Kid cleaves specific mRNAs at UUACU sites to rescue the copy number of plasmid R1

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Stability and copy number of extra-chromosomal elements are tightly regulated in prokaryotes and eukaryotes. Toxin Kid and antitoxin Kis are the components of the *parD* stability system of prokaryotic plasmid R1 and they can also function in eukaryotes. In bacteria, Kid was thought to become active only in cells that lose plasmid R1 and to cleave exclusively host mRNAs at UA(A/C/U) trinucleotide sites to eliminate plasmid-free cells. Instead, we demonstrate here that Kid becomes active in plasmid-containing cells when plasmid copy number decreases, cleaving not only host- but also a specific plasmid-encoded mRNA at the longer and more specific target sequence UUACU. This specific cleavage by Kid inhibits bacterial growth and, at the same time, helps to restore the plasmid copy number. Kid targets a plasmid RNA that encodes a repressor of the synthesis of an R1 replication protein, resulting in increased plasmid DNA replication. This mechanism resembles that employed by some human herpesviruses to regulate viral amplification during infection.

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Introduction

The copy number of many extra-chromosomal elements is tightly regulated. Examples range from bacterial plasmids and phages to human viruses such as herpes simplex virus (HSV) or human papilloma virus (HPV). Yet, in spite of major efforts on a wide range of biological systems, important aspects of copy number control remain elusive.

R1 is a low copy number plasmid of *Escherichia coli* and its maintenance in bacterial cells is very sensitive to copy number fluctuations. Thus, R1 has evolved different genetic strategies to respond to these changes and to increase its stability in the host cell (Gerdes and Molin, 1986; Gerdes *et al*, 1986; Bravo *et al*, 1988).

Replication of R1 is tightly controlled. The limiting factor for initiation of R1 replication is the protein RepA. Its gene

can be transcribed from two promoters, *PrcopB* and *PrrepA*. When plasmid copy number is normal, a bicistronic transcript is synthesized from *PrcopB*. The upstream gene product of this mRNA, CopB, represses *PrrepA* and limits plasmid DNA replication. In addition, antisense RNA *copA* limits the translation of RepA, being less effective when *PrrepA* is fully active. If copy number of R1 decreases, the concentration of the repressor CopB also decreases. This de-represses *PrrepA*, allowing R1 copy number to increase again (Figure 1) (Olsson *et al*, 2004 and references therein).

R1 also contains partition and postsegregational killing systems that act coordinately to reduce plasmid loss to frequencies below 10^{-7} (Nordström and Austin, 1989). One of these systems, *parD*, is located downstream of the basic replicon of R1 (Figure 1). *parD* encodes a toxin (Kid; 12 kDa) that inhibits proliferation of plasmid-free daughter cells, and an antitoxin (Kis; 10 kDa) that protects plasmid-containing cells. In cells containing R1, Kis binds to Kid and neutralizes it (Bravo *et al*, 1987, 1988). Protease Lon degrades Kis and this triggers toxicity of Kid, but several regulatory loops ensure production of enough antitoxin Kis to neutralize Kid if R1 is present. If the plasmid is lost during cell division, this balance shifts towards toxicity and Kid eliminates plasmid-free daughter cells (Ruiz-Echevarría *et al*, 1991, 1995a; Tsuchimoto *et al*, 1992).

Kid and Kis are members of a larger family of toxin-antitoxin pairs. They are conserved in plasmid R100, and also have chromosomal homologs in *E. coli* (Tsuchimoto *et al*, 1988; Masuda *et al*, 1993), which are functionally and structurally related to Kid and Kis (Hargreaves *et al*, 2002; Kamada *et al*, 2003). These chromosomal systems function as stress response elements and their toxins are cytostatic and reversible, which is essential for their biological function (Gerdes, 2000; Pedersen *et al*, 2002; Christensen *et al*, 2003). Interestingly, although *parD* was described as a post-segregational killing system, Kid seems to exert only a cytostatic effect (Jensen *et al*, 1995). Recent studies have shed some light on the mode of action of these toxins. They are endoribonucleases that cleave cellular RNAs and inhibit protein synthesis. However, some discrepancy exists about the type of RNAs and the specific sequences that they cleave (Christensen *et al*, 2003; Zhang *et al*, 2003; Muñoz-Gómez *et al*, 2004; Zhang *et al*, 2004, 2005).

Although the replication of plasmid R1 and the *parD* system have been extensively studied, several questions stand out. Under normal circumstances, CopB contributes very little to the control of R1 copy number (Nordström and Aagaard-Hansen, 1984; Light *et al*, 1985). It was postulated that *copB* has been kept by R1 to act as a rescue system when the plasmid copy number is very low, but how this system works remains to be established (Olsson *et al*, 2004). Similarly, the contribution of *parD* to plasmid stability seems to be relevant only in a replication defective R1 mutant, but the reasons for this are not understood (Jensen *et al*, 1995; Ruiz-Echevarría *et al*, 1995b).

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Here we show that, unexpectedly, the function of Kid is exerted in plasmid-containing cells, and not only after plasmid loss. We demonstrate that Kid becomes active and inhibits bacterial growth if copy number of R1 decreases. Moreover, we show that Kid not only targets host mRNAs but also a plasmid-encoded mRNA, cleaving them specifically at the pentanucleotide 5'-UUACU-3' instead of the less specific trinucleotide 5'-UA(A/C/U)-3'. This is a more complex target sequence than described previously (Zhang *et al*, 2004), allowing for greater specificity for individual RNA molecules. When copy number of R1 decreases, Kid cleaves two of these sites in the intercistronic region of plasmid-encoded *copB-repA* mRNA. This inhibits further synthesis of CopB and de-represses *PrrepA*. As a consequence, more monocistronic *repA*-mRNA is produced, resulting in more plasmid DNA replication that restores the copy number of R1. We show that Kid also cleaves host-encoded mRNAs, such as *dnaB* and *lon*, specifically at 5'-UUACU-3' sites. Thus, by acting on host- and plasmid-encoded mRNAs, Kid inhibits cell growth and increases the copy number of R1 simultaneously.

Our results establish a functional link between *parD* and the basic replicon of plasmid R1. They show that Kid contributes to plasmid stability by acting as a rescue system when R1 copy number decreases. Moreover, they suggest that plasmid R1 and several pathogenic eukaryotic viruses use similar molecular mechanisms to inhibit protein synthesis in their hosts and to increase their copy number simultaneously.

Kis and Kid can also function in eukaryotes, and have been used to conditionally regulate cell proliferation and cell death in these organisms (de la Cueva-Méndez *et al*, 2003). This has biomedical and biotechnological relevance, as these proteins can now be used to develop strategies for the targeted elimination of tumor cells or specific cell lineages during

Figure 1 Scheme of *oriR1* and *parD* loci. The initiator protein RepA can be transcribed as a monocistronic mRNA from promoter *repA* (*PrrepA*) or as a *copB-repA* bicistronic mRNA from the weaker promoter *copB* (*PrcopB*). Protein CopB represses *PrrepA* and in addition, an antisense RNA (*copA*) binds to its complementary mRNA sequence (*copT*, not shown for simplicity) in the *copB-repA* and *repA* transcripts, limiting the translation rate of RepA. As *PrrepA* is stronger than *PrcopB* and transcription of *copA* is constitutive, its inhibitory effect is weaker when monocistronic *repA* mRNA is being produced from *PrrepA*, which allows recovery of R1 copy number when CopB decreases. Transcription from promoter *parD* (*PrparD*) generates *kis-kid* bicistronic mRNAs. Antitoxin Kis binds to toxin Kid and neutralizes it, but rapid turnover of Kis by the host protease Lon activates toxicity of Kid. *oriR1* indicates the plasmid R1 replication origin.

development (Slanchev *et al*, 2005). Thus, a better understanding of how these proteins work also contributes to selective ablation of eukaryotic cells.

Results

Kid cleaves host mRNA at UUACU sites

In this work, we used thermo-sensitive promoters to regulate the expression of *kis* and *kid* independently (Figure 2A). Induction of these promoters inhibited cell growth in bacteria containing only *kid* but not in cells containing both *kid* and *kis* or control empty vectors (Figure 2B). Protein synthesis was severely inhibited in exponentially growing cells when transcription of *kid* was induced but not when transcription of *kis* was stimulated at the same time (Figure 2C).

PemK, the homolog of Kid in plasmid R100 is an endoribonuclease (Zhang *et al*, 2004). We analyzed whether Kid cleaves host *dnaB* mRNA, as this gene product had been previously implicated in the mode of action of Kid (Ruiz-Echevarría *et al*, 1995c). Primer extension analysis showed that Kid alone cleaves *dnaB* mRNA *in vitro*, but not in the presence of Kis (Figure 3A, left). Cleavage of *dnaB* mRNA by Kid is also observed *in vivo* (Figure 3A, right). Interestingly, in both cases Kid cleaves *dnaB* mRNA at two different sites with identical sequence (5'-UUACU-3'; Figure 3A, upper and bottom panels).

To examine cleavage of other host mRNAs by Kid, we used a mini-R1 derivative that produces a thermo-sensitive anti-toxin Kis (P18L; *kis17*), which is inactive at 42°C. Total RNA was isolated from *E. coli* transformed with this mini-R1

Figure 2 Kid and Kis can be expressed from thermo-sensitive promoters to inhibit bacterial cell growth and protein synthesis conditionally. (A) Scheme of the plasmids used to induce *kid* and *kis* expression at 42°C. *cl857* encodes a transcription factor that represses transcription from *Pr_L* at 30°C but not at 42°C. (B) Expression of Kid but not of Kid plus Kis inhibits bacterial cell growth compared to a control strain carrying empty plasmids. (C) Expression of Kid but not of Kid plus Kis inhibits protein synthesis *in vivo*, as seen by ³⁵S-Met incorporation.

Figure 3 Kid cleaves mRNA at UUACU sites. (A) Primer extension analysis of *dnaB* transcripts. *dnaB* mRNA produced *in vitro* was incubated with buffer (Ctrl), Kid or Kid and Kis proteins (left side of panel). Alternatively, *dnaB* was expressed *in vivo*, alone (Ctrl) or together with *kid* (*kid*) for 30 or 60 min (right side of panel). Upper and bottom images show the upstream and downstream 5'-UUACU-3' sites in *dnaB* mRNA, respectively. (B) Primer extension analysis of *lon* transcripts. Strains carrying mini-R1 derivatives with wild-type *parD* (*kiskid*), a thermo-sensitive *parD* (*kis17kid*) or the thermo-sensitive *parD* plus an extra copy of wild-type *kis* (*kis17kid + kis*) were grown at 42°C for 30 min. The most downstream 5'-UUACU-3' site in *lon* mRNA cleaved by Kid is shown. Relevant sequences in (A) and (B) are shown on their axis (see text for details), with the 5'-ends of Kid cleavage products denoted in bold. (C) Sequences of *dnaB* and *lon* mRNAs covered by our primer extension analysis. Cleaved 5'-UUACU-3' sites are in bold and noncleaved 5'-UU(A/C/U)-3' sites are underlined.

derivative and grown at 42°C for 30 min before harvesting. Primer extension analysis of *lon* mRNA showed that Kid cleaves this transcript *in vivo* (Figure 3B). However, no cleavage was detected in cells cotransformed with a plasmid expressing wild-type Kis (*kis17kid + kis*) or with a mini-R1 plasmid carrying wild-type copies of *kis* and *kid* (*kiskid*) (Figure 3B). Kid cleaved both *dnaB* and *lon* mRNAs at 5'-UUACU-3' sites, highlighting the sensitivity of this sequence to the action of the toxin. Strikingly, although reported previously as target sites for PemK (Zhang *et al*, 2004), we did not detect cleavage in any of the adjacent 5'-UA(U/A/C)-3' sites present in these mRNAs, apart from that embedded in 5'-UUACU-3' (Figure 3).

Kid cleaves plasmid-encoded *copB*-*repA* mRNA at UUACU sites

The *copB*-*repA* mRNA also contains two 5'-UUACU-3' sites in its intercistronic region (Figure 4A). This raised the interesting question of whether Kid also cleaves this plasmid-encoded transcript. To analyze this, we took advantage of the leaky behavior of *parD* in *kis17kid* mini-R1. In this plasmid mutant, Kis has a reduced antitoxin activity at 30°C. Thus, due to incomplete neutralization of Kid, cell growth at 30°C is reduced by 30% in bacteria carrying this plasmid (Bravo *et al*, 1987).

Cleavage of the *copB*-*repA* mRNA at 5'-UUACU-3' was detected in cells carrying *kis17kid* mini-R1 at 30°C (Figure 4B, black arrow). Longer exposure times revealed that the other 5'-UUACU-3' site in this region was also cleaved (not shown). No cleavage was detected in control experiments where wild-type Kis was coexpressed (Figure 4B; *kis17kid + kis*) or using mini-R1 plasmids with wild-type *kis* and *kid* (Figure 4B; *kiskid*) or inactive *kid* (Figure 4B; *kiskid18*).

Kid increases the *repA*/*copB* ratio and the copy number of R1

Figure 4B revealed other primer extension products in our samples. A mini-R1 derivative lacking *copB* and its promoter helped to identify the transcription initiation sites of *PrrepA* (Figure 4B; Δ *copB*), which is de-repressed due to the absence of CopB. This showed that synthesis of monocistronic *repA* mRNAs starts in a region spanning 10 bp and located downstream of the 5'-UUACU-3' sites (Figure 4B; white arrows).

Another primer extension product was detected in the *kis17kid* sample (Figure 4B; gray arrow). Two observations suggested that this signal did not arise from cleavage of the *copB*-*repA* mRNA by Kid. First, although much weaker, it could also be detected in the control samples. Second, Kid did not cleave four identical sites (5'-UAA-3') in the *lon* transcript (Figure 3B and C). Interestingly, that product lied in the short

Figure 4 Partial activation of Kid leads to cleavage of the *copB*–*repA* mRNA, de-represses *PrrepA* and increases the copy number of R1. (A) Sequence of the *copB*–*repA* mRNA intercistronic region. Open boxes and underlined sequences denote transcription start sites and cleavage sites, respectively. *SDrepA* represents the Shine–Dalgarno sequence for *repA*. The 5'- or 3'-ends of open reading frames are shown in bold. (B) Primer extension analysis of the *copB*–*repA* intercistronic region in strains transformed with mini-R1 derivatives carrying (i) a wild-type *parD* (*kiskid*), (ii) a leaky *parD* mutant (*kis17kid*), (iii) the same *parD* mutant plus wild-type *kis* (*kis17kid* + *kis*), (iv) a mutant *parD* with inactive *kid* (*kiskid18*) or (v) a wild-type *parD*, but lacking *copB* and *PrkopB* (Δ *copB*). The sequence analyzed is indicated on the axis, and the downstream 5'-UUACU-3' site in this region underlined. The primer extension product at this site is denoted with a black arrow on the image, with its 5'-end indicated in bold on the axis. Transcription start sites in Δ *copB* are indicated with a white arrow on the image and with a bent arrow and a +1 on the axis. The additional primer extension product detected in the *kis17kid* sample is indicated with a gray arrow. (C) Real-time PCR analysis of the *repA*/*copB* ratio in samples (i) to (iii) from (B). Histograms represent the average values from three independent experiments. (D) *E. coli* cotransformed with mini-R1 derivatives carrying wild type (wt) or the leaky *parD* mutant (17) and a compatible coresident plasmid (pVTRA) with *kis* (Kis) or without it (Ctrl) were grown at 30°C. Plasmid DNAs were recovered, linearized and analyzed by agarose gel electrophoresis. Ethidium bromide staining is shown. Numbers at the bottom indicate relative copy number of the mini-R1 derivative with respect to the coresident plasmid as determined by Southern blot using specific radiolabelled probes. (E) As for (D), but using a mini-oriC replicon as the coresident plasmid.

region from which *PrrepA* initiates transcription of *repA*-mRNAs in the absence of *CopB* (Figure 4B; Δ *copB*), which suggested that *PrrepA* could be de-repressed in the *kis17kid* mini-R1 derivative.

To verify this, we used real-time PCR to measure the *repA*/*copB* ratio, which should increase when *PrrepA* is de-repressed. This ratio was 38% higher in the *kis17kid* sample than in the *kiskid* control sample. Moreover, it returned to control values when wild-type *kis* was coexpressed in the *kis17kid* sample (*kis17kid* + *kis*) (Figure 4C). These results suggest that the signal detected in our primer extension (Figure 4B, gray arrow) corresponds to monocistronic *repA*-mRNA.

As *RepA* is limiting for the initiation of R1 replication, when transcription from *PrrepA* is de-repressed copy number of the plasmid increases. Thus, we measured the relative

copy number of our mini-R1 derivatives compared to that of a coresident plasmid. At 30°C, the relative copy number of *kis17kid* mini-R1 is 1.5-fold higher than that of the *kiskid* control (Figure 4D; Ctrl). This difference was abolished if wild-type *Kis* was expressed from the coresident plasmid (Figure 4D; Kis), or if *kid* was deleted from *kis17kid* (not shown). Similar results were obtained when a mini-oriC replicon was used as coresident plasmid (Figure 4E). Taken together, these results suggest that partial activation of Kid in the *kis17kid* mini-R1 increases the synthesis of *repA* and consequently the plasmid copy number.

Kid cleaves the *copB*–*repA* mRNA when R1 copy number decreases

Results in Figure 4 suggest that cleavage of the *copB*–*repA* mRNA by Kid is linked to de-repression of *PrrepA* and that

this increases the copy number of R1. Interestingly, contribution of *parD* to plasmid stability is revealed only in a replication defective R1 mutant (Ruiz-Echevarría *et al*, 1995b). Thus, we tested whether inhibiting R1 replication activates Kid and leads to cleavage of the *copB-repA* mRNA.

E. coli was transformed with a plasmid expressing *copA* from a thermo-sensitive promoter or with an empty control plasmid. These cells were then cotransformed with mR1wt (a mini-R1 derivative with a wild-type *parD*), mR1M3 (mR1wt with the 5'-UUACU-3' sites in the *copB-repA* intercistronic region mutated to 5'-UUUCU-3') or mR1Kid18 (mR1wt carrying a nontoxic Kid mutant). The copy number of all these mini-R1 derivatives decreased when transcription of *copA* was induced at 40°C (Figure 5A). Moreover, in the absence of antibiotic selection for the mini-R1 derivatives, bacterial growth was inhibited in cells carrying mR1wt or mR1M3, but not mR1Kid18, when transcription of *copA* was induced (Figure 5B). This showed that inhibition of bacterial growth was due to the activation of Kid, which happened only when R1 copy number decreased.

We examined the contribution of Kid to plasmid stability in our experimental setup. Cells from samples analyzed in Figure 5B were plated at different time points and grown at 30°C in either Amp (to determine the number of viable cells) or Amp/Kan (to measure the fraction of cells still containing plasmid R1). This showed that when R1 replication is inhibited, the number of colony-forming units (CFU) decreases with time for samples carrying mR1wt and mR1M3 compared to those carrying mR1Kid18 (Figure 5C). However, the number of plasmid-containing CFU does not decrease with time for samples mR1wt and mR1M3 compared to sample mR1Kid18 (Figure 5D). Thus, activation of Kid inhibits cell growth and impedes plasmid loss. As activation of Kid occurs in plasmid-containing cells, it is possible that it cleaves the *copB-repA* mRNA when copy number of R1 decreases.

To examine this, the *copB-repA* intercistronic region was analyzed by primer extension in our samples from Figure 5. Cleavage of both 5'-UUACU-3' sites in this region was detected in samples carrying mR1wt when expression of *copA* was induced for 4 h (Figure 6A, only the downstream 5'-UUACU-3' site is shown; black arrow). No cleavage was detected in any mR1Kid18 or mR1M3 samples, or in cells cotransformed with mR1wt and the empty control plasmid lacking *copA*. These results confirmed that Kid becomes active in plasmid-containing cells if R1 copy number decreases. They also demonstrated that this leads to specific cleavage of the 5'-UUACU-3' sites in the *copB-repA* intercistronic region.

Kid inhibits the synthesis of CopB to restore R1 copy number

Figure 6A also showed strong de-repression of *PrrepA* when copy number of mR1wt (but not of mR1M3) decreases. Interestingly, *PrrepA* of mR1Kid18 is slightly de-repressed in the same experiment (Figure 6A). However, decreasing the copy number of mR1Kid18 neither inhibits bacterial growth (Figure 5B) nor avoids plasmid loss (Figure 5C), and is not linked to mRNA cleavage (Figure 6A). These observations highlight the essential role that Kid and the UUACU sites in the *copB-repA* mRNA play for the correct functioning of the CopB rescue system (see Discussion).

Figure 5 Kid arrests bacterial growth and impedes plasmid loss when R1 copy number decreases. (A) The relative amount of mR1wt, mR1Kid18 and mR1M3 plasmids (mR1) in samples grown for 4 h in the presence (*copA*) or the absence (Ctrl) of *copA* overexpression from a coresident plasmid (pPrTs-). (B) Proliferation curves of strains used in (A), grown in liquid media in the absence of selection for the mini-R1 derivatives. (C) Relative colony-forming units (CFU) of samples overproducing *copA* analyzed in (B). (D) Relative number of CFU analyzed in (C) that still containing the mini-R1 derivative.

De-repression of *PrrepA* occurs when concentration of CopB decreases. Thus, we examined the effects of Kid on the synthesis of CopB from the bicistronic *copB-repA* mRNA.

Figure 6 Kid inhibits the synthesis of CopB and restores R1 copy number through de-repression of *PrrepA*. **(A)** Primer extension analysis of the *copB-repA* intercistronic region in samples analyzed at the 4 h time point in Figure 5B. The sequence of this region in mR1wt is shown on the axis, with its downstream 5'-UUACU-3' site underlined. The primer extension product detected at this site is denoted with a black arrow on the image, with its 5'-end indicated in bold on the axis. Transcription start sites seen in the Δ *copB* sample in Figure 4B are indicated with a white arrow on the image and with a bent arrow and a +1 on the axis. Two experiments with different loadings and exposure times (short and long exposure) are shown for samples expressing *copA*. **(B)** Kid inhibits the synthesis of CopB from a bicistronic *copB-repA* mRNA with a wild-type intercistronic region (wt) but not with a mutant intercistronic region (M3). When Kis and Kid are expressed at the same time (*kiskid*), this effect is neutralized and the synthesis of CopB returns to control values (Ctrl). Detection of pyruvate kinase (Pyk) using specific antibodies served as loading control. **(C)** Relative copy number of mR1wt, mR1Kid18 and mR1M3 in bacteria when synthesis of extra *copA* is induced for 2 and 4 h (40°C; left side of the dashed line), and subsequently repressed for another 2 and 4 h (30°C; right side of the dashed line). Each experiment was performed at least three times. The average result of three different experiments is shown in (C).

We cloned the *copB-repA* open reading frames from mR1wt and mR1M3 in our thermo-sensitive expression vectors, which allowed us to induce transcription of these mRNAs and of *kid* or *kid* and *kis* mRNAs simultaneously. Western blot showed that Kid inhibits the synthesis of CopB and that Kis neutralizes this inhibition (Figure 6B; wt). The effect of Kid on CopB synthesis depends entirely on the integrity of the 5'-UUACU-3' sites in the *copB-repA* mRNA, as no inhibition was detected using the intercistronic mutant control (Figure 6B; M3).

These results explain why cleavage of *copB-repA* mRNA by Kid de-represses *PrrepA* (Figures 4B and 6A) and the higher copy number of *kis17kid* mini-R1 (Figure 4D and E). Samples used in Figure 6A were grown at 40°C and, after 4 h, temperature was shifted to 30°C to inhibit further transcription of *copA* from the coresident plasmid. The relative copy number of each mini-R1 derivative was determined every 2 h by Southern blot (Figure 6C). This showed that, when extra *copA* is produced, the relative copy number of mR1wt decreases more slowly than that of mR1Kid18 and mR1M3. Moreover, when transcription of extra *copA* was repressed, the copy number of mR1wt was restored faster than that of mR1Kid18 and mR1M3 (Figure 6C).

Discussion

Kid cleaves host and plasmid mRNAs at UUACU sites

It had been reported that overexpression of PemK, the homolog of Kid in plasmid R100, leads to cleavage of host mRNAs at 5'-UA(A/C/U)-3' sites (Zhang *et al*, 2004). Host protease Lon is responsible for the rapid turnover of antitoxin Kis (Tsuchimoto *et al*, 1992). Another host gene, *dnaB*, had been implicated in the mode of action of Kid (Ruiz-Echevarría *et al*, 1995c). We analyzed the effects of Kid on *dnaB* and *lon* mRNAs and showed that Kid cleaves both transcripts specifically at 5'-UUACU-3' sites. Interestingly, no cleavage was observed at any of the 10 adjacent 5'-UA(A/C/U)-3' sites in these mRNAs (Figure 3). Thus, our work shows that Kid (thus, PemK) cleaves mRNA at 5'-UUACU-3' sites, a longer, more specific sequence than described previously (Zhang *et al*, 2004).

parD was identified as a postsegregational killing system contributing to the stable maintenance of R1 plasmid in bacteria (Bravo *et al*, 1987). Postsegregational killing systems eliminate plasmid-free cells (Gerdes *et al*, 1986; Engelberg-Kulka and Glasser, 1999; Gerdes, 2000; Hayes, 2003). However, we demonstrate here that Kid activation occurs in plasmid-containing cells and cleaves also the *copB-repA* mRNA, specifically at 5'-UUACU-3' sites (Figures 4B and 6A). This novel pre-segregational role of Kid is independent of ribosomes, as cleavage of 5'-UUACU-3' sites is detected both *in vivo* and *in vitro*, using a reconstituted system (Figure 3A). Furthermore, Kid cleaves the intercistronic (i.e. nontranslated) *copB-repA* mRNA region *in vivo*, which suggests that its activity is not coupled to that of translation by ribosomes (Figures 4B and 6A).

Kid becomes active in plasmid-containing cells to increase R1 copy number

We used two different approaches to analyze the consequences of Kid activation in plasmid-containing cells. First, we used a leaky mutant *parD* in a wild-type R1 replicon

context. We show that incomplete neutralization of Kid in this mutant leads to cleavage of the intercistronic region in *copB-repA* mRNA, and to de-repression of *PrrepA* (Figure 4B; *kis17kid*). We also show that in this sample the *repA/copB* ratio increases 38% (Figure 4C) and that the copy number of this mini-R1 is higher than normal (Figure 4D and E).

Using a mini-R1 with a wild-type *parD* system (mR1wt), we demonstrate that Kid cleaves the *copB-repA* mRNA when plasmid copy number decreases (Figures 5 and 6A). This depends entirely on the presence of wild-type *kid* and on the integrity its target sites in the *copB-repA* mRNA, and it also leads to de-repression of *PrrepA* (Figure 6A). Consequently, when extra *copA* is produced from a coresident plasmid, the copy number of mR1wt remains higher than that of mR1Kid18 (with inactive Kid) or mR1M3 (lacking UUACU sites in *copB-repA* mRNA). Moreover, in the absence of further extra synthesis of *copA*, mR1wt restores its copy number faster than mR1Kid18 and mR1M3 (Figure 6C).

Cleavage of 5'-UUACU-3' sites by Kid inhibits the synthesis of CopB from the bicistronic *copB-repA* mRNA (Figure 6B), which explains why Kid activation de-represses *PrrepA*. Cleavage of the *copB-repA* mRNA by Kid occurs downstream of the *copB* gene, as no other 5'-UUACU-3' sites are found in this molecule. However, endoribonucleolytic cleavage of polycistronic mRNAs often triggers exoribonucleolytic degradation of its upstream cistrons (Grunberg-Manago, 1999). Our results suggest that cleavage of the intercistronic *copB-repA* mRNA by Kid leads to the degradation of upstream *copB* by exoribonucleases.

De-repression of *PrrepA* is observed using a leaky *parD* mutant and a wild-type *parD* (Figures 4B and 6A). Transcription from *PrrepA* initiates within 10 bp located downstream of the 5'-UUACU-3' sites in the *copB-repA* intercistronic region (Figure 4B; white arrows). Activation of Kid from wild-type *parD* induces transcription from *PrrepA* at the same transcriptional initiation site that is de-repressed in the absence of *copB* (Figure 6A, upper white arrow). However, transcription of *repA* initiates at a slightly more downstream site when the leaky mutant *parD* is used (Figure 4B, gray arrow). This is probably due to differences in the way *parD* behaves in each case. In the leaky *parD* mutant, toxicity of Kid is only partial and the copy number of R1 (thus, the concentration of CopB) is higher than normal. It is possible that in cells carrying this mutant the intracellular level of CopB decreases, but remains higher than when Kid becomes active from a wild-type *parD*. This may determine the location of the initiation sites of *repA*-mRNAs within this region.

In *kis17kid* R1, transcription from de-repressed *PrrepA* starts in the sequence 5'-UAA-3' (Figure 4B, gray arrow). This sequence had been described previously as a cleavage site for PemK (Zhang *et al*, 2004). However, three experimental observations provide strong evidence that this signal corresponds to initiation of monocistronic *repA*-mRNA synthesis and not to mRNA cleavage by Kid. First, we see that three identical sites (5'-UAA-3') are not cleaved in *lon* mRNA by Kid (Figure 3B). Second, that signal is also detected in the absence of Kid activation (Figures 4B and 6A). Third, we do not observe cleavage of this site when Kid becomes active from wild-type *parD* (Figure 6A).

Kid is part of a rescue system that senses and regulates R1 copy number

A recent report suggests that CopB is kept by R1 to act as a rescue system in cells with very few copies of the plasmid. This proposal is based on the indirect observation that extra CopB increases the loss rate of R1 derivatives and strongly represses transcription from *PrrepA* (Nordström and Aagaard-Hansen, 1984; Olsson *et al*, 2004). Our results demonstrate that, although initially described as a postsegregational killer toxin, Kid is part of the CopB rescue system and has evolved to act presegregationally in a reversible manner. Indeed, our work helps to explain unsolved issues concerning the sensitivity of this system if it depends exclusively on CopB (Olsson *et al*, 2004). In cells where copy number of R1 decreases, activation of Kid leads to cleavage of host mRNAs at 5'-UUACU-3' sites and inhibits cell proliferation, which prevents plasmid loss during division (Figure 5). At the same time, Kid cleaves the plasmid-encoded *copB-repA* mRNA with the same specificity (Figure 6A). This decreases the intracellular concentration of CopB (Figure 6B) and de-represses *PrrepA* (Figure 6A), restoring R1 copy number (Figure 6C). Although dilution of CopB may contribute to activating the rescue system when R1 copy number is very low, it has been acknowledged that its sensitivity would be greater if CopB were actively degraded (Olsson *et al*, 2004). We show that reducing the copy number of mR1M3 also activates Kid, arresting bacterial growth and avoiding plasmid loss (Figure 5). However, *PrrepA* is not de-repressed in mR1M3 (Figure 6A), which indicates that dilution of CopB in this situation is not enough to activate the rescue system from the plasmid. Partial de-repression of *PrrepA* is observed when the copy number of mR1Kid18 decreases (Figure 6A). As Kid is not functional in this plasmid, it neither inhibits bacterial growth nor avoids plasmid loss (Figure 5). Thus, dilution of CopB can occur to an extent that allows partial de-repression of *PrrepA*, although at the expense of great plasmid instability (Figure 5D). This is supported by comparison of Figures 5D and 6C. The number of CFU containing mR1Kid18 (but not mR1M3) decreases after 4 h in Figure 5D. However, relative copy number of mR1Kid18 and mR1M3 are similar at this time point (Figure 6C). Thus, although fewer cells still contain mR1Kid18, they do so at higher copy numbers than those containing mR1M3, due to de-repression of *PrrepA* (Figure 6A). However, this cannot prevent plasmid loss (Figure 5D). Our results reveal that only when functional Kid and UUACU sites in the *copB-repA* mRNA are present, the rescue system can operate efficiently. Only then does Kid inhibit bacterial growth and synthesis of CopB simultaneously, providing the right conditions to dilute CopB progressively without any plasmid loss. This eventually de-represses *PrrepA* and restores the plasmid copy number.

We propose here a model to explain how *parD* acts presegregationally to stabilize R1 (Figure 7). When copy number of R1 is normal, transcription from *PrkopB* produces both CopB and RepA. CopB represses transcription from *PrrepA* and limits the replication rate of R1. In this situation, transcription from *PrparD* is also low. It produces Kis and Kid, and this neutralizes toxicity and represses *PrparD*. This equilibrium maintains the copy number of R1 and the synthesis of Kid and Kis fairly low and constant. Kis is continuously degraded by the host protease Lon, but this faster

Figure 7 Kid is part of a rescue system that regulates the copy number of R1. Transcription from *PrcopB* produces low amounts of a bicistronic *copB-repA* mRNA. CopB represses transcription from the stronger *PrrepA*, keeping the copy number of R1 low. Low levels of *kis-kid* mRNA are synthesized from *PrparD* to produce Kid and excess Kis. These proteins form a complex that neutralizes Kid toxicity and represses transcription from *PrparD*. Rapid turnover of Kis by the host protease Lon de-represses *PrparD* and further excess of Kis is periodically produced. This maintains the rescue system in a constant 'alert' state. If copy number of R1 decreases rapidly, the equilibrium is broken towards toxicity, and activates the rescue system. In this situation, remaining Kid cannot be readily neutralized, and it cleaves host- and plasmid-encoded mRNAs at UUACU sites. This arrests bacterial growth and prevents plasmid loss. At the same time, this activity inhibits synthesis of CopB, resulting in transcription from *PrrepA*. As a consequence, the replication rate of R1 increases. While copy number recovers, an excess Kis is rapidly produced from de-repressed *PrparD*, which progressively neutralizes Kid to restore the equilibrium.

turnover of Kis is counteracted by several regulatory loops that ensure neutralization of Kid. However, this balance is broken towards toxicity if copy number of R1 decreases rapidly. When this happens, degradation of Kis produces a relatively large amount of free Kid that cannot be readily neutralized by newly synthesized Kis from very few copies of the plasmid. This free Kid acts presegregationally, arresting cell growth and, simultaneously, decreasing the intracellular concentration of CopB and de-repressing *PrrepA*. As a consequence, replication of R1 is stimulated and its copy number rapidly restored. In the absence of enough Kis, *PrparD* is also

de-repressed, which produces more Kis than Kid. Thus, as copy number of R1 recovers, the intracellular concentration of Kis increases faster than that of Kid. This progressively neutralizes the effects of the toxin until the equilibrium is restored again (Figure 7). Cells that lose R1 despite the activation of the rescue system are eliminated by the prolonged, now irreversible, activity of Kid. Interestingly, the *copB* gene of plasmids R1 and R100 differ in sequence but not in function. Most interestingly, R100 conserves one of the 5'-UUACU-3' sites cleaved by Kid in the *copB-repA* intercistronic region of R1. This observation strongly supports that the

novel function of Kid described in this work is also relevant for the stable maintenance of R100 by PemK.

If Kid inhibits protein synthesis, how can it promote higher synthesis of RepA, essential to increase the copy number of R1? Conspicuously, monocistronic *repA*-mRNAs do not contain 5'-UUACU-3' sites (although they contain 19 sites with the sequence 5'-UA(A/C/U)-3', previously described as target sites of PemK; Zhang *et al*, 2004). By cleaving mRNAs specifically at 5'-UUACU-3' sites, Kid helps to dilute CopB, de-repressing *PrrepA* without inhibiting *de novo* synthesis of RepA. While this work was under revision, it was reported that a chromosomal homolog of Kid allows protein synthesis *in vivo*, but only from mRNAs lacking its cleavage target site (Suzuki *et al*, 2005). This observation strongly supports our proposal and helps to explain the apparent paradox of how RepA can be synthesized while Kid is active.

Our results differ from a report claiming that PemK (or Kid) cleaves mRNAs with less specificity. However, that study was not performed in the context of plasmid R1 stability (Zhang *et al*, 2004). Our work, which takes this into account, demonstrates that Kid has evolved to act exquisitely in plasmid-containing cells, cleaving host- and plasmid-encoded mRNAs at 5'-UUACU-3' sites, and acting in a reversible manner to regulate the copy number of R1.

The function and activity of Kid resemble viral host shutoff of herpesviruses

Human herpesviruses have acquired the ability to alter both host and viral mRNA stability. The virion host shutoff (Vhs) protein drives this process through its endoribonucleolytic activity. During lytic infection, Vhs accelerates the degradation of cellular mRNAs, leading to an overall decrease in host protein synthesis. Following the onset of viral transcription, Vhs accelerates the turnover of viral mRNAs. By shortening the half-lives of all mRNAs, Vhs redirects the cell from host to viral gene expression, facilitates the sequential expression of different classes of viral genes and stimulates the replication of the viral genome (Glaunsinger and Ganem, 2004; Smiley, 2004).

Strikingly, the mode of action of these viral host shutoff proteins shares several features with Kid. They not only cleave host mRNAs but also viral mRNAs, and, in the latter case, they seem to target intercistronic regions (Smiley, 2004). Furthermore, Vhs preferentially affects the stability of mRNAs with AU-rich elements, which contain repetitions of the sequence 5'-AUUUA-3' (Escaline *et al*, 2004). Thus, it seems that prokaryotic plasmid R1 and human herpesviruses may have evolved similar strategies to stimulate their own replication while shutting off host protein synthesis.

Materials and methods

Strains and plasmids

E. coli DH10B was used in all experiments. R1 plasmids with wild-type- (pKN1562) and thermo-sensitive *parD* (pAB17) are as in Bravo *et al* (1987). The Δ *copB* variant (pET80) is as in Ruiz-Echevarría *et al* (1995b). Alternatively, the basic replicon of R1 and the *parD* system from these plasmids were amplified by PCR and ligated to the *kan* resistance gene (R1 derivatives in Figures 4B, C, 5, 6A and C). *Kis* was cloned in pPT150 (Elvin *et al*, 1990) to produce pPrTsHCKis. The *PstI*-*EcoRI* fragment of pPT150 was cloned in pVTRA-A (Pérez-Martín and de Lorenzo, 1996) to generate pPrTsLWC (PrTs, HC and LWC stand for thermo-sensitive promoter; high copy- and low copy plasmid, respectively). The

same fragment was cloned between *EcoRI* and *XmnI* in pACYC184 to create p184PrTs. *Kid* was cloned in pPrTsLWC (pPrTsLWCKid). *DnaB* was cloned in pGADT7 (Clontech) and in pPrTsHC for the experiments *in vitro* and *in vivo*, respectively, in Figure 3A. *PrparD* and *kis* were cloned in pVTRA-A to obtain pVTRAKis. The mini-*oriC* plasmid in Figure 4E was obtained ligating a PCR fragment carrying *oriC* and its flanking *mioC* and *gldA* genes to the *chlR* resistance gene. A PCR product of *copA* was cloned in pPrTsHC for Figures 5, 6A and C. Mutagenesis of the *copB*-*repA* intercistronic region (mR1M3) was made using oligos

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5'-CTAAAGTAAAGACTTTTCTTTGTGGCGTAGC-3',
5'-GCTACGCCACAAAGAAAAGTCTTTACTTTAG-3',
5'-GGCGTAGCATGCTAGATTCTGATCGTTTGGAAATTTGTGGCT
GGCC-3' and
5'-GGCCAGCCACAAAATTCCAAAACGATCAGAAATCTAGCATGCT
ACGCC-3'.
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To introduce the inactive *kid18* mutant (Hargreaves *et al*, 2002) in mR1Kid18, oligos

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5'-CGCAGGTCATAAGCAGCAGGGAACGC-3' and
5'-GGCCGCGTTCCCTGCTGCTTATGACCTGC-3'
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were annealed and cloned into *EcoNI* and *EagI* of mR1wt. pKN1562 and mR1M3 were used as templates to amplify *c-myc*-*copB*-*repA* by PCR, which were cloned into p184PrTs for the experiments in Figure 6B.

Cell growth and protein translation

E. coli transformed with pPrTsHC plus pPrTsLWC (Ctrl), pPrTsHC plus pPrTsLWCKid (Kid) or pPrTsHCKis plus pPrTsLWCKid (Kid/Kis) were grown in LB plus Amp (100 µg/ml) and ChlR (10 µg/ml) at 30°C to an OD_{600 nm} of 0.2. Cultures were shifted to 42°C and grown exponentially in prewarmed medium. OD_{600 nm} was measured at the indicated time points. For the *in vivo* labelling experiments, 2 µCi of ³⁵S-Met were added to 500 µl of these cultures at the indicated times and incubated for 2 min before stopping the reactions with 10% TCA and 100 µg/ml of nonradioactive Met. Proteins were precipitated on ice for 1 h, collected by centrifugation and analyzed by SDS-PAGE. For the experiment in Figure 6B, cells described above were cotransformed with p184PrTs-*c-myc*-*copB*-*repA* (carrying either wt or M3 intercistronic regions) and they were grown in LB plus Amp (100 µg/ml), ChlR (10 µg/ml) and Tet (10 µg/ml) at 30°C until OD_{600 nm} was 0.4. Cultures were shifted to 40°C for 1 h and CopB expression was analyzed by Western blot using monoclonal anti-*c-myc* tag antibody 9E10. Cells cotransformed with mR1wt, mR1Kid18 or mR1M3 and pPrTsHC*copA* or pPrTsHC, and used in Figures 5, 6A and C, were grown exponentially at 40°C in LB plus Amp (100 µg/ml). OD_{600 nm} was measured at time points indicated in Figure 5B. At some of these time points, cells were plated in LB Amp and LB Amp/Kan and grown at 30°C (Figures 5D and E). For Figure 6A, temperature was shifted to 30°C after 4 h of growth at 40°C. All experiments were performed at least three times.

Primer extension and real-time PCR

All primer extension and sequencing reactions were performed using the Primer Extension System AMV Reverse Transcriptase (Promega) and the Sequenase 2.0 (USB) kits, respectively, following the manufacturer's instructions. A transcript spanning the first 465 nucleotides of *dnaB* was obtained using pGADT7DnaB digested with *BglII* as template and the MEGAscript kit (Ambion). A measure of 0.5 fmol of this RNA per sample was heated at 95°C for 5 min in 20 mM Tris-HCl (pH 7.5), 75 mM NaCl and 10 mM MgCl₂, and allowed to reach room temperature. Samples were then incubated for 10 min at 37°C with buffer or with Kid (4 pmol), either alone or with Kis (6 pmol). Proteins were purified as in de la Cueva-Méndez *et al* (2003). Samples were precipitated with ethanol and analyzed by primer extension using the oligo 5'-AGCAAACACCGACGC TATC-3'. *DnaB* was sequenced from pGADT7DnaB (Figure 3A; left). Alternatively, the same analysis was performed using identical amounts of total RNA purified from cells carrying pPrTsHCDnaB and pPrTsLWCKid and cultured exponentially at 42°C for the indicated times (Figure 3A, right). For the analysis using *lon*, cells were grown exponentially at 42°C for 30 min, and identical amounts of RNA extracted from them were used for each sample. Oligo 5'-GGTTTTCGTTATCCGCGCGAC-3' was used for primer extension

and sequencing reactions. A PCR product of *lon* was used as template for the sequencing reaction. For the analysis of *copB-repA* mRNA, cells were grown exponentially at 40°C for the indicated times. Identical amounts of RNA from these samples were used. Oligo 5'-TAAATCCACATCAGAACCAGTT-3' was used for primer extension and sequencing reactions. In Figures 4B and 6A, loading of samples was adjusted so that the signal corresponding to the bottom white arrows had similar intensity. Real-time PCR was performed in a DNA Engine OPTICON MJ Research, using SYBR Green Jump Start reagent (SIGMA). Oligo pairs

5'-ATGTCGCAGAGAGAAAATGCAG-3',
5'-CAGCGGCCATTGTTTCTCAG-3' and
5'-GTGACTGATCTTCACCAACGTAT-3',
5'-GTTTTTCGCAGAACTTCAGCGT-3'

were used to amplify bicistronic- and monocistronic-*repA* cDNA, respectively. The *repA/copB* ratio was determined following the method described in Pfaffl (2001). All experiments were performed at least three times.

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Plasmid copy number

Samples in Figures 4D, E, 5A and 6C were grown as indicated above. DNA purified from these samples was linearized, run in agarose gels and stained with ethidium bromide (Figure 4D and E). For quantification, DNA from these gels was transferred to Zeta-Probe membranes (Bio-Rad) and probed with *oriC*-, pSC101-*ori*-, and *repA*- (Figure 4D and E) or *Amp^r*- and *parD*- radiolabelled probes (Figures 5A and 6C). Labelling was performed with the Rediprime II labelling system (Amersham). Intensity of bands was quantified from X-ray films using a Fujifilm FLA-5000 densitometer. Relative copy number of the mini-R1 derivatives was determined using the intensity of the band corresponding to the coexisting plasmid as control reference. All experiments were performed at least three times.

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