JOURNAL OF BACTERIOLOGY, July 1985, p. 228–237
0021-9193/85/070228-10$02.00/0

Vol. 163, No. 1

Replication Control in Promiscuous Plasmid RK2: *kil* and *kor* Functions Affect Expression of the Essential Replication Gene *trfA*

HELEN C. SCHREINER, DAVID H. BECHHOFER,† ROBERT F. POHLMAN,‡ CALVIN YOUNG, PAULA A. BORDEN, AND DAVID H. FIGURSKI*

Department of Microbiology and Cancer Center, College of Physicians & Surgeons, Columbia University, New York, New York 10032

Received 25 January 1985/Accepted 17 April 1985

We previously reported that broad-host-range plasmid RK2 encodes multiple host-lethal *kil* determinants (*kilA*, *kilB1*, *kilB2*, and *kilC*) which are controlled by RK2-specified *kor* functions (*korA*, *korB*, and *korC*). Here we show that *kil* and *kor* determinants have significant effects on RK2 replication control. First, *korA* and *korB* inhibit the replication of certain RK2 derivatives, unless plasmid replication is made independent of the essential RK2 gene *trfA*. Second, *kilB1* exerts a strong effect on this interaction. If the target plasmid is defective in *kilB1*, sensitivity to *korA* and *korB* is enhanced at least 100-fold. Thus, *korA* and *korB* act negatively on RK2 replication, whereas *kilB1* acts in a positive manner to counteract this effect. A mutant RK2 derivative, resistant to *korA* and *korB*, was found to have fused a new promoter to *trfA*, indicating that the targets for *korA* and *korB* are at the 5′ end of the *trfA* gene. We constructed a *trfA-lacZ* fusion and found that synthesis of β-galactosidase is inhibited by *korA* and *korB*. Thus *korA*, *korB*, and *kilB1* influence RK2 replication by regulating *trfA* expression. We conclude that the network of *kil* and *kor* determinants is part of a replication control system for RK2.

The incompatibility group P (IncP) plasmids are remarkable for their promiscuity among gram-negative bacteria (8, 31). In sharp contrast to most plasmids which are confined to bacterial species only closely related to their original hosts (8, 17), the IncP plasmids can conjugally transfer to virtually all gram-negative species, and moreover, they replicate in each of these hosts. Because the IncP plasmids are able to mobilize host bacterial genes during conjugation (15) and to confer on the hosts sensitivity to various phages (32, 33, 47), they must be highly significant agents for gene exchange among gram-negative bacteria in nature. In addition, they have become important tools for genetic analysis and gene cloning in genetically uncharacterized bacterial species (10, 14, 19, 22, 40, 57).

We are studying these plasmids to understand the genetic and molecular basis of their ability to be maintained efficiently in a variety of hosts. Work in our laboratory has focused on the 56-kilobase (kb) IncP plasmid RK2 (16, 23), which is closely related, if not identical, to the other commonly known IncP plasmids RP1, RP4, R68, and R18 (4, 48, 54). Two plasmid loci essential for RK2 replication have been identified. One is the origin of unidirectional replication, *oriV*, located first by electron microscopy of replicating molecules of RK2 (24) and subsequently by genetic (11, 51, 52) and nucleotide sequence (46) analysis. *oriV* is activated by a diffusible function encoded elsewhere on the plasmid (11). This second locus was named *trfA* (51) and is now known to encode three polypeptides, at least one of which is functional in replication (21, 38, 44, 49). *oriV* and *trfA* together are sufficient to produce a replicon which will replicate in several different hosts (36). Thus, *oriV* and *trfA* represent the essential minimum for RK2 replication, and these determinants have at least some broad-host-range capability.

In addition to these essential replication loci, we found two new classes of determinants on RK2: (i) *kil* determinants which are lethal to the host cell, and (ii) *kor* (kil override genes) which control the *kil* determinants (12; for review, see reference 13). Originally, we found that RK2 encodes three *kil* loci (*kilA*, *kilB*, and *kilC*) and that each locus was controlled by a corresponding *kor* gene (*korA*, *korB*, and *korC*). We since learned that these functions are interconnected. *korA* is also required as a positive regulator of *korC* to control *kilC* (58), and *korB* is expressed in the same operon as is *korA* (3, 43; D. H. Bechhofer, Ph.D. thesis, Columbia University, New York, 1984). Furthermore, *kilB* has two components *kilB1* and *kilB2* (34). *kilB1* can be controlled by either *korA* or *korB* (13), whereas the presence of *kilB2* necessitates *korB* (34). Smith and Thomas (42) also found a second *kil*, analogous to *kilB1*, in the *kilB* region, but they named it *kilD*, with *korD* as its corresponding controlling element. A summary of these interactions is shown in Fig. 1.

Although the functions of these genes are not readily discernible from their phenotypes, we found a clue in that all of 14 different IncP plasmids tested encoded *korA* and *korB* analogs able to control the RK2 *kilA* and *kilB* determinants (12). No plasmid from any of 19 other incompatibility groups was found to encode such functions. The plasmids tested included the IncFI plasmid F, which has subsequently been found to encode host-lethal functions (27, 28, 30), and the IncN plasmid R46, whose derivative pKM101 is now known to encode *kil*- and *kor*-like genes (56). Thus these types of functions may be common among plasmids. Nevertheless, the complexity and specificity of the particular *kil* and *kor* loci from RK2 are apparently unique to the IncP group of plasmids. It was speculated that the conservation of these plasmid determinants in the IncP group might reflect their importance to plasmid maintenance, particularly in non-*Escherichia coli* hosts (12). This idea is supported by the

* Corresponding author.
† Present address: Department of Microbiology, The Public Health Research Institute of the City of New York, NY 10016.
‡ Present address: Department of Biochemistry, University of Minnesota, Minneapolis, MN 55108.

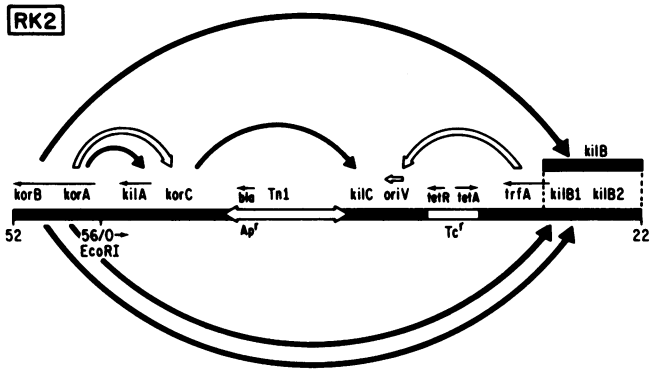

FIG. 1. Genetic interactions of plasmid RK2. A 26-kb segment of RK2 is displayed with coordinates representing distance in kb clockwise from the unique *Eco*RI site on the RK2 circular map. Positive interactions are depicted by white arcs, and negative interactions are depicted by black arcs. The direction of transcription for *trfA* (38), *korA* and *korB* (3, 43; Bechhofer, Ph.D. Thesis), *kilA* (13), and *bla* is indicated above the gene; the arrow over *oriV* shows the direction of replication. *kilB* is shown with its two determinants *kilB1* and *kilB2*. Positions of the *tet* genes are from Waters et al. (55). All other relevant references are given in the text.

finding that a host range mutation of RP4 maps in the *korA-korB* operon (1, 2).

In this report we show evidence that *kil* and *kor* determinants are directly involved in RK2 maintenance by affecting control of the *trfA* replication gene. In particular, we found that *korA* and *korB* are each able to inhibit expression of *trfA* and that *kilB1* acts in a positive manner to suppress this inhibition. These results link the *kil* and *kor* network of interactions to the control of RK2 replication. We propose that the entire *kil* and *kor* system is part of a replication control mechanism whose function may be important to the broad host range of IncP plasmids. A preliminary report of this work was presented previously (13).

MATERIALS AND METHODS

Nomenclature. Coordinates of the RK2 physical map are defined by the distance from the *Eco*RI site in kb and are designated by a prime (e.g., 50′ to 56′ region).

Bacterial strains and plasmids. *E. coli* K-12 MV10 (*ΔtrpE5 thr leu thi lacY supE44 tonA21*) is a C. Yanofsky strain. Strain RP1770 is a *korA*$^+$ *korB*$^+$ derivative of strain MV10 with the 50′ to 56′ region of RK2 integrated into the bacterial chromosome. It was isolated as a Trp$^+$ clone after transformation with the circularized *Eco*RI fragment of pRK2108 that encodes *trpE* and the 50′ to 56′ region (R. F. Pohlman, Ph.D. thesis, Columbia University, New York, 1983). Strain MC1061 is Δ(*lacIPOZY A*?)X74 *galK galU strA araD139* Δ(*araCOIBA leu*)7697 *hsdR* (37) and is from M. Casadaban. All plasmids used in this study are described in the text and shown in the figures.

Media. Media for growth and selection of bacteria have been described previously (12). Where necessary, solid media contained 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside per ml to show Lac$^+$ colonies as blue (29).

DNA methodology. Preparation of plasmid DNA and agarose gel electrophoresis were done by previously published procedures (18). Restriction endonucleases and T4 DNA ligase were purchased from commercial suppliers and used as specified. Transformation of *E. coli* was by the method of Cohen et al. (7).

Relative transformation efficiency. For comparison of transformation frequencies of different strains with the same plasmid, the number of transformants per milliliter was corrected for minor differences in competence of the test strains relative to the control strain. This was measured by transforming the strains with saturating amounts of a plasmid unrelated to the test plasmid or any resident plasmids. Variations in competence were no more than threefold and usually much less.

Relative transformation efficiency was then calculated as: Relative transformation efficiency = (corrected transformants per milliliter of test strain)/(transformants per milliliter of control strain).

β-galactosidase assay. β-Galactosidase activity of log-phase cultures was assayed by the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside as described by Miller (29).

RESULTS

The RK2 50′ to 56′ region inhibits replication of mini-RK2 plasmids in *trans*. We noticed an unexpected interaction between pRK248, a mini-RK2 replicon (Fig. 2), and pRK2108, a hybrid plasmid with the cloned 50′ to 56′ region of RK2 (Fig. 3). When pRK2108 was present in an *E. coli* host, transformation of the strain by pRK248 was at least 10-fold less efficient than that of a strain lacking pRK2108 (Table 1). The fewer transformants of the pRK2108-containing strain also showed heterogeneity in colony size.

The reduction in transformation efficiency mediated by pRK2108 was specific for RK2 replicons. Transformation by pCT5, another mini-RK2 plasmid (Fig. 2), was also affected by the presence of pRK2108 in the recipient, whereas pBR325, a plasmid unrelated to RK2, displayed no difference in transformation of the two hosts (Table 1). This was true for all other non-RK2 plasmids we tested (data not shown). Thus, some determinant on pRK2108 is responsible for interfering with the establishment of mini-RK2 plasmids specifically. For convenience, we refer to this phenomenon as interference (INF).

The INF determinant is located within the RK2 DNA carried on pRK2108, as can be seen when pRK2104 was used in place of pRK2108. These two plasmids are unrelated except for the cloned region of RK2 (Fig. 3), yet pRK2104 was as effective as pRK2108 in reducing the transformation

TABLE 1. Effect of the 50′ to 56′ region on transformation efficiency of mini-RK2 replicons

Incoming plasmid	Replicon(s)	Resident plasmid	Relative transformation efficiency[a]
pRK248	RK2		1.0
pRK248	RK2	pRK2108	0.04
pRK248	RK2	pRK2104	0.09
pCT5	RK2		1.0
pCT5	RK2	pRK2108	0.02
pBR325	pMB1		1.0
pBR325	pMB1	pRK2108	1.4
pRK2511	RK2		1.0
pRK2511	RK2	pRK2108	0.1
pRK2512	RK2, pMB1		1.0
pRK2512	RK2, pMB1	pRK2108	1.1

[a] For each incoming plasmid, values are normalized to that of the plasmidless recipient as described in the text. We arbitrarily defined inhibition as a 10-fold or more reduction in transformation efficiency.

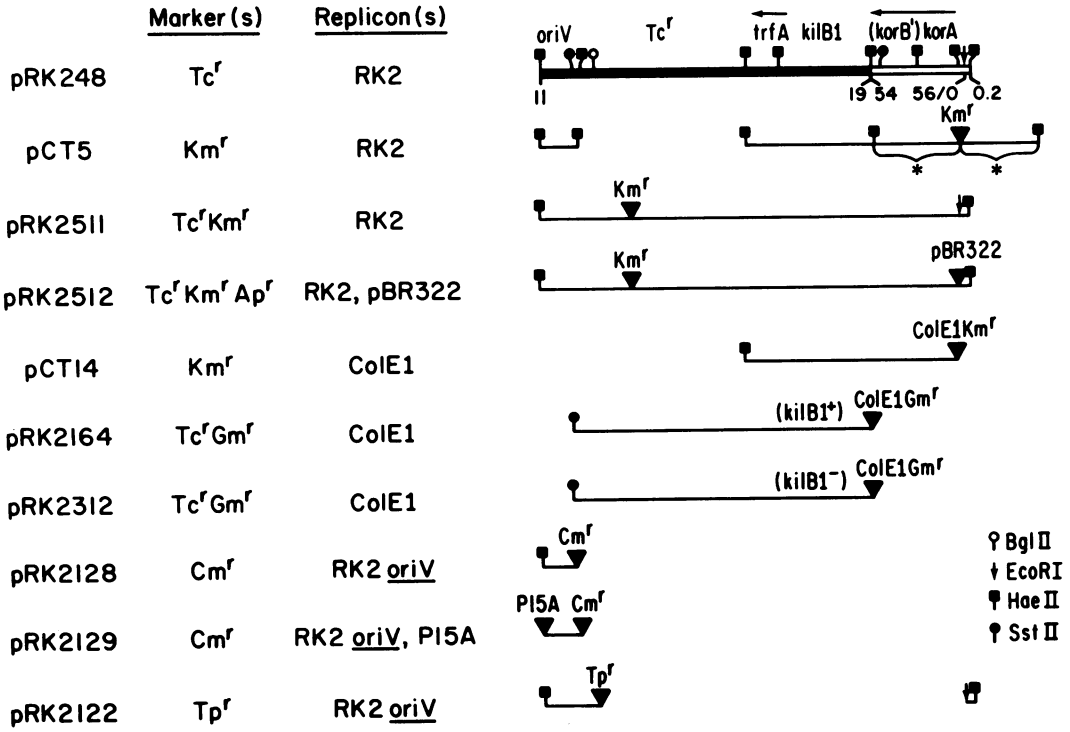

FIG. 2. Structures of pRK248 and its derivatives. The linear map on the top right shows the coordinates and location of the two RK2 segments (filled and unfilled lines) which comprise pRK248 (51). The direction of transcription is shown by arrows, *korB'* indicates that only a portion of the gene is present, and only relevant restriction sites are marked. Thin lines below show the regions of pRK248 present in each of the plasmids specified. The construction of several plasmids has been reported: pCT5 and pCT14 (51), pRK2164 (34), and pRK2122 and pRK2128 (35). pRK2129 is similar to pRK2128 except for an additional *Hae*II fragment that encodes the P15A replicon of pACYC184 (5). The asterisk in pCT5 shows a region that is duplicated. pRK2312 is the spontaneous *kilB1* mutant of pRK2164 found in strain RP1323 (34). The identity and position of the Km^r-encoding fragment in pRK2511 is identical to that of pRK2501 (18).

efficiency of pRK248 (Table 1). Thus, the INF determinant is specified by the 50′ to 56′ region of RK2.

If this interaction affects the RK2 replication system in the mini-plasmids, then covalent linkage of a non-RK2 replicon to the sensitive plasmid should suppress the inhibitory effect of the INF region. To test this, we compared the transformation efficiency of a pRK248 derivative (pRK2511) with that of a hybrid plasmid (pRK2512) consisting of pRK2511 and the unrelated replicon pBR322 (Fig. 2). Plasmid pRK2511, which is dependent solely on the RK2 system for replication, was sensitive to the INF region, as expected (Table 1). In contrast, the hybrid replicon pRK2512, which has pBR322 as a second replication system, was unaffected. Because inhibition can be bypassed by the addition of another replicon to the plasmid, we conclude that the ultimate effect of the INF determinant is inhibition of RK2-specific replication.

The target for INF is associated with the RK2 replication determinants. To locate the targets for INF, we asked whether the effect occurs with plasmids carrying only the minimal components for replication: *oriV*, the origin of replication, and *trfA*, which encodes a function that activates *oriV*.

Plasmid pRK2312 is a ColE1 plasmid with the cloned *trfA* gene of RK2 (Fig. 2). Although the region known to encode *kilB1* is also present, pRK2312 is defective in the *kilB1* determinant because it was selected as a spontaneous Kil$^-$ mutant (34). The only function known to be active in this region is *trfA*. Because *trfA* is expressed from pRK2312, it supported replication of the *oriV* plasmid pRK2128 (Fig. 2) in *trans*. This is clearly demonstrated by the ability of a pRK2312-containing strain to be transformed efficiently by the *oriV* plasmid pRK2128 (Table 2). Therefore, with these two plasmids, the RK2 replication system has been simplified to its minimal components *trfA* and *oriV*.

We asked whether the presence of the INF region in the cell would disrupt plasmid replication in this minimal system. The INF$^+$ plasmid pRK2108 completely prevented the ability of the *trfA*$^+$ plasmid pRK2312 to support the *oriV* plasmid pRK2128 (Table 2). This represents a 500-fold or more decrease in transformation efficiency. When the *oriV* plasmid contained the non-RK2 replicon P15A, as in pRK2129 (Fig. 2), there was no inhibition of transformation by the INF region (Table 2). Thus, with the basic components for RK2 replication, we can mimic the INF interaction

TABLE 2. Sensitivity of RK2 *oriV* plasmids to INF

Incoming plasmid	Replicon(s)	Resident plasmid(s)		Relative transformation efficiency[a]
		trfA$^+$ helper	Interfering	
pRK2128	RK2 *oriV*	pRK2312		1.0
pRK2128	RK2 *oriV*	pRK2312	pRK2108	<0.002
pRK2129	RK2 *oriV*, P15A	pRK2312		1.0
pRK2129	RK2 *oriV*, P15A	pRK2312	pRK2108	0.7
pRK2128	RK2 *oriV*	pCT14		1.0
pRK2128	RK2 *oriV*	pCT14	pRK2108	1.2
pRK2128	RK2 *oriV*	pRK2164[b]	pRK2108	1.0

[a] For each incoming plasmid, values are normalized to that of the strain lacking pRK2108 as described in the text.

[b] Because pRK2108 is *korA*$^+$, no other plasmid is required to control *kilB1*.

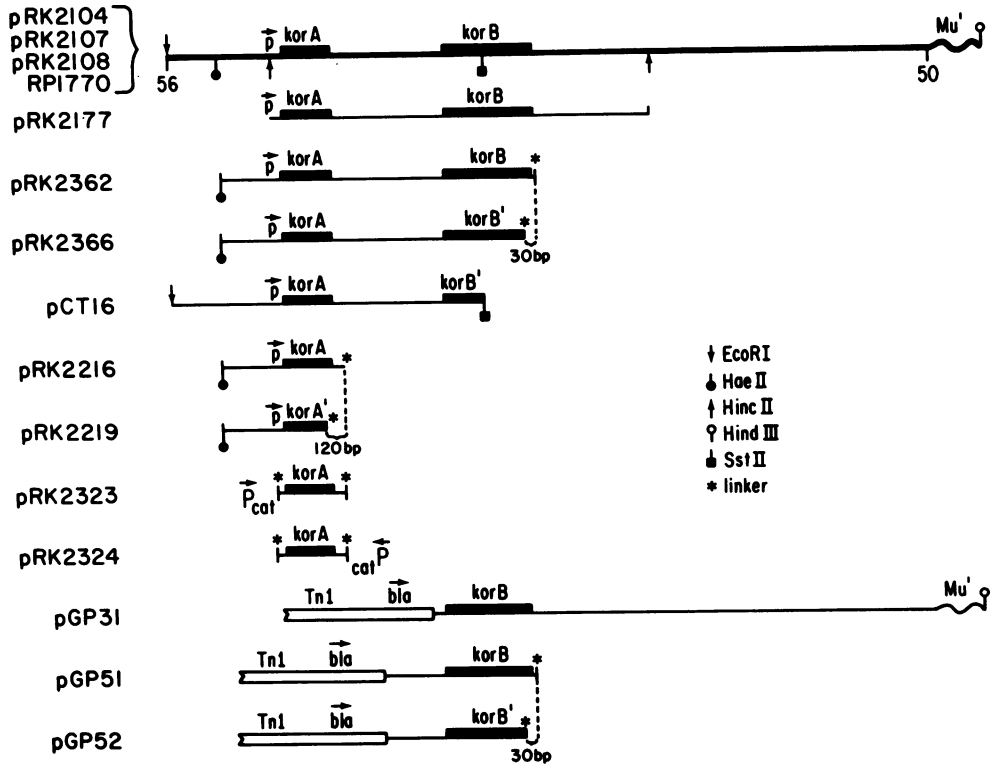

FIG. 3. Portions of the 50′ to 56′ region used to locate the INF determinants. On top is the 50′ to 56′ segment of RK2 as cloned in plasmids pRK2104 (ColE1 replicon, Ap^r), pRK2107 (R6K replicon, *trpE*$^+$), and pRK2108 (pSM1 replicon, *trpE*$^+$) (12) and integrated into the chromosome of strain RP1770. The published values for the copy number of the pSM1 replicon of pRK2108 (26) and the R6K replicon of pRK2107 (20) are consistent with our observations (data not shown). Mu′, Remnant of phage Mu. Only selected restriction sites are shown. A prime indicates that a portion of the gene is missing. The structures of pCT16 (ColE1 replicon, Km^r) (51) and pRK2216 and pRK2219 (both P15A replicon, Ap^r) (3) have been reported. pRK2323 and pRK2324 (P15A replicon, *trpE*$^+$) are identical to pRK2240 and pRK2241 (3), respectively, except for a *trpE*$^+$ fragment in the *Hin*dIII site of the Tc^r region. pRK2177 (P15A replicon, Km^r) has the indicated fragment inserted at the *Hin*cII site of pACYC177 (5). The reduced *korB* regions of pRK2362 and pRK2366 (both P15A replicons, Ap^r) were generated by BAL 31 digestion and ligation of DNA linkers (Bechhofer, Ph.D. Thesis; Bechhofer et al., manuscript in preparation). pGP31 (ColE1 replicon, Ap^r *trpE*$^+$) carries the *korB* region of pRP761-6 (2), and pGP51 and pGP52 are *trpE*$^-$ derivatives of pGP31 with the *korB* segments of pRK2362 and pRK2366, respectively. pGP63 has the *korB* region of pRP761 (2) joined to the *lac* promoter by insertion at the *Hin*dIII site of pUC8 (53). All ColE1- and P15A-based plasmids show the elevated copy numbers reported for these vehicles (5, 18).

originally observed with the mini-RK2 plasmids, and as predicted, it is suppressed by linkage of a non-RK2 replicon. This strongly suggests that the target for the INF determinant is a replication function encoded by either *trfA* or *oriV*.

***kilB1* suppresses sensitivity to INF.** The INF interaction observed for the minimal replication system is considerably stronger than that seen with intact mini-RK2 plasmids. We considered the possibility that the minimal *oriV-trfA* system might be lacking a determinant that could reduce the inhibitory effect of the INF region. To test this, we examined the effect of INF on *oriV* complementation in *trans* as before, but we used *trfA*$^+$ helper plasmids with additional functions found on mini-RK2 plasmids.

One plasmid, pCT14, carried not only *trfA* but also a functional *kilB1* and the *korA*-encoding region (Fig. 2). Except for *oriV*, pCT14 had all of the regions found in common to the INF-sensitive mini-RK2 plasmids. The *oriV* plasmid pRK2128 transformation was not at all inhibited by the INF region when pCT14 was used to supply *trfA* functions (Table 3). Thus, some determinant present on pCT14 but missing from pRK2312 accounts for their 500-fold difference in sensitivity to INF.

The following experiment shows that this determinant is *kilB1*. pRK2164 is a *trfA*$^+$ plasmid which is the *kilB1*$^+$ parent of pRK2312 (Fig. 2). We found that *oriV* plasmid pRK2128 transformation was completely insensitive to INF when *kilB1*$^+$ pRK2164 was the *trfA*-supplying plasmid (Table 2). This proves that the *korA* region is not needed to suppress INF because it is not present on pRK2164. Furthermore, the result is in striking contrast to the 500-fold reduction in transformation efficiency seen when its *kilB1*$^-$ counterpart, pRK2312, was the helper.

We also constructed a *kilB1*$^-$ mini-RK2 plasmid (pRK2319) with the same *kilB1* mutation present in pRK2312 (Fig. 4). The results with pRK2319 are considerably different from those observed for pRK248, a *kilB1*$^+$ replicon (Table 3). pRK2319 showed extreme sensitivity to INF present at high copy number (on plasmid pRK2108) or even at low copy number (in strain RP1770). The results are in complete agreement with those observed in the *trans*-complementation experiments with the *oriV* plasmid pRK2128 and the *trfA*$^+$ *kilB1*$^-$ plasmid pRK2312 in the presence of the INF region.

We conclude that the *kilB1* determinant is required to suppress the inhibitory effect of the INF region on the RK2 replication system and that mutations in *kilB1* make RK2 replication hypersensitive to INF.

***korA* and *korB* are each able to inhibit replication of *kilB1*$^-$**

TABLE 3. Sensitivity of mutant mini-RK2 replicons to INF

Incoming plasmid	*kilB1*	Relative transformation efficiency[a] in the following recipient strain		
		MV10 (no INF)	MV10(pRK2108) (high-copy INF)	RP1770[b] (low-copy INF)
pRK248	+	1.0	0.04	1.4
pRK2319	−	1.0	<0.0003	<0.0002
pRK2328	−	1.0	1.1	

[a] For each incoming plasmid, values are normalized to that of strain MV10. For other details, see the text.
[b] The 50′ to 56′ region is integrated in the bacterial chromosome.

mini-RK2 replicons. The INF region (50′ to 56′ of RK2) encodes two known *kor* genes *korA* and *korB*. They were expressed from a 3.1-kb region between coordinates 52.5′ and 55.6′ (Fig. 3), which was cloned in pRK2177. When a strain carrying pRK2177 was transformed by the *kilB1*$^+$ mini-RK2 plasmid pRK248, the frequency of transformation was significantly reduced relative to that of the plasmidless control strain (Table 4). Furthermore, a severe reduction was observed for the *kilB1*$^-$ replicon pRK2319. Therefore, the determinants responsible for INF are within the *korA-korB* region.

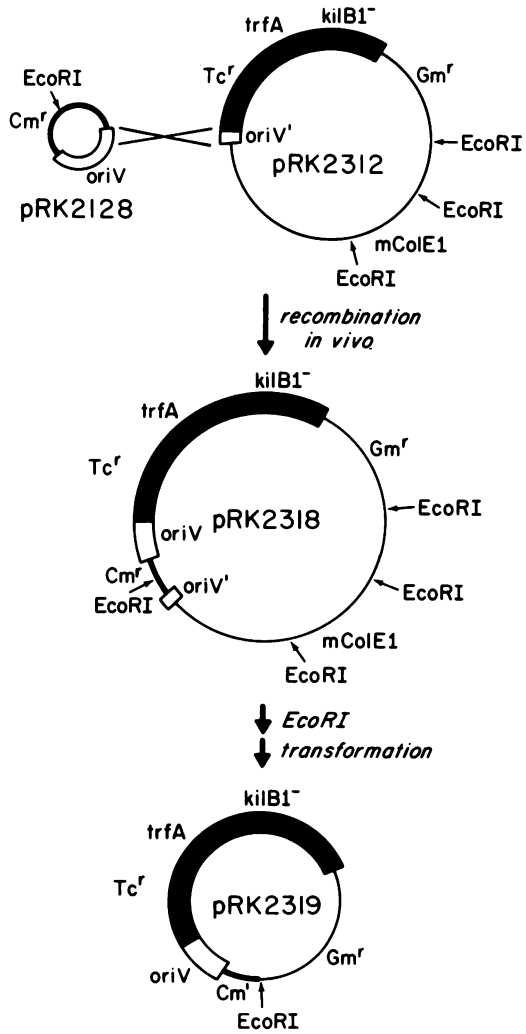

FIG. 4. Generation of *kilB1*$^-$ mini-RK2 plasmid pRK2319. A rare Cm^r transformant arising from the introduction of pRK2128 into a strain containing both pRK2312 and the INF region of RK2 yielded recombinant plasmid pRK2318. Recombination occurred within a small (<200 bp) region of homology near *oriV*. pRK2318 is insensitive to INF because it can replicate from the mini-ColE1 replicon.

TABLE 4. *korA* and *korB* mediate INF

Resident plasmid	*korA*	*korB*	Relative transformation efficiency[a]	
			by a *kilB1*$^+$ mini-RK2 replicon[b]	by a *kilB1*$^-$ mini-RK2 replicon[c]
None	−	−	1.0	1.0
pRK2177	+	+	0.02	<0.01
pRK2362	+	+	0.03	<0.01
pRK2366	+	−	1.0	<0.01
pCT16	+	−	1.0	<0.01
pRK2216	+	−	1.0	<0.01
pRK2219	−	−		0.3
pRK2323	+	−		0.01
pRK2324	−	−		1.0
pGP31	−	+	1.2	<0.01
pGP51	−	+[d]		<0.01
pGP52	−	−		0.4

[a] See the text.
[b] pRK248.
[c] pRK2319.
[d] For pGP51, there is some reduction in ability to control a high-copy-number *kilB*$^-$ plasmid, suggesting that expression of *korB* is somewhat reduced.

To determine whether the *korA* or *korB* genes are involved, we tested various mutant plasmids. The first mutants of interest were those of *korA* (Fig. 3), whose defects are known at the nucleotide sequence level (3). The results for each pair of plasmids were unequivocal (Table 4). Both *korA* mutants pRK2219 and pRK2324 had no significant effect on pRK2319, whereas the *korA*$^+$ analogs showed very strong inhibition. We conclude that *korA* alone is sufficient to inhibit replication of *kilB1*$^-$ mini-plasmids.

To test for *korB* involvement, we used pGP31, which has the *korB* region but lacks *korA* (Fig. 3). *korB* was expressed from the promoter for the β-lactamase gene (*bla*) of Tn*1*. We found that pGP31 inhibited transformation by pRK2319 and therefore does indeed mediate INF (Table 4). This demonstrates that another RK2 function is capable of inhibiting RK2-specific replication. This second determinant is clearly distinct from *korA* and maps in the region that encodes *korB*.

To determine whether the second INF determinant is *korB* itself, we used another isogenic pair of plasmids pGP51 and pGP52 (Fig. 3). The difference between the plasmids was 30 base pairs (bp) at the 3′ end of *korB*. The results show clearly that pGP51 interfered with transformation by a *kilB1*$^-$ mini-RK2 plasmid (pRK2319), whereas pGP52 did not (Table 4). Therefore, *korB* alone, like *korA*, is sufficient to inhibit the replication of a *kilB1* replicon.

***korA* and *korB* are both required to inhibit replication of *kilB1*$^+$ plasmids.** The results were very different when the mini-RK2 plasmid had a functional *kilB1* determinant (e.g., pRK248). When we tested the *korA*$^+$ *korB*$^+$ plasmid pRK2362 and the *korA*$^+$ *korB*$^-$ plasmid pRK2366 (Fig. 3), we found that only the *korA*$^+$ *korB*$^+$ plasmid pRK2362 inhibited transformation by pRK248 (Table 4). Because these plasmids differed only by 30 bp at the 3′ end of *korB*, we conclude that the *korB* gene is necessary for the inhibition. However, in contrast to the earlier results with the

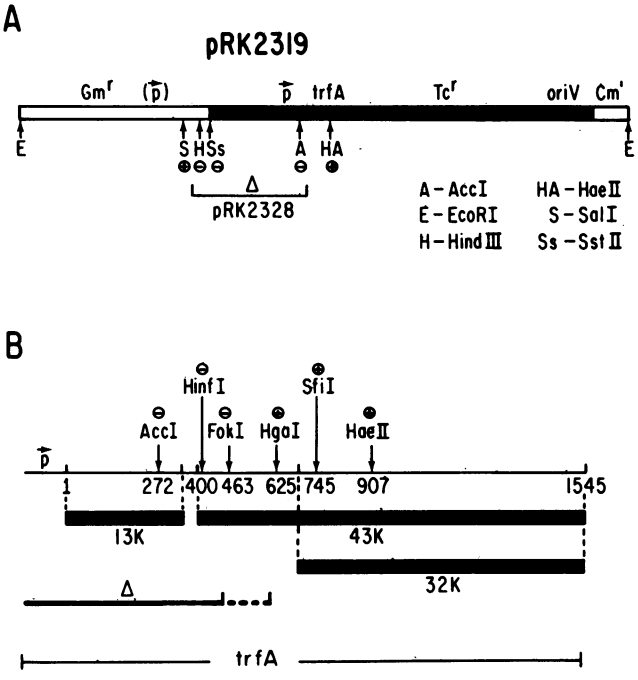

FIG. 5. Structural analysis of the *korA*- and *korB*-resistant mutant pRK2328. (A) Location of the deletion in pRK2328 relative to the map of pRK2319. Restriction sites with ⊕ are present in pRK2328 an those with ⊖ are not. The deletion has fused the Gm^r-encoding region to the structural gene for *trfA*. (p) indicates the putative promoter from the Gm^r-encoding region that is driving expression of *trfA*. (B) Fine structure analysis of the *trfA* region of pRK2328. The locations of the coding regions for the three *trfA* polypeptide products (13K, 32K, and 43K) and restriction sites are based on the published nucleotide sequence (44). Numbering begins with the first nucleotide of the first codon for the 13K polypeptide. Sites marked ⊕ are present in pRK2328, and ⊖ sites are absent. The deletion endpoint occurs within the 162-bp region between the *Fok*I and *Hga*I sites. The only intact structural gene is that for the 32K polypeptide, which has been shown to be sufficient for replication (38).

kilB1$^-$ replicon pRK2319, *korA* alone had no detectable effect on the *kilB1*$^+$ pRK248, as seen with the *korA*$^+$ *korB*$^-$ plasmids pRK2366 and pCT16 (Table 4).

To determine whether the inhibition of pRK248 by pRK2362 is due entirely to *korB*, we tested the *korA*$^-$ *korB*$^+$ plasmid pGP31. There was no reduction in transformation efficiency by pRK248 (Table 4). Therefore, only when *korA* and *korB* are both present, as in plasmids pRK2177 and pRK2362, is there a significant reduction on transformation efficiency by the *kilB1*$^+$ plasmid pRK248.

A mini-RK2 mutant resistant to inhibition by *korA* and *korB*. When the hypersensitive *kilB1*$^-$ plasmid pRK2319 was used to transform *korA*$^+$ *korB*$^+$ cells, the usual result was no colonies. On one occasion, we were successful in isolating a transformant. This strain yielded an altered plasmid, pRK2328, which showed no sensitivity to *korA* or *korB* (Table 3). pRK2328 is therefore a mutant mini-RK2 replicon in which the targets for *korA* and *korB* are either altered or missing.

To learn the nature of these targets, we studied the changes in pRK2328. We found that pRK2328 was deleted for all RK2 sequences 5′ to the structural gene for the 32,000-molecular-weight (32K) *trfA* product, including the *trfA* promoter (Fig. 5A and B). Because this replicon is dependent on *oriV* and *trfA* for replication, expression of *trfA* must be driven from a promoter located in the non-RK2, Gm^r-encoding region. *oriV* was unchanged and a normal 32K *trfA* product should be made because its structural gene and translation signals were present. Therefore, it is unlikely that either of these is a target for inhibition of replication by *korA* or *korB*.

These results led us to hypothesize that the mutation in pRK2328 made expression of *trfA* refractory to *korA* and *korB*. The simplest idea is that replacement of the natural *trfA* promoter with a non-RK2 promoter resulted in loss of sequences which respond to negative control by *korA* and *korB*.

A *trfA-lacZ* fusion is regulated by *korA* and *korB*. To test the idea that expression of *trfA* is regulated, we fused the 5′ end of *trfA* to the *E. coli lacZ* structural gene (Fig. 6). In this construction, synthesis of β-galactosidase depends on the expression signals for *trfA*. The *Sau*3A fragment fused to *lacZ* encompasses nearly the entire region shown to be deleted in the INF-resistant mutant pRK2328 characterized above and thus was expected to encode the targets for *korA* and *korB*. The source of the fragment was pRK2319 because it was both necessary and desirable to use a *kilB1* mutant. An intact *kilB1* would have required that *korA* or *korB* always be present to prevent its deleterious effect on the cells. Furthermore, the *kilB1* mutation in pRK2319 makes the plasmid highly sensitive to *korA* and *korB*, so any effect on *trfA* expression should be readily apparent.

We measured the levels of β-galactosidase synthesized from the *trfA-lacZ* fusion in the presence and absence of *korA* and *korB*. *korA* clearly had an effect (Table 5). With *korB* provided by pGP31, the result was less dramatic. There was a consistent, but slight (20%), reduction in β-galactosidase levels. In pRK2216, *korA* was expressed from its own promoter; in pGP31, *korB* was expressed by transcription readthrough from the *bla* gene of Tn*1* (Fig. 3). It seemed possible that expression of *korB* was limiting in pGP31.

To boost the expression of *korB*, we placed it downstream of the *E. coli lacZ* promoter (pGP63, Fig. 3). With this construction, regulation of the *trfA-lacZ* fusion by *korB* was

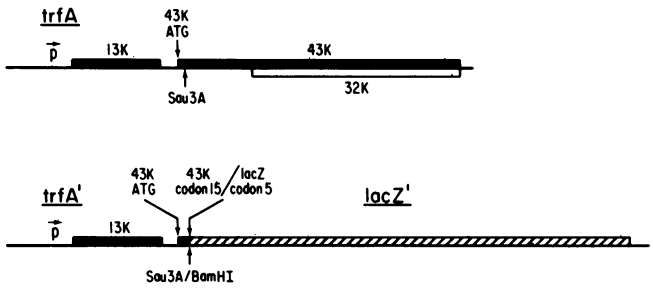

FIG. 6. A *trfA-lacZ* fusion to study regulation of *trfA* by *korA* and *korB*. The map at the top shows the configuration of the natural *trfA* determinant. Below is a gene fusion in which *lacZ* is joined in frame to the coding sequence for the 43K *trfA* polypeptide. It was constructed by ligating the large *Sau*3A fragment of pRK2319 into the *Bam*HI site of the *lacZ* fusion vector pFR109 (37). Several transformants of strain MC1061 were Lac$^+$ (blue on medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). These yielded plasmids with the appropriate structure. From one of these, the *Sal*I fragment containing the entire *trfA-lacZ* fusion was inserted into pSC101 (6) at its *Xho*I site. The resulting plasmid (pRK2386) is maintained at a low copy number and confers a Lac$^+$ phenotype on strain MC1061.

TABLE 5. Effect of *korA* and *korB* on β-galactosidase synthesis from the *trfA-lacZ* fusion

Test plasmid[a]	Relevant gene	Condition	Relative β-galactosidase activity[b]
None			1.0
pRK2163[c]	*korA* and *korB*		0.08
pRK2216	*korA*		0.06
pGP31	*korB*		0.73
pGP63	p*lac-korB*	No IPTG[d]	0.81
pGP63	p*lac-korB*	+ IPTG[d]	0.64

[a] Each plasmid was tested in strain MC1061 carrying the *trfA-lacZ* plasmid pRK2386.

[b] Values reflect the levels from a minimum of three independent cultures normalized to that of the MC1061(pRK2386) strain. Value of 1.0 is 818 U. Without pRK2386, the relative value is <0.001.

[c] pRK2163 is identical to pRK2108 (Fig. 2), except for a trimethoprim resistance marker.

[d] These cells also carried plasmid pCY5, a P15A replicon with the *lacI*^{q} allele from pHIQ6 (45). For induction of the p*lac-korB* fusion, overnight and exponential-phase cultures were grown in 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

somewhat more apparent (Table 5). Even when the *lac* promoter (p*lac*) was not induced, some *korB* was expressed and the level of β-galactosidase was lower than the control. With p*lac* induced, even more *korB* was made and consequently less β-galactosidase was synthesized from the *trfA-lacZ* fusion. Although the reduction in β-galactosidase activity was again not as strong as that observed for *korA*, it is nevertheless clear that *korB* did have an effect.

DISCUSSION

In this study, we established that *kil* and *kor* determinants of RK2 can directly participate in replication control. We found that *korA* and *korB* are each capable of acting negatively to inhibit plasmid replication, whereas *kilB1* has a strong positive effect that opposes the actions of *korA* and *korB*. Our results indicate that these effects are mediated by direct modulation of the expression of the RK2 replication gene *trfA*. These findings confirm our original hypothesis on the functions of *kil* and *kor* determinants (12).

Plasmids with only the minimal components for RK2 replication, *oriV* and *trfA*, were found to exhibit strong sensitivity to inhibition by the *korA-korB* region. This sensitivity was abolished by linkage to a non-RK2 replicon. This shows clearly that interference by the *korA-korB* region is a phenomenon related to RK2 replication and not to host cell killing because it occurs only when replication is dependent on the *oriV* and *trfA* functions of RK2.

Our studies show unequivocally that the *korA* and *korB* genes are involved. This was possible through the use of precise mutants. For *korA*, we used the smallest region of RK2 that expresses the gene and *korA*$^-$ derivatives of this region. Because we know the nucleotide sequence of these derivatives (3), we can conclude with certainty that *korA* alone is sufficient to inhibit RK2-specific replication. For *korB*, we used a minimal region which expresses *korB* without *korA*. Although the nucleotide sequence of *korB* is not yet known, we located a 30-bp region which contains the 3′ terminus of the gene (Bechhofer, Ph.D. Thesis; D. H. Bechhofer, J. Kornacki, W. Firshein, and D. H. Figurski, manuscript in preparation). Of the two plasmids which differed only by this 30-bp region, only the *korB*$^+$ plasmid inhibited RK2-specific replication. Thus, we can conclude that the effect specifically involves *korB* and not another determinant nearby.

Meyer and Hinds (25) reported an RK2 incompatibility determinant termed IncP-1(II) in the *korA-korB* region. The incompatibility mediated by IncP-1(II) is clearly different from the INF exhibited by *korA* and *korB*. IncP-1(II) is active against its target even when the target is cloned onto another replicon (25), whereas *korA* and *korB* only inhibit plasmids dependent on the RK2 replication system. Thus IncP-1(II) is functionally distinct from *korA* or *korB*, and it seems that IncP-1(II) may represent yet another replication control determinant in the *korA-korB* region.

Two complementary results revealed that *korA* and *korB* regulate *trfA* at the level of gene expression. (i) A mutant plasmid selected for resistance to *korA* and *korB* was found to have deleted the *trfA* promoter and to have fused a new promoter to the essential *trfA* coding region. This indicated that the targets for *korA* and *korB* action are in the promoter region. (ii) When the *trfA* promoter region was fused to the *lacZ* structural gene, synthesis of β-galactosidase was found to be regulated by *korA* and *korB*. It is noteworthy that inhibition of replication by *korA* and *korB* is more striking than are the effects on β-galactosidase synthesis from the *trfA-lacZ* fusion. This may reflect an expected instability of the *trfA* function. If true, its steady-state level will probably be low, and further reduction by *korA* and *korB* would have a drastic effect on replication. In contrast, the *trfA*–β-galactosidase fusion polypeptide may be much more stable, and thus even low-level expression would be detectable. We also found that *korA* plasmids are more effective inhibitors of the *trfA-lacZ* fusion than are *korB* plasmids, although both showed very strong effects on *trfA*-dependent replication. Because we do not know the absolute levels of *korA* and *korB* products produced from these plasmids, we cannot say for certain that *korB* is less effective in regulating the *trfA-lacZ* fusion. Nevertheless, it is interesting to suggest that the difference is significant.

Smith et al. (41) found two different, but related, palindromic sequences in the promoter region of *trfA* and suggested that these are operatorlike sequences which interact with *korA* and *korB* products to reduce expression of *trfA*. This is completely consistent with our results here and with recently published studies by Shingler and Thomas (39) on *galK* transcriptional fusions to *trfA*. In addition, it was shown that *korA* negatively controls the RK2 *kilA* determinant by interacting with a target in the *kilA* promoter (13; C. Young, A. Prince, and D. H. Figurski, manuscript in preparation). It seems reasonable that *korA* regulates *trfA* by the same mechanism.

Thomas and Hussain (50) recently reported studies which indicate that *korB*, together with *korA*, is a copy number control element which acts by reducing expression of *trfA*. However, without precise mutants which inactivate *korA* and *korB* specifically, the phenotype could only be mapped to regions which encode *korA* or *korB*. We show here that the *korA* and *korB* genes themselves are truly involved. With their data and ours, there can be little doubt that *korA* and *korB* are directly involved in control of RK2 replication, thus supporting the original hypothesis that these genes were likely to be involved in plasmid maintenance (12).

Plasmids with mutations in the *kilB1* determinant showed much greater sensitivity to *korA* and *korB* than does the *kilB1*$^+$ parent. This difference is not a unique property of the particular *kilB1* allele used in these experiments. Nine independent *kilB1* mutants of a *kilB1*$^+$ plasmid were isolated, and all showed the same extreme sensitivity to *korA* and *korB* (H. Schreiner, J. Minn, and D. Figurski, unpublished data).

The presence of the wild-type *kilB1* allele completely

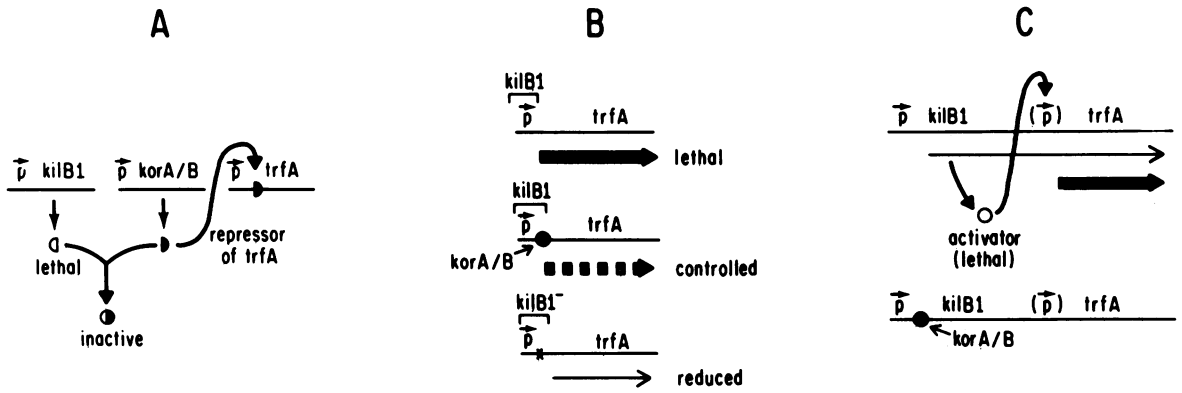

FIG. 7. Models for *kilB1*, *korA*, and *korB* action on *trfA*. (A) Mutual control by direct interaction of products. The *kilB1* product interacts with the *korA* and *korB* products to form a complex in which the *kil* moiety is not lethal and the *kor* component cannot inhibit *trfA*. (B) *kilB1* as the *trfA* promoter. The *kilB1* determinant is a strong promoter for *trfA*. Since a strong promoter may be lethal to the cell (9), the Kil$^+$ phenotype of *kilB1* may result from uncontrolled expression from this promoter. Normally, expression of *trfA* would be controlled by *korA* and *korB*. A Kil$^-$ mutation of *kilB1* could be one which reduces expression to sublethal levels in the absence of *korA* or *korB*. This low-level constitutive expression might allow synthesis of enough *trfA* product for replication. Further reduction by *korA* or *korB* would block replication by preventing sufficient synthesis of *trfA* product. (C) *kilB1* as activator. Some *trfA* expression may occur from another promoter normally controlled by *korA* and *korB*. As one example, we show *kilB1* and *trfA* to be expressed from the same promoter. The *kilB1* product might overcome the *korA* and *korB* block on *trfA* if it activated a new promoter that can express *trfA*. If *kilB1* caused a transient alteration of host RNA polymerase specificity, for example, uncontrolled expression of *kilB1* might be lethal to the cell, or the activated *trfA* promoter might itself be strong enough to be lethal. Such activation of *trfA* allows a trickle of *kilB1* expression to translate into a burst of *trfA* product sufficient to trigger replication. A *kilB1* mutant would leave *trfA* expression to the low-level promoter only, and this might be sufficient for initiation of RK2 replication at least once per cell cycle. In the presence of *korA* and *korB*, expression would be reduced severely and replication would be blocked.

eliminated the sensitivity of a plasmid to *korA* or *korB* alone. A *kilB1*$^+$ plasmid was sensitive to the presence of *korA* and *korB* together, but the severity of this interaction was dependent on the stoichiometry of *korA* and *korB* relative to *kilB1*. The low-copy-number *kilB1*$^+$ RK2 derivatives (e.g., pRK248) showed little or no sensitivity to *korA* and *korB* in the chromosome, but they were affected by *korA* and *korB* on high copy plasmids. The effect was first observed with artificially high levels of *korA* and *korB* in the cell. This provided us with an important genetic handle, and it eventually led to the finding that replication of *kilB1*$^-$ plasmids (e.g., pRK2319) is extremely sensitive even to a single copy of *korA* and *korB* in the chromosome.

This finding represents a novel twist to our perception of the *kil* and *kor* determinants. First, we learned that *kor* functions are required to prevent the lethal action of *kil* determinants on the *E. coli* host cell. Thus *kor* genes are essential to RK2 maintenance in *E. coli*. We now find the opposite relationship in the control of RK2 replication: *kilB1* is needed to oppose the inhibitory effects of *korA* and *korB*. This implies that in intact parental RK2, which encodes *korA* and *korB*, *kilB1* must be extremely important, and possibly essential, for plasmid replication.

What is the mechanism for the positive effect of *kilB1*? In considering any model, it is important to realize that *kilB1* itself can be controlled by *korA* or *korB*. If *korA* or *korB* turn off *kilB1*, how can *kilB1* prevent *korA* or *korB* inhibition of *trfA*? We suggest three conceptually different models to explain our results (Fig. 7). To be consistent with our findings, all three models predict (i) that a *kilB1* mutant will be extremely sensitive to *korA* and *korB*, (ii) that high levels of *korA* and *korB* will inhibit *trfA* expression even in the presence of *kilB1*, and (iii) that *korA* and *korB* products will prevent the deleterious effect of *kilB1* on the host cell. Of these, we consider model A to be least likely because *korA* is already known to control another *kil* determinant, *kilA*, at the level of gene expression (13; Young et al., manuscript in preparation). In support of model B, Smith et al. have shown that a *trfA* promoter mutation is *Kil*$^-$, suggesting that the promoter itself or the product of one of the genes in the *trfA* operon is the *kil* determinant (41). However, this cannot formally distinguish between model B, in which *kilB1* is the *trfA* promoter, and model C, in which a lethal *trfA* promoter is the target for *kilB1* activation. The most obvious difference is that models A and C predict *kilB1* to be *trans* acting, whereas in model B, *kilB1* is a *cis*-acting element. We have not been able to demonstrate *kilB1* complementation of a *trfA*$^+$ *kilB1*$^-$ plasmid in *trans*. This also tends to support model B, although it is theoretically possible for *kilB1* to encode a product that acts predominantly in *cis*. We prefer model B, but more work is required to choose among these possibilities.

In summary, it is now clear that at least one strategy for regulating RK2 replication is to control expression of the *trfA* gene by *kilB1*, *korA*, and *korB*. However, there are multiple *kil* and *kor* determinants in RK2, and it has been shown that their interactions form a network which seems to be unique to IncP plasmids (12, 13, 34, 58). We have now demonstrated that the network is linked to replication control. This suggests that any of several *kil* or *kor* functions may be capable of affecting IncP plasmid replication, and indeed a certain flexibility is already indicated by the finding that either *korA* or *korB* can control *kilB1* (13) and *trfA* (this work).

Why should replication control be so complex for IncP plasmids? We suggest that the reason is related to their extraordinary host range among gram-negative bacteria. The network may provide a level of adaptability to allow the plasmid to respond to differences in the various hosts and thus ensure appropriate levels of *trfA* product for stable

maintenance. We are hopeful that further analysis of the *kil* and *kor* functions will result in an understanding of the remarkable host range of IncP plasmids.

ACKNOWLEDGMENTS

We thank T. D'Amico for technical assistance and B. Hamilton for typing the manuscript. We are grateful to D. R. Helinski for providing plasmids pCT14 and pCT16, M. Casadaban for providing plasmid pFR109 and strain MC1061, and H. Shuman for helpful advice on β-galactosidase assays.

This work was supported by Public Health Service grants GM26863 and GM29085 from the National Institutes of Health, to D.F. and by Cancer Center Support grant CA13696 to Columbia University, New York. P.B. was a predoctoral fellow supported by Public Health Service training grant AI 07161 from the National Institutes of Health.

LITERATURE CITED

1. **Barth, P. T.** 1979. RP4 and R300B as wide host-range plasmid cloning vehicles, p. 395–410. *In* K. N. Timmis and A. Pühler (ed.), Plasmids of medical, environmental and commercial importance. Elsevier/North-Holland Publishing Co., New York.
2. **Barth, P. T., K. Ellis, D. H. Bechhofer, and D. H. Figurski.** 1984. Involvement of *kil* and *kor* genes in the phenotype of a host-range mutant of RP4. Mol. Gen. Genet. **197**:236–243.
3. **Bechhofer, D. H., and D. H. Figurski.** 1983. Map location and nucleotide sequence of *korA*, a key regulatory gene of promiscuous plasmid RK2. Nucleic Acids Res. **11**:7453–7469.
4. **Burkardt, H. J., G. Reiss, and A. Pühler.** 1979. Relationship of group P1 plasmids revealed by heteroduplex experiments: RP1, RP4, R68, and RK2 are identical. J. Gen. Microbiol. **114**:341–348.
5. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134**:1141–1156.
6. **Cohen, S. N., and A. C. Y. Chang.** 1973. Recircularization and autonomous replication of a sheared R-factor DNA segment in *Escherichia coli* transformants. Proc. Natl. Acad. Sci. U.S.A. **70**:1293–1297.
7. **Cohen, S. N., A. C. Y. Chang, and C. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. U.S.A. **69**:2110–2114.
8. **Datta, N., and R. W. Hedges.** 1972. Host ranges of R-factors. J. Gen. Microbiol. **70**:453–460.
9. **Davanloo, P., A. H. Rosenberg, J. J. Dunn, and F. W. Studier.** 1984. Cloning and expression of the gene for bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. U.S.A. **81**:2035–2039.
10. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. U.S.A. **77**:7347–7351.
11. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. U.S.A. **76**:1648–1652.
12. **Figurski, D. H., R. F. Pohlman, D. H. Bechhofer, A. S. Prince, and C. A. Kelton.** 1982. Broad host range plasmid RK2 encodes multiple *kil* genes potentially lethal to *Escherichia coli* host cells. Proc. Natl. Acad. Sci. U.S.A. **79**:1935–1939.
13. **Figurski, D. H., C. Young, H. C. Schreiner, R. F. Pohlman, D. H. Bechhofer, A. S. Prince, and T. F. D'Amico.** 1984. Genetic interactions of broad host-range plasmid RK2, p. 227–242. *In* D. R. Helinski, S. N. Cohen, and D. B. Clewell (ed.), Plasmids in bacteria. Plenum Publishing Corp., New York.
14. **Friedman, A. M., S. R. Long, S. E. Brown, W. J. Buikema, and F. M. Ausubel.** 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene **18**:289–296.
15. **Holloway, B. W.** 1979. Plasmids that mobilize the bacterial chromosome. Plasmid **2**:1–19.
16. **Ingram, L. C., M. H. Richmond, and R. B. Sykes.** 1973. Molecular characterization of the R factors implicated in the carbenicillin resistance of a sequence of *Pseudomonas aeruginosa* strains isolated from burns. Antimicrob. Agents Chemother. **3**:279–288.
17. **Jacob, A. E., J. A. Shapiro, L. Yamamoto, D. I. Smith, S. N. Cohen, and D. Berg.** 1977. Plasmids studied in *Escherichia coli* and other enteric bacteria, p. 607–678. *In* A. I. Bukhari, J. A. Shapiro, and S. L. Adhya ed.), DNA insertion elements, plasmids and episomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Kahn, M., R. Kolter, C. Thomas, D. Figurski, R. Meyer, E. Remaut, and D. R. Helinski.** 1979. Plasmid cloning vehicles derived from plasmids ColE1, F, R6K and RK2. Methods Enzymol. **68**:268–280.
19. **Kahn, M. L., and C. R. Timblin.** 1984. Gene fusion vehicles for the analysis of gene expression in *Rhizobium meliloti*. J. Bacteriol. **158**:1070–1077.
20. **Kolter, R., and D. R. Helinski.** 1978. Construction of plasmid R6K derivatives *in vitro*: characterization of the R6K replication region. Plasmid **1**:571–580.
21. **Kornacki, J., A. West, and W. Firshein.** 1984. Proteins encoded by the *trans*-acting replication and maintenance regions of broad host range plasmid RK2. Plasmid **11**:48–57.
22. **Meyer, R. J., D. Figurski, and D. R. Helinski.** 1977. Properties of the plasmid RK2 as a cloning vehicle, p. 559–566. *In* A. I. Bukhari, J. A. Shapiro, and S. L. Adhya (ed.), DNA insertion elements, plasmids and episomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Meyer, R., D. R. Figurski, and D. Helinski.** 1977. Physical and genetic studies with restriction endonucleases on the broad host-range plasmid RK2. Mol. Gen. Genet. **152**:129–135.
24. **Meyer, R., and D. R. Helinski.** 1977. Unidirectional replication of the P-group plasmid RK2. Biochim. Biophys. Acta **478**:109–113.
25. **Meyer, R., and M. Hinds.** 1982. Multiple mechanisms for expression of incompatibility by broad host range plasmid RK2. J. Bacteriol. **152**:1078–1090.
26. **Mickel, S., and W. Bauer.** 1976. Isolation, by tetracycline selection, of small plasmids derived from R-factor R12 in *Escherichia coli* K-12. J. Bacteriol. **127**:644–655.
27. **Miki, T., Z.-T. Chang, and T. Horiuchi.** 1984. Control of cell division by sex factor F in *Escherichia coli*. II. Identification of genes for inhibitor protein and trigger protein on the 42.84-43.6F segment. J. Mol. Biol. **174**:627–646.
28. **Miki, T., K. Yoshioka, and T. Horiuchi.** 1984. Control of cell division by sex factor F in *Escherichia coli*. I. The 42.84-43.6F segment couples cell division of the host bacteria with replication of plasmid DNA. J. Mol. Biol. **174**:605–625.
29. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Ogura, T., and S. Hiraga.** 1983. Mini-F plasmid genes that couple host cell division to plasmid proliferation. Proc. Natl. Acad. Sci. U.S.A. **80**:4784–4788.
31. **Olsen, R. H., and P. Shipley.** 1973. Host range and properties of the *Pseudomonas aeruginosa* R factor R1822. J. Bacteriol. **113**:772–780.
32. **Olsen, R. H., J. S. Siak, and R. H. Gray.** 1974. Characteristics of PRD1, a plasmid-dependent broad host-range bacteriophage. J. Virol. **14**:689–699.
33. **Olsen, R. H., and D. D. Thomas.** 1973. Characteristics and purification of PRR1, a RNA phage specific for the broad host range *Pseudomonas* R1822 drug resistance plasmid. J. Virol. **12**:1560–1567.
34. **Pohlman, R. F., and D. H. Figurski.** 1983. Essential genes of plasmid RK2 in *Escherichia coli*: *trfB* controls a *kil* gene near *trfA*. J. Bacteriol. **156**:584–591.
35. **Pohlman, R. F., and D. H. Figurski.** 1983. Conditional lethal mutants of the *kilB* determinant of broad host range plasmid RK2. Plasmid **10**:82–95.
36. **Schmidhauser, T. J., M. Filutowicz, and D. R. Helinski.** 1983.

Replication of derivatives of the broad host range plasmid RK2 in two distantly related bacteria. Plasmid **9**:325–330.

37. **Shapira, S. K., J. Chou, F. V. Richard, and M. Casadaban.** 1983. New versatile plasmid vectors for expression of hybrid proteins coded by a cloned gene fused to *lacZ* gene sequences encoding an enzymatically active carboxy-terminal portion of β-galactosidase. Gene **25**:71–82.
38. **Shingler, V., and C. M. Thomas.** 1984. Analysis of the *trfA* region of broad host-range plasmid RK2 by transposon mutagenesis and identification of polypeptide products. J. Mol. Biol. **175**:229–249.
39. **Shingler, V., and C. M. Thomas.** 1984. Transcription in the *trfA* region of broad host range plasmid RK2 is regulated by *trfB* and *korB*. Mol. Gen. Genet. **195**:523–529.
40. **Simon, R.** 1984. *In vivo* genetic engineering: use of transposable elements in plasmid manipulation and mutagenesis of bacteria other than *E. coli*, p. 125–140. *In* A. Pühler and K. N. Timmis (ed.), Advanced molecular genetics. Springer-Verlag, New York.
41. **Smith, C. A., V. Shingler, and C. M. Thomas.** 1984. The *trfA* and *trfB* promoter regions of broad host range plasmid RK2 share common potential regulatory sequences. Nucleic Acids Res. **12**:3619–3630.
42. **Smith, C. A., and C. M. Thomas.** 1983. Deletion mapping of *kil* and *kor* functions in the *trfA* and *trfB* regions of broad host range plasmid RK2. Mol. Gen. Genet. **190**:245–254.
43. **Smith, C. A., and C. M. Thomas.** 1984. Molecular genetic analysis of the *trfB* and *korB* region of broad host range plasmid RK2. J. Gen. Microbiol. **130**:1651–1663.
44. **Smith, C. A., and C. M. Thomas.** 1984. Nucleotide sequence of the *trfA* gene of broad host-range plasmid RK2. J. Mol. Biol. **175**:251–262.
45. **Snisky, J. J., B. E. Uhlen, P. Gustafsson, and S. N. Cohen.** 1981. Construction and characterization of a novel two-plasmid system for accomplishing temperature-regulated, amplified expression of cloned adventitious genes in *Escherichia coli*. Gene **16**:275–286.
46. **Stalker, D., C. Thomas, and D. R. Helinski.** 1981. Nucleotide sequence of the region of the origin of replication of the broad host range plasmid RK2. Mol. Gen. Genet. **181**:8–12.
47. **Stanisich, V. A.** 1974. The properties and host range of male-specific bacteriophages of *Pseudomonas aeruginosa*. J. Gen. Microbiol. **84**:332–342.
48. **Stokes, H. W., R. J. More, and V. Krishnapillai.** 1981. Complementation analysis in *Pseudomonas aeruginosa* of the transfer genes of the wide host range R plasmid R18. Plasmid **5**:202–212.
49. **Thomas, C.** 1981. Complementation analysis of replication and maintenance functions of broad host-range plasmids RK2 and RP1. Plasmid **5**:277–291.
50. **Thomas, C. M., and A. A. K. Hussain.** 1984. The *korB* gene of broad host range plasmid RK2 is a major copy number control element which may act together with *trfB* by limiting *trfA* expression. EMBO J. **3**:1513–1519.
51. **Thomas, C., R. Meyer, and D. R. Helinski.** 1980. Regions of broad-host-range plasmid RK2 which are essential for replication and maintenance. J. Bacteriol. **141**:213–222.
52. **Thomas, C. M., D. M. Stalker, and D. R. Helinski.** 1981. Replication and incompatibility properties of segments of the origin region of replication of broad host range plasmid RK2. Mol. Gen. Genet. **181**:1–7.
53. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13 mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
54. **Villarroel, R., R. W. Hedges, R. Maenhaut, J. Leemans, G. Engler, M. Van Montagu, and J. Schell.** 1983. Heteroduplex analysis of P-plasmid evolution: the role of insertion and deletion of transposable elements. Mol. Gen. Genet. **189**:390–399.
55. **Waters, S. H., P. Rogowsky, J. Grinsted, J. Altenbuchner, and R. Schmitt.** 1983. The tetracycline resistance determinants of RP1 and Tn1721: nucleotide sequence analysis. Nucleic Acids Res. **11**:6089–6105.
56. **Winans, S. C., and G. C. Walker.** 1985. Identification of pKM101-encoded loci specifying potentially lethal gene products. J. Bacteriol. **161**:417–424.
57. **Yakobsen, E. A., and D. G. Guiney.** 1984. Conjugal transfer of bacterial chromosomes mediated by the RK2 plasmid transfer origin cloned into the transposon Tn*5*. J. Bacteriol. **160**:451–453.
58. **Young, C., D. H. Bechhofer, and D. H. Figurski.** 1984. Gene regulation in plasmid RK2: positive control by *korA* in the expression of *korC*. J. Bacteriol. **157**:247–252.