Iteron inhibition of plasmid RK2 replication in vitro: Evidence for intermolecular coupling of replication origins as a mechanism for RK2 replication control

(copy-number control/incompatibility/replication-initiation protein/iterons/broad host range)

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ABSTRACT The broad-host-range plasmid RK2 and its derivatives are maintained in Gram-negative bacteria at a specific copy number that appears to be determined by a series of direct repeats (iterons) located at the RK2 replication origin and by the RK2 replication initiation protein, TrfA. An in vitro replication system was developed from Escherichia coli that is active with either the intact eight-iteron RK2 origin or a minimal five-iteron RK2 origin when purified TrfA protein is provided. Using this in vitro replication system, we have examined the mechanism(s) of copy-number control. It was found that two or more RK2 iterons present on a supercoiled compatible plasmid molecule are capable of specifically inhibiting in vitro the replication of either functional RK2 origin plasmid and that this inhibition is not overcome by adding increasing amounts of TrfA protein. A mutant TrfA protein, TrfA-33 (cop254D), that increases the copy number of an RK2 origin in vivo exhibits replication kinetics and activity levels in this in vitro system similar to that of the wild-type protein. However, RK2 in vitro replication initiated by TrfA-33 (cop254D) has a much reduced sensitivity to iteron inhibition. These data support a model for RK2 copy-number control that involves intermolecular coupling between TrfA-bound iterons.

Plasmid RK2 is a 60-kilobase-pair (kb) broad-host-range IncP1 plasmid that replicates at a copy number of four to seven copies per chromosome in Escherichia coli (1, 2). The in cis region required for replication was originally defined as a 700-base-pair (bp) Hae II fragment that contains a group of five 17-bp direct repeats (iterons) separated by 110-bp from a group of three 17-bp iterons (3) (see Fig. 1). In E. coli, it has been shown that a 393-bp Hpa II fragment containing the five-iteron cluster can act as a functional origin (4, 5). RK2 replication also requires a trans-acting replication initiation protein, TrfA (6–8). The trfA gene sequence encodes two proteins, a 44-kDa protein designated TrfA-44 and a 33-kDa protein designated TrfA-33 that is the result of an internal translational start codon that occurs in the same open reading frame (9–11). Either protein alone is sufficient for RK2 replication in many hosts, including E. coli, however, there is a specific requirement for TrfA-44 for stable maintenance of an RK2 origin plasmid in Pseudomonas aeruginosa (ref. 12; F. Fang and D.R.H., unpublished data). Like many other replication-initiation proteins, TrfA is a positive activator of replication that binds to the origin iterons (13). Mutants of TrfA that increase the copy number of an RK2 origin in vivo, designated “copy-up mutants,” recently have been described, and their properties indicate that this protein also plays a negative role in replication initiation (14).

The mechanism of RK2 copy-number control is largely unknown. Because the trfA gene is under complex transcriptional control in intact RK2 (1, 2), it was originally proposed that RK2 replication is primarily controlled by maintaining the TrfA protein levels in the cell at amounts rate-limiting for replication. Direct evidence against this model came with the demonstration that increasing TrfA levels in E. coli almost 200-fold resulted in only a modest (30%) increase in the copy numbers of either RK2 or a mini-RK2 origin plasmid (15). Furthermore, derivatives of RK2 have been constructed that consist of only an RK2 origin region and the trfA gene under the control of a constitutive promoter, and these plasmids also replicate at a decreased copy number (5, 16), suggesting that copy-number control must be a function of either the origin region, the TrfA protein, or a combination of the two. That copy number is controlled at least in part by the TrfA protein is indicated by the isolation and properties of copy-up TrfA mutants (14). That the origin iterons play a role in copy-number determination is suggested by the observations that the deletion of the three upstream iterons increases plasmid copy number several fold (4, 17) and that fragments of the RK2 origin that contain iterons express incompatibility against a functional RK2 origin (4).

In this study, we examine how the origin iterons and the TrfA protein might act mechanistically to achieve copy-number control. An in vitro replication system is described that is based on a soluble E. coli extract capable of replicating both five-iteron and eight-iteron RK2 origin plasmids when purified TrfA protein is provided. In this system two or more iterons, inserted into a supercoiled pUC vector, are capable of inhibiting replication from an RK2 origin, and this iteron inhibition is not overcome by increasing the amount of TrfA protein. Furthermore, we show that the TrfA protein from a copy-up mutation, trfA-cop254D, will initiate replication from RK2 origin plasmids at wild-type levels even in the presence of excess iterons. These results support a model for copy-number control of RK2 that involves the intermolecular coupling of TrfA-bound iterons (18) similar to the models suggested for the narrow-host-range plasmids R6K (19) and P1 (20).

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The E. coli strains used in this study were C600 (thr leu thi lac Y supE44 tonA), HB101 (leu pro recA ara lac Y galK Xyl mtl rpsL supE44 hsdS20), TB1 (ara Δlac-proAB rpsL φ80 ΔlacZΔM15 hsdR17), and BB3 (F pro AB lacY2 ΔM15 Te6) recA endA gyrA thi hsdR17 supE44 relA lac-5). Previously described plasmids include the functional RK2 origin plasmids pTJS26 and pTJS42 (5), the E. coli oriC plasmid pJZ101 (21), and the TrfA-overproducing plasmid pAL104 (15). Plasmids pSP7 and pSP8 contain a single copy and a properly spaced double copy of the 17-bp RK2 origin iteron sequence, respectively, cloned into the

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Sma I site of pUC19 (S. Perri, D.R.H., and A. Toukdarian, unpublished data). Plasmid pBK20 was constructed by inserting the 167-bp BspI286–HaeIII fragment containing five of the 17-bp RK2 origin tetrams (see Fig. 1) into the HindIII site of pUC19. Plasmid pBK18 contains the RK2 Hae II–EcoI fragment (eight-iteron fragment (see Fig. 1) removed from pTJS65 (16) as an EcoRI–SphI fragment and inserted into Sma I/EcoRI-digested pUC19. Plasmid pBK3-cop254D has the Sfi I–Pst I fragment from pRDL10-cop254D (14) that carries the trfA-254D copy-up mutation, in place of the Sfi I–Pst I fragment from the wild-type trfA gene within the TrfA-overexpressing plasmid pBK3 (B.L.K., unpublished data). Plasmid pBK23 was constructed by combining the BamH–Bgl II fragment carrying the RK2 replication origin of pRR10 (22) with the BamH fragment carrying the kanamycin-resistance gene from pUC5.

Preparation of Cell Extracts and Purified TrfA Proteins. To prepare E. coli extracts competent for RK2 in vitro replication, three liters of E. coli C600 were grown in Luria-Bertoni (LB) medium (GIBCO) to an OD600 of 1.0, harvested on ice, washed in buffer A (25 mM Hepes, pH 8/5 mM EDTA), suspended in 12 ml of buffer A, and subjected to the freeze/thaw lysis procedure described by Staudenbauer (23). The resulting cleared lysate was adjusted with buffer A to a protein concentration of 40 mg/ml, and 0.24 g of (NH4)2SO4 was added per ml of lysate at 0°C over a 30-min period. The mixture was stirred an additional 20 min before centrifugation at 48,000 g for 20 min. The pellet was suspended in a small amount of buffer B (25 mM Hepes, pH 8/0.1 mM EDTA/2 mM diithiothreitol) and dialyzed against 100 vol of the same buffer for 45 min before aliquots were taken and frozen at −70°C. Extract concentrations typically ranged from 60 to 80 mg/ml of protein.

Purified preparations of TrfA-33 protein, TrfA-44(98L) protein, and TrfA-44/33 mixed proteins were gifts of S. Perri and A. Toukdarian. The protein preparations were 30–50% pure on the basis of Coomassie-stained protein gels. All proteins were dialyzed into protein buffer (50 mM Hepes, pH 8/50 mM KCl/5 mM MgCl2/1 mM dithiothreitol) before use. Highly purified TrfA preparations (>90%) had the same replication characteristics as preparations that were 30–50% pure (data not shown); therefore, the less pure preparations were routinely used in the in vitro replication system. The mutant protein TrfA-33(cop254D) was partially purified from E. coli BB3(pBK3-cop254D) by using a recently developed purification scheme (S. Perri, D.R.H., and A. Toukdarian, unpublished data). The protein was estimated to be 30–40% pure.

In Vitro Replication Assay. In vitro replication reaction mixtures contained a 25-μl volume 50 mM Hepes-KOH (pH 8); 10 mM MgCl2; 20 mM creatine phosphate; 0.1 mg of creatine kinase per ml; 2 mM ATP; 50 μg of bovine serum albumin per ml; 50 μM each dNTP; (methyl-3H)UTP (150 cpm/pmol of total deoxynucleotide); 50 mM KCl, 500 μM each CTP, GTP, and UTP; 5% polyethylene glycol 6000; 0.5 mM each of template DNA; and variable amounts of native and purified TrfA protein and E. coli extract. In addition, maximal replication of the pTJS26 template (eight-iteron origin) required 50 μg of a DNAA-enriched extract that was prepared as described (24) from cells containing the DNAA-overexpressing plasmid, pLSK5 (25). Plasmid DNA was purified by alkaline lysis, centrifuged through two CsCl–dye buoyant density gradients, and run over a 25-ml Bio-Gel A-15m (Bio-Rad) column in 10 mM Tris-HCl, pH 8/0.1 mM EDTA/250 mM NaCl. The optimum amount of E. coli extract was titrated for each template DNA and each extract preparation and ranged between 200 and 300 μg of protein per reaction. The amounts of TrfA protein added are specified in each experiment. Individual reactions were combined on ice, initiated by the addition of E. coli extract, and incubated at 30°C for 90 min unless otherwise indicated. Incorporated counts were determined after precipitation with trichloroacetic acid.

In Vivo Incompatibility Tests. To determine the incompatibility between RK2 origin plasmids and RK2 iteron-containing pUC plasmids in vivo, E. coli HB101 containing an RK2 origin plasmid was transformed with an iteron-containing plasmid, and selection was maintained only for the incoming plasmid. Individual transformants were then tested by picking onto selective LB plates for the retention of both the RK2 origin plasmid and the iteron-containing plasmid.

RESULTS

Characterization of an in Vitro Replication System for RK2. The in vitro replication system described in this study utilizes a 40% (NH4)2SO4 fraction from a soluble E. coli extract and is capable of replicating supercoiled DNA containing either the 393-bp Hpa II fragment with the five-iteron minimal RK2 origin (pTJS42) or the 700-bp Hae II fragment with the eight-iteron origin (pTJS26) (Fig. 1). When supercoiled DNA template is provided, this system can duplicate the equivalent of 50–60% of the pTJS42 template DNA and 25% of the pTJS26 template DNA. In vitro replication is totally dependent on the addition of the purified replication initiation protein, TrfA (Fig. 2A). It also requires MgCl2, ATP, an ATP-regenerating system, and polyethylene glycol. Addition of a DNAA-enriched extract enhances replication of pTJS26 2–3-fold but has no effect on pTJS42 replication. There appears to be no requirement for exogenously added rTTPS other than ATP, and replication is unaffected by the addition of rifampicin (data not shown). The in vitro system described is capable of replicating RK2 and the E. coli oriC plasmid, pJZ101; however, it does not initiate replication from either CoEI or the CoEI-derived pUC plasmids.

The kinetics of replication of pTJS42 and pTJS26 were examined by using three different forms of the TrfA protein. In cells containing the plasmid RK2, the TrfA protein is synthesized as a mixture of a 44-kDa protein and a 33-kDa protein that starts at the 98th amino acid of TrfA-44 and continues in the same reading frame. TrfA-33 was purified from cells by using an overexpressing plasmid deleted for the amino-terminal portion of TrfA-44 (pBK3; B.L.K., unpublished data). A functional derivative of TrfA-44 (designated TrfA-44(98L)) was produced from cells carrying a plasmid that contains the mutant trfA gene sequence 98L, in which the ATG (methionine) start codon of TrfA-33 has been changed to a CTG (leucine), thus eliminating expression of TrfA-33 (F.

![Fig. 1. Maps of RK2 origin- and iteron-containing plasmids. Top line is the 700-bp Hae II fragment containing the RK2 replication origin that is found in pTJS26. Numbered arrows represent the 17-bp direct repeats. Black boxes indicate dnaA box homologous sequences. Downstream of iteron 1 are A+T- and G+C-rich regions, both of which are required for replication. The two restriction enzyme sites designated with an asterisk are not unique within this fragment. Restriction enzyme BspI286 is designated Bsp. The pTJS42 origin region (second line, Hpa II fragment) also contains a functional RK2 replication origin. The RK2 origin segments contained in pBK20 (third line) and pBK18 (bottom line) do not constitute functional replication origins and are cloned into pUC19 as described in Materials and Methods.](image-url)
levels -24-fold above that at RK2 25 A&g/ml. at pBK18 5 6 pBK20 pSP8 21 pUC19 and in vivo the incompatibility against number plasmid iterons pTJS42 double sufficient Furthermore, iterons themselves and cloning incompatibility containing origin previously experimentsquent or pTJS26 (Fig. 2C) and Fang protein preparation. TrfA-44(98L) shown). and iteron-containing plasmids were assayed as described in the presence of increasing amounts of a TrfA-33 protein preparation. TrfA-44(98L) and TrfA-44/33 proteins achieve maximal DNA replication levels at similar protein concentrations (data not shown). (B and C) Kinetics of pTJS42 (B) and pTJS26 (C) replication. Time courses of pTJS42 and pTJS26 replication were carried out under the assay conditions described and 0.5 µg of the TrfA-44/33, TrfA-33, TrfA-44(98L), or TrfA-33(cop254D) protein preparation per reaction.

Fang and D.R.H., unpublished data). All three of these TrfA protein preparations [TrfA-44(98L), TrfA-33, and TrfA-44/33] show similar replication kinetics with either pTJS42 (Fig. 2B) or pTJS26 (Fig. 2C) as templates; consequently, all subsequent experiments in this study utilize only the TrfA-33 protein.

In Vivo Incompatibility Expressed by RK2 Iterons. It has previously been demonstrated that DNA segments of RK2 containing origin iterons cloned into pBR322 can express incompatibility in E. coli against RK2 replicons (4). By cloning a fragment containing essentially only the five-iteron sequence into pUC19 (pBK20), we have confirmed that the RK2 iterons themselves and not adjoining sequences are responsible for expressing this incompatibility (Table 1). Furthermore, we find that a single iteron sequence is not sufficient to express incompatibility, while a correctly spaced, double copy of the iteron expresses substantial incompatibility against either RK2 origin. Increasing the number of iterons on the pUC19 plasmid to five or eight increases the severity of incompatibility. This iteron incompatibility is not eliminated or reduced by increasing the TrfA levels in vivo ≈24-fold.

Effects of RK2 Iterons on In Vitro Replication. To determine the mechanism responsible for the incompatibility expressed in vivo by the RK2 iterons, we examined the effect of adding RK2 iteron-containing plasmids to the in vitro replication system. It was first established that neither pUC19 nor the iteron-containing pUC19 plasmids served as replication templates in the in vitro replication system (data not shown). Furthermore, addition of pUC19 (up to 1 µg per reaction) had no effect on the replication of the RK2 origin plasmids being examined. The addition of iteron-containing pUC19 plasmids, however, had a marked effect on RK2 replication. When iteron-containing pUC19 plasmids were added to the in vitro replication system at a molar iteron ratio of 1:1 (i.e., 1 mol of iterons supplied by pUC19 for each mol of iterons on the RK2 template), both the two-iteron plasmid (pSP8) and the five-iteron plasmid (pBK20) significantly inhibited replication of the pTJS42 template, while the eight-iteron plasmid (pBK18) inhibited replication more strongly (Fig. 3A). When the molar ratio of pUC19-containing iterons to RK2 origin-containing iterons was increased to 2:1, replication of pTJS42 was more severely inhibited by each of the iteron-containing plasmids except for pSP7 (Fig. 3B). The single iteron plasmid (pSP7) had no effect on pTJS42 replication at either iteron concentration. Similar results were also obtained with the pTJS26 template (Fig. 3 C and D). Meanwhile, the addition of similar concentrations of the RK2 iteron-containing pUC19 plasmids to in vitro replication reactions of the oriC plasmid, pJZ101, showed no replication inhibition even when the TrfA protein was included (data not shown).

We further observed that increasing the TrfA concentration in the iteron-inhibited reactions over an 8-fold range did not relieve the iteron inhibition. However, linearizing pBK20 with EcoRI before addition to the in vitro replication system demonstrated that linear, iteron-containing DNA is not an effective inhibitor (Fig. 3B).

In Vitro Replication Characteristics of a Copy-Up Mutant TrfA Protein. A TrfA-33 mutant protein, TrfA-33(cop254D), that increases the copy number of an RK2 origin in vivo ≈16-fold has recently been described (14). We purified this protein and examined its replication characteristics in vitro. TrfA-33(cop254D) exhibits replication kinetics similar to those seen with wild-type TrfA-33 on the pTJS42 template (Fig. 2B). The amount of protein required to achieve maximal replication levels as well as the replication levels achieved are also comparable between the TrfA-33(cop254D) and the TrfA-33 proteins (data not shown).

![Figure 2](https://example.com/figure2.png)

Fig. 2. Characterization of the RK2 in vitro replication system. (A) Requirement for TrfA protein in the replication of pTJS42 (five-iteron origin) and pTJS26 (eight-iteron origin). Replication of RK2 plasmids was assayed as described in the presence of increasing amounts of a TrfA-33 protein preparation. TrfA-44(98L) and TrfA-44/33 proteins achieve maximal DNA replication levels at similar protein concentrations (data not shown). (B and C) Kinetics of pTJS42 (B) and pTJS26 (C) replication. Time courses of pTJS42 and pTJS26 replication were carried out under the assay conditions described and 0.5 µg of the TrfA-44/33, TrfA-33, TrfA-44(98L), or TrfA-33(cop254D) protein preparation per reaction.

<table>
<thead>
<tr>
<th>Incoming iteron plasmid</th>
<th>No. of iterons</th>
<th>% of E. coli colonies retaining the RK2 plasmid and the incoming plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC19</td>
<td>0</td>
<td>99 100 100 100</td>
</tr>
<tr>
<td>pSP7</td>
<td>1</td>
<td>98 100 100 100</td>
</tr>
<tr>
<td>pSP8</td>
<td>2</td>
<td>44 23 24 24</td>
</tr>
<tr>
<td>pBK20</td>
<td>5</td>
<td>6 0 0 0</td>
</tr>
<tr>
<td>pBK18</td>
<td>8</td>
<td>0 0 0 0</td>
</tr>
</tbody>
</table>

Incompatibility results are the average of two independent experiments. Antibiotic concentrations used for plasmid selection were penicillin at 250 µg/ml, tetracycline at 12 µg/ml, and kanamycin at 25 µg/ml. The iteron-containing plasmids are all pUC19 derivatives; pTJS42 is a five-iteron RK2 origin plasmid; pBK23 is an eight-iteron RK2 origin plasmid; pAL104 produces TrfA from the tac promoter at levels ≈24-fold above that produced by pBK23 (15).
two-iteron, five-iteron, or eight-iteron-containing pUC19 plasmid when replication is dependent on TrfA-33. However, when replication is dependent on TrfA-33(cop254D), the addition of the iteron-containing plasmids has little, if any, inhibitory effect (Table 2).

**DISCUSSION**

The *in vitro* replication system used in this study is capable of replicating RK2 origin plasmids when purified TrfA protein in the form of TrfA-33 only, TrfA-44(98L) only, or the TrfA-44/33 mix is provided. No difference in the replication kinetics of these proteins on either of two different RK2 origins was noted. This *in vitro* replication system differs from the one described for RK2 by Pinkney *et al.* (13) in that the latter system is stimulated by the addition of rNTPs and NAD and does not initiate replication from the intact eight-iteron RK2 origin plasmid. These differences may be the result of using somewhat different fractions prepared from the *E. coli* lysate. For both systems, however, RK2 replication does not require RNA polymerase or protein synthesis and does require DNA polymerase III and gyrase (determined by inhibitor studies; data not shown). The requirement for the DnaA protein in RK2 replication both *in vivo* (26) and *in vitro* (13) has previously been established. It is not known why *in vitro* replication of the eight-iteron origin requires the addition of DnaA-enriched extract while the five-iteron origin does not. This could be due to the presence of an extra DnaA box located upstream of the cluster of three iterons (Fig. 1), or it may reflect a structural difference involving the DnaA protein between the nucleoprotein complexes that form to initiate replication of the eight-iteron and the five-iteron origins.

*In vivo* incompatibility experiments demonstrated that it is indeed the iteron sequences that are responsible for expressing incompatibility and that the ability to express this incompatibility is affected by the number of iteron sequences present. This has also been shown for a number of other iteron-containing plasmids including F (27, 28), P1 (29, 30), pSC101 (31), R1162 (32), RSF1010 (33), Rts1 (34), and R6K (35) and supports a model in which iterons play an important role in plasmid regulation.

The *in vitro* results presented in this study indicate that RK2 iterons express incompatibility by inhibiting RK2 replication, as has been reported for the iterons of R1162 (36). Although a plasmid containing a single iteron is unable to inhibit RK2 replication *in vitro* (and unable to express incompatibility *in vivo*), plasmids containing two or more RK2 iterons cloned in tandem are able to inhibit *in vitro* replication. Furthermore, increasing the concentration of the inhibiting iterons increases the severity of replication inhibition. This iteron inhibition is specific for RK2 replicons, indicating that general host replication factors are not limit-

**Table 2. Iteron inhibition of replication of RK2 origin plasmids pTJS42 and pTJS26 initiated by TrfA-33 or mutant TrfA-33(cop254D)**

<table>
<thead>
<tr>
<th>Iteron-containing plasmid</th>
<th>pTJS42 (five-iteron origin)</th>
<th>pTJS26 (eight-iteron origin)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNA, pmol</td>
<td>Fold inhib.</td>
</tr>
<tr>
<td>None</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pSP8</td>
<td>1:2</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>1:3</td>
<td>57</td>
</tr>
<tr>
<td>pBK20</td>
<td>1:2</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>1:3</td>
<td>72</td>
</tr>
<tr>
<td>pBK18</td>
<td>1:2</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>1:3</td>
<td>3</td>
</tr>
</tbody>
</table>

The amount of iteron-containing plasmid added in each experiment was calculated to achieve the specified molar ratio of iterons on a functional RK2 origin to iterons on pUC19. The total protein added to each reaction was 1.8 μg, and the DNA synthesized per reaction in pmol is an average of at least two experiments. An inhibition (inhib.) value of 1.0-fold indicates that inhibition by the iteron-containing plasmid was not observed.
Infectious Dr. copy-number RK2 couple association in Kues, copy number in plasmid which been higher a TrfA-bound from the formation of iterons (38). The of titration models that invokes an intermolecular coupling between the TrfA-bound iterons of two different plasmids that prevents the formation of an active replication complex at the origin and, therefore, prevents replication (18). According to this model, whether a TrfA-bound RK2 origin complexes with the required host replication proteins for the initiation of replication or couples with a second TrfA-bound origin is a function of the concentration of TrfA-bound iterons in the cell. As the concentration of TrfA-bound RK2 origins increases, the probability of formation of coupled origins that are inactive also increases, and this reduces the frequency of initiation of replication from the plasmid origin. Thus, the coupling of TrfA-bound iterons provides the sensing mechanism for the regulation of RK2 plasmid copy number. Intramolecular coupling between the TrfA-bound group of three iterons and the downstream TrfA-bound group of five iterons could also occur and may explain the fact that the five-iteron plasmid is maintained at a higher copy number than the eight-iteron plasmid. A coupling model for the regulation of plasmid copy number has been proposed for plasmids RK6(K) (19) and P1 (20).

A prediction of the coupling model is that a copy-up mutant of TrfA may be copy-up because it is less effective in the protein–protein interactions required for coupling to occur. TrfA-33(cop254D), which brings about a 16-fold increase in plasmid copy number in vivo (14) is possibly this type of mutant. The in vitro results clearly establish that the copy-up phenotype expressed by the TrfA-33(cop254D) protein is not a result of substantially increased replication initiation activity. However, RK2 replication initiated by TrfA-33(cop-254D), unlike that initiated by TrfA-33, is virtually insensitive to iteron inhibition in vitro, indicating that it may be defective in TrfA-bound origin coupling. Another copy-up mutant protein recently tested, TrfA-33(cop250V), behaves similarly to TrfA-33(cop254D) in its insensitivity to iteron inhibition.

Experiments that demonstrate directly a physical association between TrfA-bound iterons at the replication origin would provide important additional support for the coupling model as a mechanism of copy-number control for RK2. Purified R6K replication initiation protein, π, has been shown to couple regions of DNA on two different DNA molecules by virtue of its binding to the iterons of the R6K origin (19, 39). RK2 is the first broad-host-range iteron-containing plasmid of Gram-negative bacteria for which it has been proposed that copy-number control involves the coupling of replication initiation protein-bound origin iterons. A closer examination of other iteron-containing plasmids might reveal a general mechanism involving origin coupling as the principal means for copy-number control for this large and diverse group of bacterial plasmids.

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