immediately followed by 19 bp repeats, separated from each other by two helix turns, over a stretch of 104 bp (Abeles *et al.*, 1984). These repeats are overlapped with the promoter of the *repA* gene, which is the site for RepA autoregulation (Abeles, 1986). Between these repeats and *repA* gene are three more DnaA boxes (Figure 1.5). The role(s) of these DnaA boxes is (are) not clear (Abeles *et al.*, 1990).

After the *repA* gene, is the *incA* region containing nine 19 bp direct repeats. These are not essential for replication, but the deletion of these iterons gave rise to an eight-fold higher copy number than the wild-type (Pal *et al.*, 1986). This *incA* copy-control locus was also shown to be a

binding site for RepA (Abeles, 1986).

The P1 replicon does not require *Poll* but it requires other host factors determined by *dnaA* (Hansen and Yamolinsky, 1986., Wickner and Chattoraj, 1987), *dnaB*, *dnaJ*, *dnaC*, *dnaG* (Scott and Vapnek, 1980) and *dnaJ*, *dnaK*, and *grpE* (Tilly and Yarmolinsky, 1989., Wickner, 1990) .

1.3.2 The models of replication control.

Five different models for control of replication of this plasmid have been considered.

- i) ***The initiation-titration model:*** As described earlier, this model cannot explain the control of replication of the P1 replicon because of the same reasons as that for the F plasmid. Control of replication by sequestration is inconsistent with the autoregulation of *repA* expression since the loss of RepA protein to the *incA* locus should be compensated by new synthesis of RepA (Chattoraj *et al.*, 1985b).
- ii) ***The two stage molecular model:*** As shown in Figure 1.6, by using a model similar to that considered for the F plasmid, Chattoraj and his colleague investigated the control mechanism(s) of mini-P1 (Chattoraj *et al.*, 1988). In the model, the initiator binds directly to different loci (*incA* and *incC*). Therefore, the concentration of repressor can be independent of that of the initiator. However, again, they were unable to show different forms of the RepA protein.

Fig. 1.6 The two-stage molecular model for the control of P1 replication.

The *cis*-acting *ori* (*incC*) includes five 19 bp repeats (arrows 10-14). The *repA* gene (stippled) codes for the initiation protein (RepA), whose promoter maps within repeats number 10-12. The control locus (*incA*), contains nine 19 bp repeats (arrows 1-9). According to the model, the repressor form of RepA binds to the repeats of the *repA* promoter. This results in autorepression of the *repA* gene. The RepA repressor form is irreversibly converted to RepA initiator. Only RepA initiator is competed by the repeats of *incC* and *incA*.

iii) **Replication control by DNA looping:** A mechanism of control that has been considered uses DNA looping (Figure 1.7 A). In this model, *incA* including the promoter for RepA and *incC* compete for RepA (shown as dimers). RepA dimers that bind to the *incA* site repeats also repress the *repA* promoter by simultaneously binding to the origin repeats. This creates looping of the intervening DNA. RepA binding would exclude RNA polymerase by steric hindrance from the origin. The nature of the binding of the RepA dimer to the two sites is not known.

iv) **Control of replication by steric hindrance:** As shown in Figure 1.7.B, the *incA* locus has two functions, to sequester RepA from *ori* (*incC*) and to directly control initiation by steric hindrance of the origin activity. By electron microscopy it was shown that the repeated sequences within the *incA* and origin loci could interact with each other in the presence of purified RepA (Chattoraj *et al.*, 1988). When the two sites are in *cis*, the interposed sequence loops. RepA is also able to pair these sites in *trans*. This can prevent initiation of replication. This direct interference of replication occurs preferentially in the presence of a complete *incA* locus ; however, Chattoraj and his colleagues showed that a single *incA* repeat was able to induce loop formation (Pal and Chattoraj, 1988). It is likely then that a single site is also able to interfere with the origin function by DNA looping.

Fig. 1.7 Control of replication by DNA looping.

The RepA protein dimer is represented by small circles. RepA binds simultaneously to the *incA* and *incC* repeats. When the both of these incompatibility sites are in *cis* the intervening DNA loops out (A). When these sites are in *trans*, a dimeric DNA structure (B) is formed (Chattoraj *et al.*, 1988).

A.

B.

Fig. 1.8 An antiparallel plasmid-plasmid pairing model for P1 replication
(Abeles and Austin, 1991).

A single copy of the plasmid initiates replication rapidly (A). When two copies are produced (B), they rapidly pair in an antiparallel configuration (C), blocking origin function of both until partition (D) resets the cycle. A typical cell cycle is illustrated below with the abscissa representing fractional divisions of the cell cycle and the ordinate representing the probability that initiation will occur. Only one initiation event can occur per cell cycle, as replication is self-blocking and is only reset by partitioning. Initiation could occur at different times in different cells, but the result at the time of cell division would always be the same. The *incA* locus and the origin are shown by solid and hatched boxes, respectively. Vertical arrows show the orientation of the *repA* gene.

v) ***Antiparallel plasmid-plasmid pairing replication control***: In the wild type plasmid, the *ori* (*incC*) and the *incA* loci are *in cis* (see Fig 1.3). Following replication these sites are *in trans* in the two daughter molecules (Figure 1.8). After replication, each daughter plasmid can be in contact with the *ori* of the other. This "handcuffing" of two molecules results in blocking of replication. Initiation of another round of replication can only occur after plasmid-partitioning to individual daughter cells. But after one round of replication the two plasmids couple and block each other at the *ori* until partition resets the cycle. This model emphasized the importance of the relationship between partitioning and copy number control.

1.4 RK2 plasmid

Plasmids belonging to the *E. coli* incompatibility groups, IncN, IncP, IncQ and IncW are capable of replication and stable maintenance in diverse unrelated Gram-negative bacteria (Thomas, 1989). Because of their ability to transfer DNA between species of bacteria and hence to allow exchange between gene pools, broad host-range plasmids have probably played a significant role in Gram-negative bacterial evolution.

The best studied IncP plasmid replicon is that of RK2. RK2 (60 kb) is conjugative, has a copy number in *E. coli* of 5-7 per chromosome equivalent, and encodes resistance to three different antibiotics (see Figure 1.9). It was isolated as a carbenicillin-resistance plasmid from

Fig. 1.9 Functional organization of plasmid RK2.

The open segments indicate the regions for stable maintenance function. The hatched sections show the conjugation functions. Resistance determinants and transposable elements are shown as solid segments (From Thomas and Helinski, 1989).

Pseudomonas in a hospital unit for the treatment of burns (Lowbury *et al.*, 1969). It has a very broad host-range. There are large segments of the plasmid coding for plasmid establishment or maintenance (i.e., so-called *kil* genes, multimer resolution system, and partitioning function). Their mechanisms are being explored (Thomas and Helinski, 1989) (Figure 1.9).

The three major groups of replication and maintenance genes are the (*oriV*, *trfA* and *trfB* regions) which function as operons (Figurski and Helinski, 1979., Thomas, 1981., Thomas and Hussain, 1984). In contrast to the narrow host-range plasmids, each of these elements are separated by three antibiotic resistance determinants (Thomas *et al.*, 1980). Replication of RK2 proceeds unidirectionally anti-clock-wise from *oriV* as shown in Fig. 1.10. The origin is activated by the polypeptide product(s) of the *trfA* (trans-acting replication factor A) as an initiator. The TrfA protein exists in 32- and 43-kDa forms, arising from translation of the same reading frame from two different start sites (Shingler and Thomas, 1984).

The molecular organization of the origin region is similar to that of P1 and F (Figure 1.10). The 617 bp *oriV* region includes the eight 17 bp iterons in two clusters and a putative promoter surrounded by DnaA boxes and a single putative IHF-binding site. Also, a 49 bp AT-rich sequence (74 % A-T) containing another DnaA box, and a 67 bp GC-rich sequence (79 % G-C) (Gaylo *et al.*, 1987., Smith and Thomas, 1985., Stalker *et al.*, 1981) (Figure 1.10). Additionally, a 17 bp iteron also exists upstream from *oriV*

Fig. 1.10 The organization of the *oriV* region of RK2.

The direct repeats are indicated as number 1 to 11. The DNA, divided into 100 bp sections, run counter-clockwise from the *Bgl*II site (Bg) at 13.2 kb on RK2. Regions showing homology to DnaA consensus sequences are shown as solid blocks. A and B represent deletions defining *incA/copA* and *incB/copB* respectively. E. c, P. p and P. a represent the minimal origins in *E. coli*, *P. putida* and *P. aeruginosa*, respectively. (From Thomas, 1989)

(Thomas *et al.*, 1984) (Fig 1.10). This iteron overlaps the promoter for two transcripts. Just after the *oriV* sequence, two degenerate repeats are present in an inverted orientation (Thomas and Smith, 1987). Three open reading frames are found within the origin region. These are involved in copy number control and incompatibility (Thomas *et al.*, 1984). For replication in *E. coli*, only a 393 bp, which includes the DnaA boxes, five iterons as well as the AT- and GC-rich region are required (Stalker *et al.*, 1981., Thomas *et al.*, 1981). However, in *Pseudomonas*, the entire 617 bp *oriV* fragment is necessary for the activation of the functional origin (Schmidhauser *et al.*, 1983). In addition, mutation in the single iteron which overlaps the promoter can be enough to prevent replication in *Pseudomonas stutzeri* (Nash and Krishnapillai, 1987).

trfA has two different functions. It is a regulator of KIL. It also, encodes two proteins of 382 amino acids (43 kDa, TrfA-43) and 285 amino acids (32 kDa, TrfA-32). As mentioned earlier, these two proteins use two independent translational starts within the same open reading frame (Kornacki *et al.*, 1984). Either TrfA-44 or TrfA-33 alone is sufficient for replication in many hosts, including *E. coli*. However, in *Pseudomonas aeruginosa*, there is a specific requirement for Trf-44 for the stable maintenance of the replicon (Durland and Helinski, 1987). TrfA-33 and TrfA-44 proteins bind specifically to the cluster of five iterons contained in the minimal origin of replication (Perri *et al.*, 1991). It was confirmed that

TrfA proteins bind to one, two, or five iterons of the origin. Both TrfA-33 and TrfA-44 proteins showed a similar affinity for the double iteron as for the minimal *oriV*, while the affinity for the single iteron was significantly lower. In addition, a plasmid containing a single iteron failed to inhibit RK2 replication *in vitro* or to express the incompatibility *in vivo* (Kittell and Helinski, 1991). It has therefore been concluded that at least two iterons (17 bp each) must be present and occupied for stabilization of TrfA-DNA complex.

Thomas and his colleagues have observed that RK2 requires DnaA, DnaB helicase, DnaG primase and PolIII for *in vitro* replication (Pinkney *et al.*, 1988). These requirements and the positioning of DnaA and DnaA boxes may be critical to each host (Gaylo *et al.*, 1987., Krishnapillai, 1986., Ogasawara *et al.*, 1985., Skovgaard and Hansen, 1987).

Despite the presence of an IHF binding consensus, there is no evidence for the requirement of IHF protein in RK2 replication.

1.5 Structural organization of R6K plasmid

R6K is a 38 kb self-transmissible PolI-independent plasmid of *E. coli* that is maintained at approximately 15 copies per chromosome. Electron microscopy analysis indicated that there are three different origins designated, α , β and γ , that are contained within a 4 kb region (Figure 1.11.A) (Kornberg and Baker, 1992). *In vivo*, the α and β origins are used

Fig. 1.11.A The structural organization of plasmid R6K and its replicon.

Map of R6K, showing the three clustered replication origins (α , β , and γ) and the asymmetric location of the terminus (*ter*). AT represent AT-rich sequence. *pir* and *bis* indicate the essential initiator protein Π and a second protein Bis, that is specifically involved in initiation from *ori* β .

(From Kornberg and Baker, 1992)

Fig. 1.11.B Struture of R6K oriγ.

Silencer activity resides in a promoter located to the right of the dnaA box, just outside the origin.

(From Kornberg and Baker, 1992)

most frequently (almost 50 % each) while *in vitro* each of the three origins is used with equal frequency. Although the α and β origins are used most frequently *in vivo*, the γ origin is required in *cis* for initiation at these other two sites. Thus, if the replication origin is defined as the *cis*-acting sequence essential for initiation, rather than the location of a replication bubble, γ would be a component of both the α and β origins. Despite the rare initiation of DNA synthesis from γ *in vivo*, it has been studied the most (Crosa, 1980., Inuzuka *et al.*, 1980., Filutowicz *et al.*, 1987).

The organization of the γ origin is similar to that of other single origin plasmids which were described previously (Figure 1.11.B). The *ori γ* sequence contains seven 22 bp direct repeats that govern the binding of the *trans*-acting replication protein called π . To the left of the repeats is a 90-bp 80 % A-T rich sequence and to the right, a DnaA box. Interestingly, the A-T rich region contains a sequence homologous to the *E. coli oriC* 13 bp repeats and binds IHF protein *in vitro* (Filutowicz and Appelt, 1988). Recently, it was proven that the DnaA protein is essential for replication of R6K γ origin plasmid *in vitro* (MacAllister *et al.*, 1991). Additionally Filutowicz and his colleague suggested that the single DnaA box (see Figure 1.11.B) is sufficient for initiation from the γ origin and might be required for the activity of the α and β origins as well (Wu *et al.*, 1992).

Though IHF protein was found to be nonessential for the propagation of plasmids containing any of the three R6K replication origins, it has been shown to induce a conformational change leading to copy number changes of the replicon (Kelly *et al.*, 1991).

In the γ origin, a single base change in the first or the sixth of the 22 bp iterons destroys the binding of π protein *in vitro* and the origin activity *in vivo* (McEachern *et al.*, 1985). It was concluded that six iterons are sufficient. Recently, it was observed that the 383 bp γ origin can be functionally separated into at least two domains: the core γ origin and the adjacent 106 bp segment, which was called the origin enhancer (Wu *et al.*, 1992). The reason why the γ origin is only rarely the site of initiation *in vivo* is likely to be the presence of an inhibitory sequence (Figure 1.11.B). This "silencer" sequence acts *in cis* and contains a weak promoter directing transcription into *ori γ* (Patel and Bastia, 1986). The details of the mechanism(s) of inhibition are not yet known but Patel and Bastia (1987) have suggested that an "activator RNA" is blocked by the antisense "silencer RNA" much as the primer RNA II of ColE1 is inhibited by the antisense RNA I (see later this chapter).

Fig. 1.12 The structural organization of pGSH500 replicon.

The long arrows indicate the three ORFs predicted from the DNA sequence. The direction of arrows indicates the predicted direction of transcription. The numbers below each ORF, represent the first and the last base pair of each ORF. The horizontal filled arrows (▸) indicate the position of the 18 iterons of $\text{rep}\beta$. The numbers indicate the position in the sequence of the iteron region within the basic replicon. The vertical arrows (↓) indicate the position of the DnaA boxes; the vertical filled arrowhead (▼) indicates the position of the potential primosome site. The AT- and GC-rich areas are also indicated. (From Da Silva-Tatley and Styen, 1993)

1.6 Structural organization of pGSH500 plasmid.

pGSH500 was obtained from a nosocomial isolate of *Klebsiella pneumoniae* in South Africa. Interest in this plasmid increased when it was shown to be associated with multiply antibiotic-resistant bacterial isolates (*E. coli*, *P. mirabilis*) from immuno-compromised patients.

This conjugative, multi-drug resistance exhibits a moderately promiscuous host-range mostly within the *Enterobacteriaceae*. The incompatibility group of this plasmid is not known. However, its DNA gave a strong hybridizational signal with an IncN plasmid DNA replicon. The size of the plasmid is about 107 kb but spontaneous deletions of pGSH500, ranging in size from 76 to 100 kb, were isolated from *E. coli* during one of the conjugation experiments (Da Silva-Tatley and Steyn, 1993).

Figure 1.12 shows the structural and functional organization of pGSH500. The *cis*-acting origin *oriV* was identified as a 245 bp sequence within the mini-replicon of pGSH500. Within the origin sequences, G-C and A-T rich (90 %) sequence occurs overlapping two DnaA boxes. There are several repeated sequences within the β *oriV*. Two identical 16 bp direct repeats (TCAACAAGCTATGGGA) and several 7 bp (TTATAAA) and 6 bp (TTATCC ; TATGGG) direct repeats are also present within the *oriV* region. Upstream from *oriV*, there are sequences corresponding to the consensus sequence for the primosome sites of ColE1 and F, which are designated *rrIA* sites (GCTCGCCGCAGTCGA, AGTGAGCG, GAGGAAGC) (Nomura *et al.*,

1982., Marians *et al.*, 1982., Imber *et al.*, 1983). These are also referred to as single-strand initiation sequences (*ssi*) (Nomura *et al.*, 1991).

Downstream from the *oriV*, there is a third DnaA box in the opposite orientation to those described earlier. There are also three major open reading frames within the mini-replicon of pGSH500 (Figure 1.12). Da Silva-Tatley and Steyn (1993) suggested that the product of ORF295 (about 30.8 kDa) is an initiator protein for the replication of pGSH500. Since this protein appears to be required for autonomous replication, it was designated RepB.

Just after the *repB* gene, there is the presence of 16 tandem direct repeats (iterons) separated from 2 similar iterons by a 40 bp gap. Among them only one iteron is 30 bp long and all other 17 iterons are 34 to 36 bp. Incompatibility is expressed by these set of iterons. But there is no association between the number of iterons present and plasmid incompatibility. Additionally, it was observed that deletion of these iterons led to derivatives of plasmids with raised copy number, suggesting that copy number control mechanism(s) may coexists with the function of incompatibility (Da Silva-Tatley and Steyn, 1993).

Fig. 1.13 Handcuffing model for control of RK2 and R6K plasmids replication.

The diagram shows the two different events when the copy number is high or low.

1.7 RK2 and R6K plasmids: replication control

In models shown for the plasmid replicons considered so far, replication is controlled by the level of an initiator, which, in turn, is regulated by a repressor, whose concentration rises directly in proportion to plasmid copy number. However, the repressors were not always identified. In the absence of such a repressor, one explanation for the inhibition of plasmid replication when the copy number rises above a certain level is offered by a hypothesis, the 'handcuffing' model. When the plasmid copy number rises, origin-Rep-Rep-origin complexes are formed and block the entry of proteins essential for initiation (Figure 1.13). This model was originally proposed for R6K where the π protein was shown to mediate interaction between the DNA molecules containing π binding sites (McEachern *et al.*, 1989) and is related to ideas developed to explain the role of iterons in copy number control of P1 (Pal and Chattoraj, 1988). Recently, it was shown that the replication of RK2 was inhibited by copies of the TrfA-binding iterons *in vitro*. Since the inhibition was not relieved by the addition of excess TrfA, it was argued that it is not due to titration of positively acting TrfA. Thus, the repeats must be having a direct negative effect (Kittel and Helinski, 1991). Since the model allows for *in cis* and *in trans* intermolecular coupling of the iterons, this may also explain why deletion of some iterons leads to a raised copy number of the plasmid. Replication regulation has not yet been studied for pGSH500.

1.8 The ColE1 plasmid and its structure.

ColE1 is the best-studied member of a group of plasmids that use the θ pattern of replication and are PolI-dependent. Other members are p15A, pMB1, RSF1030, CloDF13 (Cesareni *et al.*, 1991). These plasmids do not encode a Rep initiator protein. Their replication is initiated by the general host RNA polymerase and not DnaG primase. The transcript formed folds into specific structures, which allow the formation of an RNA-DNA hybrid at the origin. This RNA strand is then cleaved by the host-encoded RNaseH and its 3'-hydroxyl end permits leading strand DNA synthesis using PolI, that copies one strand, the leading strand while the other is displaced (Fig. 1.14). Figure 1.15 shows that lagging-strand synthesis begins when a signal named *pas* carried on the displaced strand, is exposed. This allows a primosome (an assembly of six proteins and DNA primase) to be formed. Eventually PolI (on the leading strand) is replaced by PolIII (Kornberg and Baker, 1992). The mechanism of this displacement is not known. Staudenbauer (1976) observed that these plasmids required both DNA polymerase I and III, but not polymerase II. It has therefore been possible to characterize these plasmids by their inability to replicate in *E. coli* mutants that lack or are deficient in PolI.

Fig. 1.14 Initiation of synthesis of the ColE1 continuous strand.

Early event in the initiation of replication of the leading strand of plasmid ColE1 under conditions when a DNA::RNA hybrid is formed and when such a hybrid is not formed. These alternatives are determined by the secondary structure of the transcribed RNA (not shown here) (Adapted from Kornberg and Baker, 1992).

Fig. 1.15 Initiation of synthesis of ColE1 discontinuous strand

This figure describes a continuation of the events described in Fig. 1.4 that exposes the primosome assembly site (*pas*) and the initiation of synthesis of the lagging strand. The open circles at the position of *pas* are a complex of the proteins *priA*, *priB*, *priC*, *dnaB*, *dnaC*, *dnaT* and primase. SSB is the single-strand DNA binding position (Adapted from Kornberg and Baker, 1992).

1.9 Replication and control mechanism of ColE1 and its relatives.

ColE1 replication is unidirectional and it does not encode any enzymatic functions required for replication. A 600 nucleotide DNA fragment containing the replication origin is necessary *in cis* for initiation of replication. This fragment also contains two promoters (Figure 1.16). One promoter is responsible for initiation of the replication primer RNA (RNAII). Transcription initiating at the RNA II promoter proceeds beyond the origin and terminates at different positions depending on the downstream sequence of the specific plasmid. The RNA II transcript is processed by RNaseH to yield a 550 nucleotide molecule that is efficiently used as a promoter by DNA polymerase I to initiate leading strand synthesis (Itoh and Tomizawa, 1980). In the absence of RNaseH, the 3' end of the unprocessed RNA II can still be used as a primer by DNA polymerase I, even though at lower efficiency. RNA II transcription can also start initiation of DNA replication in cells defective in both RNaseH and DNA polymerase I, by displacing the non-transcribed strand, on which lagging strand DNA synthesis (by PolIII) can then occur (Dasgupta *et al* , 1987) (Figure 1.15). Thus, generally ColE1 replication could be modulated by a diffusible molecule affecting the formation of the stable RNA II-DNA hybrid required for replication initiation. This could be done either by controlling the synthesis of RNA II or by altering its conformation. The major inhibitor is a small transcript of 108 nucleotides, called RNA I (Tomizawa and Itoh,

Fig. 1.16 The structural organization of ColE1 replicon.

Numbers refer to nucleotide positions along the ColE1 plasmid; the jagged portions are indicated to condense distance for appropriate RNA folding. The pair of preprimers are indicated as α and β . Pr represents the promoter. Solid squares are mutations causing replication defects ; open squares represent supressor of some of these replication-defective mutations. The inhibitor and target transcripts are shown as secondary structures according to computer predictions.

(From Kornberg and Baker, 1992)

ColEI

1981). RNA I binds to the nascent RNA II by complementary base pairing (Tomizawa and Itoh, 1981). The interaction between RNA I and RNA II influences the folding pathway of RNA II, such that the conformation incompatible with the formation of the stable hybrid at the origin is favored (Wong and Polisky, 1985). So, binding of RNA I to RNA II is critical in determining the effectiveness of the regulation mechanism. In general, there are three host factors (DNA polymerase I, RNaseH, PcnB) that are needed for replication control. Among them, the *pcnB* gene was identified because of its effect on ColE1 plasmid copy number (March *et al.*, 1989). The amino acid sequence of PcnB has a strong similarity to that of tRNA-nucleotidyl transferase (Cca) (Masters *et al.*, 1990). The substrates of Cca, tRNAs, resemble RNA I in size and conformation. Therefore, Wagner and his colleagues (He *et al.*, 1993) suggested that the RNA I would be a substrate for PcnB. So, PcnB could facilitate replication by interfering with the interaction between RNA I and RNA II. Alternatively, PcnB could enhance replication frequency by reducing the intracellular concentration of RNA I.

Replication of ColE1-type plasmids can also be controlled by a small ColE1-encoded protein, Rop, which consists of 63 amino acid residues; deletion of the DNA segment encoding the Rop gene increases the plasmid copy number almost two fold (Cesareni *et al.*, 1982). It was initially speculated that Rop was a repressor of RNA II transcription. However, no effect of this protein was found on the rate of RNA II transcription *in vivo*

or *in vitro* (Cesareni *et al.*, 1984., Tomizawa and Som, 1984., Lacatena *et al.*, 1984). Instead, Rop was found to accelerate the binding of RNA I to RNA II and to stabilize the complexes of the complementary RNA I - RNA II stem-loop formations *in vitro* (Castagnoli *et al.*, 1989., Tomizawa, 1986, 1990., Eguchi and Tomizawa, 1990). Based on these observations, the Rop protein has been renamed Rom (RNA one modulator), to emphasize its better-defined function.

In this thesis, it will be shown that pCU1 has a Poll-dependent replicon the origin of which has a sequence similar to that of ϕ X174 and plasmids using the RCR mode (Chapter IV).

1.10 The group of plasmids that originate in *Staphylococcus aureus*.

1.10.1 The general properties :

A number of small, multicopy plasmids from *Staphylococcus aureus* such as pT181, pC221, pE194, pC194 and pUB110 share similar replication properties (Gruss and Ehrlich, 1989). Many of these plasmids have also been shown to replicate in *Bacillus subtilis*. These plasmids encode replication initiator proteins, which have origin-specific nicking-closing activities (Novick, 1989). These proteins are directly involved in the generation of a primer for the replication of the leading strand via an

asymmetric rolling circle mechanism. A number of *in vivo* and *in vitro* studies have shown that single stranded DNA (ssDNA) is an intermediate in the replication of these plasmids. The mechanism of replication of these plasmids is different from that of the more extensively studied plasmids of *E. coli* and is in many ways similar to the replication of ssDNA bacteriophages of *E. coli* (Zinder and Horiuchi, 1985). These plasmids contain a palindromic sequence (*paiA*) whose deletion results in plasmid instability, a decrease in the plasmid copy number, and the accumulation of large quantities of ssDNA *in vivo* (Gruss and Ehrlich, 1989). Based on these observations, the *paiA* sequence has been postulated to serve as the origin of lagging strand synthesis. The *paiA* sequences of many plasmids have considerable homology (Novick, 1989). This sequence has been shown to be orientation dependent, can function at different locations, and is interchangeable among many small *S. aureus* plasmids (Gruss and Ehrlich, 1989).

1.10.2 The group of streptococcal plasmids.

Plasmids pIP501, pSM19035, and pAM β 1 (Behnke *et al.*, 1981., Behnke *et al.*, 1979., Clewell *et al.*, 1974) form a group of streptococcal plasmids which replicate in a broad host-range of Gram-positive bacteria, including *Bacillus* spp. (Buu-Hoi *et al.*, 1984., Engel *et al.*, 1980., Gonzalez and Kunka, 1983., Oultram and Young, 1985., Perez-Diaz *et al.*, 1982).

In the case of pIP501, the only two plasmid functions that are

essential for replication are the origin of replication, *oriR*, and a 57.4 kDa protein, RepR, which likely functions during the initiation of replication. The expression of *repR* is regulated by several mechanisms involving a small protein, CopR, an antisense RNA of 136 nucleotides (RNA III), and an inverted repeat which is located upstream of and overlapping promoter pII. This promoter directs the transcription of the essential *repR* gene (Brantl and Behnke, 1992). It was therefore postulated that the amount of RepR is in fact the rate-limiting factor which determines the frequency of replication initiation. There are several differences between this replicon and ColE-type replicon.

Ehrlich and colleagues have recently proposed that the ϕ smid pAM β 1, isolated from *Enterococcus faecalis* follows a different replication pathway (Gruss and Ehrlich, 1989., Le Chatelier *et al.*, 1993 and personal communication). This plasmid replicon specifies a protein called RepE but is nevertheless dependent on PolI for replication. They have proposed that PolI is required for initiation of replication as in the case of ColE1. The precise role of RepE and the mechanism by which a primer is generated is not known.

As mentioned earlier, in the RCR pattern of replication common among plasmids of Gram-positive bacteria, circular molecules with linear tails are generated. The significant feature in this pattern is that the leading strand of DNA is covalently linked to the parental template for the

Walker and co-workers showed that pKM101 had arisen from R46 by deletion of a single DNA region, approximately 14 kb in length and that the total length of pKM101 was about 35.4 kb. A related but independently derived plasmid is pR which was obtained by Battaglia and colleagues by an *in vitro* method from R46 (Gigliani *et al.*, 1981). R46 DNA was digested with *HindIII* completely. After the digestion, the resultant fragments were re-ligated in a "shot-gun" type of experiment. It was possible to derive a plasmid of 22.7 kb (and thus, a smaller derivative than pKM101 which they called pR. Anthony *et al* (1981) found that a single *BglII* fragment (about 5.5 kb) contained the plasmid replicon and part of the *mucB* gene (Winans and Walker, 1985). It was inferred that this 3.4 kb region may be the replicon region of pKM101. Recently, Battaglia and co-workers sequenced part of this 3.4 kb region called "repa-minima" (Gigliani *et al.*, 1993). Its sequence is identical to part of the sequence previously established in this laboratory for the basic replicon of pCU1 (Krishnan and Iyer, 1990). There are indications that over a larger region of the replicon of pR (called "repa-maxima"), there are sequence differences between these two otherwise similar replicons (Battaglia, personal communication to Dr. Iyer).

lagging strand.

Initiation is by covalent extension from a nick (hence, "rolling circle"). Plasmids that exhibit this pattern usually specify a nicking polypeptide all or part of which remains attached to the 5' end of the nick. Some but not all of these plasmids are from Gram-positive bacteria and have been shown to have a broad host-range as does pAM β 1. The organization of these replicons and their replication mode has been reviewed recently (Gruss and Ehrlich, 1989).

It is clear that either PolIII or PolI has an essential role in the replication of some plasmid replicons. The same cannot be said for PolII. Although this enzyme protein and its gene has been known for several years (Wickner and Hurwitz, 1974., Hughes *et al.*, 1991., Bonner *et al.*, 1992), it has not so far been possible to ascribe to it any essential role (see Table 1.3 for a comparison of the *in vitro* properties of the polymerases). However, in Chapter V of this thesis, it will be shown that some PolI-dependent replicon derivatives of pCU1 are also PolII-dependent.

1.11 The *polC* bypass pathway for the *E. coli* chromosome

Moses and his colleagues (Niwa *et al.*, 1981) have proposed that in some circumstances, the *E. coli* chromosome can dispense with the need for the α subunit of PolIII (i.e., the polymerase that normally functions in chromosome replication). They discovered a mutation called *pcbA1*

(polymerase C bypass) in the presence of which they believe that PolI can be substituted for the α subunit of PolIII in the replication complex. They call the resulting complex "RepA" to distinguish it from the normal complex which they call "RepE". A *polA*⁺*polB*⁻*polC*^{ts}*pcbA*⁻ strain replicates and survives at high temperature but an isogenic *polA*^{ts}*polB*⁻*polC*^{ts}*pcbA*⁻ strain does not. *pcbA1* appears to be an allele of *gyrB* but the mechanism by which the *pcbA1* mutation acts is not known. However, in this thesis, it has proved advantageous to use the *pcbA1*-carrying strain to isolate a particular class of replicon mutants.

1.12 Other *E. coli* genes involved in plasmid replication.

In principle, any gene required or helpful for the replication or stability of the *E. coli* chromosome or its viruses can also have a role for plasmid replication and maintenance. A complete review of DNA replication in *E. coli* is not attempted here. The reader's attention is drawn to the recent comprehensive review of Kornberg and Baker (1992). In Chapter VI, I have used some specific *E. coli* chromosomal mutants to study derivatives of the pCU1 replicon. It is therefore appropriate that information on these particular genes and functions be reviewed briefly in addition to the DNA Polymerase genes.

Integration Host factor Protein.

This *E. coli* protein is so-called because it was initially found as a protein required for the site-specific recombination of phage λ into the chromosomal attachment site. Since then, it has been found to be required for so many other functions that it has been referred to, humorously, as "a protein for all reasons" (Ogawa *et al.*, 1985). It is a heterodimer of two sub-units specified respectively by the genes *him* (or *himA*) and *hip* (or *himD*). It is known to bind to specific 13 bp sequences in DNA (called IHF binding sites) generating a DNA bend. It is not essential for the growth of *E. coli*. Mutants of either *himA* or *himD* subunit are available.

HU protein

This is also a heterodimeric DNA bending protein (HupA and HupB) but is much more abundant than IHF protein. It is rich in lysine and alanine, is basic and shows evidence of conservation among prokaryotes. Thus, it has some general features of histone proteins. It is known to cause DNA curvature and is required *in vitro* at an early stage of chromosomal DNA (*oriC*) initiation provided it is not present at too high a concentration (Funnell *et al.*, 1986). *HupA* or *HupA HupB* double mutants grow poorly. *HupB* mutants show no phenotypic effects.

PriA, B, C. proteins

These proteins also known as π' , π and π'' respectively are required for the assembly of the primosome in *E. coli*.

Strains with mutation in *priA* are defective in chromosome replication. They are also defective in the replication of plasmids F and ColE1. (Lee and Mariani, 1989).

Heat-shock proteins

The number of genes the expression of which is responsive to heat or other stimuli is probably under-estimated (Neidhardt and Van Bogelen, 1987). The ones that have been studied genetically and for which mutants have been isolated are DnaJ, DnaK, GroE, GrpE. It is believed that they may provide functions for cell growth at all temperatures and not merely at high temperatures. The promoters for these genes are not recognized by the σ^{70} subunit of RNA polymerase but by another subunit, the σ^{32} subunit (product of the *rpoH* gene). Both F plasmids and mini-F plasmids (carrying an origin and *repE*) are unstable in *rpoH* mutants (Wada *et al.*, 1986). The *gro* and *grp* genes as well as *dnaJ* and *dnaK* were initially identified as being required for the growth of bacteriophage λ but mini-F is unable to transform strains with mutations in any one of these genes (Ezaki *et al.*, 1989., Kawasaki *et al.*, 1989). Both plasmids mini-P1 and mini-Rts1 also require these genes (Tilly and Yamolinsky, 1989., Bukau and Walker, 1989).

Gyrase B This is one of the subunit of DNA Gyrase (also called Topoisomerase II). The enzyme converts closed, open circular DNA duplexes to a negative supercoil which is a more favorable form of DNA for recombination. It is needed for integrative recombination of λ . Mutations in *gyrB* are known to affect adversely chromosomal replication and probably ColE1 replication (Marians *et al.*, 1986).

As indicated in section 1.3, the *pcbA1* mutation is believed to be an allele of *gyrB* although the details of the mechanism by which this allele allows chromosomal replication in the absence or reduction of the otherwise essential PolIII α subunit is not known.

1.13 IncN group plasmids and their known properties

In addition to the plasmids and species listed in Table 1.1 (Hedges, 1972), IncN group plasmids have been isolated from *Enterobacter* and *Aeromonas* (Aoki *et al.*, 1977., Arai and Ando, 1979) and *Vibrio cholerae* (Arai *et al.*, 1980). Under laboratory conditions, they have also been introduced into *Caulobacter crescentus* (Ely, 1973), *Agrobacterium tumefaciens*, *Bradyrhizobium sp.*, *Rhizobium leguminosarum*, *Alcaligenes faecalis*, *Aeromonas hydrophila*, *Enterobacter aerogenes*, *Salmonella typhimurium*, *Salmonella enteriditis*, and *Serratia marcescens* (Krishnan and Iyer, 1988).

Restriction maps of the plasmids N3, R15, R46(pKM101) and pCU1 have been published and functions ascribed to particular parts of their DNA (Ando and Arai, 1978., Dobritsa *et al.*, 1985., Winans and Walker, 1985., Hengen, 1992). In general, there are four groups of functions although all of the regions indicated below may not necessarily be present in all members.

- i. the replicon region; single continuous region
- ii. others functions indirectly related to maintenance; scattered

regions

- . Killing of *Klebsiella* (*KikA*, *KikB*); two regions.
- . The killing of *E. coli* and regions overriding killing (*kil*, *kor*); several regions.
- . The fertility inhibition of IncP group plasmids (*fip*); a single region within 'transfer'.
- . Production of an endonuclease of unknown function (*nuc*); single region next to *fip*.
- . Mutability by UV and chemicals (*muc*); single region
- . Antirestriction (*ard*)
- iii. Region responsible for conjugative transfer (*tra*); a single long region.
- iv. Insertion sequences (i. e., IS46) and antibiotic resistance genes.

Fig. 1.17 Structural and functional maps of the IncN group plasmids

The plasmids N3 (Ando and Arai, 1981), R15 (Dobritsa *et al.*, 1985), and R46 (Brown and Willetts, 1981) shown for comparison to that of pCU1. The circular maps were converted to linear representation for clarity. The solid arrows (A,A', B,B', C,C', D,D') indicate regions of extensive sequence similarity. Abbreviations *amp*, *as*, *mer*, *spc*, *sul*, *str* indicate resistance to ampicillin, arsenate, mercuric chloride, spectinomycin, sulfonamide and streptomycin, respectively ; *Kil*, killing of *E. coli* K-12 ; *Kik*, killing of *K. pneumoniae* ; *Kor*, overriding of the effect of *kil* ; *nuc*, nuclease ; *fip*, fertility inhibition of P-group plasmids ; *stb*, stability of plasmid ; *muc*, mutability by U.V or by chemicals ; *ard*, alleviates restriction of unmodified double-stranded DNA by all families of type I restriction enzyme. The open rectangles with genetic regions that emerge from the main map are part of the map of each plasmid and placed to indicate their relative position.

N3

Plasmid N3 is a conjugative plasmid isolated from *Salmonella typhimurium* in Japan (Ando and Arai, 1978), and studied by Arai and co-workers structurally and functionally (Ando and Arai, 1980, 1981., Arai and Ando., 1980). Figure 1.17 shows its map. The size of the plasmid is 54 kb, and it is divided into 2 large *EcoRI* fragments (E1 and E2). The fragments encoding replication functions were determined by studying the ability of the fragments to replicate in the *polA* deficient *E. coli* strain p3478 (Ando and Arai, 1981). It was observed that there were two replicons, each of which can be separated as independent replicons using *EcoRI* or *BamHI*. One of these replicons (E2) was found to be unstable in the *polA* strain while the entire N3 was stable. In recent experiments in this laboratory done in collaboration with Krishnan, these two replicons were separated with selective markers conferring resistance to streptomycin (Sm^r) and spectinomycin (Sp^r). Only one of these replicons showed IncN incompatibility (Krishnan *et al.*, 1991). Thus, N3 has two distinct replicons. The occurrence of cointegrates of two or more replicons of different incompatibility groups is not unusual among naturally-occurring plasmids (Chater *et al.*, 1982., Kok *et al.*, 1984., Wolk *et al.*, 1984., Da Silva-Tatley and Steyn, 1993). The approximate copy number of the two N3 replicons was also investigated in both *polA* and wild-type strains (Ando and Arai, 1981) (Table 1.3). The E2 plasmid (the smaller fragment) had a higher copy

Table 1.3 Copy numbers of the two distinct replicons in N3

Plasmid	Copy numbers of plasmid in	
	C600	p3478
N3	2.8	2.3
E1 ^a	4.1	2.1
E2 ^a	11.6	8.9

^aE1 and E2 are E1-pBR322 and E2-ColE1 recombinant plasmids.

Adapted from Ando and Arai (1981).

number than either the wild-type or E1 recombinant plasmid. There are no further studies on this replicon.

R15

The physical and genetic structure of R15 was established by Dobritsa *et al* (1985) (Figure 1.17). R15 was isolated originally from a *Proteus vulgaris* strain (Watanabe *et al.*, 1964). This plasmid is 62.3 kb in size, conjugative and contains transposon Tn2354. It confers resistance to streptomycin (Sm^r), sulfonamide (Sul^r) and mercury (Hg^r). The *rep* genes of R15 were mapped to a 7.7 kb *Bgl*III segment, extending from coordinate 46.0-53.7 kb but the region has not been studied any further. The plasmid has a large region (Tra) for conjugative function. It also has a region of specific restriction and modification (R/M). R15 and other IncN plasmids determine *Hsp*II restriction and modification specificity (Hedges, 1972). Dubovaya and colleagues (Dobritsa *et al.*, 1985) inferred that the genes of the R-M system map between coordinates 18.2 and 23.55 kb. This conclusion was confirmed by data on phage restriction and modification in *E. coli* cells harbouring a 6.8 kb *Bam*HI-*Eco*RI fragment of pAD816 of coordinates 18.2 to 23.6 kb cloned into pBR322 (Dobritsa *et al.*, 1985).

R46 and pKM101

R46 was isolated from *Salmonella typhimurium* in a hospital environment. Initially, this plasmid had several other names (i.e., R-Brighton, R1818, TP120) (Drabble and Stocker, 1968., Datta and Kontomichalou, 1965., Grindley *et al.*, 1973). The plasmid was 51.7 kb in size, conjugative, and coded for resistance to ampicillin (Ap^r), Sm^r, Sp^r, Sul^r, tetracycline (Tc^r) (Brown and Willetts, 1981)) (Figure 1.17). An interesting property of R46 is its ability to make *E. coli* and *Salmonella typhimurium* more resistant to the lethal effects of U.V irradiation and to enhance the susceptibility of cells to mutagenesis by a variety of agents (Mortelmans and Stocker, 1979). These investigators constructed a deletion derivative of R46, by P1 phage transduction and called the resulting plasmid pKM101 (Figure 1.17).

It has been shown that some bacteriophages encode antirestriction functions which permit them to overcome host restriction (Dharmalingam *et al.*, 1982., Studier, 1975., Zabeau *et al.*, 1980). Interestingly, broad host-range plasmids also carry genes which express antirestriction functions. Belogurov and co-workers have shown that two unrelated self-transmissible plasmids, pKM101 and ColIb-P9 carry *ard* (alleviation of restriction of DNA) genes that specifically affect type I restriction in *E. coli* (Belogurov *et al.*, 1985 and 1992).

Walker and co-workers showed that pKM101 had arisen from R46 by deletion of a single DNA region, approximately 14 kb in length and that the total length of pKM101 was about 35.4 kb. A related but independently derived plasmid is pR which was obtained by Battaglia and colleagues by an *in vitro* method from R46 (Gigliani *et al.*, 1981). R46 DNA was digested with *HindIII* completely. After the digestion, the resultant fragments were re-ligated in a "shot-gun" type of experiment. It was possible to derive a plasmid of 22.7 kb (and thus, a smaller derivative than pKM101 which they called pR. Anthony *et al* (1981) found that a single *BglII* fragment (about 5.5 kb) contained the plasmid replicon and part of the *mucB* gene (Winans and Walker, 1985). It was inferred that this 3.4 kb region may be the replicon region of pKM101. Recently, Battaglia and co-workers sequenced part of this 3.4 kb region called "repa-minima" (Gigliani *et al.*, 1993). Its sequence is identical to part of the sequence previously established in this laboratory for the basic replicon of pCU1 (Krishnan and Iyer, 1990). There are indications that over a larger region of the replicon of pR (called "repa-maxima"), there are sequence differences between these two otherwise similar replicons (Battaglia, personal communication to Dr. Iyer).

pCU1

pCU1, the subject of this study, is a plasmid of 39 kb, and 6-8 copies per chromosome that specifies Ap^r, Sm^r, and Sp^r. It was obtained from an untyped *Salmonella* strain from the Laboratory Centre for Disease Control, Ottawa, Canada and transferred by conjugation to *E. coli* K12 in which it has been studied.

Figure 1.17 and 1.18 shows the entire 39 kb pCU1 plasmid. It is divided into 4 different regions each of which confer distinct phenotypes. As in the case of R46 and its derivatives, the replicon region is placed on one single *Bgl*III fragment (coordinate 21-15.6 kb). Clockwise from this regions are the Ap^r and , Sp^r/Sm^r region genes (21-25.5 kb), the *Kik*^r region (Killing of *Klebsiella*) and the region for conjugation (*tra*) (coordinate 31.5-9 kb). The *tra* region has been divided into regions necessary for the construction of a functional N group pilus and a region for conjugative DNA metabolism and the initiation of DNA transfer (Thatte and Iyer, 1983). Recently, Paterson and Iyer (1992) isolated and characterized the *oriT* (origin of transfer) region of pCU1. It was concluded that the *oriT* region is contained in the 699 bp between the 9.15 and 8.48 kb coordinates on the pCU1 map at one end of the *tra* region.

In addition, the *Klebsiella* killing gene (*Kik*), has been investigated (Rodriguez and Iyer, 1981., Hengen *et al.*,1992).

The replicon region of pCU1 is a focus of interest particularly after the

Fig. 1.18 A structural and functional map of the plasmid pCU1

A structural and functional map of the plasmid pCU1 the replicon of which (shown as a 2053 bp *PvuII*-bounded line below) is the subject of this study. The *HpaI* site at the 12.00 o'clock position is used arbitrarily as coordinate 0. Symbols outside the outmost circle are for restriction enzyme sites. A to H are known complementation unit (putative transcription units) in the *tra* region. *kikA* and *kikB* are directly or indirectly involved in the *kik* phenotype and *Sm^r*, *Sp^r* and *Ap^r* are antibiotic-resistance genes. The Rep region plasmid was derived using the *Ap^r* plasmid pCU32 as an intermediate. The bottom part of the figure shows the salient sequence features of the replicon region. The closed and open arrowheads are two different families of iterons. Vertical arrows indicate potential binding sites for the *E. coli* DnaA protein and the black dots indicate GATC sequences that can be methylated.

observation (Krishnan and Iyer, 1989) that a small derivative plasmid containing this region has nearly the same broad host-range as the parental plasmid. The *Bgl*III fragment that was referred to earlier and which contained the replicon region was further reduced to a *Pvu*II fragment of 2053 bp by Kozlowski *et al.* (1987) who also sequenced this region. Errors in this sequence were corrected by Krishnan and Iyer (1990) who drew attention to interesting structural features of the replicon. In chapter 6 of this thesis, it will be shown that this 2053 bp replicon region also has a broad host-range. Determinants of plasmid replication in *E. coli* as well as of promiscuity must therefore be specified by this region, the basic replicon of pCU1.

The features of this basic replicon shown here (Fig. 1.18) are as described by Krishnan (1989) and Krishnan and Iyer (1990) . There are three important domains.

i) An origin of replication (*cis*-acting domain) that was placed at n.t 947-1315 (368 bp). The major features of the sequence in this regions are the presence of three GATC (sites known to be methylated) sequences and a putative *dnaA* box (site known as the binding site for the *E. coli* chromosome replication initiation protein , DnaA).

ii) A domain called *rep* that may contain sites for the binding of plasmid replication-initiation protein(s) (Rep protein(s)) (ORF46, or/and ORF239). This domain also includes two 30 bp iterons (Group II iterons),

shown as open arrow heads in Fig. 1.8 and which expressed IncN incompatibility.

iii) The third domain consisting of thirteen 37 bp tandem iterons (Group I iterons shown as closed arrowheads), which also expressed IncN incompatibility when cloned separately and which negatively controlled copy number of the rest of the replicon region. Thus, this domain was regulatory rather than being essential. It is to be noted that the postulated *rep* genes and the origin overlap. Thus, the size of a mini-plasmid containing only the essential region could be remarkably small (~ 1 kb). Krishnan *et al* (1990) used site-specific oligonucleotide mutagenesis and introduced polypeptide chain termination mutations in ORF239 with the expectation that this would destroy the survival of the replicon. However, unexpectedly, these mutations did not abolish plasmid replication. Instead, they converted the replicon from being *E. coli* Polymerase I (*polI*) - independent to becoming dependent. They therefore concluded that ORF239 was a Rep protein, but only for a *PolI*-independent pathway of replication and that the basic-replicon also specified a hitherto unsuspected *PolI*-dependent pathway.

Chapter II

Materials and Methods

2.1 Bacterial strains

The bacterial strains used in this work are listed in Tables 2.1, and 2.2.

2.2 Culture media

Growth of most cultures was carried out in L-broth or M9 minimal salts medium (Miller, 1972). Tryptone broth was used for the growth of *E. coli*. SR1672.

L-broth ;

Bacto tryptone (Difco)	10 g
NaCl	5 g
Yeast extract	5 g
Distilled H ₂ O	1 litre (pH7.4)

L-agar ; LB containing 1.5 % (w/v) Difco agar.

M9 minimal salts medium;

a 5x stock of minimal salts containing 30.0 g/l Na₂HPO₄, 15.0 g/l KH₂PO₄, 2.5 g/l NaCl, and 5.0 g/l NH₄Cl (pH 7.0) was autoclaved separately from and later added to a solution of agar in dH₂O to bring the final concentration of agar to 1.5% w/v and the salt concentration to 1x.

Required amino acids, which had been filter sterilized, were added to minimal medium to give a final concentration of 30 µg/ml.

Table 2.1 List of *E. coli* strains used

Strains	Genotype / Phenotype	Source of reference
HB101	F ⁻ <i>hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1</i> λ^-	Bolivar <i>et al.</i> , 1977
C600	F ⁻ <i>thi-1 thr-1 leuB6 lacY1 tonA21 supE44</i> λ^-	Bachmann, 1972
DH5 α	F ⁻ <i>endA1 hsdR17 supE44 thi-1</i> λ^- <i>recA1 gyrA96 relA1 deoR</i> $\Delta(lacZYA-argF)$ - U169 ϕ 80 <i>dlacZ</i> Δ M15	Grant <i>et al.</i> , 1990
C1200	F ⁻ <i>rha met his</i>	Krevolin <i>et al.</i> , 1985
C2107	F ⁻ <i>polA12 rha his</i>	Krevolin <i>et al.</i> , 1985
C2110	F ⁻ <i>polA1 rha met his</i>	From M. Filutowicz
W3110	λ^- , <i>rrnD-rrnE</i>	Hill and Harnish (1981)
SF800	<i>polA1 Nal^r thy</i>	Heffron <i>et al.</i> , 1977
WA802	F ⁻ $\Delta(lac)$ 6 <i>supE44 galK2 galT22 mcrA rfbD1 met B1 mcrB1 hsdR2</i>	Yasugawa <i>et al.</i> , 1991
WA802 _{polA1}	Same as WA802 but <i>polA1</i>	Yasugawa <i>et al.</i> , 1991
SR1758	$\Delta(gal-bio)$ <i>thi-1 relA1 spoT1</i> F ⁺	Joyce and Grindley, 1984
SR1672	Same as SR1758 but $\Delta polA$	Joyce and Grindley, 1984
MC4100	F ⁻ <i>araD139</i> $\Delta(argF-lac)$ U169 <i>rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR</i>	Silhavy <i>et al.</i> , 1984
KY1603	Same as MC4100 but $\Delta rpoH30::Km^r$ <i>suhx401 zhf50::Tn10</i> (λ pF13-PrpoD _{hs} - <i>lac</i>)	Kawasaki <i>et al.</i> , 1991
MC1060	$\Delta(lacI-Y)$ 74 <i>galE15 galK16</i> λ^- <i>relA1 rpsL150 spoT1 hsdR2</i>	Casadaban and Cohen, 1980
Table 2.1	continued ...	

MC1061	<i>araD139 Δ(araA-leu), polBΔ 7697 Δ(lacI-Y) 74 galE15 galK16 λ⁻ relA1 rpsL150 spoT1 hsdR2 mcrA⁻ mcrB⁻</i>	Casadaban and Cohen, 1980
MG1655	F ⁻ wild type	Kang and Craig 1990
PK102	MG1655 with <i>ΔdnaJ::mini-Km^r</i>	Kang and Craig 1990
PK101	MG1655 with <i>Δ(dnaK-dnaJ)::mini-Km^r</i>	Kang and Craig 1990
PK12	MG1655 with <i>dnaK756</i>	Kang and Craig, 1990
DA258	CG799 <i>thr::Tn10 Ω-cat mini-Tn5</i> near <i>grpE⁺</i>	Tilly and Yamolinsky, 1989
DA259	DA258 <i>ΔgrpE::Ω-cat</i>	Tilly and Yamolinsky, 1989
AQ634	F ⁻ <i>ilv merB1 his-29 trpA9605 thyA deo pro⁻</i>	Nishimura <i>et al.</i> , 1971
AQ666	AQ634 with <i>rnh224</i>	From T. Kogoma
AQ3563	<i>ilv his-29 trp Ag605 thyA deo pro⁻ merD88 Rif^r chr::pKN500 dnaA850::Tn10</i>	Kline <i>et al.</i> , 1986
AQ3585	AQ634 with <i>rnh224 dnaA850::Tn10</i>	From T. Kogoma
BK1776	λ TL25 <i>repE⁺</i>	From B. Kline
K38	HfrC (λ ⁺)	Nurse <i>et al.</i> , 1991
K91	K38 (λ)	Nurse <i>et al.</i> , 1991
SG13107	F ⁻ <i>his pyrD</i>	Nurse <i>et al.</i> , 1991
SG13109	F ⁻ <i>his sula</i>	Nurse <i>et al.</i> , 1991
PN102	K38 <i>priA::Km^r</i>	Nurse <i>et al.</i> , 1991
PN103	K91 <i>priA::Km^r</i>	Nurse <i>et al.</i> , 1991
PN105	SG13109 <i>priA::Km^r</i>	Nurse <i>et al.</i> , 1991
CC5	HB101 GyrB _{His-136}	Contreras and Maxwell, 1992
CC6	HB101 GyrB _{Cys-136}	Contreras and Maxwell, 1992

Table 2.1 List of *E. coli* strains used (cont.)

LE234	GyrB	Contreras and Maxwell, 1992
LE316	LE234 GyrB _{Val-164}	Contreras and Maxwell, 1992
N4177	GyrB _{Cys-136, ser-171}	Contreras and Maxwell, 1992
GM1859	wild type <i>hip</i> ⁺	Dellis and Filutowicz, 1991
GM1859*	<i>hip3</i>	Dellis and Filutowicz, 1991
N99	<i>sup</i> ⁺ <i>strA</i> <i>galK2</i>	Granston <i>et al.</i> , 1988
HN678	N99 <i>himA</i> Δ 82 Tc ^r	From H. Nash
HN778	N99 Δ <i>hip</i> :: Cm ^r	From H. Nash
E582	<i>himA</i> (Δ 82:: <i>Tn10</i>)(<i>hip</i> (Δ 3:: <i>Cm</i> ^r))	From R. Weisberg
ECF2010	C2110 (<i>polA1</i>) <i>hip3</i>	Filutowicz and Roll, 1990
ENZ258	AB1157 <i>sfiA11</i>	Dri <i>et al.</i> , 1992
ENZ259	ENZ258 <i>hupA</i> ::Cm ^r	Dri <i>et al.</i> , 1992
ENZ260	ENZ258 <i>hupB</i> ::Km ^r	Dri <i>et al.</i> , 1992
ENZ262	ENZ258 <i>hupA</i> ::Cm ^r <i>hupB</i> ::Km ^r	Dri <i>et al.</i> , 1992
KH1214	<i>zaeB</i> :: <i>Tn10</i> (<i>mutD5</i>)	Degnen and Cox, 1974
M386	<i>polA1 polB polC1026</i> Leu His <i>thy pcbA1 supE</i> Tc ^r	Bryan <i>et al.</i> , 1988
M493	M386 with <i>polA12</i>	Bryan <i>et al.</i> , 1988

* The strain is not named by Filutowicz (Dellis and Filutowicz, 1991)

Table 2.2 List of other bacterial strains used

Strains	References
<i>Klebsiella oxytoca</i> (M5a1)	MacNeil <i>et al.</i> , 1981
<i>Salmonella enteriditis</i> AS1417	Tardiff and Grant, 1980
<i>Pseudomonas putida</i> 2440	Schmidhauser and Helinski, 1985
<i>Pseudomonas stutzeri</i> JM300	J. Ingraham (Lab stock)
<i>Aeromonas hydrophila</i> NRC1001	National Research Council of Canada (Lab stock)

LA was prepared by addition, before autoclaving, of 1.5 % (w/v) Bacto agar (Difco). Sterile glucose (20% w/v), and casamino acids (20 %, w/v) (Difco) were added to give the final concentrations indicated.

Tryptone broth (TYB) ;

Tryptone	5 g
Yeast extract	3 g
dH ₂ O	1 litre

TYA ; TYB containing 1.5 % (w/v) Bacto agar (Difco)

2.3 Bacterial Growth conditions

All strains were routinely grown in LB or LA at 37°C. However, *E. coli* SR1672 was grown in TY or TYA at 37°C. Also, *E. coli* M386 and other temperature sensitive strains were routinely grown at 30°C.

2.4 Storage of strains

All strains that were used or constructed were stored in 50 % glycerol (v/v) at -20°C. Strains were stored for short term on plates at 4°C.

2.5 Sterilization of media and other solutions

Sterilization was carried out by either autoclaving at 15 psi (121°C) for 20 min or where appropriate, by passage through a 0.45 µm NalGene sterile membrane filter, NalGene, USA.

2.6 Chemicals, reagents and enzymes

Antibiotics and other chemicals were purchased from Sigma Chemical Corp., USA. Isopropyl- β -D-thiogalactopyranoside (IPTG), and 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) were obtained from Boehringer-Mannheim Canada Ltd.

Deoxyadenosine 5' [α - 32 P] triphosphate, triethylammonium salt, specific activity >3000 Ci/mmol, and L - [35 S] methionine (~700 Ci/mmol) were from Dupont Canada Ltd.

Restriction enzymes and *Eco*RI, *Hind*III-linkers were purchased from New England Biolab, Beverly, MA. Klenow enzyme, T4 DNA ligase and 1 Kb MW standards of DNA were purchased from GIBCO-BRL Life Technologies Ltd., Burlington, Ont. and used as recommended by the manufacturer. Calf thymus DNA was purchased from Clone Tech Laboratory, USA.

Antibiotic solutions were prepared and sterilized by membrane filtration and added as follows :

- . Ampicillin (sodium salt) ; 10 mg/ml stock solution in dH₂O stored at -4°C, final concentration 35 μ g/ml
- . Streptomycin sulphate ; 10 mg/ml stock solution in dH₂O stored at -4°C, final concentration 25 μ g/ml
- . Spectinomycin ; 10 mg/ml stock solution in dH₂O stored at -4°C, final concentration 35 μ g/ml

- . Tetracycline HCl ; 12.5 mg/ml stock solution in 50 % ethanol (v/v) stored at -4°C, final concentration 15 µg/ml
- . Chloramphenicol ; 34 mg/ml stock solution in 100 % ethanol stored at -20°C ,final concentration 30 µg/ml
- . Kanamycin ; 10 mg/ml stock solution in dH₂O, final concentration 50 µg/ml

2.7 Plasmids and plasmid DNA

Plasmids secured from other sources or constructed as part of other studies are listed in Table 2.3. Those that were constructed as part of this study are listed in Tables in the relevant subsequent chapters.

2.8 Storage of DNA

Plasmid DNA were stored as a solution in sterile TE buffer (10 mM Tris, pH8.0, 1 mM EDTA).

2.9 Plasmid DNA analysis

Plasmid DNA was analyzed by electrophoresis through agarose gels. Mini-gels (IBI Ltd., Gel Electrophoresis Apparatus, USA) for rapid screening of DNA samples were prepared from 0.7 % - 3 % (w/v) agarose dissolved in Tris-Acetate buffer (40mM Tris-Cl, 20mM sodium acetate and 2mM EDTA pH7.6), and run at 100 mA for 1hr. All gels were stained with ethidium bromide (Sigma) (5 µg/ml) and photographed under U.V-illumination with a Polaroid Land Camera fitted with an orange filter. In the case of high concentration gels (2 % - 3 %), low-melting point agarose

Table 2.3 List of plasmids used

Plasmid	marker(s)	Nucleotide co-ordinates of the pCU1 replicon, if any and /or other replicon	Reference
pUC-4K	Ap ^r	pBR322	Yanisch-Perron <i>et al.</i> , 1985
pBR322	Ap ^r Tc ^r	pMB9	Bolivar <i>et al.</i> , 1977
pBR329	Ap ^r Cm ^r Tc ^r	pMB9	Covarrubias and Bolivar, 1982
pACYC184	Cm ^r Tc ^r	p15a	Chang and Cohen, 1978
pUC18/19	Ap ^r	ColE1	Yanisch-Perron <i>et al.</i> , 1985
pUC1318	Ap ^r	ColE1	Kay and McPherson, 1987
pUC1813	Ap ^r	ColE1	Kay and McPherson, 1987
pKlink	Ap ^r Km ^r	pIC19R	Smith and Crouse, 1989
pHP45Q	Sp ^r /Sm ^r	pBR322	Prentki and Krisch, 1984
pMAK705	Cm ^r	pSC101	Hamilton <i>et al.</i> , 1989
pLG339	Km ^r Tc ^r	pSC101	Stoker <i>et al.</i> , 1982
pLG338	Km ^r Tc ^r	pSC101	Stoker <i>et al.</i> , 1982
pCM7	Ap ^r Cm ^r	pBR327	Pharmacia Ltd.

Table 2.3 continued

pAPG10	Ap ^r Cm ^r Xyl	pUC7	Gleave <i>et al.</i> , 1990
pC ⁺ J1	Ap ^r Sp ^r /Sm ^r	parental plasmid	Konarska-Kozłowska and Iyer, 1981
pCU21	Ap ^r Sp ^r /Sm ^r	mutant of pCU1	Rodriguez, 1981
N3	Sm ^r Tc ^r Su ^r	parental plasmid	Lab stock
R15	Sm ^r Su ^r Hg ^r	parental plasmid	Lab stock
pCU45	Ap ^r Tc ^r	2 Kb mini-replicon of pCU1 (nt.1-2053'') cloned into pBR329	Kozłowski <i>et al.</i> , 1987
pCU751	Tc ^r	nt. 1-1315	Krishnan, 1989
pCU752	Tc ^r	nt. 1-1593	Krishnan, 1989
pCU773	Km ^r	nt. 296-2053	Krishnan, 1989
pRI115	Cm ^r	nt. 1-1016	Krishnan, 1989
pRI105	Ap ^r	nt. 1375-2053	Krishnan, 1989
pRI123	Ap ^r	nt. 1825-2053	Krishnan, 1989

Table 2.3 continued

pRI125	Ap ^r Tc ^r	nt. 1017-1315	Krishnan, 1989
pRI129	Ap ^r	nt. 542-2053	Krishnan, 1989
pCU717 ^a	Km ^r	nt. 1-2053	Krishnan, 1989
pCU718 ^a	Km ^r	nt. 1-2053	Krishnan, 1989
pHYK2	Sp ^r /Sm ^r	mini-replicon of N3	Krishnan <i>et al.</i> , 1991
pHYK4	Sp ^r /Sm ^r	mini-replicon of N3	Krishnan <i>et al.</i> , 1991
pHYK6	Sp ^r /Sm ^r	mini-replicon of R15	This study
pKV718	Ap ^r	ori2 plasmid of F without the helper plasmid	Kawasaki <i>et al.</i> , 1991
pFO-B	Ap ^r	ori2 of F cloned into pBR322	Murotsu <i>et al.</i> , 1984
pKV5110	Ap ^r	ori2 and repE of F	Kawasaki <i>et al.</i> , 1991

Table 2.3 **continue ..**

pLSK5	Ap ^r	pJF118EH	From W. Messer
pHC5	Ap ^r	PolII colned into overproduction vector (pUC18) under the control of <i>tac</i> promoter	Chen <i>et al.</i> , 1989
pKY-1	Km ^r	Parental plasmid	Lee <i>et al.</i> , 1982

* The region shown as Cm^r has the structural gene for chloramphenicol acetyl transferase but not the native promoter sequence.

** In this and all other cases where nt. numbers are shown, the numbers are of the pCU1 replicon region as indicated in Krishnan (1989) and can be accessed from this source or from Gene Bank (Accession Number: M18262). The complete sequence of the basic replicon is also shown as Appendix I for reading reference.

(GIBCO, BRL, USA) was used and run at 50 mA for 2 to 3 hr in a cold room (4°C).

2.10 Preparation of plasmid DNA

Three different methods were used for preparation of plasmid DNA.

2.10.1 Small scale preparation of plasmid DNA (Modified from Birnboim and Doly, 1979)

The plasmid-harboursing cells were grown overnight at 37°C in 200 ml L-broth, containing the appropriate selective antibiotics. The culture was split into two Oakridge tubes (NalGene Ltd., Oakridge Type polycarbonate bottle), and harvested by centrifugation at 5,000 g, for 15 min, and the pellet was resuspended in 10 ml of solution I, which contained 50 mM glucose, 25 mM Tris, 10 mM EDTA, pH8.0. This solution was kept warm, at 37°C in a water bath, for 30 min, and then 10 ml of solution II (0.2 N NaOH, 1 % SDS) was added. The sample mixture was mixed well with gentle agitation and left on ice for 30 min. 7.5 ml of solution III (1.8 g KOH, 4 ml dH₂O, 2.9 ml glacial acetic acid, pH4.8) was added and mixed well, and then left on ice for 30 min. The mixture was centrifuged at 5,000 g, for 20 min, at 4°C. The supernatant was taken and 0.64 volumes of isopropanol added for precipitation at -20°C for 1 hr. The precipitate was pelleted by centrifugation at 5,000 g, for 15 min. The pellet was washed twice with 70 % (v/v) ethanol and vacuum dried. The dried pellet was resuspended in 400 µl of 0.3 M sodium acetate (pH4.8) and, 10 µl of RNAase A 20 µg/ml (Sigma)

was added. The solution was incubated for 30 min at 37°C in the water bath, and then an equal volume of the phenol (BRL) was added. The mixture was vortexed, and the phases separated in an Eppendorf microcentrifuge for 10 min. The aqueous layer was collected and 800 µl of 100 % ethanol and 10 µl of glycogen (10 mg/ml) (Boehringer-Mannheim, Canada, Ltd.) were added. The mixture was left at -20°C for 1 hr, and the precipitate was pelleted in an Eppendorf Micro Centrifuge (Eppendorf Gerätebau, Ltd., Germany) for 10 min. The supernatant was discarded and the tube carefully rinsed with 100 µl of 70 % (v/v) ethanol, and centrifuged for 5 min. The ethanol was discarded, and the pellet was vacuum-dried and resuspended in 100 µl of TE buffer and analyzed by agarose gel electrophoresis.

2.10.2 Rapid, small scale preparation of the plasmid DNA from *E. coli*

Rapid analysis of plasmid DNA was done by a modification of the methods of Birnboim and Doly, (1979) and Eckhardt, (1978). The procedure was performed in the following manner ;

The plasmid containing cells were grown overnight at 37°C in 5 ml L-broth containing the appropriate selective antibiotic(s). The cells were harvested by centrifugation at 5,000 g, for 15 min, and resuspended in 100 µl of solution I. The sample was incubated in a water bath at 37°C for 15 min, then cold 200 µl solution II (see section 2.10.1) was added and mixed

well by inversion. The mixture was left on ice for another 5 min. The sample was centrifuged in an Eppendorf microcentrifuge for 10 min, and the supernatant was transferred to a sterile fresh Eppendorf tube. 10 μ l 3 M NaCl, 5 μ l glycogen (1mg/ml) and an equal volume of isopropanol at -20°C was then added to the supernatant. The resulting pellet was carefully rinsed with 70 % (v/v) ethanol. The ethanol was discarded, and the pellet was vacuum dried. The pellet was then resuspended in 10-20 μ l of TE buffer and analyzed by agarose gel electrophoresis.

2.10.3 QIAGEN plasmid Midi-preparation

QIAGEN kits, obtained from QIAGEN, USA were used to prepare plasmid DNA, and used as recommended by the manufacturer.

2.11 Restriction digestion of plasmid DNA and end-filling for linker attachment

Analysis of plasmids by restriction endonuclease was carried out using buffers and conditions as recommended by the manufacturers (New England Bio Lab or Promega, Canada, Ltd). Generally, 1 μ g of DNA was digested in a 30 μ l digestion mix for 60 min at 37°C . The reaction was halted by precipitation of the plasmid DNA. This was achieved by the addition of 3 M NaCl to a final concentration of 0.3 M and glycogen to a final concentration of 1 mg/ml followed by 2.5 volumes of cold 100 % ethanol. The mixture was held at -20°C for at least 20 min and the plasmid

DNA recovered by centrifugation in a microcentrifuge for 15 min. The resulting pellet was washed carefully in 70 % ethanol (v/v), dried and redissolved in TE buffer or sterile dH₂O. The digested DNA fragments in the samples were "end-filled" using all four dNTPs in a Klenow fragment-catalyzed reaction and ligated with blunt-end *EcoRI* or *HindIII*-linker and the samples were then used for further study.

2.12 Ligation of DNA fragments

DNA molecules were ligated when required by the use of T4 DNA ligase (BRL) using the buffer recommended by the manufacturer. Ligation was carried out at 15°C for 16 hr.

2.13 Recovery of DNA from agarose gels

Plasmid DNA was extracted from agarose gels as described by McDonnell *et al* (1977). After agarose gel electrophoresis and staining with ethidium bromide (Sigma), the required DNA fragment was cut from the gel with a razor blade using a U.V transilluminator to visualize the DNA band(s). Then the gel slice was transferred to a clean Eppendorf tube. The DNA was recovered from the slice of agarose gel using the 'GeneClean' kit purchased from Bio101 Inc., La Jolla, CA following the protocol recommended by the manufacturer. Routinely, the slice of agarose was dissolved in NaI buffer. The DNA was precipitated with "Glassmilk". After these procedures, the precipitate was washed twice with cold NEW (NaCl, ethanol and dH₂O) buffer. Then the DNA was eluted in TE buffer or sterile

dH₂O. Lower m.w DNA (below 500 bp) was prepared using the Mermaid kit (Bio 101 Inc., La Jolla, CA). The procedures used were the same as those used with the Geneclean kit.

2.14 Bacterial Transformation

Transformation with plasmid DNA was done by the method of Hanahan (1987). A single colony from L-agar plate was used to inoculate 50 ml of L-broth. The cells were grown, with vigorous aeration, at 37°C overnight. The culture was diluted to an OD_{650nm} 0.1 with fresh, prewarmed L-broth. Aeration was continued and the OD_{650nm} of the culture monitored at 30 to 60 min intervals. When an OD_{650nm} 0.5 was reached, the cells were transferred into cold sterile plastic tubes and harvested by centrifugation at 5,000 g, for 10 min. The pellet was resuspended by vortexing in 1/2 volume of cold sterile 0.1 M CaCl₂. After induction of competence by successive treatment with 10 mM MgCl₂ and 100 mM CaCl₂, 200 µl of cells were incubated with the DNA for 30 min. The mixture was heat-shocked at 42°C for 2 min then incubated at 37°C for 90 min after the addition of 1 ml of L-broth. Transformations were selected by spreading aliquots of the transformation mixture onto nutrient agar plates containing the relevant antibiotics followed by overnight incubation at 37°C.

2.15 Segregational stability and Incompatibility test.

Bacterial strains containing plasmids to be tested for stability of maintenance were inoculated into L-broth containing the appropriate antibiotic(s) from single colonies on selective agar and incubated at 37°C until they reached a cell density of 5×10^9 per cell. The culture was diluted ($\times 10^6$) into fresh M9 medium (with supplementary nutrients if needed) and incubated at 37°C. After each of 20 generations of growth (approximately 12 hr), the culture was again diluted ($\times 10^6$) into fresh non-selective medium (M9 salts). This procedure was continued for at least 100 generations of growth in non-selective medium. At each dilution, aliquots were plated on non-selective solid medium for viable count determination, and 100 colonies from each experiments were tested for the presence of the plasmid marker by patching to antibiotic containing plates.

Incompatibility tests were carried out in a similar fashion. Test plasmids were introduced into plasmid-containing *E. coli* HB101 by transformation. Transformation mixtures were plated on media selective for markers on the incoming and resident plasmids. Colonies of individual transformant clones from the double-selection plates were suspended in L-broth, diluted 10^4 in the same medium, and subsequently incubated without selective pressure. The culture were then diluted and plated on non-selective medium (L-agar) for single colonies. To determine the loss of one or the other plasmid during the incubation period, colonies were then patched

onto appropriate selective media. Also, in some cases, the transformation mixtures were plated on media selective for the donor plasmid. Colonies of the transformants were then tested for the retention of the marker associated with the previous-resident plasmid.

2.16 Electroporation procedure:

Precultures were inoculated from a single colony into 100 ml of LB, and incubated with shaking at 180 rpm overnight at 37°C. This preculture was inoculated into 1 liter prewarmed LB and incubated again until early mid-log phase (O.D. 0.5, wave-length 650nm). The cells were then chilled on ice for 30 min and pelleted for 15 min at 4000 rpm at 4°C, resuspended in 1 liter of distilled sterile water, again centrifuged and resuspended in 500 ml of cold dH₂O. This suspension was pelleted again, and the cell pellet was washed twice in 20 ml of 10 % (v/v) sterile glycerol. Finally, the cells were resuspended in 3 ml of 10 % glycerol or cold sterile dH₂O. This cell suspension was used immediately, or "aliquoted" and stored at -70°C.

Electroporations were done with a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA, U.S.A.) coupled to a parallel resistance selector (Bio-Rad Pulse Controller). For electroporation, 100 µl of bacterial cells were thawed on ice and mixed at 0°C with DNA solution in water or TE buffer. The cell suspensions were transferred to 2 mm wide plastic cuvettes (Bio-Rad electroporation cuvettes), and submitted to a single electric pulse. For

most bacterial strains, the electroporation apparatus was set at 12.5 kV / cm, 5 msec.

After electroporation, the cells were recovered with 1 ml of LB broth, and incubated for 90 min at 37°C with shaking. Subsequently, 100 or 250 µl of cells were plated on appropriate selective LB-agar plates.

2.17 Mutagenesis and screening for the mutated DNA

By using the *mutD5* mutator strain, mutations were introduced *in vivo* and mutants were screened with the M386 strain as shown below :

- i) The plasmid pCU999 was transformed into the *mutD5* strain.
- ii) A single Km^r colony was selected and transferred to L-agar
- iii) It was incubated further for 30 days.
- iv) Plasmid DNA was isolated from the strain.
- v) It was used to transform strain M386 (*pcbA1 polA1 polB100 polC^{ts} supE*). Transformants were selected at 30°C.
- vi) Colonies were screened by replication and growth at 30°C and 44°C on both LA and kanamycin agar.
- vii) Desired colonies that failed to replicate on media with or without the antibiotic at 42°C were saved.

2.18 In vitro transcription and translation assay

An in vitro prokaryotic DNA directed translation kit (Promega, Canada, Ltd.) was used to express the open reading frames identified in the mini-replicon. ^{35}S -methionine labelled products were separated electrophoretically on 15 % SDS-polyacrylamide gel (Laemmli, 1970). The gels were stained with Coomassie blue, dried and autoradiographed at room temperature (see section 2.20). The size of the expression products was determined by comparison with protein molecular weight markers from Bio-Rad (low molecular weight marker : Phosphorylase B 97.4 kD, Serum albumin 66.2 kD, Ovalbumin 45.0 kD, Carbonic anhydrase 31.0 kD, Trypsin inhibitor 21.5 kD, Lysozyme 14.4 kD).

2.19 Gel retardation assay

A polyacrylamide gel (5 %) retardation assay modified from Fried and Crothers (1981) was used to detect the DNA-binding activity of the IHF protein. The IHF protein (1.1 mg/ml) was provided by Dr. H. Nash (NIH, U.S.A.). The protein was diluted with IHF binding buffer (50 mM Tris-HCl pH7.4, 70 mM KCl, 10 % Glycerol, 1mM EDTA, 1 mM β -mercaptanol, 7 mM MgCl_2 , 3 mM CaCl_2 , 200 $\mu\text{g/ml}$ BSA). The binding reaction proceeded with 5 ng of end-labelled (α - ^{32}P dATP) *Hind*III DNA fragment (from pLK336; nt. 263-598 of the basic replicon), 100 ng of non-specific competitor carrier Calf thymus DNA (Clone Tech Laboratory) and 3 ng of diluted IHF protein. The reactions were incubated 20 min at room temperature. After the incubation,

each sample was allowed to run in 5 % polyacrylamide gel with Tris-glycine electrophoresis buffer (1 M Tris-Cl, pH7.9, 1M sodium acetate, pH7.9, 0.5 M EDTA, pH8.0), at 35 mA about 2 hr. Then the gel was transferred onto 3 MM Whatman filter paper and dried under vacuum. The dried filter was autoradiographed and the protein-DNA complex visualized after overnight exposure without an intensifying screen.

2.20 Polyacrylamide gel electrophoresis

Two different gel casts were used for polyacrylamide gel electrophoresis (BRL Model V16-2 Vertical Electrophoresis Apparatus, and Sturdiel Slab gel unit, SE400, Hoeffer Scientific instruments, San Francisco, CA, U.S.A). Gels were run with a 20 to 35 mA constant current at 4°C or at room temperature overnight until the dye (Bromophenol Blue) front had migrated to the bottom of the gel.

The solutions used were as follows :

i) Non-gradient SDS acrylamide gels

Stock solution (15 % gel)

3M Tris-HCl (pH8.8)	13.23 ml
Distilled H ₂ O	29.47 ml
10 % SDS (w/v)	1.05 ml

40 % acrylamide (BRL) + 0.5 % bisacrylamide (Bio-Rad)	26.25 ml
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The final volume was 70 ml. This mixture was then degassed using a water pump vacuum for 30 min.

Immediately before pouring 25.5 μ l N',N',N',N'-tetramethylenediamine (TEMED) and 252 μ l fresh 10 % (w/v) ammonium persulphate were added.

10 $\times$ running buffer stock

Tris base (25 mM final conc.)	60.4 g
glycine (192 mM final conc.)	288.0 g
SDS (0.1 %, w/v, final conc.)	20.0 g

Made up with distilled H₂O to 1 litre (pH8.3).

The method used for preparing gels was that of Hancock *et al* (1977). The non-gradient SDS acrylamide gel was poured until it was 3-4 cm from the top of the front gel plate. The surface was overlayed with a small volume of butan-2-ol, and the gel left to set for 3 hr. Traces of butan-2-ol

were washed out, and the stacking gel layered on top. After pouring the stacking gel solution, a 9 or 12 well Teflon gel comb was inserted into the stacking gel. The gel was allowed to set for 30 min, the comb was removed and the gel transferred to the tank for loading. Mr estimations were carried out by the preparation of a calibration curve utilising standards of known Mr (by plotting $\log_{10}$ Mr against distance migrated). The standards used were Bio-Rad low molecular weight standards.

Stacking gel solutions and SDS sample buffer were made as follows :

10 % (w/v) stacking gel acrylamide

acrylamide	10.0 g
bisacrylamide	0.5 g

Stacking gel buffer

Tris base	5.98 g
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(The pH was adjusted to 6.8)

Stacking gel mixture

Stacking gel acrylamide	3.0 ml
Distilled H ₂ O	4.4 ml
Stacking gel buffer	2.4 ml

The final volume was 10 ml. This solution was degassed as described, and 5 μ l of TEMED, 100 μ l of 10 % (w/v) ammonium persulphate added immediately before casting the gel.

SDS sample buffer

Distilled H ₂ O	5.25 ml
Stacking gel buffer	1.25 ml
100 % (v/v) Glycerol	1.00 ml
10 % (v/v) SDS	2.00 ml
β-mercaptoethanol	0.50 ml
0.1 % (v/v) Bromophenol Blue	0.10 ml

2.21 Staining of SDS polyacrylamide gels

After electrophoresis, the gels were stained with the following solution:

Methanol	225 ml	45 % (v/v)
Acetic acid	50 ml	10 % (v/v)
Page Blue 83	1 g	0.2 % (w/v)
Distilled H ₂ O	225 ml	

This solution was filtered before use. The fixed gels were destained for 4-6 hr with the following mixture :

Methanol	225 ml	45 % (v/v)
Acetic acid	50 ml	10 % (v/v)
Distilled H ₂ O	225 ml	

2.22 Gel drying and autoradiography

Gels were mounted on a double layer of Whatman 3MM chromatography paper and dried under vacuum at 80°C for 1 hr on a Bio-Rad dual temperature slab gel dryer (Bio-Rad). For autoradiography of ^{35}S or ^{32}P -labelled gels, the dried gel was placed in an X-ray cassette with Kodak X-OMAT AR diagnostic film. The film was exposed at room temperature initially for 12 hr. Autoradiographs were developed using an AFP Imaging 14XL X-ray film processor. Developer and Fixer for this machine were purchased from Picker Chemicals.

2.23 Nucleotide sequencing

Double-stranded sequencing was done by using either the modified Wang and Sodja (1991) method or Sequenase version 2.0 provided in a sequencing kit purchased from US Biochemical Corporation, Cleveland, Ohio, U.S.A. The method was based on Sanger *et al* (1977). Synthetic oligonucleotide primers specific to the basic-replicon were described before (Kozlowski *et al.*, 1987., Narang, 1990., see Table 2.4).

For the double-stranded sequencing, the mixture of plasmid DNA and primer were heated in boiling water for 5 min and immediately cooled in ice water. Then this mixture was used for the sequencing reaction according to the manufacturer's suggested protocol. The labelling reaction, was started

Table 2.4 List of oligonucleotide primers used.

Primer	DNA Sequence	Source / Reference
P1	5'-CAATGAGTTATATTAGCTCT	Kozlowski <i>et al.</i> , 1987
P2	5'-TTCGCTTACTGAAGGCTGTG	"
P3	5'-AATTGAGCAAGACTTTGCAT	"
P4	5'-AATTCGCCCATCTTCATGAC	"
P5	5'-AGGTATAATTAAAGTTATGG	"
P _A	5'-CGCGGTTCTAGGGGTCGACCGAGCC	Narang, 1990
P _B	5'-TCTATGGGATCCACCCACCTTAC	"
P _C	5'-TGGTAGTGTAGAGAGTAA	"
P _D	5'-GTTCTGCGGTAAGCTTTTACCCCA	"
P _E	5'-TATACCTTTGTGTTATTTGTGGAT	"
P _F	5'-ATCAGCACACATGAGCCTG	"

by combining 0.1 M DTT, dGTP labelling mix, ^{35}S -ATP and sequenase. The mixture was centrifuged briefly in a microcentrifuge and left at room temperature for 5 min. When the labelling reaction was complete, aliquots of 2.7 μl were distributed to the rim of each of 4 tubes containing a respective ddNTP termination mix. Then tubes were recentrifuged briefly and incubated at 37°C for 5 min. The reactions were stopped by the addition of 4 μl of stop solution (Stop solution : 95 % Formamide, 20 mM EDTA, 0.05 % Bromophenol Blue and 0.05 %, Xylene Cyanol FF). Samples were stored at -20°C until required.

The BRL sequencing gel unit MS2 and polyacrylamide gels were used to fractionate DNA. 8 % polyacrylamide gels were prepared from 'Gelmix' (GIBCO-BRL) following the addition of 450 μl of 10 % w/v ammonium persulfate to facilitate polymerization as directed by the supplier. The 0.3 mm Mylar side-spacers and "shark-teeth" combs were inserted before allowing polymerizing and forming of wells. Electrical current was supplied by an LKB2297 macrodrive 5 constant power pack. The samples were then loaded and the gels run at 1200-1500 V as required.

2.24 Computer analyses of DNA sequence

The DNA sequence was analyzed for the location of restriction sites, ORFs and consensus nucleotide stretches with a Zenith data systems IBM PC-XT compatible hardware and the 'Microgenie' software program

developed and sold by Beckman Instruments Inc., Palo Alto, CA, U.S.A.

2.25 Electron microscopy

i) General technique :

The samples for electron microscopy were prepared as described by Burkhardt and Pühler (1988). Formvar coated grids (400 mesh) were allowed to touch the cytochrome-DNA monolayer, and then the DNA samples molecules were stained with uranyl acetate and shadowed with platinum-palladium. Micrographs were taken with a Philips EM201 microscope at magnification of $\times 45,000$.

ii) Procedures used for observing the binding of protein to DNA :

The procedures and techniques were adapted from Williams (1977). First of all, IHF protein (1.1 mg/ml., prepared by Dr. Nash, NIH, U.S.A.) was diluted with binding buffer (see section 2.19) and prepared to a concentration slightly less than 1 $\mu\text{g/ml}$. The diluted protein was preincubated for 15 min at 30°C. After cooling to room temperature, 0.25 nM of the purified DNA fragment (isolated Gene clean treatment) was added. The mixture was incubated for 2 more hours at room temperature. Meanwhile, the grid was prepared (400 mesh). After 8 μl polyglycine treatment, they were left at room temperature about 30 sec. Then, the solution was removed by vacuum suction system and 6 μl of samples added. The samples were incubated for 1 min and washed with dH_2O . After the washing procedure, samples were treated with 5 μl of 5 % aqueous uranyl

acetate for 15 sec. After a further wash with distilled water the samples were used for electron microscopy.

2.26 Polymerase Chain Reaction (PCR)

The polymerase chain reaction was used to identify the presence of small DNA fragment inserts (less than 500 bp) that were cloned into pUC18 or pUC19 vector system. The two reverse and forward primers (5'-TAACAATTTTCACACAGG, 5'-CCCAGTCACGACGTTGT ; 6.7 pmol/ μ l) were purchased from Queen's University, Kingston, Ontario or New England Bio Lab, U.S.A. The Taq polymerase was purchased from Promega. The reactions were performed in a 500 μ l microfuge tube in a final volume of 102 μ l. In this volume, 20 pmol/ μ l of each oligonucleotide primer and 2 unit/ μ l Taq DNA polymerase were mixed in 25 mM $MgCl_2$, 100-200 mM dNTP, 1 μ l of DNA (dilution of 1 μ g/ μ l sol.), 10 $\times$ PCR buffer (Promega), and 75 μ l mineral oil (0.01 %, w/v). Cycling reactions were performed in a Perkin-Elmer Cetus DNA Thermal Cycler. Each cycle consisted of denaturation at 94°C for 30 sec., annealing at 55°C for 30 sec., and extension at 72°C for 1 min. Thirty cycles were performed. The completed reaction products were extracted once with an equal volume of a 1:1 mixture of phenol and chloroform and once with a two-fold volume of butanol. Then the DNA was digested with appropriate restriction enzyme(s) and allowed to run in a gel containing high percentage (2-3 %) of low-melting point agarose.

Results and Conclusions

Chapter III

The DNA polymerase I-independent replicon

The basic replicon of pCU1 has been shown to be contained within a 2053 base pair *PvuII* fragment that has been completely sequenced (Krishnan and Iyer, 1990 ; Gene Bank Accession Number: M18262). Its location in relation to the entire parental plasmid pCU1 is shown in Figure 1.18. Krishnan *et al* (1990) also showed that this replicon could replicate by a *Poll*-independent pathway. They then proceeded to introduce two polypeptide chain termination mutations one near to the N terminal end of ORF239 and another near its C terminal end. The first of these mutations (at nt. 602) was also designed in such a way as to not affect the overlapping ORF46. The second mutation was at nt. 1118. In a coupled transcription-translation system (*in vitro*) no ORF239 polypeptide was detected with the first mutation and a truncated polypeptide was detected with the second. Each mutant converted the plasmid to becoming *Poll*-dependent. It was therefore inferred that the basic replicon could use two pathways of replication only one of which required polymerase I (*Poll*). The focus of this chapter is the identification of plasmid determinants that are sufficient for the *Poll*-independent pathway and of some host genes that are required or which enhance this pathway.

3.1 Deletion derivatives of the basic replicon

Fragments of DNA that cannot replicate by themselves in *E. coli* but which carry genes conferring antibiotic-resistance to *E. coli* do not yield antibiotic resistant transformants even when this DNA is circularized prior to transformation (except very rarely by recombinational rescue). However, if such a fragment is linked *in vitro* to a DNA sequence that has the properties of a replicon, it will yield transformants for the antibiotic marker gene. The choice of the particular marker fragment is determined usually by such features as the antibiotic-resistance phenotype of the recipient and the restriction sites at the borders of the antibiotic-resistance fragment. For example, the fragment called omega (Ω) (Prentki and Krisch, 1984, see Fig. 3.1.A) that was used extensively in this study consists of a selectable marker fragment conferring streptomycin and spectinomycin resistance (Sm^r/Sp^r) flanked by restriction enzymes sites and prokaryotic signals for the termination of both transcription and translation. The presence of these signals helps prevent read-through from the Sm^r/Sp^r fragment into the test sequences. The Ω fragment was linked at suitable restriction endonuclease sites (Fig.3.1.A) to fragments of DNA representing deletions of the basic replicon (Fig.3.1.B) and tested for their ability to yield transformants. Another antibiotic-resistance fragment that was also used in this way is pKlink (Fig. 3.1.B) (Table 3.1).

Table 3.1 Procedures used for the construction of pCU1 replicon-derivatives that were Poll-independent*

Plasmids	Procedure used for construction (in brief)
pCU999/ pCU999Ω	pCU45 (<i>PvuII</i>) fragment linked to <i>PvuII</i> site of Km ^r fragment from pKlink/ pCU999 (<i>PvuII</i>) fragment linked to <i>PvuII</i> fragment of Ω (<i>Sm^r/Sp^r</i>) from pKlinkΩ
pCU998	pCU999Ω (<i>HincII-EcoRI</i>) fragment (nt. 263-2053) + <i>EcoRI</i> -linker and connected to Ω (<i>EcoRI</i>) fragment
pCU923	pCU773 (<i>PvuII-EcoRI</i>) fragment (nt. 296-2053) + <i>EcoRI</i> -linker and connected to Ω (<i>EcoRI</i>) fragment
pCU952	pCU752 (<i>PvuII-EcoRI</i>) fragment (nt.1-1593) + <i>EcoRI</i> -linker and connected to Ω (<i>EcoRI</i>) fragment
pCU953	pCU999Ω, <i>HindIII</i> fragment (1-2053) isolated and digested again with <i>BspHI</i> (nt. 1-1362) + Tc ^r fragment (<i>NcoI-HindIII</i>) from pACYC184
pCU951	pCU751 (<i>EcoRI-PvuII</i>) fragment (nt. 1-1315) + <i>EcoRI</i> -linker and connected to Ω (<i>EcoRI</i>) fragment
pCU996/ pCU996Ω	pCU999 (<i>HaeII</i>) fragment (nt.198-1394) linked to <i>HaeII</i> site of Km ^r fragment from pKlink/ pCU999Ω (<i>NarI</i>) (nt.195-1391) ligated to pUC-4K (<i>AccI</i>) (see Fig.3.1.B) and isolated again as <i>EcoRI</i> fragment (nt. 195-1392) and connected to Ω (<i>EcoRI</i>) fragment
pCU952-1	pCU952 (<i>HincII-EcoRI</i>) (nt. 263-1593) + <i>EcoRI</i> -linker and connected to Ω (<i>EcoRI</i>) fragment
pCU995	pCU996Ω (<i>HincII-EcoRI</i>) (nt.264-1392) + <i>EcoRI</i> -linker and connected to Ω (<i>EcoRI</i>) fragment
pCU953-1	pCU951 (<i>HincII-HindIII</i>) (nt.263-1362) + <i>HindIII</i> -linker and connected to Ω (<i>HindIII</i>) fragment
pCU950	pCU951 (<i>HincII-EcoRI</i>) (nt. 263-1315) + <i>EcoRI</i> -linker and connected to Ω (<i>EcoRI</i>)
pCU949	pCU751 (<i>DraI-HindIII</i>) (nt.382-1315) + Tc ^r fragment (<i>DraI-HindIII</i>) of pACYC184

* In all cases, the fragment shown within the first parenthesis is derived from the pCU1 basic replicon. Maps of relevant parental plasmids used and their relevant restriction sites are shown in Fig 3.2 A. Maps of Ω and pKlink are shown in Fig 3.1.A and B.

A number of deletion derivatives of the replicon that had been constructed by Kozlowski and by Krishnan (Kozlowski *et al.*, 1987., Krishnan and Iyer, 1990) were also used. The source of the basic replicon of 2053 bp was the *PvuII* fragment contained in pCU45 (Krishnan and Iyer, 1990) (Fig.3.2.A). pCU999 and pCU999 Ω (Fig.3.1.B) are derivatives in which the basic replicon is linked to the Km^r and Sm^r/Sp^r fragments, respectively. Figure 3.1.B also illustrates how some of the deletion derivatives were generated and Table 3.1 is a complete listing of all deletion derivatives and a brief description of their construction. In all cases, a test fragment that was to be linked to the antibiotic marker fragment was recovered from agarose gels as described in MATERIALS and METHODS and used for ligation and transformation.

Each of the derivatives obtained in these experiments was used to transform *E. coli* HB101. In this way, it was possible to define regions smaller than the basic replicon (n.t 1-2053) that were sufficient for plasmid DNA replication. To obtain assurance that each such construct was as intended or inferred, the structure of the construct was determined by digestion with appropriate restriction enzymes followed by agarose gel electrophoresis and the sizing of resulting fragments. Examples are shown in Fig. 3.3.A to 3.3.C.

Plasmid DNA of each of the deletion derivatives was used to transform appropriate isogenic pairs of strains W3110 and SF800, C1200

Fig. 3.1 Derivation of deletion derivatives of the basic replicon.

A diagrammatic illustration of the derivation of the complete basic replicon derivatives containing the 2053 base pair of the replicon region of pCU1 and of its deletion derivatives. In most cases, the Sp^r/Sm^r Ω fragment was linked to these derivatives. In a few cases, the Km^r fragment derived from pKlink (Martin and Crouse, 1989) was used.

Fig 3.1.A is a detailed description of Ω taken from Prentki and Krisch (1984). $\sim\sim\sim$ is the promoter and direction of transcription of the the *aadA* gene conferring Sp^r/Sm^r. ● are termination sites for transcription and translation. Other symbols with lines are standard abbreviations for restriction enzymes and their sites. The thick arrow heads indicated three stop codon at each sides.

In Fig 3.1.B, Ap^r, Km^r and Tc^r are for resistance to ampicillin, kanamycin and tetracycline respectively. Arrows with letters leading from one circle to another indicate the source of the fragment that was gel purified after using the restriction enzyme indicated (P, *Pvu*II ; He, *Hae*II ; N, *Nco*I ; E, *Eco*RI ; Hc, *Hinc*II ; A, *Acc*I). Arrows inside circle indicate the direction of transcription when this is known.

The source of all fragments not shown in this figure is shown in Fig 3.2 (see also Table 3.1).

Ω **B**

Fig. 3.2 A and B Source of DNA used for various plasmid constructs

Diagrammes to illustrate the source of DNA used in the construction of the various derivatives described in Tables 3.1 and 3.2 and in Figures 3.1.B, 3.3 and 3.4.

The open triangles are multicloning sites within which only the relevant sites are shown. Plasmid pUC-4K is shown in Fig 3.1 (bottom right). In B, the numbers with vertical arrow leading from them are nt. numbers of the basic replicon. The three boxes at the bottom are origins of replication as defined by electronmicroscopy of replicating DNA molecules (Barnerjee et al., 1992 ; Appendix 2). Other features shown were described in Fig. 1.18 except for the dashed lines between nt. 405-424 which is a sequence that is repeated 12 times in inverted orientation between nt. 1399 and 1852.

Fig. 3.3.A-C Confirmation of the structure of deletion derivatives

Display of restriction fragments generated from pCU999 (A) and from pCU998 (B) after their isolation and digestion with *EcoRI* to confirm the structure of the respective clones. The procedures for electrophoresis and staining are as described in Materials and Methods. Numbers indicate the size of fragments in kilobase pairs. Fragment patterns on the right (A) or left (B) are size markers using the commercially available 1 kb standards. Some sizes are indicated.

A

-3.0
-2.0
-1.6
-1.0
-0.5

B

2.0
1.6

1.8

Fig.3.3.B

Display of restriction fragments generated from pCU996 Ω (Lane 2), pCU952 (Lane 3), pCU951 (Lane 4), pCU995 (Lane 6), pCU950 (Lane 7) after each plasmid DNA preparaton was digested to completion with *EcoRI* or *HindIII*. The procedures for electrophoresis and staining were as described in Materials and Methods. (0.8 % agarose gel). The fastest migrating bright bands of RNA.

Lane	1	1 Kb ladder (From the bottom 298 bp, 344 bp, 394 bp, 516 bp, 1 Kb, 1.6 Kb, 2.0 Kb, 3.0 Kb, 4.0 Kb)
	2	<i>EcoRI</i> digested pCU996 Ω
	3	<i>EcoRI</i> digested pCU952
	4	<i>EcoRI</i> digested pCU951
	5	<i>HindIII</i> digested pCU951
	6	<i>EcoRI</i> digested pCU995
	7	<i>EcoRI</i> digested pCU950
	8	1 Kb ladder

Fig. 3.3.C

Agarose gel electrophoresis (1%) of pCU949 DNA (a plasmid displaying segregational instability upon release of a antibiotic selection) after growth in the antibiotic media and extraction of plasmid DNA.

A. Lanes 1 and 2 shows pCU949 digested with *Hind*III.

B. Lanes 2 and 3 shows pCU949 digested with *Dra*I-*Hind*III

Lane 3 and lane 1 of each panel show the 1 kb ladder.

A.

B.

Fig. 3.4 Sequences and sequence features in deletion derivatives

The top half of this figure illustrates the sequence features of the basic replicon as described by Krishnan and Iyer (1990). It should be noted that in addition to the ORFs, there are 3 groups of iterons shown as open arrowheads (30 bp), closed arrowheads (37 bp) and dashed lines (19 bp). The first two are direct repeats. The cluster of the third group of iterons is non-tandem and direct but the member at 405-424 is inverted. pCU999 and pCU999 Ω are plasmid derivatives in which the pCU1 basic replicon region is linked to DNA fragments conferring kanamycin-resistance or spectinomycin/streptomycin resistance respectively.

The series of lines shown in the bottom half of the figure represent sequences from the basic replicon (nt. 1 to 2053) present in various deletion derivatives the designations of which are shown at the right. The numbers at the end(s) of the lines indicate the first and last nucleotide of the basic replicon present in each derivative. The top and bottom halves of the figures are aligned so that sequence features present or absent in a particular plasmid derivative will be apparent. Other symbols are ; ∇ , putative DnaA protein-binding sites ; $\bullet$, IHF protein-binding sites.

and C2110, SR1758 and SR1672, WA802 and WA802_{polA1}. In each pair of these strains, the latter strain carries the *polA1* amber or null mutant which is known to prevent replication of plasmid pBR322 (PolI-dependent plasmid) which was used as a control. All deletion derivatives transformed both members of each isogenic pair. It is concluded that all determinants for plasmid replication either with or without PolI are contained within n.t 382 to 1315 (i.e., in plasmid pCU949 and in the deletion derivatives larger than it) (Figure 3.4). This conclusion is confirmed by the results of experiments in a following section (section 3.3).

3.2 Segregational Stability of deletion derivatives

The segregational stabilities of pCU999 Ω containing the basic replicon and of its deletion derivatives were studied in *E. coli* HB101. In each case, cells from a single colony from a selective agar plate were inoculated and grown overnight in 5ml of L-broth containing an appropriate antibiotic(s). 100 μ l of this culture was then used to inoculate 5ml of M9-glucose minimal medium (Miller, 1972) supplemented with other nutritional requirements for the strain. After 12 hrs growth (approximate 20 generations) the culture was diluted 500-fold and regrown. This was repeated at least 10 times. Samples were collected at each interval and plated on L-agar. Colonies that came up on L-agar plates after overnight incubation at 37°C were replicated on streptomycin and spectinomycin containing L-agar plates. The

Fig. 3.5 Stability of deletion derivatives

Segregational stability of the derivatives shown in Fig 3.4 in the absence of 'antibiotic-selection' for the retention of the plasmid. Shown in the figure are results with pCU950 (closed triangles) [a similar observation was made with pCU949 using the Tc^r marker] and pCU999 Ω (open circles). A result similar to that of pCU999 Ω was obtained with all other derivatives shown in Fig. 3.4.

Table 3.2 Procedures used for the construction of pBR322, pUC18 pUC1318 and pACYC184 clones containing different regions of basic replicon of pCU1. The sequences derived from sources other than the pCU1 basic replicon are shown in Fig 3.2.A. The nt. coordinates shown in parenthesis are of the basic pCU1 replicon region

Plasmids	Procedure of construction used for pK1-plasmids
pK192/93	pCU45 (<i>Dra</i> I) (382-2053) cloned into <i>Pvu</i> II site of pBR329 in two orientation
pK1129	pR1129 (<i>Eco</i> RI- <i>Hind</i> III) (nt. 542-2053) fragment of pCU45 cloned into same sites of pUC18
pK11010	pCU998 (<i>Nla</i> III- <i>Eco</i> RI) (nt. 1363-2053) fragment cloned into the <i>Sph</i> I- <i>Eco</i> RI sites of pUC1813
pK1128	pR1129 (<i>Bsp</i> HI- <i>Eco</i> RI) (nt. 542-1358) fragment cloned into <i>Dra</i> I- <i>Eco</i> RI of pACYC184 and releaved with <i>Dra</i> I- <i>Sca</i> I then added the <i>Hind</i> III-linker. This <i>Eco</i> RI- <i>Hind</i> III fragment then cloned into pUC18.
pK1198	pK1129 (<i>Eco</i> RI- <i>Bsp</i> HI) (nt.542-1358) fragment cloned into <i>Dra</i> I- <i>Nco</i> I sites of pACYC184.
pK1953	pCU953 (<i>Dra</i> I- <i>Bsp</i> HI) (nt. 382-1358) fragment cloned into <i>Dra</i> I- <i>Nco</i> I sites of pACYC184.
pK1949	pCU951 (<i>Dra</i> I- <i>Hind</i> III) (nt. 382-1315) fragment connected with <i>Tc</i> ^r (<i>Hinc</i> II <i>Hind</i> III) fragment of pACYC184
pK1336	pCU995 (<i>Eco</i> RI- <i>Nla</i> III) (nt. 263-598) fragment cloned into <i>Eco</i> RI- <i>Sph</i> I sites of pUC18
pLK336	Same as pK1336 but fragment was cloned into pUC1813
pK1412	pCU950 (<i>Fnu</i> DII) (nt.273-684) fragment cloned into <i>Eco</i> RV site of pBR322
pK1213	pK1129 (<i>Eco</i> RI- <i>Rsa</i> I) (nt. 542-756) fragment cloned into <i>Eco</i> RI- <i>Nru</i> I sites of pBR322
pK1168	pK1532 (<i>Eco</i> RI- <i>Rsa</i> I) (nt. 599-756) fragment, added the <i>Eco</i> RI-linker and cloned into pUC18
pK1167	pK1532 (<i>Eco</i> RI- <i>Hin</i> FI) (nt. 599-765) fragment endfilled and added <i>Eco</i> RI-linker. Then cloned into <i>Eco</i> RI site of pUC18.
pK1532	pCU995 (<i>Nla</i> III) (nt. 599-1130) fragment cloned into <i>Sph</i> I site of pUC1318
pK1365	pK1532 (<i>Hin</i> FI- <i>Eco</i> RI) (nt. 765-1130) fragme. V endfilled and added <i>Eco</i> RI-linker. Then cloned into <i>Eco</i> RI site of pUC18.
pK1232	pK1532 (<i>Fnu</i> DII) (nt. 685-918) fragment cloned into <i>Eco</i> RV site of pBR322.
pK122	pK1532 (<i>Fnu</i> DII) (nt. 685-1040) fragment cloned into <i>Eco</i> RV site of pBR322.
pK1363	pCU950 (<i>Alu</i> I- <i>Eco</i> RI) (nt. 951-1315) fragment cloned into <i>Eco</i> RI- <i>Pvu</i> II sites of pBR322
pK1439	pCU952 (<i>Alu</i> I- <i>Eco</i> RI) (nt. 951-1391) fragment cloned into <i>Eco</i> RI- <i>Pvu</i> II sites of pBR322.
pK1150	pK1336 (<i>Eco</i> RI- <i>Hin</i> FI) (nt. 263-399) fragment enfilled and added <i>Eco</i> RI-linker. Then cloned into <i>Eco</i> RI site of pUC18.
pK1186	pK1353 (<i>Eco</i> RI- <i>Nla</i> III) (nt. 410-598) fragment cloned into <i>Eco</i> RI- <i>Sph</i> I sites of pUC18
pK156	pK1129 (<i>Eco</i> RI- <i>Nla</i> III) (nt.542-598) fragment cloned into <i>Eco</i> RI- <i>Sph</i> I sites of pUC18
pK1353	pCU950 (<i>Hin</i> FI) (nt. 410-765) fragment endfilled and added <i>Eco</i> RI-linker. Then cloned into <i>Eco</i> RI site of pUC18.

Fig. 3.6 Derivatives containing sequences from the basic replicon cloned into pUC- or pBR- type vectors.

The top half of this figure is as in Fig 3.4. The series of lines shown in the bottom half of the figure are sequences of the basic pCU1 replicon present in these derivatives. Unlike the derivatives shown in Fig 3.5, these derivatives are regions cloned into vectors such as pBR322. All derivatives with the prefix pKI were constructed as part of this study. The procedures used for construction are described in abbreviated form in Table 3.2. pRI129 and pKI129 have the same pCU1 sequences. In the former, they are linked to pBR322 (Krishnan and Iyer, 1990) and in the latter to pUC18 (this study).

382	2053	pKI92/93
542		pRI129 (pKI129)
947		pRI104
1362		pKI1010
1374		pRI105
542	1358	pKI 128
	1825	pRI123
1	1016	pRI115 (pKI115)
542	1362	pKI198
382	1362	pKI953
382	1315	pKI949
1017	1315	pRI125

proportion of plasmid-bearing cells was determined by picking at least 200 colonies after each subculture. The results are shown in Figure 3.5.

All derivatives with the exception of pCU950 and pCU949 were stable. About forty per cent of the HB101(pCU950) cells lost the plasmid after 7 subcultures. Therefore, a region determining plasmid stability may exist within n.t 1-262. However, pCU998 (nt. 263-2053) and pCU951 (nt. 1-1315) are stable. Therefore, the deletion of the direct repeats (Group I iterons) in addition to the first 262 base pairs may be necessary to cause this instability.

3.3 pBR322 clones containing sequences from the basic replicon

The plasmid pBR322 and its close relatives require *E. coli* Polymerase I for replication (Kingsbury and Helinski, 1970., Goebel and Schrempf, 1972., Grindley and Kelly, 1976) and will therefore not transform a *polA* deletion or amber mutant. With this vector, derivatives containing different regions of the pCU1 basic replicon were constructed and used to transform the *polA1* mutant(s). A listing of these derivatives and the method used for the construction of each is shown in Table 3.2. Figure 3.6 shows the sequences from the basic replicon of pCU1 present in these derivatives. These plasmids were always maintained with selection; stability upon release of selection was not studied.

3.4 Isolation and properties of origin regions that can function in a Poll-independent manner.

The experiments described in section 3.1 led to the conclusion that one of the smallest derivatives of the basic replicon that was both Poll-independent and stable was plasmid pCU996 Ω containing n.t: 195 to 1391 of the basic replicon (see Fig. 3.4). Since all known replicons have distinctive regions where replication is initiated (origins of replication), it was inferred that the region between n.t 195 to 1391 must contain at least one such origin region. Electronmicroscopic studies on replicating molecules of pCU996 Ω that were undertaken concurrently with these studies, have identified and mapped three distinguishable regions called *oriB* (Brahma), *oriS* (Shiva) and *oriV* (Vishnu) of which *oriB* and *oriS* but not *oriV* were identified as Poll-independent origins (Banerjee *et al.*, 1992). The open boxed region in Figure 3.7 shows the nucleotides spanning each region within limits defined by electronmicroscopy (For details, see Appendix 1 of this thesis). The experiments described in this section were intended to detach the Poll-independent origin regions away from the rest of the replicon and to study their properties in such a circumstance. Also described are experiments where an origin region is transplaced to a location in the replicon which is not its native location.

Fig. 3.7 Additional derivatives containing origin sequences from the basic replicon cloned into pBR- or pUC- type vectors.

Derivatives (clones in pBR- or pUC- plasmids) constructed to test for replication origin function in the absence of Polymerase I. The construction of these plasmids is described in Table 3.2. The top half of the figure is similar to Fig 3.5 and 3.6 except that the region between nt. 195 and 1391 is expanded and the three origin regions (as defined by electron microscopy of replicating molecules) are shown.

The bottom part of the figure shows the sequences present in the various clones. T' indicates the number of transformants when each derivative was used to transform a single batch of competent *polAΔ* strains already carrying pCU996Ω [i.e., SR1672 (pCU996Ω)]. The approximate number of transformants were as follows :

+++, > 1000
++, > 100 but < 1000
+, > 10 but < 100
-/+ , < 10
-, not detected

			T*
pKI336 (pLK336)	263 — 598		+++
pKI412	274 — 698		-/+
pKI213	542 — 756		-
pKI168	598 — 756		-
pKI167	598 — 756		-
pKI532	598 — 1130		+
pKI365	766 — 1130		-
pKI232	892 — 918		-
pKI22	918 — 1040		-
pKI363	952 — 1215		-
pKI439	952 — 1281		-
pKI150	263 — 410		++
pKI186	410 — 598		++
pKI56	542 — 598		-
pKI353	410 — 766		+

3.4.1 Construction and test of clones or sub-clones from regions containing the origins.

The sources of DNA for such constructs were one of the following: the *EcoRI* fragment of pCU996 Ω , pCU951 (Fig.3.4) and pKI129 (Fig.3.6). All three origins were expected to be present in each DNA (from the electronmicroscopic studies). A list of the clones or subclones generated and the method used for their construction is shown as part of Table 3.2. Generally, desired clones were selected or screened by insertional inactivation of the tetracycline resistance gene in the cloning vector pBR322 or by inactivation of the *lacZ* gene in pUC18. The presence of the desired insert in an isolated clone was confirmed by restriction enzyme digestion and electrophoresis in a 2 or 3% low melting point agarose gel. The cloned and characterized derivatives were each tested by transformation into the *polA* deletion strain SR1672 and into SR1672 carrying pCU996 Ω (to provide all plasmid-specified trans-acting functions). In the latter case, selection was simultaneously for both resident and incoming markers. None of these derivatives cloned in the *Poll*-dependent vectors pBR322 or pUC18 could transform SR1672. This confirmed the results of the previous two sections ; that is, none of the derivatives shown in Fig 3.7 are *Poll*-independent replicons. The results of transformation experiments in which SR1672 (pCU996 Ω) was transformed with these same derivatives is shown on the right in Figure 3.7. Several of the derivatives did not give rise to any

transformants. pKI336 carrying n.t 263 to 598 of the basic replicon and which spans partly *oriB* and partly *oriS* gave rise to a larger number of transformants than some others. From further experiments on pKI336, it will be shown that the *oriB* region can be reduced to n.t 263 to 399 (as represented by pKI150 in Figure 3.7) with only partial loss of function (i.e., reduction in transformation efficiency). In the light of these results, the negative or doubtful results with pKI412 (n.t 273 to 684) must be noted (see Fig.3.7). Other plasmid derivatives that yielded some transformants in SR1672 (pCU996 Ω) are pKI532 (n.t 599 to 1130, extending from the *oriS* region into part of *oriV*), pKI353 (n.t 410-764, *oriS* region) and pKI186 (n.t 410 to 598; part of the *oriS* region). In this context, it should be noted that other plasmids containing sequences from the *oriS* region defined by electronmicroscopy (i.e., pKI412, pKI213, pKI168, and pKI167) did not yield any transformants.

Strain SR1672 is a *polA*(Δ) but a recombination-proficient derivative (*RecA*⁻ *PolA*⁻ *E. coli* are not viable ; Monk and Kinross, 1972, Bates *et al.*, 1989). pCU996 Ω and the various pKI origin derivatives (Fig.3.6., Fig.3.7) show sequence homology in their respective origin regions. Recombination between pCU996 Ω and a pKI origin derivative is a distinct possibility and can account for some or all of the transformants obtained with each derivative. However, the observation that some of these derivatives gave rise to transformants while others did not suggests that those plasmids that

gave rise to transformants perhaps did function for some generations in the *polAΔ* mutant as elements replicating autonomously without being linked to the helper plasmid, pCU996Ω. The observation that pKI363 (nt. 951 to 1315) and pKI439 (nt. 951 to 1391) did not give rise to any detectable transformants supports this reasoning. The origin region present in these plasmids is *oriV* (Figure 3.7). The *oriV* region in pCU996Ω that contains all three origin regions was not utilized in the *polA* mutant but was utilized in the wild-type strain (Barerjee *et al.*, 1992).

In the following section, experiments are described in which attempts were made to isolate and further characterize selected plasmids that were inferred to carry *oriB* or *oriS*.

3.4.2 Plasmid pKI336, pLK336 and pOriB

i. Construction

The procedure used for the construction of the above *oriB*-containing plasmids is outlined in Fig.3.8. Plasmid pCU995 (carrying n.t 263 to 1391 of the basic replicon) was digested completely with *EcoRI*. The digested DNA was electrophoresed in a 0.8% agarose gel to separate the *EcoRI* fragment from the omega fragment. The fragment was extracted, purified and digested with *NlaIII*, and the small 336 bp *EcoRI-NlaIII* fragment isolated from a 2.0% low melting-point agarose gel. This small fragment was purified using the Mermaid Kit, Bio 101, La Jolla, California, USA) cloned into the

Fig. 3.8. Cloning of a 336 bp *oriB* region

Procedure used for the isolation of the pUC18 and pUC1813 clones containing the 336 bp *oriB* region (nt. 263 to 598) of the basic replicon (see Fig. 3.7).

The *EcoRI* fragment in pCU995 shaded heavily) contains the basic replicon and the lighter shaded part, the Ω fragment. From the purified large (darkly-shaded) *EcoRI* fragment, an *EcoRI*-*NlaIII* (E-NI) fragment was cloned separately into the *EcoRI*-*SphI* (Sh) sites of pUC18 or pUC1813. The resulting plasmids were called respectively pKI336 and pLK336 and their structure was confirmed as shown in Fig. 3.9. Ha, *HaeII* sites.

Fig. 3.9.A Restriction fragments generated from pKI336

Plasmid DNA extracted from a candidate colony which was expected to contain a recombinant pUC18 derivatives carrying the 335 bp *oriB* region and pCU996Ω.

Lane 1 consists of standard molecular weight fragments (1 kb ladder)

Lane 2 pKI336 DNA extracted from HBi01 and digested with *EcoRI*

Lane 3 pCU996Ω DNA extracted from SR1672 and digested with *EcoRI*

Lane4 Plasmid DNA extracted from the derivative called SR1672 (pCU996Ω) (pKI336) and digested with *EcoRI*.

Electrophoresis was in 3 % low-melting point agarose. Arrows a, b and c point respectively to the linearized DNA of fragment of pKI336, the Ω fragment and the basic replicon fragment of pCU996Ω.

1 2 3 4

← a
← b
← c

Fig. 3.9.B Confirmation of the structure of pKI336.

A. pKI336 DNA digested to completion with *Eco*RI and *Hind*III

(Lane 2-5). Lane 1: 1 Kb molecular weight standards (the ruler indicate the migration distances, i.e., 17.5 mm indicate 335 bp).

B. Analysis of the product of the polymerase chain reaction that was used to amplify the 335 bp in pKI336. Primers used are indicated in Materials and Methods. Lane 2-4 ; undigested products after increasing number of cycles of reaction
Lane 1 ; 1 Kb ladder.

(Electrophoresis in 2.5 % low-melting point agarose gel in both cases)

A.

B.

EcoRI-SphI site of pUC18 (see Figure 3.8) and transformed into DH5 α . Colonies were screened on LBA with X-gal and ampicillin. A number of candidate white colonies were screened for plasmid DNA and their structure determined (see Fig 3.9.A). This structure was confirmed by (a) digesting pKI336 in a separate reaction with *EcoRI* and *HindIII* and analyzing the restriction products (Fig 3.9.B) and (b) by amplifying the DNA using Polymerase chain reaction and analyzing the products of amplification (Fig 3.9.B). The construct pLK336 (Fig 3.8) permitted the retrieval of the 336 bp fragment from pUC1813 as a single *HindIII* fragment. Both pKI336 and pLK336 were rescued with pCU996 Ω in *E. coli* SR1672 (Fig 3.7). These two derivatives were believed to carry *oriB* and the origin of the respective pUC plasmids, the latter not functioning in the *polA* deletion mutant. Next, and using a similar procedure, pKI336 was digested with *HaeII* and its 370 bp *HaeII* fragment removed. This fragment contains the origin of pUC18 (see Fig. 3.8). The remaining DNA fragment was re-ligated and transformed into *E. coli* strain HB101 (pCU996 Ω). Colonies were screened with double selection for resistance markers on the two plasmids. 35 colonies were obtained and screened. Plasmid DNA was extracted from one such colony i.e., SR1672 (pCU996 Ω) (pOriB). It was digested with *EcoRI* to yield three fragments, one of which was linearized pOriB and the other two derived from pCU996 Ω . The OriB fragment was extracted from the gel, ligated and analyzed further as shown in Fig 3.10. Upon digestion this molecule with

Fig. 3.10 Analysis of the *oriB* plasmid

Confirmation of the presence of the 335 bp *oriB* region in the plasmid pOriB. pOriB plasmid DNA was isolated from WA802_{polA1}(pCU996 Ω), digested with *EcoRI* and electrophoresed in 3 % agarose gel (low-melting point) (Lane 3 and 4). Lane 1 consists of molecular weight marker fragments. Lane 2 shows non-digested of pCU996 Ω and pOriB. The arrow indicate the 335 bp region carrying *oriB* and the fragment that is migrating slowly indicates the rest the *oriB* plasmid fragment.

EcoRI and *HindIII*, an expected fragment of 336 bp was released and could be detected (Fig 3.10). pKI336, pLK336 and pOriB plasmids transformed SR1672 (pCU996 Ω) but not SR1672. They are thus dependent on *trans*-acting functions provided by pCU996 Ω .

ii. Stability

The stability of plasmid pKI336 was tested in *E. coli* strain SR1672 (pCU996 Ω). A single colony of SR1672 (pCU996 Ω) (pKI336) from LA containing both ampicillin and spectinomycin was inoculated into 5ml of Tryptone-Broth and incubated overnight without any antibiotic. After 12 hrs, the culture was diluted and spread on Tryptone Agar. A 10^{-6} dilution (0.1 ml) was also used to reinoculate fresh Tryptone Broth (5 ml) and the cycle repeated eight times. Colonies were replica-plated on ampicillin and streptomycin / spectinomycin agar plates separately.

The pKI336 marker was lost from 60% of the population after 4 such subcultures, but no further loss of the plasmid was observed after a further 8 subcultures. The stability of the pCU996 Ω marker was increased when pKI336 was co-existing in the same *E. coli* strain (Figure 3.11). As indicated previously, recombination between pKI336 and pCU996 Ω in some of the cells is a possibility.

Fig. 3.11 Stability of pKI336 and pCU996 Ω in the *polA* Δ strain carrying both plasmids.

Experiment to test the stability of markers associated with pKI336 and pCU996 Ω respectively under conditions when, after construction, SR1672 (pCU996 Ω) (pKI336) was grown in Tryptone Broth without any antibiotic added. Open circles : pKI336 marker, closed squares: pCU996 Ω marker in SR1672 ; closed triangles, pCU996 Ω marker in SR1672 (pCU996 Ω) (pKI336)..

Fig. 3.12 Sequence features of the *oriB* region (nt 263-598).

The figure highlights features that can be observed in the *oriB* region and which have been discussed in the text. DnaA and IHF are sequences that can serve as binding sites for these host proteins. The gray bars are the two 30 bp iterons with only the leading part of one repeated. The five black boxed lines are sequences the properties of which have been confirmed by gel-shift and DNA foot printing experiments with the Rep protein (product of ORF239) (Papp personal communication). P_2 is the inferred promoter for ORF239. A promoter (P_1 ?) must also be present upstream of ORF46 as this polypeptide has been detected. (●●●●●) indicate the **ACAGG** sequence that has been detected in other plasmid origins. RBS indicates the sequence of a putative ribosome binding site.

240 CTTGTTTCGGGTTCTGAGGGTTGACCGGCGGGAAGTGGTAAGCGATGATATGCACAATATCCA DnaA
260 280

320 A-T rich 340 360
CAGGCAATATTTTAAAGGTATTTTATAGATTTTATCTTTTAAAGTCTTTTAGAGCTATATAA

HF 380 400 420
CTCATTGATTAAATCATAAATAAGTGTTATCTCTGGGAATCCGCCACCTTGTTATGGGAATTG P₁(?)

440 460 480 500
GCCCACCTATCTATGGGAACACCCCCACCTTACTATGGGAATTAGCCCCACCTTGTTATGGGAATTG ORF46

520 540 560 DnaA
GCCCACCTTAGACGAACTGTAAATAATGATTTACTTGTGTTTGAACCTTGTGGTAGTGTGGAGAGT P₂

-10 580 600
AATTTTAACCCACAAGGCAAGGCTCATGGA ORF239
DnaA RBS

iii. Structural features.

The sequence features of this 336 bp (n.t 263 to 598) present in pKI336 and providing *oriB* are described here (Figure 3.12).

An AT-rich (87%) sequence occurs between nucleotide 311 to 360. The 336 bp sequence includes 2 overlapping DnaA boxes with the sequences **ATATGCACA** and **ATATCCACA** (n.t 290-306). Two identical non-tandem 30 bp directly-repeated DNA sequences (nucleotide 415-444 and 481-510) separated by a spacer region are present in the region with the above two DnaA boxes flanking them on one side. The second of this 30 bp iteron is partially repeated in tandem. Two other DnaA boxes [**CTCTCCACA** (n.t 567-559), **TAACCCACA** (n.t 575-583)] appear before the start of ORF239 (Figure 3.3). A 19 bp sequence partially contained within one of the members of this non-tandem iteron and partially outside it (n.t 406-423) is repeated in an inverted fashion on the complementary DNA strand elsewhere in the basic replicon and straddling each member of the Group I iterons (i.e nt. 1399-1417, 1436-1454) (Krishnan, 1990). The group I iterons are not shown in Fig.3.12 as they are outside the region of the origins but they are shown in Fig.3.3. Another feature of the *oriB* region is the presence of an IHF (Integrational Host Factor) protein binding consensus site **ATAACTCATTGATTTA** at n.t 367-382.

Analysis of other plasmids containing direct repeats at their origins has revealed that the majority of these plasmids contain the sequence

ACAGG in the immediate proximity of their direct repeats (Filutowicz *et al.*, 1985). The *oriB* region of pCU1 has this **ACAGG** sequence overlapping the first DnaA box (n.t 304-308) (Figure 3.12). A further feature (shown as black boxed lines) is the presence of five 20 bp sequences that have partial similarity to repeated sequences in the moderately broad host-range complex plasmid pGSH500 (Da Silva-Tatley and Steyn, 1993). Recently, these sequences were proven to be the Rep protein (product of ORF239) binding sites (Papp, personal communication).

3.4.3 The plasmid pKI150

Since plasmid pKI336 showed origin activity in a *polA*⁻ *E. coli* strain harbouring pCU996 Ω , a further deletion study was done with this plasmid. pKI336 was digested with *EcoRI*-*HindIII* and its 336 bp *oriB*-carrying fragment isolated on 2% agarose gel. After purification of the DNA, the 336 bp fragment was digested again with *HinfI* and allowed to run in a 3% low melting agarose gel. From the gel, a 136 bp *EcoRI*-*HinfI* (nt. 263 to 399) fragment was isolated, filled in with dNTP and the Klenow polymerase, and an *EcoRI*-linker was added and ligated. This ligation mixture was digested again with *EcoRI* and cloned into the *EcoRI* site of pUC18 plasmid. The procedure used is illustrated in Fig. 3.13. Transformation was into *E. coli* DH5 α and transformant colonies were screened on X-gal and ampicillin-containing LBA plates. Four colonies showed a white colour. All four

Fig. 3.13 Construction of pKI150, a derivative smaller than pKI336.

Illustration of the procedures used for the construction of pKI150, a pUC18 clone containing 150 bp of the oriB region derived from pKI336. (For confirmation of the structure of pKI150, see Fig 3.14). The circle represents the DNA of pUC18. The insert into the circle represents the 336 bp oriB fragment of pKI336 (dark shade) and the polylinker.

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Fig. 3.14 Confirmation of plasmid pKI150

EcoRI restriction enzyme digestion of pKI150 plasmid DNA and agarose (3 %) gel electrophoresis of the resulting fragments. The arrow points to the *oriB*-containing fragment and the rest of the plasmid is presumably near the well (Lanes 2 and 3). Marker fragments (1 Kb ladder) are in Lane 1.

1 2 3

516 -

220 -

154 -

142 -

colonies showed the presence of the desired insert in pUC18 and analyses of two are shown in Figure 3.14. In order to test pKI150 for origin activity, it was transformed into *E. coli* SR1672 (pCU996 Ω). As shown in Fig. 3.7, pKI150 was rescued by pCU996 Ω (125 colonies appeared on the double selective media). Therefore, the 136 bp (nt. 233-399) may be sufficient for *oriB* activity. This region contains 2 DnaA boxes the IHF binding-site, and the AT-rich region. However, none of the other repeated sequences are included in this clone and it should be noted that pKI336 (nt. 263-598) gave rise to a relatively larger number of transformants. Sequences present in pKI150 may not be optimum for *oriB* activity.

3.4.4 Plasmids containing the *oriS* region.

Figure 3.7 showed that pKI532 contains *oriS* and part of *oriV* but not the *oriB* region (i.e., 599-1130). When used to transform SR1672 (pCU996 Ω), this plasmid gave rise to transformant colonies (on the double-selection plate) in the range of 10-100. The procedures used for the construction of this plasmid and smaller derivative that do not contain any part of *oriV* is shown in Figure 3.7 and Table 3.2. The only one of these smaller derivatives that showed any transforming activity was pKI353 (Fig.3.7). This plasmid gave rise to transformants in a range which is similar than that of pKI532 (10-100 transformants). An even smaller derivative, pKI167 (containing nt. 599-765) gave no transformants. Thus, nucleotide sequence 410 to 598

seems to be essential for *oriS* activity. It is likely that these sequences which include the Group II iterons (possibly Rep protein binding site) and the two DnaA boxes (n.t 567-559, 575-583) (see Figure 3.12) may have an important role(s) for *oriS* activity. For pKI532 or pKI353, it has not been possible to obtain physical evidence for autonomously occurring plasmids i.e, evidence of the type obtained for the *oriB*-containing pKI336.

3.4.5 Repositioning the *oriB* region

As described in the previous section, in the basic replicon of pCU1, the Poll-independent origins are close to one another (*oriB* : nt 263-399) and *oriS* : nt 410-765). It seemed possible that in the native replicon, this close spacing is relevant to the utilization of one or both origins. In the experiments described in this section, a plasmid was constructed in which the position of *oriB* was altered.

The plasmid pRI129 is a pBR322 clone containing nucleotides 542 to 2053 from the pCU1 replicon. This region contains *oriS* and *oriV* but not *oriB* (nt. 195 to 399). Previous studies (Krishnan and Iyer, 1990) have shown that pRI129 is not Poll-independent. In the present experiments, a pUC18 derivative containing the same cloned sequences and called pKI129 was used after confirming that this was also Poll-dependent. The structure of this plasmid is shown in Figure 3.15. This plasmid was digested with *HindIII* and the 336 bp *oriB*-carrying *HindIII* fragment originating from

Fig. 3.15 Procedure used for altering the relative position of origins.

Procedure used to construct a derivative containing the basic replicon in which the positions of *oriB* relative to *oriS* and *oriV* has been altered. *ori* is the origin of the pBR322 plasmid vector (shown as a single line). The region shown with double lines is derived from the basic replicon with the origin indicated. H, *HindIII* ; E, *EcoRI*.

Table 3.3 Transformation of an otherwise isogenic pair of *PolA*⁺ and *PolA*⁻ strains by derivatives containing *oriS*, *oriV*, Group I iterons or *oriS*, *oriV*, Group I iterons and *oriB* (in this relative order) (Note; In the parental replicon or in pCU45 that contains the pBR322 vector the order is *oriB*, *oriS*, *oriV*, Group I iterons)

Plasmids	Strains [*]	
	SR1758	SR1672
pKI129 (<i>oriS</i> + <i>oriV</i> + IT [*])	+	-
pCU129 (<i>oriS</i> + <i>oriV</i> + IT [*] + <i>oriB</i>)	+	+
pUC18	+	-
pBR322	+	-
pLG338 (pSC101)	+	+

+ ; >100 transformants

- ; no transformants detected

* Transformations were done with other *PolA*⁻ strains also (i.e., SF800, C2110, WA802_{*polA1*}).

IT^{*} = Group I iterons

pLK336 (Fig. 3.8) cloned into the *Hind*III site of pKI129 and transformed into HB101. In the recombinant plasmid, pCU129 (Fig.3.16), the *oriB* region has been transplaced from its normal position to a different one. pCU129 but not pKI129 could transform the *polA* deficient strains SR1672, SF800, C2110 and WA802_{polA1} (Table 3.3). Thus, and under these conditions the *oriB* region is required for PolI-independent replication and altering its position relative to the other two origins (and other sequence features outside the 336 bp *oriB* region) does not prevent this mode of replication.

In a previous section (3.3.4), it was shown that two clones carrying *oriS* and *oriV* (pKI532) or *oriS* alone (pKI353) could transform the *polAΔ* strain carrying pCU996Ω. It was inferred that *oriS* was functioning as an origin in this circumstance. In this section (3.4.5), it was indicated that pKI129 carrying *oriS* and *oriV* and not *oriB* does not replicate in *polAΔ* strain. A difference between these two sets of experiments is that pKI532 and pKI353 do not carry the Group I iterons while pKI129 does. This will be considered further in Chapter V.

3.5 Detection of promoter regions.

The potential open reading frames in the basic replicon are displayed in Fig.3.4. In a previous study (Krishnan and Iyer, 1990), a coupled *in vitro* transcription and translation system was used to detect polypeptides specified by the basic replicon. Two polypeptides, the molecular weights of

Fig. 3.16 Detection of the promoter P_2 .

Construction of pKI337, a pKI336 derivative (pUC18::oriB region) that expresses chloramphenicol-resistance when the promoter-probe chloramphenicol gene cartridge from pCM7 was cloned into its *Hind*III site.

The derivation of pKI336 was shown and described in Fig 3.8 and Table 3.2. In pCM7, the region shown as Cm^s and bounded by *Hind*III site (H), carries the chloramphenicol-resistance cartridge without its promoter and is therefore phenotypically Cm^s . The derivative pKI337 is Cm^r . The promoter inferred to be present in the *ori*B region of pKI336 is shown as P and will be referred to elsewhere in the text as P_2 . The shaded circle shown as ori is the origin of pUC18.

which were consistent with those determined by ORF239 and ORF46 were detected. Subsequent sequencing of the N terminal ends of the synthesized polypeptides has served to confirm this conclusion (Watson and Iyer, unpublished observation). Krishnan *et al* (1990) then introduced mutations in ORF239 by site-directed mutagenesis. The resulting mutants survived but now required Polymerase I thus implicating the ORF239-determined protein in the PolI-independent pathway of replication. Since ORF239 begins with nucleotide sequence 596 (see Fig. 3.4), its promoter is likely to be upstream of this sequence. This upstream sequence of ORF239 overlaps the *oriB* region, which was described in section 3.4. In this section, experiments to identify potential promoters in this region will be described.

3.5.1 Isolation of promoter regions.

The promoterless Tn9 chloramphenicol acetyltransferase gene (CAT) present in a *HindIII* cartridge in plasmid pCM7 confers chloramphenicol resistance only when it is provided with a functional promoter. This promoter-probing cartridge was isolated from pCM7 with *HindIII*, ligated into the *HindIII* site of pK1336 (n.t 263-598) and transformed into HB101. Transformants were selected for Cm^r and Ap^r. A resulting transformant was called pK1337 (Figure 3.16). As described in section 3.4, plasmid pK1129 (n.t 542-2053) cannot replicate in *polA*⁻ *E. coli* hosts. However, if the *oriB* (n.t 242-598) fragment was cloned into this plasmid to give pCU129, this

Fig.3.17 Detection of promoter P₃.

Construction and use of derivatives to demonstrate the presence of a promoter between nt. 599 to 756 of the basic replicon.

pKI532, the source of the above sequences, is described in Table 3.2 and Fig 3.7. The nt. 599 to 756 region was first recovered from this plasmid, gel-purified and linked to a replicon fragment of pBR322 (*EcoRI*-*Sal* fragment) to yield pKI168. The source of the Cm-resistance promoter probe fragment (*PstI* fragment) was pKM7, the construction of which is described in Fig. 3.18. Insertion of this promoter-probe fragment at the *PstI* site of pKI168, activated the expression of Cm^r in pKI339. A promoter P₃ is therefore shown to be present in the nt 592-756 region. E, H, Nl, Sh, Ra, Sc and P are restriction sites for *EcoRI*, *HindIII*, *NlaIII*, *SphI*, *RsaI*, *Sal* and *PstI* respectively.

Fig. 3.18 Derivatives constructed as intermediates to detect promoters P_2 and P_3 .

Diagramme to illustrate the construction of pKM7 that was used in the experiments described in Fig. 3.17 and the text. pKM7 was also used for the construction of pK1338 that is described in section 3.5.2. E, Sh, Nl, H and P are restriction sites for *Eco*RI, *Sph*I, *Nla*II, *Hind*III and *Pst*I respectively.

plasmid replicated in the *polA⁻* *E. coli* host. This suggested that pKI129 produces a trans-acting factor(s), which may be the initiation protein for *Poll*-independent replication. This reasoning implied the existence of a promoter before the ORF239 sequence. ORF239 begins at nt. 596. Therefore, it is possible that the promoter that activated the heterologous *cat* gene and which was inferred to be between nt. 542 to 595 is the promoter for ORF239.

There are two other ORFs (ORF203 and ORF188) initiating from within ORF239 and in frame with it (see Fig. 3.4). The potentiality for either of these ORFs having a separate promoter was examined by separating the sequences between 599 to 756 from plasmid pKI532 with *EcoRI* and *RsaI* (Figure 3.17). The resulting 157 bp fragment was linked to the *EcoRI*-*ScaI* replicon fragment of pBR322. The recombinant molecule was isolated and called pKI168.

Before the *EcoRI*-*RsaI* fragment (nt. 599 to 756 of the basic replicon of pCU1) present in pKI168 could be tested for promoter activity, a plasmid carrying the chloramphenicol gene cartridge bounded by suitable restriction sites needed to be constructed. The plasmid constructed for this purpose (and other purposes) is called pKM7 and its construction is shown in Fig. 3.18. The *PstI* sites in pKM7 (see Fig. 3.18) were used to recover the chloramphenicol-resistance promoter-probe from pKM7. When this cartridge was linked to pKI168 at its *PstI* site, the resultant derivative (pKI339, see

Fig. 3.17) showed a Cm^r phenotype. This leads to the conclusion that the region between nt. 592 and 756 of the basic replicon of pCU1 can also have promoter activity.

The two promoters uncovered in these studies using the heterologous chloramphenicol-resistance cartridge are called P_2 and P_3 in anticipation of an expected promoter, P_1 , upstream of ORF46 (see Figure 3.4) that has yet to be identified. A polypeptide the size of which is consistent with that specified by ORF46 has been identified (Krishnan and Iyer, 1990., and this study, Chapter IV).

3.5.2 Comparative analysis of the promoters P_2 and P_3 to other promoters.

Particular sequences upstream of the putative transcription initiation sites of ORF 239 and ORF 188 show high homology to the consensus sequences of the *E. coli* σ^{32} , σ^{70} and σ^{54} promoters (Hawley and Mclure, 1983., Mclure, 1985., Thomas and Franklin, 1989). The sequences of these promoters also show homology to other *rep* gene promoters (Sugiura *et al.*, 1990., Kelly and Bastia, 1985., Abeles *et al.*, 1989., Itoh *et al.*, 1987) (Fig.3.19). Interestingly, promoters from the *rep* genes of mini-F (f5) (Wada *et al.*, 1987) and Rts1 (Terawaki *et al.*, 1990) are controlled by the σ^{32} RNA polymerase, even though they resemble the -35 and -10 region of σ^{70} -controlled promoters.

Fig. 3.19 Sequence homology of the region of the P_2 promoter of the basic replicon with other plasmid rep gene promoters.

(Adapted from Gammie and Crosa, 1991)

		-35 Region		-10 Region
pColV-K30	RepI	ATATACT	+17	<u>T</u> AAACT
pSC101	RepA	ATTGACT	+16	<u>T</u> ATAGT
R6K	II	ATTGACT	+16	<u>T</u> AAGAT
P1	RepA	GTTGCTA	+18	<u>T</u> AGGAT
Rts1	RepA	AATGTGA	+16	<u>T</u> AGCAT
mini-F(f5)	RepE	ATTGACT	+23	<u>T</u> AAAAA
pCU1	ORF239	TTTGAAC	+19	<u>T</u> AATTT
σ^{32}		CTTGAA	+16/19	<u>C</u> ATTTA
σ^{70}		TTGACA	+16/18	<u>T</u> ATAAT

Table 3.4 Transformation of the RpoH-deficient *E. coli* strain by plasmids that do or do not carry a functional ORF239 gene.

<i>E. coli</i> strain			
Plasmids	Functional ORF239	MC4100 (w.t)	KY1603 (Δ rpoH)
pCU999 Ω	+	+	-
pCU996 Ω	+	+	-
pCU718	-	+	+
pCU717	-	+	+

+, indicates 10^2 - 10^3 transformant colonies

-, indicates that no transformants were detected

A single batch of competent cells of each strain was used for all the DNA samples tested. The experiments were also repeated.

Table 3.5 Transformation of the RpoH-deficient *E. coli* strain by the different promoter-bearing derivative plasmids for chloramphenicol-resistance.

Plasmids	MC4100 (w.t)	KY1603 ($\Delta rpoH$)
pKI337	+	+/-
pKI338	+	-
pKI339	+	+
pUC18	+	+

+ Transformants selected for Ap^r and Cm^r

+ = > 100 transformants

- = no transformants detected

+/- = 10 to 100 transformants

See also the legend of Table 3.4.

Fig. 3.20 Sequence homology of P_3 and a σ^{54} promoter.

Py ; Pyrimidine

Pu ; Purine

N₄ ; Any 4 sequences without specificity

	-26 Region		-10 Region
Consensus	CTGGPy _a PyPu	+N ₄	TTGCA
pCU1-P ₃	CTGGTTAT G	+4	ATGCA
<i>R.meliloti</i> (nifH)	CTGG CAC G	+4	TTGCA

Thus σ^{32} plays a critical role(s) in replication of certain plasmids in addition to its primary role in the heat shock response (Wada *et al.*, 1986, 1987).

In *E. coli*, transcription of the heat shock genes is under control of the σ^{32} promoter. This σ factor is encoded by *rpoH* (*htpR*, *hin*) (Grossman *et al.*, 1987., Straus *et al.*, 1987). Therefore, strains lacking σ^{32} because of a deletion or insertion in *rpoH* show no transcription from heat-inducible promoters *in vivo*.

The basic pCU1 replicon and its Poll-independent derivatives failed to replicate in the $\Delta rpoH$ strain (Table 3.4). Also, the plasmid pKI338 (n.t 542-598) carrying the promoter P_2 (see Fig. 3.19) did not show chloramphenicol resistance in the $\Delta rpoH$ strain (Table 3.5). It is therefore probable that the P_2 promoter can be controlled by the σ^{32} RNA polymerase. One other observation lends indirect support to this reasoning. Krishnan *et al* (1990) isolated 2 mutations that were mapped by sequencing to the ORF239 sequence and showed that these resulted respectively in a truncated ORF239 polypeptide or no detectable polypeptide. These mutants were called pCU717 and pCU718. These two derivatives were able to replicate in the $\Delta rpoH$ strain (Table 3.4). Thus, under conditions when the ORF239 polypeptide is required, a functional σ^{32} RNA polymerase is also required. Under condition when the ORF239 is absent (as in pCU717 and pCU718) the σ^{32} was not required.

The promoter P_3 (upstream of ORF188) shows high similarity to the σ^{54} promoter (Figure 3.20). This sigma factor was discovered as a result of studies on the nitrogen fixation genes which exist in several species of Gram-negative bacteria, including *Klebsiella pneumoniae*, *Azotobacter* spp., *Rizobium* spp. (Magasanik, 1982). Since the mini-replicon of pCU1 showed a broad host-range in Gram-negative bacteria (Krishnan and Iyer, 1988), this sigma factor may play an important role(s) in the replication and maintenance of the replicon in hosts other than *E. coli* K-12.

3.6 Identification of some trans-acting factors for origin function

For plasmids that can replicate in the absence of Poll, there are generally at least two classes of trans-acting factors that are needed for origin function and its regulation. First, they need an initiator protein(s), that is normally encoded by the plasmid and called a Rep protein. This Rep protein binds to specific DNA sequences, in the origin region, together with other host factors. The binding of Rep opens the DNA strand to allow proper access and activity of other factors. A second class of factors that may or may not be absolutely essential is often host-specified and has other molecular roles. Often they assist plasmid origin function. The number and nature of all potential host factors is not always established. Here, I show the role of two of these factors in addition to the role of the *E. coli* DnaA protein that has been established previously (Krishnan and Iyer, 1990).

3.6.1 Cloning and testing of putative rep sequences.

In section 3.3, it was shown that pCU996 Ω could rescue the *oriB*-containing and *oriS*-containing plasmids in *trans*. To determine which part of pCU996 Ω was fulfilling this role, it was necessary to subclone parts of the nt 195 to 1391 replicon sequence present in pCU996 Ω and to do so in a Poll-independent vector. A derivative of the plasmid pSC101 called pl.G338 (Stoker *et al*, 1982) was chosen as the most convenient for this purpose. Figure 3.21 shows the construction of the clone pKim-1, pKim-2, and pKim-3 which contain (n.t 262-1315; ORF46, 239, 203, 188), (n.t 1-1016; ORF46) and (n.t 381-1315; ORF46, 239, 203, 188, $\Delta oriB$) respectively. The restriction patterns of pKim-1 and pKim-3 DNA after digestion with *EcoRI*, *HincII* are shown in Fig. 3.22 (see also Fig. 3.20 for the position of the relevant sites). To determine if these hybrid plasmids contain an essential pCU1 gene(s) that would support the replication of pKI336 or pKI150, the experiments carried out were similar to those described in section 3.2. The results are shown in Table 3.6. They indicate that the ORF239 and ORF46 regions are together sufficient for providing *trans*-acting functions for the Poll-independent use of *oriB* and that the ORF46 region alone is insufficient. The plasmids pCU718 and pCU717 (Krishnan *et al.*, 1990) do not produce the ORF239 polypeptides and could replicate in *polA*⁺ *E. coli* but not in *polA*⁻ *E. coli*. To determine whether these derivatives could provide the *trans*-acting factors or not, two more deletion derivatives were constructed.

Fig. 3.21 Construction of pKim-1, pKim-2 and pKim-3 using the pSC101 replicon carrying vector pLG339.

The plasmid pCU751 was digested with *HincII* and *EcoRI* (nt. 263-1315) or *DraI-EcoRI* (nt. 382-1315). These two fragments were cloned separately to replace fragments in pLG339 (*HincII-EcoRI*, or *PvuII-EcoRI*). The former recombinant molecule was called pKim-1 and the latter called pKim-3.

The plasmid pRI115 was digested with *HindIII* and purified the 1060 bp fragment (nt. 1-1060) purified. This purified fragment was cloned into the *HindIII* site in pLG339, and called pKim-3. Pv, H, E, Dr, Hc are restriction sites for *PvuII*, *HindIII*, *EcoRI*, *DraI*, *HincII* respectively.

Fig. 3.22 Analysis of pKim-2 and pKim-3.

Agarose gel (0.8 %) electrophoresis of restriction fragments generated by *EcoRI* + *HincII* digestion of pKim-1 (A) and *EcoRI* digestion of pKim-3 (see Fig. 3.21). In each case, the adjacent lane is a display of 1 Kb marker fragments.

A

B

First, plasmids pCU718 and 717 were digested completely with *Hae*II. The resultant plasmid DNA was run in a 0.8% agarose gel. From the gel, the 1.2 Kb *Hae*II fragment was isolated and purified. In a similar manner, the 1.4 Kb *Cm*^r fragment from the plasmid pACYC184 was isolated. The two were recombined *in vitro* as described in Materials and Methods, transformed into HB101 and transformants selected on chloramphenicol plates. Plasmid DNA from a transformant was purified by the alkaline lysis method and its structure confirmed by *Hae*II restriction and agarose gel electrophoresis. These two derivatives were named pCU918 (derived from pCU718) and pCU917 (derived from pCU717) (Figure 3.23). Unexpectedly, these two derivatives were found to replicate in the *polA*⁻ *E. coli* strain even though their parent plasmids did not. This observation is described, extended and considered in Chapter 5. In the present context, the results in Table 3.6 and 3.7 show that pCU918 and pCU917 could not provide a needed *trans*-acting function to pKI336 in strain SR1672.

3.6.2 Can other IncN group plasmids provide an ORF239-like function?

Several plasmids belonging to the IncN group have been reported in the literature (see Chapter 1). Two such plasmids whose restriction maps are available are N3 and R15. The IncN group replicons from these two plasmids were isolated and called pHYK2 and pHYK6 (Krishnan *et al.*, 1991), respectively (see Figure 3.24). Both replicons were *Poll*-independent and they were maintained stably in HB101, and SR1672.

Fig. 3.23 *Hae* II restriction fragments generated from pCU917 and pCU918

Agarose (1.0 %) gel-electrophoresis of restriction fragments generated by *Hae*II digestion of pCU917 (lane 3) and pCU918 (lane 2) to confirm their structure. In each case, the slower-migrating fragment contains the antibiotic marker. Lane 1 is of 1 kb standard fragments.

Table 3.6 Derivatives of the basic pCU1 replicon region that were tested for the rescue of the *oriB* plasmids.

SR1672 or WA802 _{polA1}	Plasmids tested for their ability to be rescued			
	pKI336	pKI150	pBR322	pUC18
pKim-1	+	+	-	-
pKim-2	-	-	-	-
pKim-3	+	+	-	-
pCU996 Ω	+	+	-	-
pCU917	-	-	-	-
pCU918	-	-	-	-

+ = 200-300 transformants

- = $< 10^{-7}$ transformants

pCU917 and pCU918 are mutant plasmids that are defective in the production of the ORF239 polypeptide. pHY15 is a mutant derivative that is *Poll*-dependent and which will be described in the next chapter.

Table 3.7 Transformation of the polAA strain carrying different plasmids with the putative *oriS* plasmids.

SR1672 with	Tested for activity yield transformants				
	pKI532	pKI186	pKI353	pKI167	pKI336
pCU917	-	-	-	-	-
pCU918	-	-	-	-	-
pCU996 Ω	+	+	++	-	+++

+ : indicates 10-100 transformants

++ ; indicates > 100 transformants

+++ ; indicates > 10³ transformants

- ; no detectable transformants

pCU996 Ω carries nt 195-1391 of the wild-type replicon

pCU917 and pCU918 carry the same region as pCU996 Ω but have chain-

terminating mutations in ORF239. pHY15 is a derivative of a mutant that requires

Poll for replication.

Table 3.8 shows that pHYK2 and pHYK6 are both able to provide a needed trans-acting function to pKI336 and pKI150. Presumably, ORF239 or an ORF239-like analogue is conserved in this group of plasmids.

3.6.3 The heat shock proteins DnaK and DnaJ enhance the activity of *oriB*.

As part of an effort to identify host proteins required for origin activation, the *oriB* plasmid was tested in *E. coli* mutants that were defective in the heat shock genes *dnaJ* or *dnaK*.

Plasmid pOriB was isolated from SR1672 (pCU996 Ω). The plasmid DNA population (which may have included some pCU996 Ω DNA) was digested with *Sph*I, to linearize only pCU996 Ω DNA. The mixture was then used to transform *dnaJ* and *dnaK* mutant strains that already harboured pCU996 Ω .

Table 3.9 shows that the DnaK and DnaJ proteins have a role in enhancing *oriB* activity.

3.6.4 The IHF protein is essential for *oriB* activity.

As described in Banerjee *et al* (1992), the two P_{OII} -independent origins, *oriB* and *oriS* each contain an IHF binding site that has very high sequence homology with that of the IHF binding site of phage λ . In addition, *E. coli* mutants deficient in IHF protein (Him^- or Hip^- mutants) are transformed very inefficiently (1000 fold less than the isogenic IHF $^+$ strain) by plasmid pCU996 Ω , which indicates that the function of any of these origins requires this DNA bending protein (Landy, 1989) (see Chapter 4).

Fig. 3.24 Agarose gel-electrophoresis of pHYK2 and pHYK6 (Krishnan *et al.*, 1991).

Lane 1 ; 1 Kb ladder

Lane 2,3,4 ; non-digested pHYK2 plasmid

Lane 5 ; non-digested pHYK6 plasmid

Lane 6, 7 and 9 ; *EcoRI* digested pHYK2

Lane 11 to 14 ; *EcoRI* digested pHYK6

Table 3.8 The ability of the replicon regions of other IncN group plasmids to support the replication of the *oriB* plasmid.

SR1672 with	Tested for ability to yield transformants	
	pKI336	pKI150
pHYK2 (N3)	+	+
pHYK4 (N3)	-	-
pHYK6 (R15)	+	+
pCU996 Ω	+	+
pBR322	-	-
pACYC184	-	-
pCU918	-	-
pCU917	-	-
pSC101	-	-

Designations in parenthesis are to the parental IncN plasmid

+ = > 300 transformants

- = < 10⁻⁷ transformants

Table 3.9 Transformation of *dnaJ* and *dnaK* mutants with pCU996 Ω and pOriB.

Strains and Genotype or Phenotype	Number of transformants	
	With pCU996 Ω	With pOriB
MG1655 (<i>dnaK</i> ⁺ <i>dnaJ</i> ⁺)	2500	
PK12 (<i>dnaK</i> 756)	1800	
PK102 (<i>dnaJ</i>)	1950	
PK101 (<i>dnaJ</i> - <i>dnaK</i>)	1100	
MG1655 (pCU996 Ω)		1156
PK12 (pCU996 Ω)		560
PK102 (pCU996 Ω)		422
PK101 (pCU996 Ω)		56

Each number indicates the actual number of colonies that were observed on selective media in one experiment.. The experiment was done 3 times and showed similar trends in colony numbers each time i.e., the colony numbers were at least 2-fold larger when the strain carried the wild-type *dnaJ* and *dnaK* alleles.

To test the possibility that the IHF protein was binding to these region(s) electron microscopic and gel-shift experiments were done. Plasmid pRI115 (nt. 1-1016 of the pCU1 replicon carried by pACYC184) was digested to completion with *HindIII* and the linearized DNA was isolated and purified from agarose.

The IHF protein was diluted with IHF-binding buffer (Inamoto *et al.*, 1990). Dilutions of the IHF protein were pre-heated at 30°C for 15 min. After this, the purified DNA was added, mixed well and the mixture of DNA and protein incubated for two more hours at room temperature. 8µl polyglycine was added to a grid, and left at room temperature for 30 sec and then suctioned gently. 6µl of each sample was then mounted on the grid and incubated further at room temperature for 60 sec. The grid was washed with a few drops of distilled water. Other details of the techniques are described in Chapter 2.

Figure 3.25 shows the binding of pRI115 DNA by the IHF protein. To further confirm binding, a gel mobility-shift assay was performed using the same pure IHF protein preparation. The *oriB* plasmid pLK336 was digested with *HindIII* 3µl of dTTP, dCTP, dGTP, ³²P dATP, and 1µl of T4 DNA polymerase (New England Bio Lab) was added to the digested DNA with 3µl of IHF binding buffer. The volume of the solution was made up to 50µl with distilled water. The mixed solution was incubated further at 37°C for 30 min. Samples were allowed to run in a polyacrylamide gel. A 336 bp labelled

Fig. 3.25 Electronmicrograph supporting the evidence that the IHF protein binds to a region of the basic replicon.

An electronmicrograph of pRI115 DNA to which the IHF protein had been added and incubated as described in Materials and Methods.

(magnification ; x 45,000)

Fig. 3.26 Gel-retardation of pLK336 DNA upon its exposure to the IHF protein.

Retardation of the migration of pLK336 linearized DNA in a 15 % polyacrylamide gel as a consequence of interaction with purified IHF protein. The direction of migration is from the top to the bottom. Lane 1 to 6 contains a constant amount of the DNA and increasing concentrations of protein (from left to right).

DNA was separated from the gel and purified with phenol and chloroform treatment. The binding reaction was in the presence of Calf thymus carrier DNA at room temperature for 30 min. Figure 3.26 indicates the retardation in the migration of the DNA fragment as a result of reaction with the protein.

3.7 Complementation studies with the mini-F (f5) plasmid.

The replication initiator protein RepE of the mini-F plasmid plays an essential and specific role in initiating replication from one of its origins called *ori-2* (Maki *et al.*, 1984., Tolun and Helinski, 1982, Watson *et al.*, 1982). This initiator protein has a molecular mass of 29 kDa and binds to the repeated sequence of 19 bp within the *ori-2* region (Masson and Ray 1986, Tokino *et al.*, 1986). Despite the lack of DNA sequence homology between the basic replicon of pCU1 and mini-F (f5), there is an overall structural resemblance to one another (Figure 3.27). To investigate the possibility of a functional similarity between ORF239 and RepE, complementation experiment between heterologous protein-encoding regions and origin regions was done.

pKV718 is a defective derivative of plasmid F carrying the *ori-2* region of mini-F that can replicate only when RepE protein is supplied in *trans*. It was constructed from pFO-B, a pBR322-based plasmid carrying the *XhoI-HinI* segment of mini-F (Murotsu *et al.*, 1984). pFO-B DNA was

digested with *Apa*L1, and the 4.1 kb fragment containing *ori*-2 and *bla* was circularized to obtain pKV718 in *E. coli* strain BK1776 (λ TL25 *repE*⁺) (Kline *et al.*, 1986). The plasmid pKV718 and pFO-B derived from mini-F on the one hand, and pOriB and pKI336 derived from pCU1 on the other hand, are all unable to replicate in *polA*1 deficient *E. coli*. However, they can each be rescued in the same *E. coli* if *trans*-acting functions are provided by other portions of the plasmids from which they were derived.

These plasmids could therefore be tested for complementation *in trans* by *trans*-acting functions provided by the heterologous plasmid.

pCU996 Ω , pKV5110 (*ori*-2, *repE*⁺, *bla*) (Kawasaki and Yura, 1991) or other plasmids (see Table 3.10) were transformed into competent SR1672 to construct various derivatives like SR1672 (pCU996 Ω) and SR1672 (pKV5110), etc. These strains were then regrown and re-transformed with the origin-containing plasmids (i.e., *ori*-2 of F or *oriB* of pCU1) Transformants containing both the incoming and resident plasmids were selected and maintained on L-agar containing appropriate antibiotic markers.

As shown in Table 3.10, pCU996 Ω and pKim-3 could rescue pKV718 and pFO-B *in trans*. However, in the reciprocal experiment, the *repE* gene containing pKV5110 plasmid could not rescue the *oriB* plasmid of pCU1.

Fig. 3.27 Comparisons of the structure of the pCU1 basic replicon to the f5 replicon of plasmid F.

A diagrammatic comparison of the overall structure of the f5 replicon of plasmid F and the basic replicon of pCU1. The arrows and or arrow-heads shown within the rectangle for pCU1 are potential binding sites for a Rep protein (s) (i.e., ORF239 or ORF239 and ORF46) based on information derived from binding studies between similarly-organized regions on other plasmids.

Table 3.10 The ability of ori-2 (F) and oriB (pCU1) plasmids to transform the polAA strain carrying plasmids providing trans-acting functions for own origins.

SR1672 with	Incoming origin containing plasmids			
	pKV718	pFO-B	pKI336	pOriB
pCU996Ω	+	+	+	+
pKV5110	+	+	-	-
pKim-2	-	-	-	-
pKim-3	+	+	+	+
pCU918	-	-	-	-
pCU917	-	-	-	-

+ ; indicates $> \text{ or } 10^2\text{-}10^3$ transformants

- ; indicates $< 10^{-7}$ transformants

Table 3.11 Plasmid incompatibility tests

Plasmid*	HB101 (pCU996Q)	HB101 (pCU951)
pBR322	-	-
pUC18	-	-
pKI336	+	+
pKI412	+	+
pKI213	-	-
pKI168	-	-
pKI167	-	-
pKI232	-	-
pKI353	-	-
pKI150	+	+
pKI186	+	+
pCU996	+	+
pCU918	+	+
pCU751	+	+

+ indicates that 100 % of the transformants had lost the resident plasmid. - indicates that 100 % of the transformants retained the resident plasmid.* The pCU1 replicon sequences present in plasmids designated pKI- and pCU- are shown in Fig 3.4 and Fig 3.6 respectively.

3.8 The *oriB* and *oriS* regions each express N incompatibility.

A previous study (Krishnan and Iyer, 1990) has defined two regions of incompatibility in the basic replicon of pCU1. One of these was contained in nt. 1-1315, which is now shown to include *oriB* and *oriS*. The construction of plasmids carrying each of these origins (section 3.4) has permitted experiments to determine whether each of these origins expresses IncN incompatibility. The results of these experiments are shown in Table 3.11. It can be seen that pKI336 carrying the functional *oriB* region and pKI186 carrying the functional *oriS* region both express incompatibility to pCU996 Δ or pCU751. In contrast, pKI86 (nt. 542-598) which did not express Poll-independent origin function does not express N incompatibility.

3.9 Conclusions

1. It is demonstrated that nucleotide sequences between nt. 381 to 1315 (934 bp) of the basic replicon (nt. 1 to 2053) are sufficient to express the Poll-independent pathway of replication. However, a plasmid containing this region (or nt. 263 to 1315) was not maintained stably in HB101 when selection pressure was released. Thus, nucleotide sequence 1 to 381 may contain some maintenance-enhancing function for Poll-independent replication. The known important sequences within nt. 1-381 are two *dnaA* boxes, an IHF binding consensus, an A-T rich region, and the pentanucleotide sequence 5'-ACAGG-3' (Filutowicz *et al.*, 1985., Stalker and

Helinski, 1985).

2. The data presented in this chapter support the observation (Banerjee *et al.*, 1992) that within nt. 1 to 1315, there are two *Poll*-independent origins of replication. Each origin is activated individually and located only a short distance from each other. These two distinct origins could be separated from each other physically as core origins and each could be activated detectably when unlinked from the other. However, to replicate efficiently each core origin may need neighbouring sequences that extend into another origin. In that case, these two origins may share a need for all or part of the sequences between nt. 263 to 598. However, in section 3.4.5 (Fig. 3.15), it was shown that repositioning nt. 263 to 598 (containing *oriB*) so that the sequence of the three origins is changed from *oriB-oriS-oriV*-Group I iterons (*oriV* is the *Poll*-dependent origin) to *oriS-oriV*-Group I iterons-*oriB* resulted in a plasmid (pCU129) that is *Poll*-independent. Origin usage in pCU129 needs to be compared to origin usage in the plasmid (pCU996Q) carrying the parental replicon as a first step in exploring the relationship if any between *oriB* and *oriS*.

3. The IHF protein of *E. coli* is shown to bind to the *oriB* region and in Chapter VI, it will be shown that the *Poll*-independent plasmid derivatives transform bacteria containing mutant IHF proteins less efficiently than bacteria producing wild-type IHF proteins. Recently, (Williams *et al.*, 1991), it was shown that conformational changes induced by binding of IHF

protein at the γ origin of plasmid R6K, can regulate initiation at this origin.

4. Although the role of *oriB* and of the ORF239 polypeptide in Poll-independent replication is demonstrated a major conclusion of this chapter (that is considered further in Chapter V) is that in the absence of any one of these components, the plasmid can replicate without DNA Polymerase I provided the Group I iterons are absent. This reveals a novel role for the iterons.

5. Two distinct promoters have been identified upstream of ORF239, 203 and ORF188 (P_2 ; nt. 542-596, P_3 ; nt. 596-756). These promoters show homology to σ^{32} and σ^{54} promoters respectively. P_2 is shown to require a functional *rpoH* gene. Since all Poll-independent derivatives that were tested failed to transform the *rpoH* mutant (but Poll-dependent derivatives did), a role for P_2 in the Poll-independent mode of replication is suggested. It is shown additionally that the heat shock proteins (DnaK, DnaJ) enhance replication using the Poll-independent pathway (Table 3.9). The role and mechanism(s) of the P_3 promoter is not clear. It has some sequence homology with promoters that use the σ^{54} RNA polymerases (Magasanik, 1982). It can be speculated that this promoter may be utilized in Gram negative bacteria other than *E. coli* for the transcription of one of the ORFs that are in frame with ORF239 (i.e., ORF188). This may contribute to the promiscuity of pCU1.

6. The N-terminal end of the RepE protein of F and the ORF239 protein of pCU1 show similarity although these two plasmids are believed to be unrelated (Da Silva-Tatley and Steyn, 1993). The plasmids belong to different incompatibility groups. From the results presented in Table 3.8 and Table 3.10, it can be concluded that the ORF239 protein of pCU1 can substitute for the RepE protein of F *in trans* to initiate replication from the *ori-2* origin of F. The converse did not however occur. The basis of this lack of reciprocity needs to be explored further.
7. The plasmids containing the isolated *oriB* and *oriS* regions are each able to express IncN group incompatibility to the basic replicon.

Chapter IV

The Polymerase I-dependent replicon

Two polypeptides of 27 kD and 5.5 kD were detected by Krishnan and Iyer (1990) using an *in vitro* coupled transcription-translation system and with the DNA of the basic wild-type replicon as the template. The sizes of these polypeptides are consistent with sizes expected for polypeptides encoded by ORF239 and ORF46 respectively. Furthermore, two independent polypeptide chain-terminating mutations within ORF239 abolished or truncated this polypeptide depending on their position. The effect of both mutations was to convert plasmid replication from being PolI-independent to becoming PolI-dependent. In a subsequent study, Banerjee *et al* (1992) showed by electronmicroscopy of replicating DNA molecules that a particular origin, *oriV*, of three origins of replication was not used in a *polA* deletion mutant. These observations implicated DNA Polymerase I but not the ORF239 polypeptide in *oriV* usage and the PolI-dependent pathway of replication. This chapter is concerned with the determinants of this PolI-dependent pathway of replication.

4.1 Using the PolI-independent but temperature-sensitive plasmid pMAK705 (pSC101^{ts}) to identify a region from the basic replicon sufficient for the PolI-dependent pathway.

pMAK705 (Hamilton *et al.*, 1989) is a PolI-independent replicon of pSC101. It carries a mutation which makes its replication temperature-sensitive. As shown in Figure 4.1, a 816 bp (n.t 542-1358)

Fig 4.1 Construction of a clone of a *polA*-independent pSC101^{ts} vector carrying a region of the basic replicon.

The *rep* region in pMAK705 and pKK128 is the pSC101^{ts} replicon that fails to replicate at 42°C. pKK128 contains nt. 542 to 1358 of the basic replicon (*Nco*I to *Eco*RI) and was used to replace the *Hinc*II-*Cl*aI *lacZ* fragment of pMAK705 to give rise to pKK128.

pRI129(E- *Bsp* HI)+pACYC104(E- *Nco* I)

Table 4.1 Transformation of PolA⁺ and of PolA mutant strains by different plasmids

Plasmids	<i>E. coli</i> Strains					
	C1200 or SR1758 (PolA ⁺)		C2110 or SR1672 (PolA Δ)		C2107 (PolA ^{''})	
	30°C	44°C	30°C	44°C	30°C	44°C
pMAK705	+	-	+	-	+	-
pKK128	+	+	+	-	+	-
pLG338	+	+	+	+	+	+
pUC1813	+	+	-	-	+	-

+ ; indicates approximately 3×10^2 transformants.

- ; indicates that transformants were not detected ($< 10^{-7}$)

pLG338 is a derivative of pSC101 that is not temperature-sensitive, it is otherwise similar to pMAK705.

pUC1813 has been used as a control.

fragment from the basic replicon of pCU1 was cloned into the *Clal-HindIII* site of pMAK705. The resultant molecule pKK128 has the pSC101^{ts} replicon and part of the pCU1 replicon respectively. The plasmid pKK128 was transformed into different *E. coli* strains and tested for survival at 30°C and 44°C using the plasmid harbouring antibiotic markers as an indicator of survival. The results are shown in Table 4.1. They show that pKK128 but not pMAK705 survives at 44°C in the wild-type PolA⁺ strain but not in PolA⁻ strains. Therefore, it was concluded that a 816 bp fragment (nt 542-1358, see Fig.4.1) is sufficient for the PolI-dependent pathway of replication. This region does not contain a complete ORF46 or ORF68. However, it does contain *oriV*, *oriS* the three overlapping ORFs (ORF239, ORF203 and ORF188) and the promoters P₂ and P₃. It is likely that a replicon for the PolI-dependent pathway but not the PolI-independent pathways is contained within this 816 bp region.

4.2 Isolation of a PolI-dependent replicon and its derivatives

The parental plasmid pCU1 confers a phenotype that can function independently from its replicon and called Kik (Killing of *Klebsiella*). To isolate mutants of plasmid DNA that were Kik⁻, Rodriguez (1981), used *in vitro* hydroxylamine mutagenesis of plasmid DNA. One of the mutants she isolated unintentionally was called pCU21 and showed a PolI-dependent mode of replication (Table 4.2). Rodriguez isolated pCU21 plasmid DNA and examined its restriction pattern after digestion with various restriction

enzymes. She found that the size of pCU21 plasmid DNA was approximately the same as the parental plasmid pCU1 (39 Kb) but that their restriction fragments were distinguishable. Fig 4.2 shows pCU21 and pCU1 DNA digested separately with three different enzymes and electrophoresed. With *HpaI* and *HincII*, the patterns are dissimilar. No differences were observed with *XmnI*. In addition, since pCU21 can not replicate in a *PolA* Δ strain (SR1672), it suggested that a mutation of the replicon had occurred. This was investigated further.

In order to isolate the minimal replicon of pCU21, all *BglII*-generated fragments of pCU21 were first linked to the non-replicating kanamycin-resistance DNA fragment (from pKlink) randomly and transformed into the *PolA*⁺ HB101 to determine if any of the *BglII* fragments could rescue the *Km*^r fragment. This yielded clones pHY36 and pHY25, that were next tested by transformation into isogenic *PolA*⁺ and *PolA*⁻ hosts. These derivatives were *polA*-dependent (Table 4.2).

pHY36 and pHY25 were then digested with *PvuII*, and a resulting 1.6 kb *PvuII* fragment was linked to the *PvuII* site of the Ω fragment as described previously (see Chapter 3, Figure 3.1). The resulting plasmid (called pHY16) could transform the *PolA*⁺ strains (HB101, WA802, SR1758)) but not the *PolA*⁻ strains (SR1672, WA802_{*polA*}) (Table 4.2). A deletion derivatives of pHY16 was next made by a procedure similar to that shown in Chapter 3 (Figure 3.1). The resulting smaller plasmid called pHY15 also

Table 4.2 The ability of pCU21 and its derivatives to transform a PolA⁺ and a PolAΔ strain.

Plasmids	<i>E. coli</i> strains	
	SR1672 (<i>polA</i> Δ)	SR1758
pCU21	-	+
pCU1	+	+
pHY36	-	+
pHY25	-	+
pHY16 (nt. 1-1382 and 1863-2053)	-	+
pHY15 (nt. 195-1382 and 1863-1976)	-	+
pHY14 (nt. 263-1382 and 1863-2053)	-	+

+; transformants observed (approximately 10³)

- ; no transformants observed. nts shown in parenthesis are of the basic replicon of pCU1.

**Fig 4.2 Restriction fragment patterns generated from pCU1 and pCU21,
DNA to confirm physical differences.**

Odd-numbered lanes contain pCU1 fragment and even-numbered
lanes, pCU21. Enzymes used in order from left to right are *HpaI*, *XmnI* and
HincII.

HB101 harbouring	Incoming plasmids		
	pHY15	pHYK532	pHYK365
pCU996	+	+	+
pCU999 Ω	+	+	+
pCU951	+	+	+
pCU950	+	+	+
pKim-4	+	+	+
pSC101	-	-	-
pUC18	-	-	-
pKY-1	-	-	-

+ indicates that 100 % of the transformants had lost the resident plasmid.

- indicates that 100 % of the transformants retained the resident plasmid.

pKim-4 contains nt. 1363 to 2053 of the basic replicon (see Chapter V).

pKY-1 is an unrelated Poll-dependent plasmid (Lee et al., 1982).

Fig 4.3 Nucleotide alteration in the plasmid pHY16 that is Poll-dependent

Portion of the sequencing gel showing the C-T alteration (*) at the nt position 707 (see Fig 4.4).

Fig 4.4 Sequence comparison of the region of the mutation in pHY16 to the similar region in the wild-type replicon.

w.t nt DNA sequence	;	5'- ATG CAG ACG TAT TTC
aa sequence	;	Met Gln Thr Tyr Phe
mutant nt DNA sequence	;	5'- ATG TAG ACG TAT TTC
aa sequence	;	Met End Thr Tyr Phe

replicated in the PolA⁺ strains but not in PolA⁻ strains. In addition, a *HincII* deletion mutant of pHY15, named pHY14, also showed the same phenotype. Thus, a minimal replicon of the PolI-dependent replicon was isolated (Table 4.2). The nucleotide sequences (of the basic pCU1 replicon) expected to be present in these derivatives are also shown in Table 4.2.

Incompatibility tests showed that the derivatives expressed IncN incompatibility with pCU996 or pCU996 Ω (Table 4.3).

4.3 Sequence analysis of the replicon region in pHY16.

The replicon region in pHY16 was sequenced as described in Materials and Methods. The primers and sequencing strategy used is also shown in Materials and Methods. A sequence change at nucleotide 707 (C-T) that has occurred is shown in Fig. 4.3. Interestingly, this mutation resulted in a stop codon that is in the N terminal part of ORF239 and ORF203 (Figure 4.4) (in the second codon of ORF203). It is also possible that the mutation affected promoter P₃ although this has not been tested directly. It is also to be noted that pHY16 does not contain nt 1383 to 1862 of the basic replicon, a region that had almost all of the Group I iterons.

4.4 In vitro analysis of protein(s) determined by pHY15

The expression of the putative products determined by pHY15 was investigated using a pHY15 DNA directed coupled transcription-translation system as described in Materials and Methods.

The expressed products were analysed in 15% SDS-polyacrylamide gels. Polypeptides with a size consistent with those determined by ORF46 and ORF68 could be detected (Figure 4.5). In similar earlier experiments using the basic wild-type replicon or the mutant Poll-dependent plasmid pCU718, an ORF68 - determined polypeptide was not detected (Krishnan *et al.*, 1990). The ORF239 polypeptide was detected in the wild-type but not the mutant replicon in plasmid pCU718. However, I show in the next chapter that when nt. 197 to 1396 from pCU718 are linked to a marker fragment, the resultant plasmid, pCU918 is Poll-independent. pHY16, the construction of which is described here has the sequences nt. 198 to 1380 and 1863 to 1976 and a chain-terminating base substitution at position 707 within ORF239 which is shared by ORF239 and ORF203 but not by ORF188. The reason why a putative ORF68 polypeptide is detected in the *in vitro* experiment with this plasmid pHY15 as template but was not detected with pCU718 (Krishnan *et al.*, 1990) is not known. Apart from the different positions of the base-substitutions in pCU918 (nt. 602) and in pHY15 or pHY16 (nt 707), the replicon regions present in these two plasmids are similar but not identical. Yet, pCU918 is Poll-independent while pHY15 is Poll-dependent.

Since mutations have not been introduced in ORF46 or ORF68, no firm conclusions can be drawn at present concerning the need or otherwise of these two small polypeptides for the Poll-dependent replicon pHY16.

Fig 4.5 Analysis of polypeptides synthesized from pHY15 in an in vitro coupled transcription-translation system.

Lane a ; pCU996 Ω

b ; pHY15

c ; pCU918

d ; pCU718

Arrows indicate the position at which polypeptides determined by ORF68 and ORF46 are expected to migrate.

a

b

c

d

30 —

18 —

→

6 —

→

An absolute need for ORF68 in PolI-independent replication was excluded by the observation that both pCU751 (Krishnan and Iyer, 1990) and pCU951 (Chapter 3, this thesis) are truncated at the N terminal end of ORF68 (Figure 3.4) and yet PolI-independent. It should also be noted that although the potential product of ORF188 has never been detected, a role for this polypeptide in PolI-dependent replication has not been rigorously excluded.

4.5 Use of a *polC* bypass mutant of *E. coli*. to isolate mutants affecting the PolI-dependent replicon.

In *E. coli*, it is now well established that there are three different DNA polymerases PolI, PolII and PolIII and that PolIII which is a complex of several proteins is the replicating polymerase (Kornberg and Baker, 1992). This polymerase is also required for the replication of most plasmids in *E. coli*. Despite this general observation, a chromosomal mutant called *pcbA1* (polymerase C bypass) has been described which requires little or no Polymerase III for its survival under laboratory conditions (Niwa *et al.*, 1981). The primary observation is that a *polA1*, *polB100*, *polC^{ts}*, *pcbA1* strain (strain M386) or a similar strain with the *PolA⁺* allele (M351) survives at 42°C while *polA^{ts}* *polB100*, *polC^{ts}* *pcbA1* (strain M386) strain does not. These observations and subsequent studies by Moses and collaborators (Bryan *et al.*, 1988) have led to the hypothesis that in *polA⁺* or

polA1 polC^{ts} pcbA1 strains growing at 42°C, the PolI polypeptide that is available is able to interact and form a functional replicating complex with the subunits that normally form such a complex with the PolIII polypeptide (α subunit). The mechanism by which this may occur is not yet known. However, these observations have been confirmed in this laboratory and the availability of this strain suggested a means by which mutations affecting selectively the PolI-dependent pathway of the basic pCU1 replicon could be isolated (It may be noted that in normal strains, selection for mutants affected in this pathway would not be practical because the basic pCU1 replicon also has the PolI-independent pathways).

The scheme used for isolating the desired mutants is described in Materials and Methods. Mutants were obtained by introducing the plasmid pCU999 containing the basic replicon linked to the kanamycin resistance marker fragment into the mutator strain carrying the *mutD5* mutation. After maintenance in this strain, pCU999 molecules were extracted from it (some of which were expected to be mutant) and transformed into the *pcbA1* strain M386. Five thousand transformant colonies were screened for growth at 32°C and 42°C on media with and without kanamycin. 6 colonies did not grow on kanamycin agar at 42°C but did grow at 32°C. Surprisingly, these colonies did not also grow on the agar plates at 42°C in the absence of the antibiotic. The plasmid from one of these strains was called pGK-1 and the probable basis of its inability to grow without the antibiotic at 42°C is

considered further in Chapter V. In the present context, purified pGK-1 DNA was digested with *Hae*II and the large fragment (nt. 198-1395 of the pCU1 replicon) purified and linked to the kanamycin resistant fragment as described in the construction of pCU996 (Chapter III). This derived plasmid was called pGK-2 (nt. 198-1395) and transformed into M386 and its parent-like PcbA⁺ strain SF800. M386 (pGK-2) grew on LA at both 32°C and 42°C but on LA+kanamycin it did not grow at 42°C. This mutant plasmid derivative thus had the properties expected of a plasmid defective in the Polymerase I-dependent pathway. The PcbA⁺ strain carrying pGK-2 grew normally at both temperatures.

4.6 Sequence analysis of the replicon region in pGK-1

The mutated 2053 bp region in pGK-1 was sequenced using the double-strand sequencing method (Materials and Methods). The primers of the top and bottom strands used for sequencing were as described by Krishnan, 1989., and Narang, 1990. The strategy used is shown in Materials and Methods (see Table 2.4). Figure 4.6 and 4.7 show the relevant section of the autoradiogram of the sequencing gel and the position of the mutation in the basic replicon. No sequence alterations were detected elsewhere in the sequence including the Group I iterons. The phenotypic loss of growth at 42°C in M386 (i.e. putative loss of replication using the Poll-dependent pathway) is therefore likely to be caused by this mutation. The mutated sequences lie within the region defined previously by

Fig 4.6 Sequence alteration in pGK-1 and pGK-2

An autoradiogram of the relevant portion of the sequencing gel is shown. The sequence changes are shown by an asterisk and Fig 4.7 indicates the nature of the change.

GAT C

T
G
T
T
C
A
G
C
C
T
C
G

Fig 4.7 Position and nature of the sequence alteration in pGK-1 (pGK-2)

Diagram illustrating that the position of the nucleotide alteration in pGK-1 (and pGK-2) is within *oriV* and indicating the nature of the change. It can be noted that the position of the mutation is within ORF239.

pGK-2

pGK-1

GCT CCG CGA TTGT

GCT CCG A C TTGT

Fig 4.8 Sequence comparison of *oriV* to origins known to replicate by a rolling circle mode.

The sequence for the *oriV* region of pCU1 is from this study, for an *ori* of pR from Gigliani *et al* (1993) and for all other origins from Gruss and Ehrlich (1989).

φX174	aTg TgC TccCccAa CTTGATA tTA
pCU1	gcc TTC TTTaacATC CTTGATA gcct
pR	gcc TTC TTTaacATC CTTGATA gcct
pC194	CTTGTC TTTCTTAT CTTGATA ATA
pUB110	CTTGTC TTTCTTAT CTTGATA CATA
pBAA1	tcgGgTctTTTCTTAT CTTGATA C TA
pCB101	TT TTC TTTCTTAT CTTGATA ATA
pLP1	gTT TTC TTCTTAT CTTGATA C TA

electronmicroscopy as the Poll-dependent *oriV* (nt.890-1200) and they presumably affect the use of *oriV* in pGK-2 (nt. 198-1395) at least when replicating in M386.

The mode of replication from *oriV* is controversial. In the electronmicroscopic studies on replicating molecules Banerjee *et al* (1992), found that when pCU996 Ω replicated in a wild-type strain, *oriV*-initiated replication occurred bidirectionally by a theta mode. Recently (Gigliani *et al.*, 1993) another IncN group plasmid replicon called pR has been described. A partial sequence of this replicon shows that it is identical to the corresponding region in pCU996 and contains at least the *oriS* and *oriV* sequence. Evidence from genetic experiments using this plasmid suggested that an origin in it was being replicated by a rolling circle mode or a mechanism that generates single-stranded DNA. In Fig 4.8, the sequence of the *oriV* region of pCU1 is compared to that of pR and other replicons known to be able to replicate by a rolling circle (RCR) mechanism (Gruss and Ehrlich, 1989). The comparison suggests that there is the potentiality for *oriV* of the pCU1 replicon to replicate by an RCR mode. This needs further investigation.

4.7 Isolation of a smaller region sufficient for the Poll-dependent replicon

In Chapter 3, a derivative of the basic replicon called pKI532 (nt. 599-1130) was described (see Figure 3.6). As shown in Figure 3.7, plasmid

pKI365 (765-1130) was constructed from pKI532 using *HinfI*-*EcoRI* digestion. After purifying and further addition of an *EcoRI*-linker, the 366 bp fragment (nt. 765-1130) was cloned into the *EcoRI* site. This was done because it would allow the origin region of pUC-plasmids including pUC1813 to be deleted with *HaeII*, thus enabling one to determine whether regions cloned into it can function as replicons. As shown in Figure 4 9 when the replication origin of pUC1813 and pUC1318 were deleted with their *HaeII* fragment, and used to transform HB101, transformants were recovered but the reisolated plasmid DNA that was recovered in each case in HB101 showed a DNA rearrangement. In contrast to this observation, the *Poll*-dependent plasmid pHY15 (containing nt. 195 to 1381 and 1863 to 1976 of the basic replicon and a base substitution at nt. 707) is structurally and segregationally stable. It is therefore possible that in addition to the *oriV* region, the *Poll*-dependent replicon requires other regions present in pHY15 for stable maintenance. Alternatively, the base substitution at nt. 707 might confer this stability.

4.8 Construction of pHYK532 and pHYK365

The Ap^r marker in pKI532 and pKI365 has limitations when used to screen for transformants of many other bacterial species. One purpose in constructing small *oriV* replicons was to use them in host-range studies (Chapter VI). For this reason and because of the structural instability of pKI532 (Δori) and pKI365 (Δori), plasmids carrying additional and

Fig 4.9 Structure of pKI532 (Δori) and pKI365 (Δori) DNA isolated from the HB101.

Panel A , Lane 1 ; 1 kb ladder

2 and 3 show two independent cases of isolation of the pKI532 (Δori) plasmid after digestion with *EcoRI*.

Panel B , Lane 1 ; 100 bp ladder (bottom band indicate 100 bp).

2 to 9 show independent cases of isolation of the pKI365 (Δori) plasmid after digestion with *EcoRI*.

In each case, DNA was isolated from a single colony on Ampicillin agar.

(In all the cases, 2.5 % low-melting agarose gel was used)

A.

1 2 3

B.

1 2 3 4 5 6 7 8 9

Fig 4.10 Construction of pHYK532, a derivative of pKI532 carrying selection markers in addition to the Ap^r.

The *oriV* region from pKI532 was excised as a *Bam*HI fragment after a *Bam*HI digest of pAPG10. Transformants were selected for Ap^r Cm^r to give pHYK532, the structure of which was analysed using appropriate restriction enzymes. pHYK365 was constructed from pKI365 in a similar manner.

potentially more useful markers were constructed. The procedure used for the construction of these two plasmids called pHYK532 and pHYK365 is shown in Fig 4.10. As shown in this figure, a *Bam*HI fragment containing the appropriate *ori*V-containing sequences from each of pKI532 or pKI365 was isolated and inserted into the *Bam*HI site of pAPG10 (Gleave *et al.*, 1990), a pUC vector with suitable markers for the selection or screening of transformants.

4.9 The *ori*V-bearing PolI-replicons are PolII-dependent

DNA polymerase II (product of the *pol*B gene) represents only a small amount of the DNA polymerase activity present in *E. coli* (Moses and Richardson, 1970). Mutations in this gene have not produced a detectable phenotype (Campbell *et al.*, 1972., Hirota *et al.*, 1972). Recently, Moses and his colleagues also observed that the *E. coli* strain MC1061 previously described by Casadaban and Cohen, (1980) did not have this gene and yet had no phenotypically observable differences from wild type *E. coli*.

pHYK532 and pHYK365 were found to be maintained in HB101 without any change in their structure. These two plasmids and the larger but stable pHY15 and pHY16 were used to transform the *pol*B Δ strain MC1061 and MC1061 carrying a cloned *pol*B⁺ region (i.e., pHC5, Chen *et al.*, 1989). The results are shown in Table 4.4. They lead to conclusion that these four plasmid replicons carrying *ori*V of the pCU1 replicon are

Table 4.4 Transformation of PolII-deficient strains by various PolI-dependent plasmids 251

Plasmids	<i>E. coli</i> strains		
	MC1060 (w.t)	MC1061 (<i>pol</i> BA)	MC1061 (pHC5) ^{***}
pBR322	+	+	+
pACYC184	+	+	+
pHY16	+	-	+
pHY15	+	-	+
pHYK532	+	-	+
pHYK365	+	-	+
pAPG10 [*]	+	+	+
pUC18	+	+	+
pKY-1 ^{**}	+	+	+

+ ; $> 10^3$ transformants

- ; $< 10^{-7}$ transformants

* pAPG10 is a vector based on pUC7 with Cm^r/Xyl selective markers (see Materials and Methods)

**pKY-1 is PolI-dependent plasmid isolated from *Shigella sonnei* (Lee *et al.*, 1982).

***When MC1061 (pHC5) was used as the recipient selection was simultaneously for Ap^r and Cm^r.

PolII-dependent. However, there are reservations concerning pHYK532 and pHYK365 (see 4.10-4.12). These observations are the first report of PolII-dependency in any known replicon and now establish a function for this polymerase. The question of why pHYK532, pHYK365, pKI532 and pKI365 (all plasmids containing additional regions derived from pUC plasmids) are structurally stable while pKI532 (Δori) and pKI365 (Δori) are not stable remains to be explored. It is unlikely to involve the function of *ori* of the pUC region as an origin because pHY15 and pHY16 that do not contain any sequence from a pUC vector are structurally stable and PolII-dependent. Other plasmid derivatives that may be expected to be PolII-dependent but have not yet been tested are pKI532, pKI365, pCU718 and pCU717.

4.10 Incompatibility

Incompatibility tests were done with the PolI-dependent replicon-bearing plasmids pHY15, pHYK532, and pHYK365 (Table 4.4). These plasmids express IncN incompatibility towards the basic replicon (pCU996 Ω) towards plasmids not containing the Group I iterons (pCU996, pCU951, pCU950) and the clone carrying only the Group I iterons (pKim-4). pHYK532 and pHYK365 carry the replicon of the pUC vector in addition to *oriV* as do the plasmids pKI532 and pKI365.

4.11 Segregational Stability

All four derivatives that were PolI-dependent and PolII-dependent were tested for their stability in *E. coli* HB101. Stability tests were done as

described before (Materials and Methods). None of the plasmids lost their stability over approximately 100 cell generations for which they were tested. pCU718 and its derivative plasmid pCU918 are also very stable in this strain.

4.12 Conclusions

1. The Poll-dependent replicon of pCU1 was isolated from the basic replicon specifying both the Poll-independent and dependent modes of replication and confirmed to be Poll-dependent. Its sequence features are described. The structural organization of the *cis*-acting *oriV* region within this replicon shows some feature in common with that of the replicon of ϕ x174 phage and some plasmids that produce single-strands during replication or replicate using the RCR rather than the θ mode (Gruss and Ehrlich, 1989) (see Chapter VII).
2. The ORF239 polypeptide shown to be required for *oriB* Poll-independent function, but not apparently for *oriS* function (Chapter III and V) may not be required for *oriV* function as a plasmid, pKI365 (Δ *ori*), that carries only the *oriV* region as a replication origin was apparently able to replicate in HB101. This suggests that *oriV* of pCU1 is totally dependent on host-encoded factors. However, this plasmid derivative was structurally unstable which raises reservations about total host dependency.

3. A large Poll-dependent plasmid pHY15 (carrying nt. 195-1382 and 1863 to 1976) of the basic replicon and carrying a base-substitution at position nt. 707) is Poll-dependent but structurally stable. The reason why this plasmid is Poll-dependent may be related to the base substitution at nt. 707.

4. In the coupled *in vitro* transcription-translation system, pHY15 DNA did not direct the production of a detectable ORF239 protein. As this polypeptide has been shown to be needed for Poll-independent *oriB* function (Chapter III), it is consistent with the loss of Poll-independency in pHY15 even though the *oriB* region is present in this plasmid. In pHY15, the base substitution (nt. 707) is within the *oriS* region (nt. 526-836 as defined by electronmicroscopy). It is also within the promoter region P_3 (nt. 599-758). The mutation may therefore have inactivated *oriS* directly or indirectly by inactivating the synthesis and availability *in vivo* of a putative polypeptide dependent on P_3 . (It should be noted however that such a polypeptide has not been detected *in vitro*.)

5. The simple expectation from the observations that pHY15 is Poll-dependent is that it depends on the usage of the Poll-dependent *oriV*. When the *oriV* region from pHY15 was cloned into pUC-based vectors, (Figure 4.8)

the resulting plasmids (like pHY15) were also PolII-dependent. In contrast, the other unrelated PolI-dependent plasmids described in the literature (like the pUC and pBR) are not. Thus replicons dependent on the usage of *oriV* of pCU1 have the distinctive and unique property of being PolII-dependent.

A surprising observation for which there is currently no explanation is that although pUC18 is PolII-independent the two derivatives containing the pUC replicon and the *oriV* replicon of pCU1 (i.e., pHYK365 and pHYK532) are both PolII-dependent.

6. A plasmid mutant called pGK-2 was isolated and shown to be unable to replicate under conditions when the host is expected to use PolI for its replication (i.e., in strain M386). This mutant plasmid was found to have a mutation within *oriV* an observation which is consistent with the above phenotype. It would be of interest to know whether *oriV* is or is not utilized when this mutant plasmid replicates in wild-type *E. coli* and in PolII mutants.

Chapter V

New roles for Group I iterons

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In chapters III and IV, it was indicated that the introduction of two different mutations in a plasmid containing the basic replicon (nt. 1 to 2053) resulted in plasmids pCU718 and 717 that no longer replicated in strains in which DNA Polymerase I was not available (Krishnan and Iyer, 1990). This conversion from Poll-independence to dependence was correlated with the loss of the ability to synthesize the ORF239 polypeptide. An unexpected observation that was the basis of the experiments described and discussed in this chapter is that when the *Hae*II fragment (nt. 198-1395) from each of these mutant plasmids was subcloned, with a non-replicating marker fragment, both derivatives (pCU918 and pCU917 respectively) were now Poll-independent. This has suggested new roles for the Group I iterons, the subject of this chapter.

5.1 Construction and properties of pCU918 and pCU917

The procedure used for the construction of these two plasmids was similar to that used for pCU996 (Fig 3.1, Table 3.1) except that the source of the DNA was pCU717 or pCU718. The resulting plasmids are called pCU917 and pCU918. Both these plasmids transform the *pol*IA Δ strain SR1672 efficiently (Table 5.1). Hence, deletion of the first 195 bp and the Group I iterons led to the suppression of two independent mutations that had initially made the plasmid replicon Poll-dependent.

Table 5.1 Transformation of the wild-type strain and the *polA* Δ mutant by plasmids with the mutation in ORF239.

Plasmids	Strains	
	SR1758 (w.t)	SR1672 (<i>polA</i> -null)
pCU999 Ω	+	+
pCU717	+	-
pCU718	+	-
pCU918	+	+
pCU917	+	+
pBR322	+	-
pLG338	+	-
pUC1813	+	-

+, 10^2 - 10^3 transformants colonies.

-, $< 10^{-7}$ transformants.

5.2 The Poll-independent pathway used by pCU918 and pCU917.

The observations of Krishnan and Iyer (1990) and of Narang (1990) indicated that the Group I iterons have two roles ; copy number control and incompatibility. The results described in Chapter III and IV suggested a third role ; namely, in determining a particular pathway of replication. Since ORF239 had already been shown to be required for a pathway that utilized *oriB* (Chapter III, section 3.6), the Poll-independent pathway of pCU918 or pCU917 is most probably one that utilizes *oriS* (The third origin, *oriV* was shown to be Poll-dependent).

pCU949 has nt 385 to 1315 of the basic replicon linked to a Tc^r marker fragment (Table 3.1). It has neither the *oriB* region nor the Group I iterons and although unstable it could transform *polA* mutants (Table 5.2). Presumably, it utilizes *oriS*. In pKI92 and pKI93, nt. 381 to 2053 of the basic replicon of pCU1 are cloned into pBR329 (Figure 5.1). Unlike pCU949, these plasmids have the Group I iterons and are not Poll-independent (Table 5.2). They are also stable. This suggests that the presence of the Group I iterons in these derivatives prevents the utilization of *oriS* and also confers stability to the plasmid. It is also pointed out that in all three of these plasmids, the first two *dnaA* boxes and the first IHF binding consensus site are deleted.

The results shown in Table 5.2 are in agreement with those shown in Table 5.1 in which experiments, a different *polA* mutant was used.

Fig. 5.1 Construction of pKI92 and pKI93 plasmids

1671 bp (*Dra*I) of basic replicon of pCU1 cloned in two different directions into pBR329 (*Pvu*II), and called pKI92 and pKI93. Black box indicates the portion of pBR329. Shaded box indicate the 1671 bp DNA fragment.

pV=*Pvu*II

Table 5.2 The ability of various plasmid derivatives to transform a PolI-deficient strain.

Plasmids ^a	Strains	
	WA802	WA802 _{polA1}
pCU751	+	+
pCU951	+	+
pCU950	+	+
pCU949	+	+
pCU996Ω	+	+
pCU999Ω	+	+
pKI92	+	-
pKI93	+	-
pBR329	+	-
pUC1813	+	-
pLG339	+	+
pKI10918	+	-

+, 10^2 - 10^3 transformants ($< 10^3$).

-, 10^{-7} transformant.

* unstable ; all other derivative were stable.

a ; The region from the basic replicon present in the first eight plasmids are shown in Figure 3.4 and Figure 3.6. pKI10918 is described in this chapter.

Since pCU949 does not contain nucleotides greater than nt. 1315 and could transform the PolA⁻ strains, it rules out a role for ORF68 in the replication of this plasmid in the PolA⁻ strains.

5.3 Re-linking the Group I iterons to pCU918

To confirm the role of the Group I iterons as pathway determinants, the 13 Group I iterons were relinked to pCU918 as follows. The iteron region was cloned into pUC1813 to give pKI1010 and into pSC101 to give pKim-4 (see Fig 5.2 and Fig 5.3). pCU918 was digested with *EcoRI* and cloned into the same site in pKI1010 and named pKI10918. Then, this plasmid was transformed into WA802 and WA802_{pcbA1}. As shown in Table 5.2, pKI10918 carrying the iterons was PolI-dependent as was pCU718. This confirms that the iterons can act as determinants for the alternative PolI-independent pathways. When the iterons were provided *in trans* with pKim-4, the results were not conclusive because of the high degree of incompatibility between the iteron region and the other regions of the basic replicon.

5.4 A proposed role for the Group I iterons in inhibiting the pcbA1 pathway.

In Chapter IV, section 4.5, the isolation of a mutant of the basic 2053 bp replicon called pGK-1 was described. In pGK-1, the mutation was found

Fig. 5.2 Cloned derivatives containing variable regions of the Group I iterons.

The vectors and the construction of these derivatives are shown in Table 5.5. The gaps shown for some vectors indicate missing nucleotides and iteron numbers. For example, pKI1074, is missing iteron number 5 to 12 and nt. 1829 is adjacent to nt. 1531. The results of incompatibility tests using these derivatives is shown in Table 5.6.

Fig. 5.3 Construction of pKim-4, a derivative carrying the Group I iterons cloned into a pSC101-replicon vector.

to map within *oriV*. pGK-2 was derived from pGK-1 by subcloning the *Hae*II fragment from pGK-1 (nt. 198-1395) with a kanamycin-resistance marker fragment. pGK-2 unlike pGK-1 contains very little of the Group I iterons (28 bp, nt. 1367 to 1394 of the first iteron). The phenotype of pGK-1 was distinguishable from pGK-2 in the following way. In strain M386 (pGK-2), a strain that is believed to use PolI for the replication of its chromosome by virtue of the presence of the *pcbA1* mutation, the cells replicated and formed colonies on LA at 43°C despite carrying a *polC*^{ts} mutation. However, these same colonies did not replicate on kanamycin agar at 43°C. It was inferred that this was because the mutation in the plasmid was within the PolI-dependent *oriV*. A different observation was made when M386 (pGK-1) was used. In this case, colonies did not appear at 43°C even on LA. This implied that the presence of the Group I iterons in pGK-1 was probably inhibiting replication of the M386 host DNA replication. To explain this observation, I postulated that the iterons compete directly or indirectly for a factor(s) needed for chromosomal replication by the *pcbA1* pathway. A likely candidate was the DnaA protein as there are several putative DnaA-binding sites in the iterons; that is, in strain M386, the DnaA protein may be directly or indirectly a limiting factor for host DNA replication. In the presence of the iterons and their *dnaA* boxes, not enough DnaA protein may be available for chromosome replication. To test this possibility, a derivative plasmid in which the DnaA protein can be overproduced was used. Plasmid

Fig.5.4 Inhibition of the growth of M386 at 42°C by plasmids into which the Group I iterons had been cloned.

Panel A ; inhibition of strain M386 at 42°C (non-selective LA plate) with pKim-4 plasmid

Panel B ; M386 on 30°C (non-selective LA plate) with pKim-4 plasmid

pLSK5 (Messer *et al.*, 1988) has the *dnaA* gene under control of a *tac* promoter and is compatible with pKim-4 (the pSC101 derivative carrying the iterons). pKim-4 or pKI1010 (pUC1813 containing the iterons) was transformed into strain M386 selecting the transformants at 30°C. These colonies were tested, by replication, for growth on LA at 30°C and 42°C (Table 5.3) (Fig 5.4). Table 5.3 shows that the Group I iteron-containing plasmids reduced the survival of M386 at 42°C. Table 5.4 shows that in similar experiments, when the strains were made to also carry the DnaA-overproducing plasmid pLSK5 growth at 42°C is restored under conditions of DnaA overproduction. This is consistent with the reasoning that the binding of the DnaA protein is responsible for this effect.

5.5 Group I iterons and plasmid incompatibility

It has been shown previously (Krishnan and Iyer, 1990) that the Group I iterons are N group plasmid incompatibility determinants. In these experiments, it had been found that pRI105 carrying nt. 1374-2053 (inclusive of the last 12 iterons) cloned into pBR322 expressed incompatibility to the basic replicon. In the experiments described here, incompatibility tests were done using derivatives with different regions between nt. 1367 to 2053 cloned into suitable multicopy vectors. The cloned regions present in these Group I iteron derivatives are shown in Fig 5.3 and the construction of each is outlined in Table 5.5. Incompatibility tests were

Table 5.3 Inhibition of the growth of M386 at 42°C by plasmids into which the Group I iterons had been cloned.

Plasmids	Presence of Group I iterons	Growth* of M386 carrying the respective plasmids on LA at :	
		30°C	42°C
pLG339	-	+	+
pKim-4	+	+	-
pKI1010	+	+	-
pUC1813	-	+	+
None	-	+	+

*200 colonies from the selection plates (at 30°C) was replicated on LA and incubated at 30°C or 42°C, + indicate growth of all replicates. - indicates no growth.

Table 5.4 Restoration of the growth of plasmid-bearing M386 strains at 42°C upon overproduction of the DnaA protein.

Plasmids in strain M386	Growth Temperature*	
	30°C	42°C
pKim-4 + pLSK5 (non-induced) *	+	-
pKim-4 + pLSK5 (induced)*	+	+
pLG339 + pLSK5* (induced and non-induced)	+	+
pLSK5 (induced and non-induced)	+	+
pKim-4	+	-

From the selective media 200 colonies were replica-plated and incubated at the two temperatures with or without the IPTG (final concentration 1mM).

+ growth.

- no growth (of all tested colonies)

* selection for markers of both plasmids.

Table 5.5 Outline of procedures used in the construction of clones carrying different members of the Group I iterons.

Plasmids	Procedure of construction used for the iteron-bearing plasmids
pKI1010 (pKim-4)	pCU999 Ω (<i>EcoRI-NlaIII</i>) (nt. 1363-2053), a 690 bp <i>NlaIII-EcoRI</i> fragment cloned into <i>SphI-EcoRI</i> sites of pUC1813 vector plasmid (Ap ^r) (also see Fig. 5.3)
pRI105	iteron-containing fragment (nt. 1375-2053) cloned into pBR322 (Krishnan, 1989) (Ap ^r)
pKI1052	pCU952 (<i>BspHI-HindIII</i>) (nt. 1359-1593) fragment cloned into <i>BspHI-HindIII</i> sites of pACYC184 (Cm ^r)
pKI1074	pRI145 (nt. 1-1532, 1829-2053) (<i>BspHI-PvuII</i>) fragment (nt. 1359-1532, 1829-2053) cloned into (<i>NcoI-PvuII</i>) sites of pACYC184 (Tc ^r)
pKI1032	pCU32 _{copI} (copy number mutant of pCU1, Narang, 1990) (<i>PvuII-BspHI</i>) (nt. 1359-1448, 1820-2053) cloned into <i>NcoI-PvuII</i> sites of pACYC184 (Tc ^r)
pRI123	iteron-bearing fragment (nt. 1825-2053) cloned into pBR322 (Krishnan, 1989) (Ap ^r)

done between these cloned members and selected plasmids that were described in previous chapters. In a set of experiments, HB101 derivatives carrying these plasmids were transformed with each of the derivatives shown in Fig 5.5. Transformants were selected for the respective markers on the transforming DNA and about 100 transformant colonies replicated for the presence of the resistance marker on the previously-resident plasmid. The results are shown in Table 5.6. pKI1010 or pRI105 carrying all or nearly all complete sequences of the Group I iterons (Fig 5.2) expressed incompatibility to all tested derivatives. This confirm and extends previous results of Krishnan (1989) in which he had tested incompatibility between pRI105 and the entire basic replicon.

It can be noted that the vectors carrying the group I iterons not only express incompatibility to pCU996 Ω , a plasmid known to utilize *oriB*, *oriS* and *oriV* but also to pCU918 and pCU949 that are believed to use only *oriS* and *oriV*. It would be useful to extend these incompatibility tests in strains that do not have a functional *polA* gene and thus cannot use a pathway dependent on *oriV*. It has been shown by Krishnan (1989) that there are minor sequence variations between the different group I iterons but that the first six iterons (present in pKI1052) can between them account for all such variations. The present data (Table 5.6) therefore leaves open the question of why these six iterons are insufficient for the expression of incompatibility to all of the plasmid tested. A further difficulty in interpreting the results is

Plasmids	pKI1010	pRI105	pKI1052	pKI1074	pKI1032	pRI123
pCU918	+	+	-	-	-	-
pCU917	+	+	-	-	-	-
pCU718	+	+	-	-	-	-
pCU717	+	+	-	-	-	-
pCU996 Ω	+	+	-	-	-	-
pCU951	+	+	-	-	-	-
pCU949	+	+	-	-	-	+
pHYK532	+	+	-	-	-	-
pHYK365	+	+	-	-	-	-

HB101 derivatives carrying the plasmids listed vertically were used as recipients in transformation experiments with the DNA of plasmids shown horizontally. Selection was for the markers of the horizontally-shown plasmids. Transformants were then replicated on LA containing the antibiotic to which the previously resident plasmid conferred resistance. + indicates that 100 % of the transformants had lost the resident plasmid. - indicates that 100 % of the transformants retained the resident plasmid.

Table 5.6 which relates to the behaviour of the two pHYK plasmids. The conclusion from the experiments of Chapter IV, was that these replicons are not dependent on any trans-acting plasmid functions. Here (Table 5.6), it appears that the thirteen cloned Group I iterons (pKI1010) prevent their stable maintenance but the first six alone (pKI1052) do not. A hypothetical explanation that could be considered is similar to the one put forward in Chapter III, where it was shown that survival of the host M386 at 42°C was affected by the introduction of a cloned derivative carrying the thirteen iterons. The hypothesis to explain that observation was that the DnaA protein essential for the plasmid and the host was limiting and that the DnaA-binding sequences present in the iterons made less of it available for the host chromosome (only in strain M386 at 42°C). Since the first six iterons do have three out of the seven putative DnaA-binding sites in the iterons, this hypothesis is weakened. It is also to be expected that other unrelated plasmid replicons that have DnaA-binding sequences and are DnaA-dependent will be similarly affected. This needs to be tested. The derivative pCU949 has been shown to be inherited unstably even when present as the sole plasmid. The apparent incompatibility between pCU949 and pRI123 may therefore be only a reflection of this instability.

5.6 Conclusions

1. In the absence of Polymerase I, there are two potential pathways of plasmid replication. One of these requires *oriB* and the ORF239 polypeptide (and possibly, ORF46). The second is inferred to require neither ORF239 nor *oriB*. It probably utilized *oriS*.
2. Since *oriB* has been shown to require the ORF239 polypeptide (Chapter III), it is reasonable to assume that in the absence of this polypeptide, the only other PolI-independent origin that would be available for use in a *PolA*⁻ strain is *oriS*. However, its apparent use occurs only if the Group I iterons are deleted. Thus, the Group I iterons seem to inhibit *oriS*-usage in circumstance where *oriB* cannot be utilized (because of the absence of the ORF239 protein or when *oriB* is deleted). This was confirmed by reintroducing the iterons *in cis* and demonstrating the inhibitory effect. The mechanism of this inhibition is unknown.
3. Taken together with earlier published data (Krishnan and Iyer, 1990), the Group I iterons seem to have several possible roles : (a) They regulate plasmid copy number because their deletion resulted in an increase in copy number, (b) they express N group incompatibility, (c) they can influence the utilization of an origin (*oriS*), one of the two origins that can function in the absence of Polymerase I, (d) in a particular circumstance

(i.e., in the *pcbA1* strain M386 when using PolI for its replication), they can prevent chromosomal replication and cell viability.

4. Krishnan and Iyer (1990) raised the possibility that the Group I iterons may have sequences that participate in the binding of the ORF239 protein. Recent *in vitro* evidence (Papp, personal communication) supports this hypothesis. The Group I iterons also contain 7 potential DnaA protein-binding sites and in this chapter there is some circumstantial evidence that these binding sites can be utilized *in vivo*.

Chapter VI

**Host-range of replicon derivatives in some *E. coli* mutants and
other Gram-negative bacteria**

6.1 Host range in chromosomal mutants of *E. coli*

6.1.1 IHF and HU deficient mutants

Two related histone-like proteins IHF, which is a sequence-specific DNA-binding protein (Craig and Nash, 1984), and HU, which binds DNA nonspecifically (Rouviere-Yaniv and Gros, 1975), play an accessory role in a variety of DNA-protein interactions (Friedman, 1988). Even though binding is different for the two proteins, they seem to have functionally similar role(s) of bending the DNA. Also, it has been observed that, at low concentrations, either HU (Dixon and Kornberg, 1984) or IHF protein (Bramhill and Kornberg, 1988., Baker and Kornberg, 1988) can stimulate primase-dependent initiation at *oriC* (origin of replication of the *E. coli* chromosome) *in vitro*. However, replication is inhibited at *oriC* *in vitro* when the concentration of HU is high (Ogawa *et al.*, 1985). As shown in Chapter III (Fig 3.), the basic replicon of pCU1 contains 3 consensus sequences of IHF binding sites one within each of the origins, *oriB* and *oriS* and a third close to *oriV*. The results in Chapter III also suggested that purified IHF protein could bind *in vitro* to the IHF site within *oriB* and possibly also to the site within *oriS*. The results of transformation experiments in which mutant strains defective in IHF proteins or the HU proteins were transformed with a range of derivatives of the basic replicon are shown in this section. Transformation experiments were as described in Chapter 2. After the transformation reaction, colonies were selected on antibiotic(s)

containing media. Experiments were repeated twice and the transformant values were similar. The numbers shown in Tables 6.1, 6.2, and 6.3 are averages.

As shown in these tables, all Poll-independent and Poll-dependent replicons are dependent on IHF but not on HU. However, with the exception of some of the strains shown in Table 6.3, these strains that are deficient in the IHF proteins are *PolA*⁺ and could therefore be using any of the replication pathways available to them. One interesting observation is that the Poll-independent replicon pCU996 became Poll-dependent when the IHF protein was absent (Table 6.3). A similar observation has been made for the *oriC* plasmid (a plasmid containing the replication origin of the *E. coli* chromosome) (Filutowicz and Roll, 1990).

6.1.2 DnaA and RNase H mutants

There are 14 *dnaA* boxes in the basic replicon of pCU1 (Krishnan and Iyer, 1990). It was also observed (Krishnan, 1989) that pCU1 itself was dependent upon the host-encoded protein, DnaA. Here, the *dnaA* dependency was tested using the basic replicon (i.e pCU999) as well as its various derivatives. These derivatives and the basic replicon were also tested with and without the *rnh* (ribonuclease H deficient) mutation. Table 6.4 shows the results. It is concluded that both the Poll-independent and the Poll-dependent pathways show a dependency on the DnaA protein.

Table 6.1 Transformation frequencies of Poll-independent and Poll-dependent minireplicons of pCU1 in *E. coli* strains containing *himA* or *hip* mutations.

Plasmid	Recipient strain			
	¹ <i>himA</i> ⁺ <i>hip</i> ⁺	² <i>himA</i>	³ <i>hip</i>	⁴ <i>himAhip</i>
pCU999Ω	1.5×10 ⁻⁴	8×10 ⁻⁷	7×10 ⁻⁷	<10 ⁻⁹
pCU996Ω	1.4×10 ⁻⁴	<10 ⁻⁹	<10 ⁻⁹	<10 ⁻⁹
pCU717	6.0×10 ⁻⁵	<10 ⁻⁹	<10 ⁻⁹	<10 ⁻⁹
pCU718	8.0×10 ⁻⁵	<10 ⁻⁹	<10 ⁻⁹	<10 ⁻⁹
pCU918	1.1×10 ⁻⁴	<10 ⁻⁹	<10 ⁻⁹	<10 ⁻⁹
pHY16	1.5×10 ⁻⁴	1.7×10 ⁻⁶	<10 ⁻⁹	<10 ⁻⁹
pHY15	1.8×10 ⁻⁴	5.0×10 ⁻⁶	<10 ⁻⁹	<10 ⁻⁹
pHYK532	2.0×10 ⁻³	1.8×10 ⁻⁸	<10 ⁻⁹	<10 ⁻⁹
pHYK356	1.8×10 ⁻³	3.0×10 ⁻⁸	<10 ⁻⁹	<10 ⁻⁹
pUC18/19	4.0×10 ⁻⁴	1.5×10 ⁻⁴	2.0×10 ⁻⁴	3.8×10 ⁻⁵

. Transformation frequencies are with 1 microgram of each DNA.

¹ GM1859, ² HN678, ³ HN778, ⁴ E582 all strains are listed on Materials and Methods chapter.

Table 6.2 Number of transformants relative to those on
the HupA⁺HupB⁺ strain for pCU999Ω

Plasmid	Recipient strains			
	¹ <i>hupA</i> ⁺ <i>hupB</i> ⁺	² <i>hupA</i>	³ <i>hupB</i>	⁴ <i>hupA</i> <i>hupB</i>
pCU999Ω	1.0	0.9	0.9	0.9
pCU996Ω	0.9	0.8	0.9	0.8
pCU918	0.6	0.5	0.5	0.5
pCU917	0.7	0.5	0.6	0.6
pHY16	0.8	0.8	0.6	0.6
pHY15	0.8	0.8	0.8	0.7
pHYK532	0.9	0.9	0.9	0.9
pUC18/19	0.9	0.9	0.9	0.8
pGK-1	0.8	0.8	0.8	0.8

Relative transformants are indicated as the ratio of transformants relative to those obtained with pCU999Ω.

¹ENZ258, ²ENZ259, ³ENZ260, ⁴ENZ262

Table 6.3 Transformation frequencies of *E. coli* strains containing *hip3* and *polA* mutants.

Plasmids	Strains			
	¹ PolA ⁺ <i>hip</i> ⁺	² PolA ⁺ <i>hip3</i>	³ PolA <i>hip</i> ⁺	⁴ PolA <i>hip3</i>
pCU996	6.0×10 ⁻⁴	3.0×10 ⁻⁷	4.5×10 ⁻⁵	<10 ⁻⁹
pCU718	3.8×10 ⁻⁵	2.0×10 ⁻⁸	<10 ⁻⁹	<10 ⁻⁹
pCU717	4.2×10 ⁻⁵	2.2×10 ⁻⁸	<10 ⁻⁹	<10 ⁻⁹

Transformation frequencies are expressed per viable recipient cell.

¹ GM1859, ² GM1859⁺, ³ C2110, ⁴ ECF2010

Table 6.4 Transformation efficiencies of the basic replicon and its derivatives.

Plasmids	Relative transformation frequency ^a			
	¹ <i>dnaA</i> ⁺ <i>rnh</i> ⁺	² <i>dnaA</i> ⁺ <i>rnh224</i>	³ <i>dnaA</i> <i>rnh</i> ⁺	⁴ <i>dnaA</i> <i>rnh224</i>
pCU999Ω	1.0	0.9	< 0.001	< 0.001
pCU996Ω	1.0	0.8	< 0.001	< 0.001
pCU718	0.8	0.2	< 0.001	< 0.001
pCU918	0.8	0.7	< 0.001	< 0.001
pCU717	0.9	0.1	< 0.001	< 0.001
pCU917	0.9	0.7	< 0.004	< 0.001
pGK-1	0.9	0.9	< 0.002	< 0.001
pHY16	0.7	0.2	< 0.001	< 0.001
pHY15	0.7	0.3	< 0.001	< 0.001
pHY14	0.6	0.1	< 0.001	< 0.001
pHYK532	0.9	0.2	< 0.001	< 0.001
pHYK365	0.9	0.1	< 0.001	< 0.001
pUC18/19	0.8	0.6	0.5	0.4

^a; The frequencies shown are relative to those for pCU999Ω for which the frequency was 3×10^{-3} .

¹AQ634, ²AQ666, ³AQ3563, ⁴AQ3585

For the Poll-dependent derivatives of the pCU1 replicon (pCU717, pCU718, pHY16, pHY15, pHY14, pHYK532, pHYK365) but not for the Poll-independent derivatives, the transformation efficiency is reduced in the Rnh^- DnaA⁺ derivative (Table 6.3).

6.1.3 DnaK, DnaJ, GrpE, RpoH mutants

Chapter 3 described that one of the promoters (P_2) detected in the basic replicon has structural and functional similarity to the σ^{32} type promoter. It was shown that the Poll-independent pathway was deficient in an *rpoH* (Δ), (gene for σ^{32} production). In addition, it was shown that *oriB* may need heat-shock proteins for enhancing its activation (see Chapter III, Table 3.10). Table 6.5 shows the replication ability of plasmid derivatives in mutants deficient in host-encoded factors that are known to be relevant to the heat-shock response. The result show that plasmids inferred to be capable of using *oriB* and ORF239 (pCU999 Ω , pCU996 Ω and pGK-1) need these host factors for enhancing their replication activity while those that do not need ORF239 and rely on *oriS* or *oriV* usage do not seem to require these factors. One exception to this is *grpE*, the absence of which had no marked effect on any of the derivatives (Table 6.5).

Table 6.5 Transformation efficiencies of the basic replicon and its derivatives in *DnaJ*, *GrpE*, and *RpoH* deficient *E. coli* strains.

Plasmids	Relative transformation efficiency ^a			
	¹ <i>dnaK</i> ⁺ <i>dnaJ</i> ⁺	² (Δ) <i>dnaJ</i>	³ <i>dnaK</i>	⁴ Δ <i>dnaKdnaJ</i>
pCU999 Ω	1.00	0.55	0.60	0.04
pCU996 Ω	0.98	0.60	0.54	0.03
pCU717	0.75	0.72	0.75	0.66
pCU718	0.77	0.76	0.77	0.71
pCU917	0.88	0.85	0.81	0.86
pCU918	0.98	0.89	0.87	0.82
pGK-1	0.99	0.54	0.55	0.08
pHY16	0.83	0.83	0.82	0.82
pHY15	0.88	0.88	0.81	0.72
pHYK532	0.98	0.91	0.98	0.92
pHYK356	0.97	0.94	0.91	0.88
pUC18/19	0.99	0.98	0.98	0.98

^a values shown are relative to the efficiency of the plasmid carrying the basic replicon (pCU999 Ω) in the *dnaK*⁺*dnaJ*⁺ strain. In this strain and with pCU999 Ω , the number of transformants was 3×10^3 .

¹MG1655, ²PK102, ³PK12, ⁴PK101

Plasmids	Relative transformation efficiency ^a		
	¹ <i>grpE</i> ⁺	² Δ <i>grpE</i>	³ Δ <i>rpoH</i> ⁺
pCU999 Ω	1.00	0.98	< 0.001
pCU996 Ω	0.98	0.97	< 0.001
pCU717	0.87	0.87	0.85
pCU718	0.88	0.81	0.78
pCU917	0.89	0.85	0.001
pCU918	0.97	0.77	0.001
pGK-1	0.99	0.87	< 0.001
pHY16	0.99	0.88	0.89
pHY15	0.99	0.89	0.97
pHYK532	0.98	0.90	0.96
pHYK356	0.99	0.95	0.93
pUC18/19	0.99	0.90	0.88

a; Values shown are relative to pCU999 Ω on the *grpE*⁺*rpoH*⁺ strain.

(Number of transformants = 3×10^3) ¹DA258, ²DA259, ³KY1603

* MC4100 is the wild-type strain of this mutant. Results with it are not shown separately as they were similar to DA258.

Table 6.6 Transformation of *PriA*⁻ and *GyrB*⁻ mutants of *E. coli* by different derivatives of the basic replicon.

Plasmid	Mutants strains			
	¹ <i>priA</i> ⁺	² <i>priA</i>	³ <i>gyrB</i> ⁺	⁴ <i>gyrB</i>
pCU999Ω	+	+	+	+
pCU996Ω	+	+	+	+
pCU918	+	+	+	+
pCU917	+	+	+	+
pHY15	+	-	+	+
pGK-1	+	+	+	+
pHYK532	+	-	+	+
pHYK356	+	-	+	+
pUC18/19*	+	+	+	+

+ : > 100 transformants

- : < 1 transformant

* did not check the stability of these plasmids in those mutant strains.

¹K38, K91, SG13107, SG13109 ²PN102, PN103, PN105 ³HB101, LE234,

⁴CC5, CC6, LE316, N4177

Table 6.7 Relative transformation efficiencies of different replicon derivatives in two different Gram-negative bacteria.

Plasmids	Relative number transformants*	
	<i>Klebsiella oxytoca</i>	<i>Salmonella enteritidis</i>
pCU999Ω	1.0	1.0
pCU996Ω	0.9	0.9
pACYC184	0.9	< 0.001
pHY15	< 0.001	< 0.001
pHYK532	< 0.001	< 0.001
pHYK356	< 0.001	< 0.001
pCU718	0.6	< 0.001
pCU918	0.1	< 0.001
pCU917	0.2	< 0.001

* Numbers are relative to those of pCU999 in *K. oxytoca* and *S. enteritidis* respectively (approximately 10^3)

Table 6.8 Number of transformants resulting from electroporation of some Gram-negative bacteria other than *E. coli*.

The numbers are the average of four independent experiments with the range shown in parenthesis.

Species	Average number of electrotransformants*					
	pHY15	pCU918	pHYK532	pHYK356	pUC18	pCU9962
<i>Klebsiella</i>	< 0.01	45.0	< 0.01	< 0.01	79.3	91.2
<i>oxytoca</i>		(40-48)			(75-85)	(87-94)
<i>Salmonella</i>	< 0.01	< 0.01	< 0.01	< 0.01	12.5	95.2
<i>enteriditis</i>					(9-15)	(70-120)
<i>Pseudomonas</i>	42.5	38.0	30.2	22.0	0.8	72.0
<i>putida</i>	(39-46)	(37-42)	(25-34)	(18-25)		(60-85)
<i>Pseudomonas</i>	65.2	< 0.01	71.4	< 0.01	< 0.01	0.4
<i>stutzeri</i> JM300						
<i>Aeromonas</i>	12.5	20.2	22.5	18.2	< 0.01	87.2
<i>hydrophila</i>	(7-20)	(17-23)	(20-26)	(14-21)		(84-92)

* The numbers shown are the average of four experiments ; the figures shown in parenthesis indicate the range (i.e., minimum and maximum number)

6.1.4 PriA and GyrB mutants

The Poll-dependent replicon is contained within the sequence of ORF239. Within this replicon there is one possible primase binding consensus region (nt.1013-1036) (Kornberg and Baker, 1992).

Transformation experiments were done with the *priA* mutant and its wild-type strain. In addition, a mutant of the *gyrB* gene that is responsible for supercoiling and decatenation of the DNA was also used.

Table 6.6 indicates that the Poll-dependent replicon is dependent on *priA* while the independent replicons are not. The mutation in *gyrB* had no observable effect on either type of replication.

6.2 Host-range of mini-replicons and its derivatives in five different Gram-negative bacteria

The host-range of a small plasmid that included the basic replicon was known from previous studies (Krishnan and Iyer, 1988). Since an *oriB* and an *oriS* pathway of Poll-independent replication as well as a Poll-dependent pathway is now shown to coexist in the basic replicon, it was of interest to test the host-range of appropriate plasmid derivatives of the basic replicon in Gram-negative bacteria other than *E. coli*. It must be noted however that these bacteria are likely to specify and produce forms of DNA Polymerase I similar to Poll of *E. coli*. By using two different gene transfer methods (transformation and electroporation), this was determined in five different Gram-negative bacteria (Table 6.7, 6.8).

The transformation procedure used was according to Hanahan (1987), and has been described in Materials and Methods. *K. oxytoca* and *S. enteriditis* were used as hosts in these experiments. The results (Table 6.7) indicate that with one exception (pCU718) the Poll-dependent replicons of pCU1 do not transform either *K. oxytoca* or *S. enteriditis*. The basis of the ability of pCU718 to transform *K. oxytoca* while the other Poll-dependent replicons do not do so, has not been studied. pCU918 and pCU917 give a relatively low frequency of transformants (even lower than pCU718). It may be recalled that these plasmids unlike the basic replicon, do not use *oriB* in *E. coli*.

Further host-range studies were done by using an electroporation method. Table 6.8 shows the host-range differences between the different plasmids in 5 Gram-negative bacteria. The results with the Poll-dependent replicons and *K. oxytoca* and *S. enteriditis* are similar to those obtained with transformation. These plasmids yielded electrotransformants in *P. putida*, *A. hydrophila* and except for pHYK356, in *P. stutzeri*. pCU996Q that can potentially use both the *oriB* and *oriS* pathway of Poll-independent replication as well as the Poll-dependent pathway yielded electrotransformants in all five species. In contrast, with pCU918, electrotransformants were not detected in *S. enteriditis* and *P. stutzeri*.

The results reported in this last section (6.2) should be considered as suggestive for future experiments rather than being conclusive for the following reasons : (1), only one natural isolate of each species was used ; to draw a firm conclusion that a particular replicon derivative cannot replicate in a particular species, several members of the species will need to be used. (2), in most of the isolates tested (an exception is the *K. oxytoca* strain M5a1 in which no plasmid has been found), it is not known whether they already carried a plasmid or a plasmid-region conferring IncN incompatibility ; if this were the case, the absence or lower frequency of transformants would not be directly related to differences in host chromosome-encoded functions. (3), the genetics of the species used is not characterized ; most likely, they produce DNA polymerases similar to Polymerase I of *E. coli*. In order to draw any firm conclusions concerning the ability or otherwise of the PolI-dependent or PolI-independent pCU1 replicons to function in these hosts, it will be necessary to isolate mutants of the presumptive PolI-like genes in these hosts. (4), Potential DNA restriction systems indigenous to these natural isolates would affect the outcome of the transformation experiments.

6.3 Conclusions

1. All plasmids require host factors for their replication and maintenance and it is now well-established that plasmids or plasmid groups vary in these requirements. In *E. coli*, several of these factors have been identified although the list is probably still incomplete. The pCU1 replicon derivatives surmised in the previous chapters to use one or more of the different replication pathways were tested for their requirement for different host factors. This was done by using available strains that are mutant in these factors.
2. All of the replicon derivatives used showed some dependency on the Integration Host Factor proteins defined by *him* and *hip* mutations.
3. The most distinguishing feature of pCU999 Ω that is absent in pCU996 Ω is the presence of the Group I iterons. pCU999 Ω gave rise to a small but detectable number of transformants in strains mutated singly in *him* or *hip* (7.8×10^{-7}) while pCU996 Ω did not give any.
4. While pCU996 Ω transforms a Pol⁻ Hip⁺ cells efficiently, and PolA⁺ Hip⁻ cells inefficiently (frequency 3×10^{-7}), it yields no detectable transformants in PolA⁻ Hip⁻ cells. This could occur if in the absence of the

Hip protein, the Polymerase I-dependent pathway was used exclusively.

Mutations of the *hupA* and *hupB* genes have only a small effect on the transformation efficiencies of the different replicon derivatives.

5. It is confirmed that the requirement for the host DnaA protein is absolute regardless of which replicon derivative is used. The replicon derivatives that are PolI-dependent transform the RNase H-deficient strains less efficiently than the derivatives that are PolI-independent even though all strains had the potentiality to produce PolI (i.e., were PolA⁺).

6. All replicon derivatives that could use PolI-independent pathways were markedly affected by the deletion of the *rpoH* gene (σ^{32}) while those that were PolI-dependent were not. Mutation in *dnaJ*, *dnaK* and *grpE* had little effect.

7 The effect of the mutation in *priA* was the opposite to that of the *rpoH* mutation in (6) above i.e., the PolI-dependent derivatives did not yield transformants while the PolI-independent derivatives did. The mutation in *gyrB* had no effect on any of the derivatives.

8 Conclusions concerning the ability of these different derivatives to replicate in species other than *E. coli* are subject to the reservations stated at the end of section 6.2. However, the following points can be made

a The host-range of the basic replicon and of pCU996 Ω (the major feature of which is the absence of the Group I iterons) includes all species tested. This is in general agreement with previous results (Krishnan and Iyer, 1989) in which a larger replicon derivative that also contained *oriT* was used.

b pCU996 Ω is known to use pathways that involve *oriB*, *oriS* or *oriV* (Banerjee *et al.*, 1992) while pCU918 cannot use a pathway dependent on *oriB*. pCU918 does not transform *S. enteriditis* or *P. stutzeri* but pCU996 Ω does. None of the Poll-dependent derivatives (inferred to use *oriV*) are also able to transform *S. enteriditis*. The clear inference is that only the *oriB* pathway can function in *S. enteriditis*. Assuming that *S. enteriditis* produces its own DNA Polymerase I, a second inference would be that there is some specificity, direct or indirect between *oriV* and DNA polymerases of the Poll type.

c rHY15 and pHYK532, two Poll-dependent replicons electrotransformed *P. stutzeri* even more efficiently than pCU996 Ω . (It is not clear why pHYK356 did not) This suggests that in some hosts, the Poll-dependent pathway may be more efficient.

d In Chapter III, it was shown that there is in the basic replicon, two other ORFs (ORF203, ORF188) in frame with ORF239, the product of which was detected in the *E. coli* system. It was also pointed out that upstream of ORF188, there is a σ^{54} promoter that could potentially function to produce a replication protein in other hosts.

Some of the observations of Krishnan and Iyer (1989) suggested that plasmid host-range determinants probably exist outside the basic replicon. The experiments described here indicate that such determinants already exist within the basic replicon.

Chapter VII

Discussion

The results of experiments presented in Chapters III to VI have revealed that the 2053 base pair replicon of pCU1 has a greater degree of complexity than was suspected when this study was begun. The major elements of this complexity are :

- (a) two origins of Polymerase I-independent replication that appear to be used under different circumstances.
- (b) A group of iterons that are suggested to prevent the use of one these origins when it is the sole origin that can be used.
- (c) two promoters and indirect evidence for a third
- (d) a PolI-dependent replicon within the basic PolI-independent replicon and the further observation that this replicon is also PolII-dependent.
- (e) differences in the host-range of plasmid derivatives in which origin usage or replicons may be distinguishable.

Each of these aspects is discussed briefly and further here in such a way as to (a) indicate further work that will be necessary and (b) where possible, to draw comparisons to the information on other plasmids that was reviewed in Chapter I. To serve as a reference point in this discussion, the top in Fig. 3.4 that needs to be consulted.

Fig 7.1 The structural similarity between basic replicon of pCU1 and the basic replicons of pGSH500 and mini-F.

cis-oriV indicates the minimal *cis*-acting region required for replication in F and pGSH500. In F plasmid, the *cis-oriV* is also designated *incB*. *rep*, indicates the essential domain for autonomous replication. ORF239, *repB* and *repE* indicate the genes for essential proteins but in the case of pCU1, we do not as yet know whether the ORF46 protein is dispensable. The *oriB*, *oriS* and *incB* shown here are region present in pKI336 (Fig. 3.12). The *inc/cop* domains of *rep β* and mini-pCU1 encode plasmid incompatibility and copy number control determinants. *incC* is the region in F with similar function. The iterons are marked ($\blacktriangleright$). DnaA boxes (1 or 1) and direct repeats at the start of *repB* are indicated ($\blacktriangleright$). Direction of transcription is also shown (∇). (Adapted from Da Silva Tatley and Steyn, 1993)

pCU1
(mini-replicon)

pGSH500
(repB)

F
(repFIA)

7.1 The two polymerase I-independent origins.

The initial evidence for the existence of the two origins called *oriB* and *oriS* came from electronmicroscopic mapping of replicating molecules of a near-minimal plasmid (pCU996 Ω) maintained in a *polA* deletion mutant (Banerjee *et al.*, 1992 : Appendix 2). In this thesis, support for this evidence has come from experiments in which these origin replicons cloned separately in a *Poll*-dependent vector (pUC-) could transform the *polA* mutant only if the mutant carried another plasmid providing the plasmid-specified trans-acting functions. This indicates that both the *oriB* and *oriS* regions are dependent on such functions i.e., they are not replicons. Naturally-occurring plasmids with multiple origins have been described in the literature. The best-studied are R6K (Section 1.5) and the f5 replicon of F (Section 1.2). However, it is not known whether any of these origins are *Poll*-dependent.

The *oriB* region is adjacent to plasmid regions providing the *trans*-acting functions. Such a replicon organization has been observed for other plasmid replicons known to have only single origins. In Fig. 7.2, a comparison is made with the origin regions of two such replicons, *ori-2* (*oriS*) of F and *oriB* of pGSH500. In the case of *ori-2* of F, there is now biochemical evidence that the RepE proteins of F binds to *ori-2* in the region, depicted as the four direct repeats in Fig. 7.2 (Kawasaki *et al.*, 1992). For pCU1, the preliminary biochemical evidence is that the ORF239

Fig 7.2 α -helix-turn- α -helix region in three different Rep proteins.

(*) represent amino acids conserved in all 3 sequences, whereas (·) indicate neutral substitutions. Asterisk and dots represent non-polar and hydrophobic amino acids, respectively.

(From Da Silva Tatley and Steyn, 1993)

Protein	Position	Sequence																			
		1	5	10	15	20															
		α -Helix			Turn	α -Helix															
		I																			
		. . . * A . . * G * V . . * * .																			
		L																			
ORF239	66	V	A	D	Y	Q	Q	I	F	Q	V	S	R	N	Q	A	I	K	D	V	K
RepB	102	V	S	D	Y	V	K	Y	F	N	V	A	T	S	V	A	S	R	D	V	K
RepE	64	V	A	K	Y	A	E	I	F	G	L	T	S	A	E	A	S	K	D	I	R

protein does bind to the *oriB* region (Papp, personal communication). This is consistent with the observations in this thesis that ORF239 is an absolute requirement for the rescue of *oriB*-bearing small plasmids in *polA*-strains. However, using the coupled *in vitro* transcription-translation system and different plasmid derivatives as templates, the small ORF46 protein has been observed (Krishnan and Iyer, 1990 and this study). A role for this protein (in addition to ORF239) in replication or its regulation is therefore possible. Mutations affecting this protein, but not other features in this region, have yet to be isolated. Biochemical evidence of this nature is not available for pGSH500. However, Da Silva-Tatley and Steyn (1993) have drawn attention to similarities between ORF239 of pCU1, RepE of F and RepB of pGSH500 (Fig. 7.2). Although F and pCU1 are considered to be unrelated (different histories, different incompatibility groups, one a narrow host-range and the other a broad host-range plasmid), the above similarity as well as the observation in this thesis (Section 3.7) that ORF239 of pCU1 will rescue *ori-2* of F is intriguing. It would be of interest to study the binding of the ORF239 protein to *ori-2* of F and to construct and study hybrids of the respective origins and replication proteins. The *oriB* region plasmids derived from pCU1 could also be rescued by other plasmid replicons of the N group (Section 3.6.2) suggesting that the ability to produce an ORF239-like protein is conserved in the entire group of N replicons. It is not known whether these other N group plasmids contain an

oriB region although this would be the simple expectation.

The second *Poll*-independent origin, *oriS* of pCU1 is adjacent to *oriB* and there is evidence in this thesis that some sequences present in *oriB* and in *oriS* may participate in the optimum utilization of each (Section 3.4). Nevertheless, *oriS* and *oriB* can be physically and functionally separated and unlike *oriB*, *oriS* is within the codons of the ORF239 protein. As it has been found that certain mutations in ORF239 of the basic 2053 bp replicon abolished *Poll*-independency, it was a surprise to find that nt. 198-1394 from these same mutants were *Poll*-independent. Since *oriB* usage has been shown to require the ORF239 protein, this implies that in these derivatives *oriS* can probably be utilized without it. It is also relevant that a plasmid derivative (pCU949, Fig. 3.4) from which *oriB* has been deleted will still replicate in *polA*⁻ mutants. Electronmicroscopic studies on origin usage in these derivatives are in progress (Banerjee, personal communication) and can provide independent tests of these conclusions on *oriS*. By analogy with other better-studied origins, it seems likely that *oriB* usage will require the participation of the ORF239 protein, and the DnaA protein of *E. coli* protein and probably other host proteins some of which were considered in Chapter VI. Indirect evidence was also presented (Section 3.5) for a suggestion that the promoter for the ORF239 protein is P₂ and that it uses the σ^{32} heat-shock subunit of RNA polymerase. However, the putative promoter for ORF46 has not been experimentally identified. A transcript determined by

this promoter could be polycistronic and determine both ORF46 and ORF239. The codon sequence of ORF239 has indicated the possibility that two other polypeptides called ORF203 and ORF188 that are in-frame with it but which terminate at the same position (i.e., homologous to C terminal portion of ORF239) could be produced *in vivo* and therefore be relevant to the use of *oriS*. However, the smaller polypeptides corresponding to ORF203 and ORF188 have not been detected in the *E. coli in vitro* systems used. An essential role for an ORF68 product in *oriS* utilization is unlikely because pCU949 (nt. 382-1315) has a deletion of the N terminal part of ORF68. An essential role for the detected small ORF46 polypeptide in *oriS* usage is a possibility. It may be significant that the *oriS* region contains several binding sites for the DnaA protein, the protein that has a central role in initiating replication at the *E. coli* chromosome origin.

7.2 The Group I iterons.

In *E. coli*, derivatives of the basic plasmid replicon from which these iterons are completely deleted, will replicate and be maintained in a stable manner. They are therefore not essential components for plasmid maintenance in this host. It has also been observed that the complete cloned iterons are incompatible with plasmid replicon derivatives that do not contain them (Section 5.5) and that in the absence of the iterons, plasmid copy number increases (Krishnan and Iyer, 1990). Both these latter

observations are consistent with a regulatory role for these iterons. It has been postulated that there may be binding sites for the ORF239 protein within this iteron region in addition to such sites in the Poll-independent origin region(s). As Poll-independent replication is observed in the absence of the iterons and *oriB* (i.e., by pCU949), *oriS* is postulated to be used in these circumstances. This expectation can be tested directly by electron microscopy. If true, the implication would be that the iterons when present as additional components in derivatives of the above two plasmids, prevent *oriS* function. In a derivative containing both of these functional origins but not the iterons, the frequency of *oriB* and *oriS* utilization was 1 : 2 (Banerjee *et al.*, 1992). it would be of interest to determine whether this ratio changes when the iterons are present. Between them, these iterons contain seven putative DnaA protein-binding sites. As mentioned earlier there are at least 3 such sites in *oriS*. Assuming that ORF203 and ORF188 are also not produced *in vivo*, these three DnaA binding sites may together be necessary and sufficient for initiation from this origin, that is, no additional plasmid specified proteins may be needed. Alternatively, the ORF46 protein may be necessary in addition to DnaA. When the iterons with their additional DnaA-binding sites are present, occupation of these sites may directly or indirectly prevent access of the DnaA protein to *oriS*. *oriS* utilization can be examined under condition when the DnaA protein is overproduced. In a comparable but special situation (i.e., in the *pcbA1 polB*

polC^{ts} strain carrying the mutant pGK-1 plasmid), the cells did not grow at 42°C but did so when the same strain contained a plasmid that overproduced the DnaA protein (Section 5.4). In that situation, loss of cell viability was also attributed to the ability of the Group I iterons to bind the DnaA protein. The implication of these experiments and reasoning in its simplest form is that incompatibility expressed by the cloned iterons is not specific to the N group of plasmid replicons but rather to replicons that are dependent on the DnaA protein. This hypothesis is testable.

7.3 The PolI- and PolII-dependent replicon.

Although the existence of a PolI-dependent origin of replication within the basic replicon was known from previous studies, the demonstration of a PolI-dependent replicon required the fortuitous isolation of a mutant (Rodriguez, 1981) in which the PolI-independent pathways (i.e., those utilizing *oriB* and *oriS*) were inactivated. The reason for the inactivation of both independent pathways has been speculated on in section 4.4 in which it was shown that a 1300 bp fragment derived from the mutant and called pHY15 also behaved like a stable PolI-dependent replicon. Furthermore and uniquely, this plasmid (or a slightly smaller derivative, pHY14) was also dependent on PolII. This derivative is therefore potentially useful in further studies on this polymerase. Smaller derivatives of pHY15 or pHY14 were found to be structurally unstable and are therefore not

considered to be replicons. However and surprisingly, when these potentially unstable regions were linked to a pUC vector (which is PolII-independent), the resultant cointegrates were stable and PolII-dependent. The basis of this suppression of the PolII-independency of the pUC vector as well as the basis of stability is not understood. It will first require authentication of their structure by detailed sequence analysis. Before this is done, it is premature to consider models of replication for this replicon.

7.4 Host-range

One of the motivations for the study of the IncN replicon was to obtain insight into its broad host-range. Among the features of this replicon that could contribute to host-range are the distinguishable P_2 and P_3 promoters, the distinguishable chromosomal genes required for the optimum function of the PolI-dependent and PolI-independent replicons and the separate ways by which *oriB* and *oriS* usage can apparently be affected. Preliminary studies have also suggested that these differences correlate with host-range in species other than *E. coli*. Thus the PolI and PolII dependent replicon electrotransformed *P. stutzeri* more efficiently but were unable to transform *S. enteritidis*. The latter species could also not be transformed by the derivative that was unable to produce the ORF239 protein and devoid of the Group I iterons while the basic replicon could. The presence in the basic replicon of the three overlapping codons ORF239, 203

and 188 that are in frame also raise the possibility that although protein determined by the smaller ones were not detected in the *E. coli* system, they may be detected and functional in other species. These observations encourage a more extensive analysis of the host-range of different derivatives of the basic replicon.

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Three Clustered Origins of Replication in a Promiscuous-Plasmid Replicon and Their Differential Use in a PolA⁺ Strain and a Δ PolA Strain of Escherichia coli K-12 (NOTES)

S.K. Banerjee, B.T. Luck, H.-Y. Kim, and V.N. Iyer

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