Replication-induced transcription of an autorepressed gene: The replication initiator gene of plasmid P1

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Communicated by Donald R. Helinski, University of California, San Diego, La Jolla, CA, April 26, 2000 (received for review December 12, 1999)

The replication origin of plasmid P1 contains an array of five repeats (iterons) that bind the plasmid-encoded initiator RepA. Within the array lies the repA promoter, which becomes largely repressed on RepA binding (autorepression). One might expect that extra iterons produced on plasmid replication would titrate RepA and release the repression. The promoter, however, is induced poorly by extra iterons. The P1 copy number is reduced by extra iterons in the presence of the autorepressed repA gene but not when additional RepA is provided from constitutive sources. It has been proposed that the iteron-bound RepA couples with the promoter-bound RepA and therefore maintains repression. Although not the product of replication, we find that the act of replication itself can renew RepA synthesis. Replication apparently cleans the promoter of bound RepA and provides a window of opportunity for repA transcription. We propose that replication-induced transcription is required to ensure initiator availability in a system that is induced poorly when challenged with additional initiator binding sites.

The copy number of bacterial plasmids often is regulated by controlling initiator protein concentration (1). Plasmids where this type of regulation is clear use antisense RNA to reduce initiator synthesis. In certain other plasmids, including the plasmid P1, replication is controlled by ~20-bp repeats, called iterons (Fig. 1). Iterons bind plasmid-encoded initiator proteins and invariably are found in the origin of replication. Their saturation by initiators is considered essential for replication initiation. Iterons also can be found outside the origin. Their deletion increases the plasmid copy number. Conversely, the presence of extra iterons in trans reduces copy number. These observations led to the proposal that iterons reduce plasmid copy number by titrating initiators that are assumed to be limiting for replication (2). The model was challenged when increasing initiator concentration from trans sources did not increase the copy number significantly (except for plasmid R1162, ref. 3) (2, 4). A second property of initiators, that they increase the copy number significantly (except for plasmid P1) with 14 iterons; ref. 7). The promoter of the repA gene is downstream of the incA locus. RepA binds to the origin represses the promoter (autorepression) ≥99%, causing the low basal level of the protein. The concentration of active RepA must be even lower because the capacity of RepA to bind iterons depends on activation of the protein by chaperones (8). Under physiological conditions, RepA is not present in excess, because the copy number does increase, albeit to a small extent (~1.4-fold), when extra RepA is provided (5). The most intriguing observation is that the autorepressed promoter is poorly induced by extra iterons in trans (titration) (9). Because extra iterons reduce copy number efficiently, one of the mechanisms of copy number reduction thus could be by limiting new initiator synthesis. This view was supported by the finding that the copy number reduction by excess iterons could be overcome by increasing initiator concentration from constitutive sources (5, 10, 11). Thus, iteron-mediated copy number control in P1 plasmid may involve limiting RepA supply.

The tight autorepression of the P1repA promoter taken together with its poor induction on titration prompted us to seek an alternate induction mechanism (12). We argued that unfettered access of RNA polymerase to the promoter may be possible only when the replication fork releases the promoter of bound RepA. A burst of transcription activity after replication (replication-induced transcription) was found. This may be the natural mechanism for RepA supply because an increase of iteron concentration after replication is likely to maintain the repA promoter repression at a level inadequate for reinitiating replication. This has been confirmed by varying iteron concentration within a range that most likely occurs in the natural replication cycle of the plasmid and by measuring the repA promoter activity.

Materials and Methods

Plasmids. pVM11 (~3 kb, SpecB) (Table 1) is derived from a miniP1, pSP102 (4.3 kb, Cm63) (13), by exchanging its BamHI–PstI fragment containing the cat gene with a BamHI–PstI fragment containing the spec gene of plasmid pSE418 (S. Elledge, personal communication), and deleting about 1 kb DNA between PvuII sites in repA (P1 coordinate 849) and one in front of the spec gene. pVM11 replicates only when provided with RepA in trans. pSM113 (see Fig. 4) was made by ligating a 1.7-kb BglII–EcoRV fragment containing the promoter-less luciferase gene from pSP-luc+ vector (Promega) with the BamHI–NeuI (partial) digested pSP102. In pSM113, the luciferase gene is downstream of repA translational stop codon.

Bacteriophages. xincCreP4-laZ phage (ADK336) (Table 3) was constructed first by cloning a NeuI–BamHI fragment of pSP102 (P1 coordinates 503-1529) between the Smal–BamHI sites of the vector pMLB1109 to generate the plasmid pRJM380. The vector has a promoterless lacZ gene downstream of the cloning sites. The incCreP4-laZ region next was transferred to ARS45 (14) to generate ADK336, xincCreAincA-laZ phage (ADK369) was constructed similarly from plasmid pDKC422. The plasmid is identical to pRJM380 except that the 331-bp SfiI–BamHI region from the C-terminal end of repA was replaced with an 804-bp SfiI–BamHI region (P1 coordinates 1201–2001) of pRJM370 (9) to include the incA locus.

Plasmid Copy Number. Volumes of exponentially growing cultures were adjusted to contain four OD600 units of cells. A 10-μl

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Article published online before print: Proc. Natl. Acad. Sci. USA, 10.1073/pnas.130189497.

Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.130189497
aliquot of OD_{600} = 15 cells of DH5Δlac/pNEB193 (New England Biolabs) was added to each culture (recovery control). Plasmid DNA from the mixture was isolated by using a Qiagen Miniprep Kit (Qiagen, Chatsworth, CA) and recovered in 50 µl of both thiamine and thymine. At OD600 = 0.2, the cultures were transferred to a 42°C bath and, after 60 min, returned to the 30°C bath. Aliquots were removed and added to an equal volume of ice-cold quenching solution (30 mM each of KCN, Na3N3, and EDTA). Two OD600 units of cells were collected by centrifugation. The cell pellet was washed in 50 µl of 10 mM potassium phosphate buffer (pH 7) and resuspended in the same buffer. Total cellular RNA was isolated by using the RNeasy Mini Kit (Qiagen). For S1 assay, probes for the rpoA gene were end-labeled and 1 ng of the repA probe was used for hybridization to 20 µg of total cellular RNA and subsequently treated with S1 as recommended (Ambion). The products were separated on a 6% sequencing gel by running at 60 W until the blue dye migrated 32 cm. The band intensities were recorded in a PhosphorImager (Fuji).

In Fig. 3 and Table 1, RNA was quantified by using a Primer Extension Kit (Promega). Total RNA was isolated as above. To monitor recovery, a culture of DH5Δlac/pALA96 (10) was added to the experimental cultures in 1:50 (in OD600 units) before RNA isolation. Typically, 20 µg of total cellular RNA was hybridized to 5' end-labeled repA probe (KP4, 5'CTGCCTAT- AAGAATATCGGA-3', P1 coordinates 710–692) and bla-1 probe (5'AGGGCCGACACGGAAAATGTTGAATACCTACTCA-3', pBR322 coordinates 4126–55; ref. 17). The mixture was heated at 65°C for 20 min and then left at room temperature for 20 min before proceeding with one round of primer extension. The entire reaction was resolved on a 6% sequencing gel, and the repA and bla messages were quantified as above.

Luciferase Assays. These were performed by using the Luciferase Assay system (Promega). To 50 µl of experimental culture, 40 µl of a PC2 culture (OD600 = 0.4) (carrier cells) and 10 µl of 1 M K3HPO4 (pH 7.8) + 20 mM EDTA solution were added. The mixture was placed on dry ice for 5 min and then allowed to equilibrate to room temperature for 15 min. The cells then were lysed with 300 µl of a lysis mix, and 1–2 µl of the lysed extract was added to 100 µl of the substrate in wells of a white opaque microtiter plate (EG & G, Salem, MA). Light emission was measured immediately at a reading speed of 1 sec/well in a 1420-Victor Multilabel Counter (Wallace, Gaithersburg, MD).

Results
Expression of repA mRNA in a Replication-Synchronized Culture. The autorepressed repA promoter is induced poorly in the presence of a 10-fold excess of iterons (9). The induction is undetectable when the excess is 2-fold. Therefore, it is unlikely that a doubling of plasmid copy number in a cell generation would be effective in induction. This prompted us to explore whether replication

Quantification of repA mRNA. In Figs. 2 and 3, total RNA was isolated from cultures of PC2 (dnaCts), and repA mRNA was quantified by S1 nuclease protection or by primer extension. The array of iterons in ori is called incA that also contains ~35 and ~10 of repA promoter. P1 coordinates are from ref. 28.

Table 1. Levels of repA mRNA from autorepressed promoters: Effect of iteron numbers

<table>
<thead>
<tr>
<th>Integrated prophages in DH5Δlac</th>
<th>λincCrePA-lacZ</th>
<th>λincCrePAincA-lacZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>lacZ activity (Miller units)*</td>
<td>14 ± 1.2</td>
<td>28 ± 2.6</td>
</tr>
<tr>
<td>Number of prophages (iterons)</td>
<td>1 (5)</td>
<td>2 (10)</td>
</tr>
<tr>
<td>Phage yield by ter assay†</td>
<td>&lt;10⁴</td>
<td>7 × 10⁵</td>
</tr>
<tr>
<td>Relative repA mRNA¹</td>
<td>1</td>
<td>1.5 ± 0.10</td>
</tr>
<tr>
<td>Transforms with pSP102</td>
<td>7 × 10³</td>
<td>4 × 10³</td>
</tr>
<tr>
<td>Transforms with pVM118</td>
<td>7 × 10³</td>
<td>4 × 10³</td>
</tr>
</tbody>
</table>

*The activity was measured from log phase cultures grown in L broth (30) and units were used to estimate prophage numbers. The units may not be compared between the two prophages because a transcription terminator in incA and a damaged par promoter precedes lacZ in the incA carrying prophage (unpublished results).
†The assay helps to discriminate between mono- and poly-lysogens (13).
RNA was measured by primer extension exactly as described for Fig. 3 by using aliquots from the same culture used for measuring lacZ activity.
As opposed to overnight for others, these colonies appeared after 48 hr at 37°C.
⁵pVM11 = ΔrepAΔincA whereas pSP102 is ΔincA only.
with nuclease S1 (Fig. 2). There was a second burst of repA expression at around 30–60 min after the shift to 30°C as assayed (a normalizing control) mRNA-specific probes, and analyzed by S1 protection. Lane 1: RNA of PC2 without any plasmid assayed with repA probe only, showing no hybridization of the probe to host messages. Lanes 2 and 3: RNA from PC2/pSP102 (the plasmid is an overproducer of RepA; ref. 29) assayed with repA (lane 2) and repA (lane 3) probes separately to mark positions of repA and rpoA signals. The 65- and 55-nt bands represent the unhybridized (full length) repA and rpoA probes, respectively, that escaped S1 digestion. The 45- and 35-nt bands represent probes that were protected from S1 digestion because of specific hybridization to repA and rpoA messages, respectively. The ladder of bands above 45- and 35-nt bands represents incomplete digestion products of the hybridized probes as explained in Materials and Methods. Lanes 4–17: RNA from PC2/pSP102 assayed at indicated times with both the probes. The normalized levels of repA message from these lanes are presented graphically (Lower, ○). The relative copy number of pSP102 at indicated times is also determined (▲).

Expression of repA-luciferase Fusion in a Replication-Synchronized Culture. To confirm replication-induced transcription by an alternate assay, a transcriptional fusion was made between repA and the firefly luciferase gene. The enzyme Luciferase is monomeric and heat sensitive, does not require processing for activity, and can be detected in femtogram quantities (19, 20). The assay was therefore ideal to record low levels of gene activity characteristic of repA, and induction in temperature-shift experiments because pre-existing luciferases are inactivated during incubation at 42°C.

Upon shift-down to 30°C, the Luciferase activity increased also with a burst (Fig. 4A). The peak of the burst appeared at 15 min after the shift-down, about 5 min later than that of the repA mRNA (Figs. 2 and 3). Similar to the repA mRNA expression, there was a second burst of enzyme activity 35–45 min after the shift-down. The resumption of Luciferase activity in bursts after the second burst if the plasmid goes through two rounds of replication in one generation like the PC2 chromosome (16, 18). During this period, the copy number increased a little more than over 2-fold. The results were similar when mRNA was quantified by primer extension (Fig. 3). From three experiments, the burst was 7 ± 2-fold between 5 and 10 min after shift-down. There was also a second burst around 30 min. These results indicate that replication induces repA expression. That the expression depends on replication is also evident from the drop in repA mRNA at 42°C (Figs. 2 and 3).
The replication-induced transcription also was originating from two halves. One was maintained at 30°C throughout (Fig. 4). Luciferase activity being heat sensitive is lost irreversibly on incubation at 42°C. It resumes with two peaks on return to 30°C (Fig. 4). The burst required synchronization of RepA promoter (30°C) but not in control cultures when the enzyme is made from a vector promoter (8), or when cells were complemented with a dnaC plasmid to prevent replication synchronization (C), or when repA promoter was repressed with a trans source of RepA (repA) (D). Luciferase units are machine units per mg of total protein.

Fig. 4. Expression of repA-luciferase transcriptional fusion in a replication synchronized culture as in Fig. 2. pSM113 is identical to pSP102 except for the luciferase gene downstream of repA. Cultures of PC2/pSM113 were split into two halves. One was maintained at 30°C throughout (C) and the other was subjected to temperature shifts (A). Luciferase activity being heat sensitive is lost irreversibly on incubation at 42°C. It resumes with two peaks on return to 30°C (A) but not in control cultures when the enzyme is made from a vector promoter (B), or when cells were complemented with a dnaC plasmid to prevent replication synchronization (C), or when repA promoter was repressed with a trans source of RepA (repA) (D). Luciferase units are machine units per mg of total protein.

the shift-down is consistent with replication-induced transcription of the luciferase gene.

The burst cannot be characteristic of Luciferase activity measurements as the vector alone failed to show a similar pattern of expression (Fig. 4B). The burst required synchronization of DNA replication, because when the host was converted to dnaC+ by complementation with a pDnaBdnaC plasmid, the activity returned to the steady-state level without visible spikes (Fig. 4C). The replication-induced transcription also was originating from the repA promoter. When the promoter was repressed by providing RepA from a constitutive source (ADK300 prophage; ref. 5), the bursts were not apparent, and the Luciferase activity failed to reach the steady-state level within the time scale of the experiment (Fig. 4D). A simple explanation of our results is that when the replication fork crosses the origin iterons, it cleans the promoter of bound RepA and allows a transient burst of repA transcription.

Inefficient Induction of repA mRNA by Extra Iterons. To understand the significance of replication-induced transcription in plasmid replication, we wanted to test the level of repA induction with increasing iteron concentration. The repA mRNA concentration was measured in isogenic monolysogens and dilysogens of λ carrying a single copy of repA with its associated promoter region, incC (Fig. 1). The repA mRNA concentration increased significantly (1.5-fold) in dilysogens (Table 1). However, no further increase in mRNA level was apparent when the prophages also carried the incA region, i.e., 4–18 extra iterons. This nonlinear response was seen earlier when induction was followed indirectly by reporter gene activity or immunoblotting of RepA (9). As we interpreted previously (9), the low level of initial induction appears to be caused by titration but when the concentration of RepA-iteron complexes become significant (here in incCrepA dilysogens) to favor handcuffing, no further induction appears to occur. These results support the view that repA induction can be limited because of handcuffing, necessitating repA synthesis to precede handcuffing. The initial small but significant induction (1.5 ± 0.1-fold) suggests that the free RepA concentration is indeed low, approaching that of iterons.

Iterons Can Block MiniP1 Replication by Blocking repA Induction. On several occasions, blockage of replication by extra iterons has been overcome by extra RepA supplied from foreign promoters (5, 10, 11). An intriguing feature of these experiments is that the extra RepA was required even though the autorepressed RepA source was present. These experiments suggested that the repA promoter was not adequately induced in the presence of extra iterons. To confirm the notion under more natural concentrations of RepA and iterons, a single copy of the autorepressed repA gene was used as a trans source of RepA and replication of a ΔrepA miniP1 was measured. The natural repA source supported replication but not optimally as the plasmids were unstable (Table 2). Extra RepA from constitutive sources increased copy number and stability (compare pDKC426 and pDKC424). As expected, extra iterons reduced copy number (3-fold) despite the presence of the autorepressed RepA source (compare pDKC426 and pALA96). The increase in iteron concentration was in a range (less than 2-fold) that could occur by replication normally. The reduction in copy number was overcome when the extra RepA was provided (compare pALA96 and pALA177). The results were qualitatively similar whether or not the miniP1 plasmid carried the incA locus. These results confirm that the autorepressed promoter responds poorly to added iterons under near normal conditions, and a boost of initiator supply (from foreign promoters) can restore iteron-mediated copy number reduction but cannot increase the copy number too far beyond the normal.

Role of Replication-Induced Transcription in Copy Number Control. To test the relevance of transcribing repA with a burst in copy number control, we tried to dampen RepA synthesis by mutating G to A (P1 coordinate 599) at the start site of repA transcription, and GGA to TTT (P1 coordinates 650–654) in the Shine-Dalgarno sequences preceding the repA translation start codon in pSP102. The repA mRNA level increased about 2-fold in both the mutant plasmids (pSM114 and pSM116, respectively) compared with the wild-type plasmid. pSP102, but RepA protein concentration and plasmid copy numbers were not significantly changed (data not shown). These results suggest that the autoregulatory synthesis of RepA is robust and damping of RepA synthesis rate is not critical for copy number control at or below the physiological iteron concentration.

We next compared miniP1 stability by supplying RepA in cis...
RepA* (source name) & Extra iterons & Copy number (stability) of mini-P1† & \( \triangle \) Copy number (stability) of mini-P1†
\hline
None (pDKC426) & None & 1.0 \( \pm \) 0.23 (10) & 4.2 \( \pm \) 0.39 (86) \\
0.4\times (pDKC425) & None & 1.1 \( \pm \) 0.15 (22) & 4.7 \( \pm \) 0.26 (100) \\
3\times (pDKC424) & None & 1.3 \( \pm \) 0.10 (72) & 5.1 \( \pm \) 0.18 (100) \\
None (pALA96) & #9–#8 (partial) & 0.39 \( \pm \) 0.08 (1) & 2.1 \( \pm \) 0.09 (1) \\
0.4\times (pALA178) & #9–#8 (partial) & 0.54 \( \pm \) 0.11 (1) & 3.3 \( \pm \) 0.25 (8) \\
3\times (pALA177) & #9–#8 (partial) & 0.93 \( \pm \) 0.13 (1) & 4.6 \( \pm \) 0.25 (98) \\
\hline

*RepA amounts are relative to the physiological level as determined previously (7). pDKC426, pDKC425, and pDKC424 are isogenic to pALA96 (10), pALA178, and pALA177 (29), respectively, except that the latter set has extra 45 bp containing iteron #9 and 15/19 bp of iteron #8. Both the iterons bind RepA in vivo (13). The latter set of plasmids thus supplied both extra iterons and extra RepA.

†Mini-P1 plasmids were pRJM384 (\( \Delta \)repAincA) and pRJM345 (\( \Delta \)repA\( \Delta \)incA). All copy numbers are relative. The host was DH5\( \Delta \)lac(\( \Delta \)incCrepA-lacZ).

‡Numbers represent % of cells that retained plasmids after 24 hr growth on L + 50 \( \mu \)g/ml ampicillin plates at 37°C.

§Colony sizes on L plates containing 10 \( \mu \)g/ml chloramphenicol and 50 \( \mu \)g/ml ampicillin were small in the case of all unstable plasmids. For example in pRJM384-carrying cells, the sizes were roughly 1, 0.3, 0.4, and 0.8 in the presence of pDKC424, pALA96, pALA178, and pALA177, respectively. The copy numbers determined after drug selection are most likely overestimates in the case of unstable plasmids.

and in trans. An integrated prophage (ADKC267) was used as a trans source of RepA where repA was transcribed constitutively but the RepA level was similar to that produced by the wild-type plasmid, as determined by immunoblotting. To measure stability, a miniP1 plasmid with a repA(Am) mutation (ADKC252 = \( \lambda \)-P1:5RepA4am) and a suppressor negative host (N100) were used. The stability of ADKC252 in N100 (ADKC267) was very similar to that of the wild-type plasmid (ADKC235 = \( \lambda \)-P1:5R) in N100 where RepA was only produced in cis. There was less than 1% loss after 14 h growth at 37°C on L agar plates without selection in both cases. Thus, as long as RepA is available in near physiological concentrations, the requirement of the transcriptional burst does not seem to be obligatory. The burst is seen apparently because of replication requirement for induction (note the drop in repA transcription during 42°C incubation in Figs. 2 and 3).

Discussion
We show here that replication can induce the autorepressed repA gene of plasmid P1 (Figs. 2–4). An alternate induction mechanism, RepA titration by iterons, is ineffective (Tables 1 and 2). In what follows, we discuss how the plasmid copy number can be regulated by varying RepA concentration. In particular, we propose that RepA plays a positive role in replication by reversing handcuffing, believed to be the primary mode of negative control of replication.

A positive regulatory role of RepA is evident when plasmid replication is reduced by extra iterons: by an integrated copy of the wild-type plasmid, \( \lambda \)-P1:5R (5, 10), \( \lambda \) clones of incC (5), pACYC184 and pUC19 clones of incC (11), or pBR322 clones of 1–2 iterons (Table 2). The role is also apparent in Table 1, where an integrated copy of the incCrepAincA region did not allow the establishment of an incoming miniP1 but not when it carried its own source of RepA (pVM11 vs. pSP102). Thus if iteron-mediated reduction of replication (incompatibility) is mediated by handcuffing, as is currently believed, RepA can overcome it. How this happens can only be speculated because how handcuffing blocks replication is not clear yet. Our understanding of handcuffing is as follows. (i) It appears to operate at suboptimal RepA concentration. When the repA promoter is partially repressed, by supplying RepA below the physiological level, it cannot be induced fully by replication most likely because of handcuffing (9). (ii) Handcuffing appears to be a reversible process because RepA stimulates formation of multimeric fragments in the presence of ligase, even when the fragment carries a single iteron (21). For example, a trimeric fragment is formed when RepAs bound to a dimeric fragment uncouples and one of them recouples with a new RepA bound fragment. (iii) Handcuffing appears to cause steric hindrance to subsequent RepA

![Diagram of P1 plasmid replication requiring replication-induced boost of repA transcription (curved arrows) synthesis to saturate origin iterons with RepA (●). The model shows that handcuffing occurs with unsaturated daughter origins which shuts off repA transcription. The transcription therefore precedes handcuffing. Handcuffing is effectively reversed by increase in cell volume and increase in RepA concentration because of chaperone-mediated activation of newly synthesized RepA. When extra iterons are provided by using foreign vectors, handcuffing prevents new RepA synthesis and, hence, origin saturation. The replication under these conditions therefore depends on an external source of RepA.](image-url)
binding because handcuffing-defective RepA mutants can have increased on-rates of binding (22). With these characteristics of handcuffing in mind, we envisage the following cycle for P1 plasmid replication.

We propose that the replication is regulated primarily by two opposing reactions: RepA-mediated saturation of origin and steric hindrance to saturation by handcuffing (Fig. 5). The initiation occurs on saturation of origin iterons. After replication, repA is transcribed maximally but replication is delayed because newly synthesized initiators are inactive and the pre-existing initiators get distributed to daughter origins (titration). The partially saturated origins get handcuffed readily thus preventing further RepA synthesis and inhibiting saturation by steric hindrance. Increase in cell volume may help to increase plasmid monomer fraction by the laws of mass action (5). Iterons make handcuffing too stable for RepA to saturate the iteron concentration apparently because further increases of constitutive sources helps because its synthesis does not depend on saturation, and hence, replication. In the absence of replication, no new RepA is synthesized. Additional RepA from constitutive sources helps because its synthesis does not depend on P1 replication. P1 can cope with only a limited increase of iteron concentration apparently because further increases of iterons make handcuffing too stable for RepA to saturate the origin. This may explain how RepA is more efficient in bringing copy number back to normal than it is in increasing the number to above normal (Table 2). We note that the model for replication control discussed here does not exclude other mechanisms such as origin sequestration (24) and plasmid partition (25).

Iteron-mediated incompatibility can operate at three levels: by removing RepA (titration), repressing RepA synthesis, and interfering with RepA access to origin (handcuffing). The contribution of titration as such appears to be minor compared with its role as an intermediate in the handcuffing reaction. In fact, the term titration seems to be a misnomer because iterons do not act as sinks for RepA. The proteins can still participate in handcuffing. In contrast, initiator limitation, the basis of the titration model, still appears to be a part of the normal control system. Nevertheless, the mechanism of negative control appears to be handcuffing if it is the basis of initiator limitation. Mechanisms such as replication-induced transcription and chap-erone-activation are there to positively control RepA activity so that handcuffing is overcome at a desired frequency.

The autorepressed dnaA gene of Bacillus subtilis behaves similarly to the P1repA: the gene is not induced by titration but by replication (26). The concentration of the active form of DnaA also is believed to trigger initiation in Escherichia coli (27). Initiator limitation appears to be a common mode of replication control and not exclusive to plasmids under antisense control.

We are grateful to Deepak Bastia for the pDNA1βDNAc plasmid, Bruce Patterson for help with Luciferase assays, and Michael Yarmolinsky, Kyusung Park, Joe Bogan, Michael Lichten, and the reviewers for thoughtful comments on the manuscript.