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# Analysis of copy number control elements in the region of the vegetative replication origin of the broad host range plasmid RK2

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**Broad host-range plasmid RK2 is able to replicate in a controlled manner in most Gram negative bacterial species. To analyze the elements of its control mechanism, we have measured the copy number in *Escherichia coli* of mini-RK2 replicons isogenic except for defined deletions in regions adjacent to the vegetative replication origin, *ori*$V_{RK2}$, which have previously been implicated in copy number control because of their expression of plasmid incompatibility. The results indicate that while the previously defined 700-bp *Hae*II *ori*$V_{RK2}$ fragment carries one copy control element (*cop*A), a second (*cop*B) lies at least partly outside this fragment towards the tetracycline resistance genes of RK2. Deletions affecting both these regions give a mini replicon with a copy number of 35–40 compared with 4–7 for parental RK2. Further incompatibility experiments indicate that targets for both *inc*A (*cop*A) and *inc*B (*cop*B) lie within the 700-bp *Hae*II *ori*$V_{RK2}$ fragment.**
*Key words:* DNA replication/plasmid RK2/copy number control/incompatibility

## Introduction

Bacterial plasmids are covalently closed circular DNA molecules that are capable of autonomous replication within their bacterial host. Individual plasmids replicate in a controlled way so as to maintain a certain average copy number of plasmid DNA molecules per chromosome equivalent or per bacterial cell in a bacterial population. The mechanisms by which naturally occurring plasmids regulate the frequency of initiation of their DNA replication cycle, which determines their copy number, have been intensively studied in recent years, but the components of the replication control system have been worked out in *Escherichia coli* only for ColE1-type plasmids (Twigg and Sherratt, 1980; Itoh and Tomizawa, 1980; Tomizawa *et al.*, 1981; Tomizawa and Itoh, 1981; Muesing *et al.*, 1981; Lacatena and Cesareni, 1981; Cesareni *et al.*, 1982) and IncFII plasmids (Molin *et al.*, 1981; Stougard *et al.*, 1981a; Light and Molin, 1983; Brady *et al.*, 1983). In both of these cases there seem to be two components to the control circuits involved: a plasmid-encoded protein which is a negative regulator for a promoter, the transcript from which is required positively for replication, and an RNA transcript synthesized from the opposite strand to the above transcript that acts negatively to regulate the function of this transcript, either as a primer for leading strand initiation or to produce an essential replication initiation protein.

Analysis of replication control in broad host-range plasmid RK2 (belonging to *E. coli* incompatibility group IncP; RK2 is similar or identical to RP4, RP1 and R68, Burkhardt *et al.*, 1979) is of interest because RK2 is capable of regulated replication in a wide range of Gram negative bacterial species (Datta and Hedges, 1972; Olsen and Shipley, 1973; Beringer, 1974; Cho *et al.*, 1975). An understanding of its copy number regulation may be useful for development of various types of broad host-range cloning vector. Also, the DNA sequence of the vegetative replication origin of RK2, *ori*$V_{RK2}$ (Stalker *et al.*, 1981), is very different from the equivalent regions of ColE1-type (Oka *et al.*, 1979) or IncFII plasmids (Stougard *et al.*, 1981b; Ryder *et al.*, 1982) and may therefore represent a rather different type of control circuit.

RK2 is 56.4 kb in size (for a general review, see Thomas, 1981a) and is maintained at ~4–7 copies per chromosome equivalent (Figurski *et al.*, 1979). Two regions of RK2 are essential for replication (Thomas, 1981b); the vegetative replication origin, *ori*$V_{RK2}$, located between coordinates 12.0 and 12.7 kb (defined clockwise from the unique *Eco*RI site) and the *trf*A gene, located between coordinates 16.0 and 17.5 kb, which provides a protein necessary for plasmid replication. There are no reports which describe clearly which part of RK2 carries the determinants for plasmid copy number control. However, expression of incompatibility has been mapped to certain regions. Plasmids which cannot be maintained stably together in a cell line without selection are termed incompatible and are thought to share replication and/or segregation functions which cause the plasmids to compete for stable maintenance. The regions responsible for certain aspects of these processes can be identified by cloning plasmid fragments into a compatible plasmid and testing for elimination of the parent plasmid by these hybrids. This could be due to competition for, and possibly titration of, essential replication or segregation functions, or to the production of negative regulators of replication initiation. Studies in *E. coli* on cloned segments of RK2 indicate that the *ori*$V_{RK2}$ region but not the *trf*A region, shows very strong incompatibility, suggesting that if a negative regulator of copy number exists then it is likely to be in or adjacent to *ori*$V_{RK2}$ (Meyer, 1979; Thomas *et al.*, 1980, 1981).

In studies on the DNA segments necessary for *ori*$V_{RK2}$ activity and expression of incompatibility (Thomas *et al.*, 1981) the smallest deletion which substantially reduced incompatibility expression removed ~50 bp and this deletion did not destroy *ori*$V_{RK2}$ function. This deletion lies in a group of three direct sequence repeats (Stalker *et al.*, 1981). Other deletions indicate that the series of eight direct repeats (Figure 4) may all be involved in incompatibility. To determine whether or not this incompatibility results from the action of copy number control functions we have constructed mini RK2 replicons isogenic except for the small *inc*$^-$ deletion referred to above. We present accurate mapping of this deletion, and determination of its effect on the incompatibility and copy

number of mini replicons into which it is incorporated. These studies have led to the discovery of a second plasmid copy number determinant which is also described.

## Results

### *Accurate determination of the deletion in pCT7.5*

It has previously been established (Thomas *et al.*, 1981) that a deletion of ~50 bp, which removes the two closely spaced *Hinc*II sites in the *Hae*II *ori*$V_{RK2}$ fragment (Figure 4), results in a considerable reduction in the incompatibility expressed towards an independent RK2 replicon by this fragment when cloned in a high copy number ColE1-derived vector (pCT7 has wild-type *ori*$V_{RK2}$, pCT7.5 has the deletion mutation). It was proposed that this deletion (△7.5) arose by homologous recombination between the directly repeated DNA sequences on either side of the *Hinc*II sites (Stalker *et al.*, 1981). To determine whether or not this is the case, the DNA sequence of this region (which has already been determined for the wild-type, Stalker *et al.*, 1981) was determined for the deletion mutant, pCT7.5, by labelling of the 3′ overhanging ends at the convenient *Sst*II site adjacent to *ori*$V_{RK2}$ (Figure 4), as described in Materials and methods. Part of one of the autoradiographs obtained covering the point of divergence between wild-type and mutant sequences is shown in Figure 1. The sample of pCT7.5 plasmid DNA was quite old because we wanted to use DNA as closely related as possible to the original isolate of this plasmid. Nicking during prolonged storage is therefore likely to be the cause of the positions where bands appear in all five sequencing tracks. Nevertheless, the assignment of the point of divergence of the two sequences is unambiguous and shows that the deletion mutant has lost 56 bp and retains only one of the group of three direct repeats adjacent to the minimal *ori*$V_{RK2}$ (Figure 1 and Figure 4). The exact point of recombination cannot be determined because of the identical sequences of the direct repeats.

### *Effect of the pCT7.5 deletion on mini RK2 plasmid copy number*

To determine the effect of the pCT7.5 deletion (△7.5) on mini RK2 plasmid copy number, pCT460 and pCT461 were constructed such that they are isogenic except for the △7.5 deletion in *ori*$V_{RK2}$ (Figure 2; Thomas, 1983). These plasmids are similar to pRK2501, Figure 4 (Kahn *et al.*, 1979), which is derived from the mini RK2 plasmid pRK248 (Thomas *et al.*, 1980). pRK248 consists of two RK2 segments each obtained by partial *Hae*II digestion. The *ori*$V_{RK2}$, $Tc^r$ *trf*A and *kil*D segment maps from coordinate 12.0 to 18.7 on RK2 while the *trf*B, *kor*A and *kor*D segment maps from 54.0 to 0.3 kb. In pRK2501 a 1430-bp *Hae*II fragment, originally from Tn903 (Oka *et al.*, 1981) and carrying $Km^r$, has been inserted into pRK248 between the *Bgl*II site near *ori*$V_{RK2}$ and the $Tc^r$ determinant. The construction of pCT460 (with the wild-type *Hae*II *ori*$V_{RK2}$ fragment) and of pCT461 (with the △7.5 deletion mutant *Hae*II *ori*$V_{RK2}$ fragment) means that these plasmids lack the RK2 DNA carrying the *Bgl*II site which lies between the *Hae*II *ori*$V_{RK2}$ fragment and the point of insertion of the *Hae*II $Km^r$ fragment in pRK2501 (Figure 4). This is because in pCT7 and pCT7.5 these two *Hae*II fragments are contiguous, while pCT141 carries the *Hind*III to *Eco*RI fragment of pRK2501 carrying $Tc^r$, *trf*A and *trf*B (Figure 2).

The copy numbers of pCT460 and pCT461 were determined as described in Materials and methods by both ethidium bromide/caesium chloride separation of total [$^3$H]thymine-labelled DNA giving a cccDNA/total DNA ratio, and also by comparison of the ratio of labelled pBR322 to test plasmid in alkaline SDS plasmid DNA extracts. Initially we assumed a copy number of 50 copies per chromsome equivalent for pBR322 and although it appears that the pBR322 copy number varies with growth conditions (Stueber and Bujard, 1982) the copy number values were very similar for both methods. We therefore ensured that growth of cultures during labelling was standardized and continued to assume a copy number of 50 for pBR322. The results are shown in Table I. Two points are clear. First, the △7.5 deletion results in an ~2-fold increase in plasmid copy number. Second, pCT460, which was constructed as a 'wild-type' plasmid has a copy number considerably higher than expected for pRK2501 or pRK248 which exist at copy numbers of 6–10 copies per

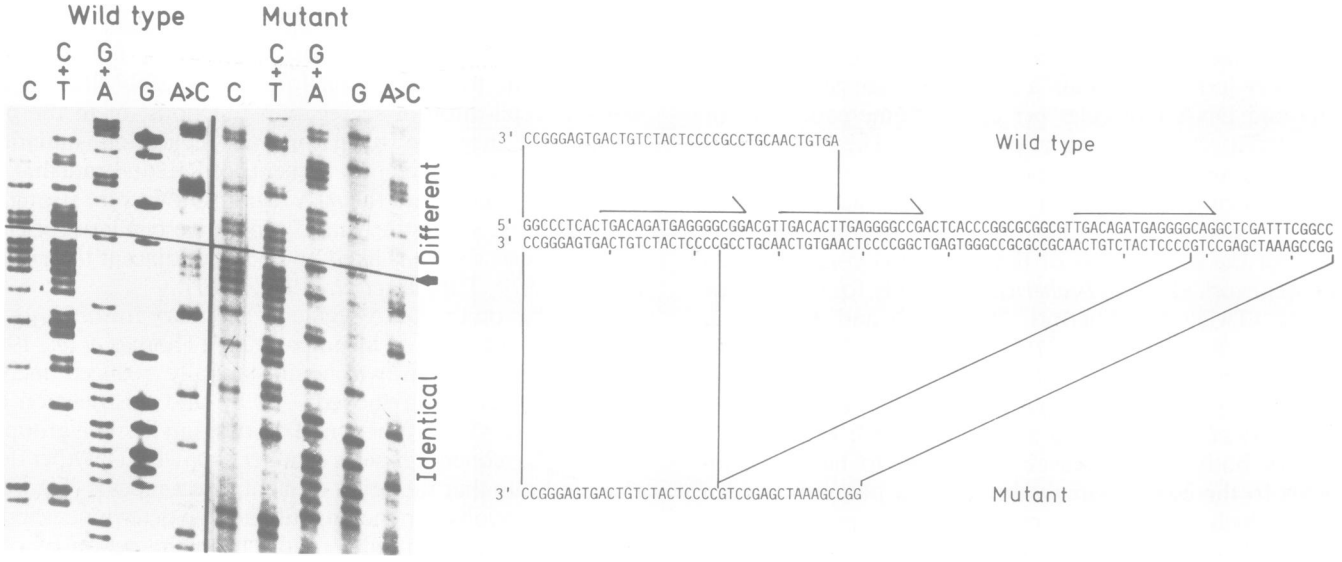


**Fig. 1.** DNA sequence analysis of the region of the △7.5 deletion. Fragments were radioactively labelled and sequenced as described in Materials and methods. Shown is the part of an autoradiograph of a gel covering the region where wild-type and mutant *ori*$V_{RK2}$ sequences diverge (marked with an arrow) due to the △7.5 deletion. The sequence of this region is also shown along with the sequence of wild-type and mutant which can be seen on this bit of gel. This shows clearly that the △7.5 deletion removed 56 bp and leaves only one copy of the direct repeat (→).

chromosome equivalent (Thomas *et al.*, 1980, 1981). It appears possible that the discrepancy between the copy number of pCT460 and pRK2501 may be due to the loss of the information between the *Hae*II *ori*$V_{RK2}$ and the *Hae*II Km$^r$ fragments.

*Effect on mini RK2 plasmid copy number of RK2 sequences clockwise from the HaeII ori$V_{RK2}$*

To test whether sequences clockwise from *Hae*II *ori*$V_{RK2}$ affect plasmid copy number, pCT539 and pCT543 were constructed, so as to carry these additional sequences and to be isogenic except for the presence of the △7.5 deletion, Figure 3. The plasmids consist of the *Eco*RI to *Sst*II *ori*$V_{RK2}$ fragments of pCT7 and pCT7.5, the *Sst*II to *Sal*I Km$^r$ fragment of pRK2501, and the *Sal*I to *Eco*RI *trf*A and *trf*B fragment of pCT141. pCT539, which has the 'wild-type' *ori*$V_{RK2}$ from pCT7, should be identical to pRK2501, while pCT543 differs only by the 56-bp deletion (△7.5) in the three direct repeat regions of *ori*$V_{RK2}$. Copy number determinations (Table I) show that pCT539 has a copy number of 11, close to that expected for pRK2501 and pRK248 (6–10 copies per chromosome equivalent, as measured by ethidium bromide/caesium chloride gradient; Thomas *et al.*, 1980, 1981),

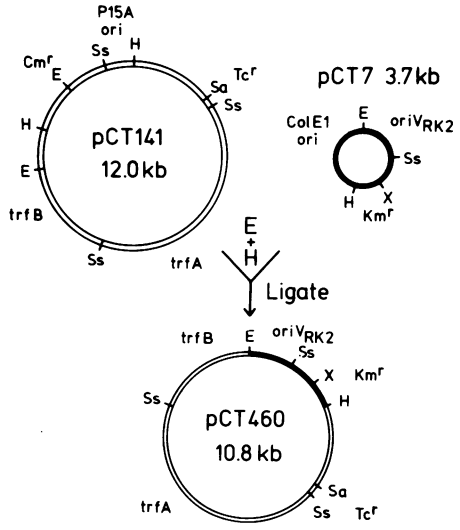


**Fig. 2.** Construction of pCT460 and pCT461. To construct pCT460, DNA of plasmids pCT7 and pCT141 was digested with *Eco*RI and *Hind*III and then, after ligation, transformed into MV10 selecting Tc$^r$Km$^r$. Transformants were screened for Cm$^s$ and plasmid DNA analysed from Tc$^r$Km$^r$Cm$^s$ bacteria. pCT461 was constructed in a similar way from pCT7.5 and pCT141. Restriction sites are represented by: *Eco*RI, E; *Hind*III, H; *Sal*I, Sa; *Sst*II, Ss; and *Xho*I, X.

**Table I.** Copy number of mini RK2 plasmids

| Plasmid | Plasmid copies/chromosome equivalent | |
|---|---|---|
| | Method A[a] | Method B[b] |
| pCT460 | 17 ± 1.0 | 18.4 ± 1.43 |
| pCT461 | 35 ± 6.7 | 42.5 ± 1.84 |
| pCT539 | | 11.04 ± 1.5 |
| pCT543 | | 17.14 ± 2.0 |

The numbers given are mean values of two independent determinations, each carried out in duplicate with their maximal deviations.
[a]CsCl/ethidium bromide separation of test plasmid cccDNA molecules from chromosomal DNA.
[b]Comparison of pBR322 and test plasmid in alkaline SDS extracts.

while pCT543 has a copy number of 17 copies per chromosome equivalent, ~60% higher. Part of the discrepancy between the observed copy numbers of pCT539 and pRK2501 may be explained by the slightly lower estimates obtained from density gradient separation when compared with the pBR322 comparative technique, as illustrated by the levels of both pCT460 and pCT461 (Table I). Thus it does seem that the sequences directly clockwise of the *Hae*II *ori*$V_{RK2}$ fragment do play a role in copy number control of mini RK2 plasmids. The effects of deleting either these sequences or the sequence deleted in △7.5, separately or in combination, where there is an almost directly additive effect (Table I), suggest that these additional sequences represent a second distinct copy control element. We propose to call the copy control element which lies entirely within the *Hae*II *ori*$V_{RK2}$ fragment *cop*A and the second element which lies at least partly outside this fragment, *cop*B.

*Analysis of incompatibility relationships between different ori$V_{RK2}$ segments*

The results presented for the △7.5 deletion show a clear connection between *ori*$V_{RK2}$ incompatibility expression and copy number control. For the *cop*B element however it is possible that this represents a *cis*-acting element, for example, a promoter or a transcription terminator, which might not express incompatibility. To test this and to determine, if possible, the general site(s) of action of these controlling elements, *ori*$V_{RK2}$ sequences were joined to pBR322 and tested for incompatibility. The *ori*$V_{RK2}$ fragment bounded by the unique *Eco*RI site and the *Xho*I site in the Km$^r$ gene, from pCT460, pCT461, pCT539 and pCT543 were joined to the large *Eco*RI to *Sal*I fragment of pBR322 creating plasmids pCT544, pCT545, pCT546 and pCT547, respectively. These plasmids were transformed into strains carrying pCT460, pCT461, pCT539 and pCT543 and the strength of incompatibility

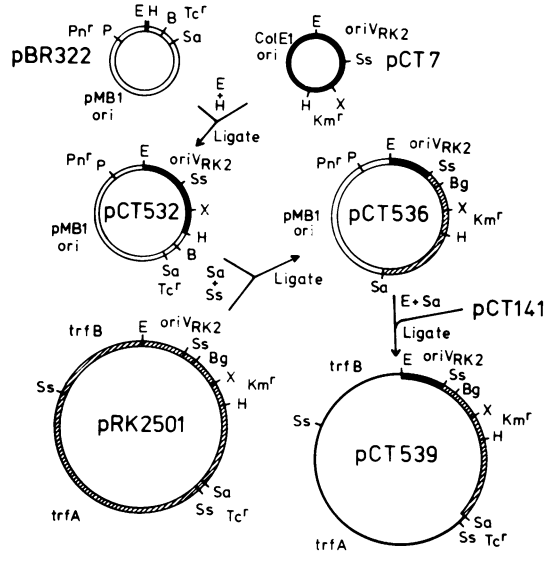


**Fig. 3.** Construction of pCT539 and pCT543. pCT539 was constructed by first joining the *ori*$V_{RK2}$ *Eco*RI to *Hind*III fragment of pCT7 to the large *Eco*RI to *Hind*III fragment of pBR322 giving pCT532 which is Pn$^r$Tc$^r$. A Pn$^r$Tc$^s$Km$^r$ plasmid (pCT536) was then obtained by digestion of pCT532 and pRK2501 with *Sal*I and *Sst*II and ligation. Finally, *Sal*I and *Eco*RI double digestion of pCT536 and pCT141 followed by ligation gave a Pn$^s$Tc$^r$Km$^r$Cm$^s$ plasmid, pCT539. pCT543 was constructed identically starting with pCT7.5 instead of pCT7 and utilizing the intermediates pCT533 and pCT540. Restriction sites are represented by: *Bam*HI, B; *Bgl*II, Bg; *Eco*RI, E; *Hind*III, H; *Pst*I, P; *Sal*I, Sa; *Sst*II, Ss; *Xho*I, X.

**Table II.** Incompatibility exhibited by different *ori*$V_{RK2}$ regions attached to high copy number vectors, towards various mini RK2 plasmids

| Incoming plasmid | Proportion of transformants retaining resident after 8 gen.[a] | | | |
|---|---|---|---|---|
| | pCT460 | pCT461 | pCT539 | pCT543 |
| pBR322 | 1.02 | $6.8 \times 10^{-1}$ | $8.5 \times 10^{-1}$ | 1.0 |
| pCT544 | $2.0 \times 10^{-4}$ | $5.0 \times 10^{-4}$ | $1.3 \times 10^{-4}$ | $1.2 \times 10^{-4}$ |
| pCT545 | $3.5 \times 10^{-1}$ | $8.0 \times 10^{-1}$ | $5.0 \times 10^{-2}$ | $1.3 \times 10^{-1}$ |
| pCT546 | $1.2 \times 10^{-4}$ | $1.4 \times 10^{-4}$ | $1.9 \times 10^{-4}$ | $1.2 \times 10^{-4}$ |
| pCT547 | $4.6 \times 10^{-5}$ | $2.8 \times 10^{-4}$ | $6.4 \times 10^{-5}$ | $1.1 \times 10^{-4}$ |
| pDS3 | 1.0 | $5.6 \times 10^{-1}$ | $7.5 \times 10^{-1}$ | 1.3 |
| pCT160 | $1.8 \times 10^{-3}$ | $3.5 \times 10^{-2}$ | $5.0 \times 10^{-4}$ | $1.5 \times 10^{-3}$ |

[a]This figure is taken from the graph obtained from data as described in Materials and methods. The rate of loss of the resident plasmid is proportional to the strength of incompatibility expressed by the incoming plasmid. The 8 generation point was chosen because the figures accurately reflect the trends of the graphs.

tested as described in Materials and methods. pBR322 was used as a negative control. In addition, a $Cm^r$ plasmid pCT160, where the *Hae*II *ori*$V_{RK2}$ fragment is inserted into a $Cm^rTc^s$ pACYC184 derivative was tested in the same way and pDS3, a $Cm^rTc^s$ pACYC184 derivative was used as a control.

The results (Table II) of these incompatibility tests are presented as the proportion of transformants retaining resident genetic markers eight generations after transformation. They indicate a number of points. First, in confirmation of the results of Thomas *et al.* (1981) the △7.5 deletion reduces the expression of incompatibility by the *Hae*II *ori*$V_{RK2}$ towards wild-type mini RK2 plasmids. We call the locus affected by this deletion *inc*A. Second, with the *ori*$V_{RK2}$ region cloned at high copy number, the clockwise adjacent sequences express a second incompatibility function, specified by *inc*B, which is evident in the presence of, and not affected by, △7.5 and which expresses incompatibility as strongly as wild-type *inc*A. Therefore, *cop*B does appear to be associated with an incompatibility function and therefore is likely to be a *trans*-acting rather than a *cis*-acting copy control element. Third, pCT460 is susceptible to both *inc*A and *inc*B indicating that both of these elements can act within the *Hae*II *ori*$V_{RK2}$ fragment. Fourth, there is some evidence that pCT461 is slightly less susceptible than pCT460 to both *inc*A and *inc*B. This is less clear for *inc*A than for *inc*B – the altered susceptibility towards *inc*A being only significantly observed when it is pCT160 that carries *inc*A. However, for neither incompatibility function does the △7.5 deletion totally abolish susceptibility, indicating that it has only a partial or an indirect effect on the site(s) of action of *inc*A and *inc*B. Fifth, the presence of *cop*B in mini replicons increases their sensitivity to *inc*A or mutant *inc*A. Thus pCT539 is more susceptible to mutant *inc*A on pCT545 than is pCT460. One possible explanation of this effect would be that *cop*B regulates some function which is itself the target of *cop*A (*inc*A). Then if this function is unregulated its level may rise to above the threshold where the negative action of the mutant *inc*A element can have an effect on replication large enough to result in resident plasmid instability. It appears, therefore, that both *cop*A and *cop*B have equivalent incompatibility loci (*inc*A and *inc*B) and that both can act on targets within the *Hae*III *ori*$V_{RK2}$. These targets may be interrelated in some way.

## Discussion

Previous attempts in this laboratory (unpublished) to obtain RK2-derived plasmid copy number mutants have been unsuccessful. As a means of avoiding a direct selection for increased copy number, a mutation in the *ori*$V_{RK2}$ region which has previously (Thomas *et al.*, 1981) been shown to reduce *ori*$V_{RK2}$-expressed incompatibility, *inc*A, has been incorporated into a mini RK2 plasmid. Comparison of the copy number of otherwise isogenic plasmids indicates that this 56 bp deletion, which apparently has occurred by homologous recombination between the outside copies of the three direct repeats in the *Hae*II *ori*$V_{RK2}$ fragment, results in a 60–100% increase in plasmid copy number. This strongly suggests that *inc*A, whose expression is affected by this deletion, represents part of a copy number control system. Since copy number control and incompatibility expression are measured in different ways and may not always behave in the same way, we designate the equivalent copy number control gene *cop*A. This finding is in line with other systems where incompatibility often seems to represent copy control elements.

The results presented indicate that not all the elements required to regulate the copy number of RK2 are present within the *Hae*II *ori*$V_{RK2}$ fragment. Thus, plasmid pCT460 has a copy number of 17–20 copies per chromosome equivalent, compared with the estimated copy number of RK2 which is 4–7 copies per chromosome equivalent (Figurski *et al.*, 1979). The copy number of pCT460 is similar to, although slightly higher than, the range of 10–16 reported previously for the *Hae*II *ori*$V_{RK2}$ mini plasmid pCT45 (Thomas *et al.*, 1981; Thomas, 1981b) but differences could be due to the exact genetic context of the *Hae*II *ori*$V_{RK2}$ fragment. An important regulatory element appears to lie at least partly outside the *Hae*II *ori*$V_{RK2}$ fragment in the clockwise direction, i.e., between *ori*$V_{RK2}$ and $Tc^r$. Thus pCT539, which should have all of the DNA normally lying between *ori*$V_{RK2}$ and $Tc^r$ has a copy number of ~11 copies per chromosome equivalent. While this is higher than the previously reported figures of ~6 copies per chromosome equivalent for pRK2501 (Thomas *et al.*, 1981) and its parent pRK248 (Figurski *et al.*, 1978; Meyer, 1979; Thomas *et al.*, 1980) a number of the more recent determinations for pRK2501 and pRK248 are rather higher than this, being in the range 8–10 copies per chromosome equivalent. While there is no evidence that insertion of a *Hae*II fragment carrying $Km^r$ into pRK248 to give pRK2501 alters plasmid copy number it is quite possible that both these plasmids lack some regulatory element present in parental RK2. Although there may be problems in accurate copy number determination for RK2 due to its size and existence partly as a relaxation complex (Guiney and Helinski, 1979), which could lead to an underestimate, determinations for indistinguishable plasmids like RP4 (N. Grinter, personal communication) have suggested that the lower end of the range, 4–7 copies per chromosome equivalent, is probably more realistic. Therefore, pRK248 may have a copy number about twice that of RK2, a factor as large as that caused by deletions in either *cop*A or *cop*B. It is possible that as yet uncharacterized control elements lie elsewhere on RK2. Indeed, other weakly acting incompatibility loci have been located (Sakanyan *et al.*, 1978; Meyer and Hinds, 1982; K. Ellis and P. Barth, personal communication; A. Hussain and C.M. Thomas, in preparation), although these may reflect partitioning functions rather than replication control.

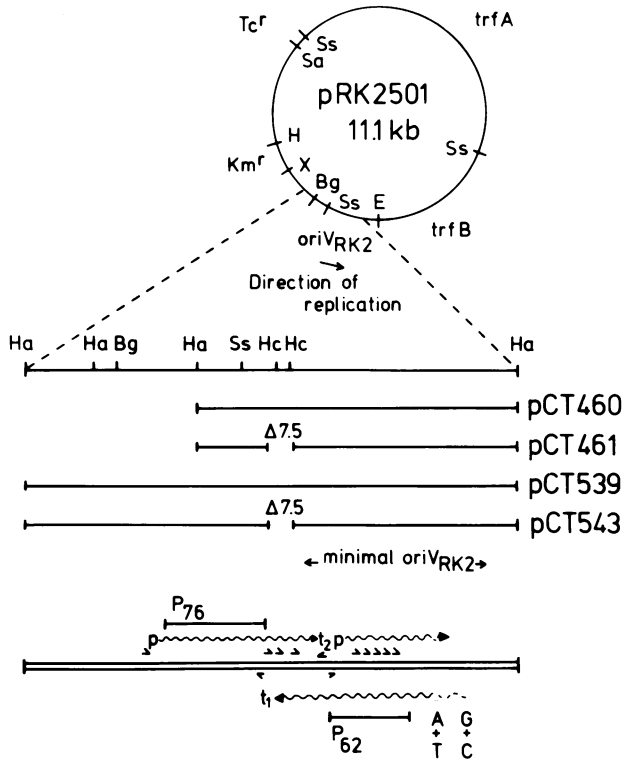


**Fig. 4.** Structure of mini plasmids used for copy number determination and schematic representation of potentially important features of the *ori*$V_{RK2}$ region as deduced from the DNA sequence (Stalker *et al.* 1981; Waters, 1982; Waters *et al.*, 1983). All mini plasmids are similar to pRK2501. Expanded is the segment between $Km^r$ *Hae*II fragment insertion site and the end of *Hae*II *ori*$V_{RK2}$. The extent of deletions is shown. Restriction sites are shown as: *Bgl*II, Bg; *Eco*RI, E; *Hae*II, Ha; *Hinc*II, Hc; *Hind*III, H; *Sal*I, Sa; *Sst*II, Ss; *Xho*I, X. Features of the *ori*$V_{RK2}$ region are shown as: direct repeats, →; putative promoters, p; putative transcription terminators, t; open translational reading frames, $P_{76}$, $P_{62}$; A+T- and G+C-rich DNA segments, A+T, G+C.

The way in which *cop*A and *cop*B act is as yet unknown. Both of these elements express incompatibility suggesting that they are not simply *cis*-acting functions. It is possible that their incompatibility is due to titration of some positively required function. However, since the minimal *ori*$V_{RK2}$, which might be expected to bind such a function, does not express this sort of incompatibilty (Thomas *et al.*, 1981) it seems more likely that *cop*A and *cop*B are due to *trans*-acting products with site(s) of action within the *Hae*II *ori*$V_{RK2}$ fragment. Results with pCT40 (Thomas *et al.*, 1981) which contains only the 393-bp *Hpa*II minimal *ori*$V_{RK2}$ fragment, suggest that the *cop*A (*inc*A) element acts within this minimal *ori*$V_{RK2}$ region (Figure 4). Various factors may of course modulate the sensitivity to both *cop*A (*inc*A) and *cop*B (*inc*B) as illustrated by the fact that the presence of *cop*B in *cis* increases the sensitivity of a mini replicon to mutant *inc*A (*cop*A), Table II. At present we are able to construct hypotheses about *cop*A and B using the data in this paper combined with the nucleotide sequence of this region. In discussing the nature of *cop*B it is of interest that the region of RP1 (very similar if not identical to RK2, Burkhardt *et al.*, 1979) surrounding $Tc^r$ and extending to give overlap with *ori*$V_{RK2}$ has been determined recently (Waters, 1982; Waters *et al.*, 1983). As shown in Figure 4, there are a number of features of possible significance in the region required for *cop*B. First, there is an extra single copy of the *ori*$V_{RK2}$ repeated sequence close to the *Bgl*II site. Second, there is an open reading frame proceeding from close to the *Bgl*II site towards *ori*$V_{RK2}$ which would code for a polypeptide of 76 amino acids ($P_{76}$, mol. wt. 8435 daltons). This open reading frame is preceded by a possible Shine-Dalgarno ribosome-binding site (GAG) and a putative promoter sequence (−35, TTGACA −10, GAAATT) with considerable homology to the consensus sequence (Hawley and McClure, 1983). The extra direct repeat overlaps the −35 sequence and could be of regulatory significance. The putative polypeptide would be rich in positively charged amino acids, particularly arginine, which would be consistent with it binding to DNA in a negatively regulatory capacity. Studies are underway to test the existence of $P_{76}$ and to determine whether it is required for *cop*B function.

One or more copies of the directly repeated sequence appear in both regions so far identified as *inc* or *cop* loci, although the largest group of direct repeats do not on their own express incompatibility under all circumstances (Thomas *et al.*, 1981). Direct repeats occur in other replication systems: R6K (Stalker *et al.*, 1979), miniF (Tolun and Helinski, 1981; Murotsu *et al.*, 1981); P1 (A.S.A. Abeles, D. Chattoraj and M. Yarmolinsky, reported at EMBO Workshop, 1982) and lambda (Grosschedl and Hobom, 1979; Moore *et al.*, 1979). In the case of R6K, miniF and P1 at least some of the direct repeats have been implicated in incompatibility expression and possibly copy number regulation. For R6K, at least, expression of incompatibility can be increased by raising the level of transcription across the direct repeats (A. Shafferman, S. Young and D.R. Helinski, reported at EMBO Workshop, 1981). In the case of *ori*$V_{RK2}$ the *cop*A region does not coincide with any likely open reading frame identified from DNA sequence (Stalker *et al.*, 1981). Preliminary experiments (Thomas, unpublished) indicate the existence of transcription in a direction opposite to replication through the groups of five and three direct repeats. This may terminate at a putative rho-independent transcription terminator, $t_1$ (Figure 4). Thus an RNA transcript across the direct repeats could be the basis of *cop*A but exactly how it would regulate initiation remains to be determined. The direct repeat in the *cop*B region is intriguing but its significance also remains to be determined.

Until the RNA and protein products of the *ori*$V_{RK2}$ region are fully characterized it will be impossible to say how similar the copy number control circuits of RK2 are to those of ColE1-like plasmids and plasmids of the IncFII group (for references see Introduction). it is tempting to speculate, however, that *cop*A of RK2 is a negatively acting RNA molecule that interferes with RNA primer function (see Figure 4) and that *cop*B may be a negatively acting protein that interferes either with initiation of the primer transcript or with the action of *trf*A or host specified proteins in the *ori*$V_{RK2}$ region. Current experiments are in part aimed to test this hypothesis.

Previously it has been suggested that *trf*A may play a role in copy number control by being regulated to give a standard amount of *trf*A protein per cell which would determine the rate of replication (Thomas *et al.*, 1981). The results in this paper indicate that copy number control is primarily located close to *ori*$V_{RK2}$ in the broad host-range plasmid RK2.

**Table III.** Plasmids used in this study

| Plasmid | Size (kb) | Selective markers | Other properties | Source of reference |
|---|---|---|---|---|
| pBR322 | 4.363 | $Tc^rPn^r$ | pMB1 replicon | Bolivar *et al.*, 1977 |
| pDS3 | 2.5 | $Cm^r$ | P15A replicon (pACYC184 derivative) | D. Stalker, unpublished |
| pRK2501 | 11.1 | $Tc^rKm^r$ | RK2 replicon | Kahn *et al.*, 1979 |
| pCT4.3 | 7.0 | $Km^r$ | ColE1 and RK2 replicon | Thomas *et al.*, 1980 |
| pCT7 | 3.7 | $Km^r$ | ColE1 replicon/*ori*$V_{RK2}$ | Thomas *et al.*, 1980 |
| pCT7.5 | 3.65 | $Km^r$ | ColE1 replicon/*ori*$V_{RK2}$ | Thomas *et al.*, 1981 |
| pCT141 | 12.1 | $Tc^rCm^r$ | pDS3/*trf*$A^+$ *trf*$B^+$ | Thomas, 1981b |
| pCT160 | 3.0 | $Cm^r$ | P15A replicon/*ori*$V_{RK2}$ | Thomas, 1981b |
| pCT460 | 10.8 | $Tc^rKm^r$ | RK2 replicon | Thomas, 1983 |
| pCT461 | 10.75 | $Tc^rKm^r$ | RK2 replicon | Thomas, 1983 |
| pCT539 | 11.1 | $Tc^rKm^r$ | RK2 replicon | This study |
| pCT543 | 11.05 | $Tc^rKm^r$ | RK2 replicon | This study |
| pCT544 | 5.1 | $Pn^r$ | pMB1 replicon/*ori*$V_{RK2}$ | This study |
| pCT545 | 5.05 | $Pn^r$ | pMB1 replicon/*ori*$V_{RK2}$ | This study |
| pCT546 | 5.4 | $Pn^r$ | pMB1 replicon/*ori*$V_{RK2}$ | This study |
| pCT547 | 5.35 | $Pn^r$ | pMB1 replicon/*ori*$V_{RK2}$ | This study |

## Materials and methods

*Bacterial strains, plasmids and growth conditions*

*E. coli* K12 strains C600△*trp*E5 (*thr leu thi lac*Y *sup*E *trp*E5) designated MV10 and C600*thy* (*thr leu thi lac*Y *sup*E *thy*), designated CR34, were used. Plasmid constructions were carried out using MV10 while labelling of DNA with [$^3$H]thymine for plasmid copy number determinations was carried out in CR34. Plasmids used in this study are listed in Table III. Routine bacterial growth was carried out in L broth (Kahn *et al.*, 1979) or on L agar plates (L broth with 1.5%, w/v, agar). Labelling of DNA was carried out during growth in M9.CAA medium (Kahn *et al.*, 1979). For growth of CR34, medium was normally supplemented with thymine at 20 μg/ml except during labelling with [$^3$H]thymine when 5 μg/ml was used (see below). Medium was supplemented with antibiotics to select for the presence of plasmids carrying resistance genes: chloramphenicol, 25 μg/ml, for $Cm^r$; kanamycin, 50 μg/ml, for $Km^r$; penicillin, 300 μg/ml in L agar, 150 μg/ml in L broth for $Pn^r$ and $Ap^r$ (ampicillin resistance); tetracycline, 20 μg/ml, for $Tc^r$. All bacteria were grown at 37°C.

*Bacterial transformation*

Bacteria were made competent for transformation as described by Meyer *et al.* (1977) except that bacteria were grown to exponential phase in L broth which was inoculated from a fresh stock plate.

*Isolation and analysis of plasmid DNA*

Plasmid DNA was extracted from bacteria – both exponentially growing and saturated cultures – by the akaline SDS extraction procedure of Birnboim and Doly (1979) with minor modification as described by Smith and Thomas (1983). This DNA was suitable for both gel analysis and *in vitro* recombination. Agarose gel electrophoresis was carried out as described by Kahn *et al.* (1979).

For DNA sequence analysis, DNA was isolated by the Triton X-100 cleared lysate method (Kahn *et al.*, 1979) and then further purified by caesium chloride/ethidium bromide equilibrium density gradient centrifugation as described by Meyer *et al.* (1977).

*Restriction endonucleases and in vitro recombination*

Enzymes were purchased from either Bethesda Research Laboratories, Cambridge Uniscience Limited or Boehringer Chemicals Limited, and used under conditions similar to those recommended by the manufacturers.

*DNA nucleotide sequence analysis*

Caesium chloride/ethidium bromide gradient purification DNA of plasmids pCT4.3 and pCT7.5, containing respectively wild-type and deletion mutant *ori*$V_{RK2}$, was digested with *Sst*II and the fragments labelled at the 3′ end using terminal transferase and [$\alpha$-$^{32}$P]cordycepin triphosphate (from Amersham International at a specific activity of 2000 – 5000 Ci/mmol). After secondary cleavage with *Eco*RI, fragment separation by agarose gel electrophoresis and electroelution of the required bands from gel slices, the nucleotide sequences of singly end-labelled fragments were determined by the method of Maxam and Gilbert (1980), with some modifications. The A > C hot alkali reaction was used in addition to the C-, (C + T)-,(A + G)- and G-specific reactions, to increase the certainty of A/G assignments. After the base-specific reactions the samples were re-precipitated twice, washed three times with ethanol and lyophilized before the piperidine cleavage step. Incubations at 90°C were carried out with samples sealed in glass capillaries.

*Plasmid copy number determination*

Plasmid copy number was estimated using separation of plasmid cccDNA from total DNA on ethidium bromide/caesium chloride gradients as previously described (Thomas *et al.*, 1980).

A second method employed was to introduce the test plasmid into C600*thy*(pBR322), pBR322 being present as an internal standard. After labelling of total DNA with [$^3$H]thymine as for the previous method, plasmid DNA was extracted by the alkaline/SDS method. Plasmid DNA was digested with *Eco*RI, separated by 0.9% agarose gel electrophoresis, the bands cut out and total $^3$H radioactivity determined by melting each band in 1 ml $H_2O$ using a microwave oven, mixing with 9 ml aquasol scintillant (New England Nuclear) and counting. Background counts were estimated from a gel slice taken from between the two plasmid bands. The ratio of counts in each plasmid, together with their size and an estimated copy number of pBR322, allowed the test plasmid copy number to be estimated.

*Determination of plasmid incompatibility*

The incompatibility tests carried out involved segments of DNA carrying *ori*$V_{RK2}$, linked to a compatible vector, being transformed into a strain carrying a mini RK2 plasmid. Strain MV10 carrying a mini RK2 plasmid was freshly constructed by transformation. Transformants were purified by streaking and used to make competent bacterial suspensions which were transformed with freshly prepared plasmid DNA. After heating shock and addition of L broth to allow expression of transformed markers, the cells were incubated at 37°C for 2 h before being diluted 10-fold into L broth containing antibiotics selective for the incoming plasmid. These cultures were grown with shaking at 37°C. Immediately, and after a further 3 and 5 h, samples were taken, serially diluted and spread on appropriate selective agar plates to determine numbers of transformants and the proportion of these retaining the resident plasmid.

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